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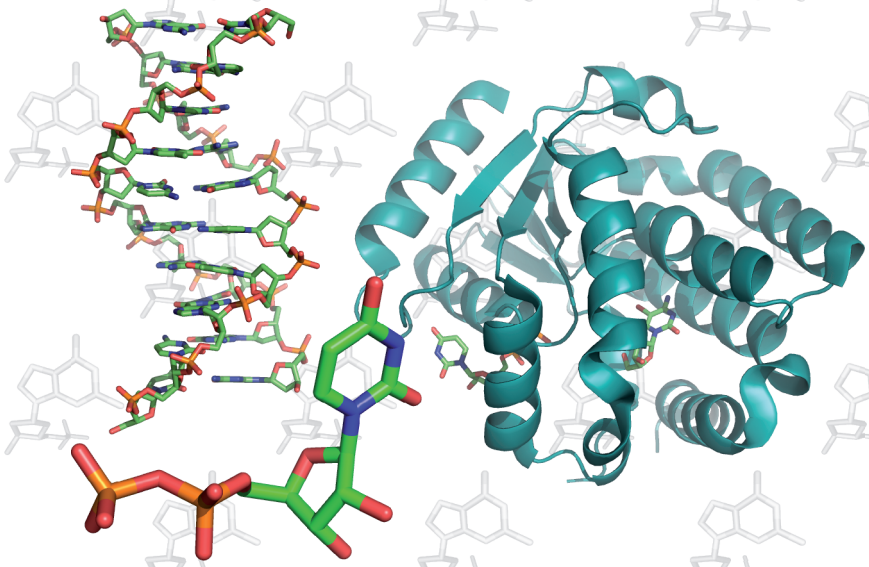
Symposium Series

Český Krumlov
June 5–10, 2011

XVth Symposium on
Chemistry of
Nucleic Acid Components



Volume 12
2011

COLLECTION
Symposium Series

Chemistry of Nucleic Acid Components

Editor
Michal HOCEK

Managing Editor
Bohumír VALTER

Volume 12, 2011, ISBN 978-80-86241-37-1

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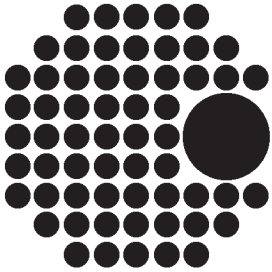

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Printed in Czech Republic – Visit

FOREWORD

The history of the Symposia on the Chemistry of Nucleic Acid Components goes back to 1969 when the first conference of the series took place at the Liblice Castle near Prague. It was organized by the Nucleic Acid Chemistry group in the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences in order to enable meeting of scientists from both parts of politically divided Europe. Since then, the series continued in regular three-year intervals without interruption. Originally it addressed synthesis, transformations, physical chemistry and biological activity of nucleosides, nucleotides and oligonucleotides. During the time aspects of biochemistry and recently also chemical biology in broadest sense also became important topics to be covered. However, the unifying view of this interdisciplinary area was always that of an organic chemist.

Originally meant as a get-together platform for European nucleic acid chemists, the Symposia gradually adopted more international character and nowadays the participants come from all around the globe. Personal contacts and friendships which originated from the encounters of the participants proved to be of particular importance. Emphasis has always been places upon an active role of participants and on the attendance of students and younger scientists. Many of those who attended our earlier conferences are now recognized as prominent scientists in academia or in industrial research. It is comforting to see new faces among us again – it witnesses the appeal which nucleic acids chemistry in its mature age still has for the fundamental research and its applications both in pharmacotherapy and in chemical biology.

It has always been the policy of these Symposia to give an opportunity to many participants including young scientists to present the results orally and to give enough space for informal discussions. In addition to that, several plenary lectures from distinguished scientists are presented to emphasize major developments in the field. Also this time we will have plenary and short lectures covering four major themes: (i) organic synthesis of nucleosides, nucleotides and oligonucleotides, (ii) medicinal chemistry and pharmacology, (iii) biochemistry and chemical biology and (iv) physical chemistry and biophysics of nucleic acids.

The NAC Symposia were held successively at the Liblice Castle, at the Bechyně Castle, at the Třešť Castle, and in the Harmony Hotel in Špindlerův Mlýn. The last 14th Symposium in 2008 was for the first time organized in the unique venue of Hotel Růže in medieval city of Český Krumlov. Rebuilt from the renaissance palace from 16th century, this venue has a special authentic historical atmosphere which perfectly contrasted with the state-of-the-art science presented during the Symposium. After receiving so much positive feedback for this venue from participants, we could have hardly moved anywhere else. Therefore, it is our pleasure to welcome you once again in Český Krumlov for the jubilee 15th Symposium.

The Organizers sincerely hope that once again it will be positively accepted by the participants and wish all of them ultimate scientific satisfaction and a pleasant time.

June 2011

Michal Hocek

Editorial Comments

Proceedings of the XIVth Symposium on the Chemistry of Nucleic Acid Components are published as a special volume of Collection Symposium Series. Please note that it is not a regular issue of the international peer-reviewed journal Collection of Czechoslovak Chemical Communications.

The appropriate reference to the contribution published in this issue is the following:

Authors in: *Collection Symposium Series* (M. Hocek, Ed.), Vol. 12, p. xxx. Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague 2011.

The contributions were not peer-reviewed. The Organizers and the Editor are not responsible for the originality and quality of the contributions.

All authors are cordially invited to submit any time in the future a full-paper or a review version of their contributions for publication (subject to peer-review) in the journal *Collection of Czechoslovak Chemical Communications*.

Michal Hocek
Editor-in-Chief
Collection of Czechoslovak Chemical Communications
and
Editor of Proceedings

František Šorm Memorial Award,

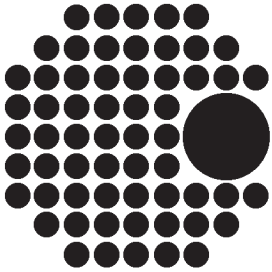
a special distinction established to commemorate the late Professor František Šorm, the founder and the first Director of the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic in Prague and a leading personality in Czech science and the Academy, is awarded by the Director and the Scientific Council of the Institute to prominent foreign scientists in appreciation of the merit of their scientific achievements. Besides honoring scientific qualities of the awardees in a broader sense, it is especially intended to be an appraisal of their important collaboration with the Institute in its traditional research topics (organic synthesis, natural compounds chemistry, steroid chemistry, and the chemistry, biochemistry and molecular biology of peptides, proteins and nucleic acid components). It is handed over to the laureate in person on the occasion of his or her memorial lecture either at the Institute or at an international conference organized by the Institute in the Czech Republic.

At the XVth Symposium on the Chemistry of Nucleic Acid Components held in Český Krumlov, Czech Republic on June 5–10, 2011

Professor Dr. Thomas Carell

Ludwig-Maximilians-University, Munich, Germany

was presented with the František Šorm Memorial Award in recognition of the merit of his scientific achievements in the area of nucleic acid chemistry and chemical biology.



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Oral Communications

Posters

THE CHEMISTRY OF GENOME MAINTENANCE

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DNA is continuously damaged. The formed lesions are often mutagenic or they induce cell death. Organisms have developed a number of defense strategies to ensure the stability of the genetic system. One major defense strategy is based on DNA repair enzymes. These scan the DNA for the content of lesions and initiate repair. Repair can involve the direct reversal of the lesion, removal of the lesion from the DNA material or removal of a whole DNA fragment around the lesion. Next to repair, special polymerases are present in each cell, which help organisms to copy their genetic material in the presence of lesions. These lesion tolerance polymerases are needed in order to allow cells faithful copying of their genes even if lesions are present. Here I am discussing the DNA repair enzyme (6-4) photolyase and the lesion tolerance polymerase Polymerase eta. Crystal structures are reported that show both proteins in complex with lesion containing DNA in the process of repair and translesion synthesis. Finally the quantification of hydroxymethylcytosin (hmC) is reported. This compound was previously thought to be an oxidative lesion but has now been shown to be a natural nucleobase of still unknown function.

INTRODUCTION

Our genome is constantly damaged by various endogenous and exogenous events. Particularly problematic is oxidative DNA damage. It is induced by reactive oxygen species, which are produced daily due to our need to oxidize carbon sources with oxygen to CO_2. This process of respiration, which takes place in our mitochondria leads mostly to the formation of peroxyl-radicals, which attack the genetic material not only in the mitochondria but also in the cell nucleus. Another very important DNA damaging agent is UV light[1]. UV irradiation is the ubiquitous part of sun light. UV light is absorbed by nucleobases particularly by the pyrimidine bases, which react to give a number of defined DNA lesions such as cyclobutene pyrimidine dimers, (6-4) lesions or spore photo products. Another aspect of DNA lesioning is important in respect to a typical cancer therapy. During such a therapy most patients are treated with cytotoxic compounds such as cisplatinum or gemcitabine, which both form covalent adducts with DNA strands. These adducts are supposed to block DNA replication and transcription in order to initiate cell death. As such these compounds are DNA damaging agents that are affecting cell growth not only of tumorgenic cells but of all cells that have a high replication cycle. All these DNA lesions are

efficiently repaired in our cells with the help of a variety of DNA repair enzymes. Since DNA repair is a rather slow process, particularly in non-transcribed areas of our genome, cells posses in addition a lesion tolerance mechanism. This lesion tolerance process is established by special DNA polymerases so-called Y-family polymerases or low fidelity polymerases, which are able to replicate genetic material in the presence of lesions. This replication allows cells to cope with DNA damage. In the course of a cancer therapy however, these lesion tolerance and repair processes counteract the wanted medicinal effect of cytostatic compounds.

In my group we are investigating DNA repair and DNA lesion tolerance processes in more detail. Specifically we are synthesizing DNA lesions and lesion analogues. We insert these building blocks into oligonucleotides using standard phosphoramidite chemistry and we investigate the mutagenic potentials of these lesions as well as the reparability. To this end the group is overexpressing the required DNA polymerases and repair enzymes with the final goal to obtain a crystal structure of the DNA lesion containing DNA in complex with the DNA polymerase or repair enzyme.

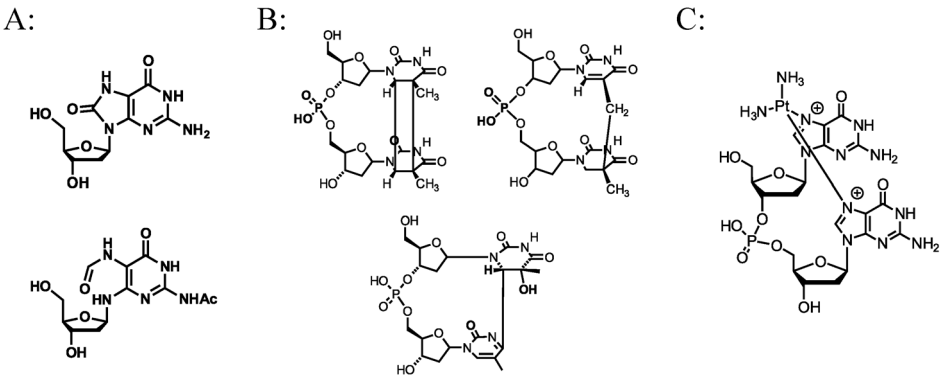

FIG. 1
Depiction of various DNA lesion formed by reactive oxygen species (A), UV-Irradiation (B) and DNA damaging agents (C)

RESULTS AND DISCUSSION

Repair of UV-Induced Lesions

In the first part of the lecture I am going to describe the repair of UV induced lesions by DNA photolyases[2]. UV irradiation generates two major lesions cyclobutene pyrimidine dimers[1] and (6-4) lesions (Fig. 1B). Both are

repaired in many organisms by special enzymes called DNA photolyases. These enzymes utilize long wavelength sun light in order to drive the repair reaction. As such they have a mechanism close to photosynthesis. Light energy is absorbed by one co-factor mostly methanyltetrahydrofolate or a deazaflavine. This light gathering antenna chromophore transfers the energy to reduce the deprotonated flavine which acts as an electron injector. Upon further excitation of the flavine co-factor an electron injection occurs into the lesion followed by a subsequent rearrangement reaction that leads to repair. This repair mechanism which is not operating in humans but in plants, marsupials, rattle snakes and many fish is one of the major defense systems of nature against UV induced lesions and is supposed to be one of the most ancient repair enzyme systems that have enabled life to develop exposed to sunlight. We have developed a new synthesis of (6-4) lesion containing DNA. Based on the discovery that UV irradiation of purine rich DNA strands with the pyrimidine pyrimidine dinucleotide sequence which leads to the exclusive formation of UV lesions at the dipyrimidine site we are now able to prepare (6-4) lesion containing DNA at defined positions in oligonucleotides. Today we can generate DNA strands with up to 16 base pairs, which possess at a predefined site a (6-4) lesion. We furthermore cloned and over expressed (6-4) photolyases from various sources and finally found out that the (6-4) photolyase from the eukaryot *Drosophila melanogaster* is particularly suited for further studies. The protein is 56 kDa large and contains an FAD-co-factor. The protein was overexpressed with

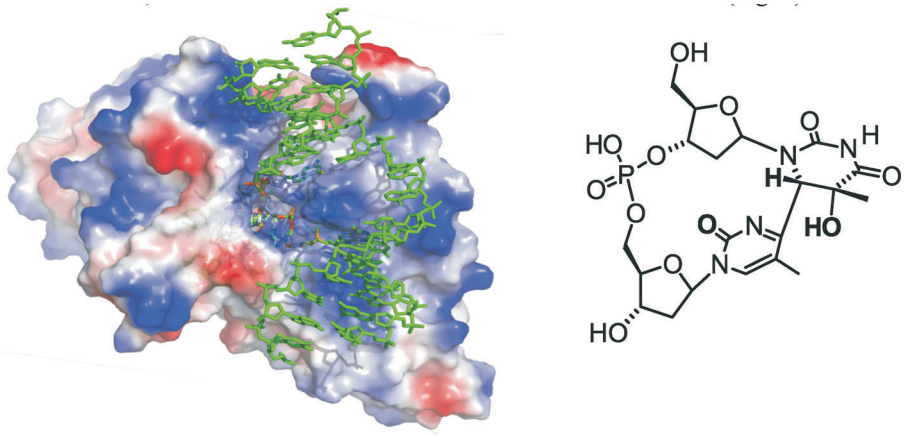

FIG. 2
Depiction of the (6-4) photolyase crystal structure in complex with (6-4) lesion containing DNA and structure of the (6-4) lesion

the help of a streptag in *E. coli* and purified to homogeneity. Finally we were able to crystallize the (6-4) photolyase in complex with (6-4) lesion containing DNA and found out that the protein interrogates the DNA duplex with the help of a critical arginine side chain and that it subsequently turns the lesion out of the duplex into the active site, where it comes in close contact to the flavine co-factor (Fig. 2)[3].

Reduction of the flavine and photoirradiation initiates an electron transfer between the flavine and the lesion, which leads to lesion reversion and hence repair. With the help of biochemical study and mutagenesis investigations we were able to develop a new mechanism for this repair reaction which is similar to ribonucleotide reductase[3,4]. We think that the flavine injects an electron into the lesion (intermediate II) and that the lesion is subsequently losing a water molecule (III) that is re-attacking the lesion at the acylimine unit (IV), followed by fragmentation of the lesion radical (V) as depicted in Fig. 3.

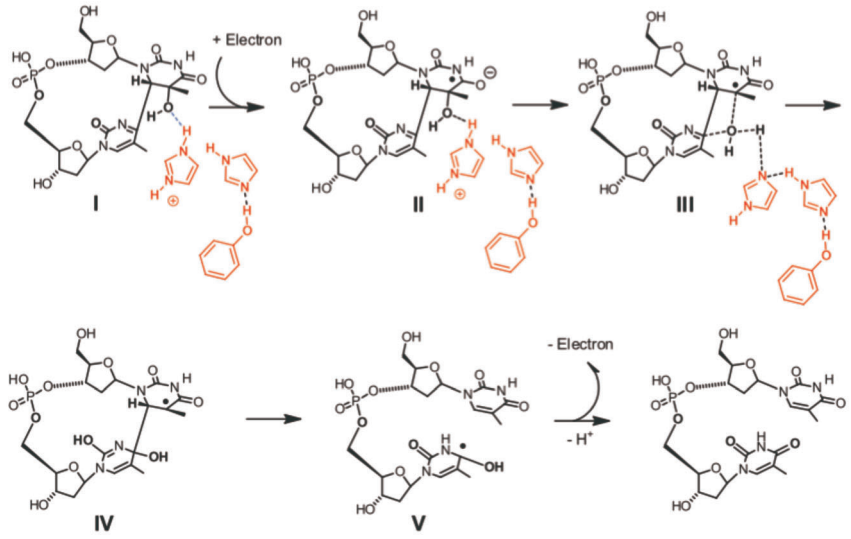

FIG. 3
Proposed (6-4) photolyase repair mechanism

Lesion Tolerance Processes

In the second part of the lecture I am going to discuss how lesion tolerance mechanisms are at work helping cells to override bulky adduct lesions[5–7]. Bulky adduct lesions are mostly formed from aromatic amines which are ac-

tivated in the liver. These activated amines form on the liver bisacetylated compounds. Loss of an acetyl group gives a highly reactive nitreniumion, which is able to attack DNA mostly at the guanine bases. Here these adducts react with the C8-position to form adducts such as the acetylaminofluorene and aminofluorene C8-guanine adduct. These adduct are highly mutagenic they block replication and transcription. Despite this it was found that cells are able to replicate that genetic material in the presence of these lesions with the help of special DNA polymerases called low fidelity, lesion tolerance polymerases. In collaboration with the group of Zvi Livneh we were able to indentify that the low fidelity polymerase η plays a major role in this process. In order to study the lesion tolerance mechanisms, in more detail we synthesized a bulky adduct acetylaminofluorene-dG-phosphoramidite and the corresponding aminofluorene-dG-phosphoramidite in large quantities and synthesized DNA strands containing these lesions in a predefined site with the help of solid phase phosphoramidite chemistry. The corresponding protein pol η was overexpressed in *E. coli*. We used for our studies the yeast polymerase η and here only the catalytic domain. Finally we were able to obtain the protein in sufficient quantities for biochemical and structure biology analysis using again strep tag purification of the protein. Particularly interesting is the fact that pol η is not only able to copy through the bulky adduct but that it will also base pair a bulky adduct dG lesion with the corresponding cytosine base. This is particularly surprising because NMR studies suggested that the acetylaminofluorene-dG-adduct existed mostly in the so-called syn-conformation, where Watson–Crick face of the dG part of the lesion is not available for hydrogene bonding. In order to solve this mystery we crystallized pol η in complex with DNA containing diacetylaminofluorene-dG lesion and various primers[8]. Shifting the dG acetylaminofluorene lesion back and forth inside the DNA polymerase we obtained several snapshots of the lesion bypass process, which allow us today to clarify the lesion bypass process with atomic resolution (Fig. 4)[8].

The large active site of pol η allows the DNA primer construct to rotate freely in the polymerase cleft. For the bypass reaction the DNA strand containing AAF-dG lesion rotates in the active site in an unusual conformation allowing the polymerase to base pair the AAF-dG with a cytosine even with the AAF-dG lesion adapting syn-conformation. What is detected is a large move of the primer strand around the templating lesion that allows the triphosphate to access the lesion for proper base pairing. Subsequently the 3′-hydroxygroup is able to attack the triphosphate in order to perform the required primer extension reaction. This example shows that the low fidel-

ity polymerases have a very large open active site that enables them not only to accommodate DNA lesions but also provides freedom to rotate primer and template in the active site in order to enable efficient lesion bypass.

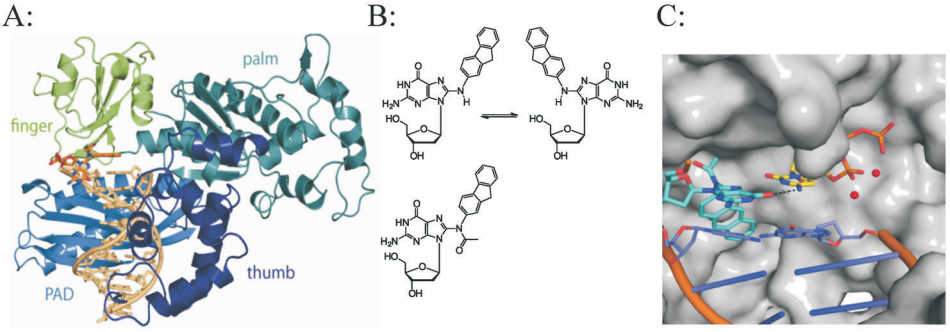

FIG. 4
A: Structure of Pol η in complex with DNA containing the AAF-dG lesion. B: AF-dG lesion in *syn*- and *anti*-equilibrium. C: Structure of the bypass complex showing how Pol η base pairs the AAF-dG lesion in the active site with an incoming dCTP

Nature has developed a variety of DNA repair and lesion tolerance processes in order to cope with DNA lesions. Although these mechanisms are absolutely essential for the survival of life on earth they also establish a harmful effect when patients are under anticancer treatment. The next major goal in the field must be to develop special inhibitors that can be given in combination with cytostatic compounds in order to block replication and repair of DNA lesions. These cytotoxic compounds have the potential to revolutionize modern chemotherapy. Work in this direction will also be covered in the lecture.

The New 6th Base Hydroxymethalcytosine

Our DNA material is not only constructed from the four canonical bases plus DNA lesions, which are present in DNA for a short period of time until repair has removed them. It also contains few modified nucleobases like for example 5-methylcytosine. These 5-methylcytosines are important for epigenetic programming of cells. Epigenetics is the science of the information content in DNA beyond the pure sequence information. Different cells possess the same genetic material however they have to perform vastly different functions. This is achieved by selective silencing of certain genes. In

fact, cell differentiation is on a chemical level the selective methylation of cytosine bases to give 5-methylcytosines. This methylation reaction silences specific genes. During cellular development more and more genes are specifically silenced until a highly specialized cell is produced, which possesses a unique set of active genes. Very recently a new DNA modification 5-hydroxymethylcytosin was detected. Its function is currently unclear but hydroxymethylcytosine is currently one of the hottest topics in epigenetic research[9]. In order to quantify the amount of this new base in different tissues and in certain brain parts we have developed isotope labeled methyl-C and hydroxymethyl-C derivatives. Our isotope-labeled compounds possess two or three deuterium atoms instead of hydrogenes. As such these compounds have the same physico-chemical properties like the natural methyl-C and hydroxymethyl-C bases, however they are two to three molecular weight units heavier. Using these isotope labeled compounds it was possible to establish an HPLC/MS based quantification assay. In this assay we take tissues and cells from various sources[10]. The DNA is subsequently isolated and totally digested down to the nucleotide level with the help of certain digestion enzymes. Finally the isotopically labeled methyl-C and hydroxymethyl-C derivatives are added at defined quantities and the whole hydrolysate is pumped through an HPLC system directly into a mass spectrometry unit. While methyl-C and the isotopically labeled methyl-C derivatives have the same retention times, in the mass spectrometer they provide two different mass signals. Because the amount of isotopically labeled compound is known it is possible to calculate the concentration of the natural methyl-C base in the sample. The same is true for hydroxymethyl-C. Using this isotope labeling method we are able to quantify the amount of hydroxymethylcytosine in different tissues and particularly in different brain parts. It was discovered that all organs contain hydroxymethyl-C but while the methyl level is with 4.5% constant the hydroxymethyl-C varies strongly (Fig. 5). Some organs possess very low levels of hydroxymethyl-C others particularly those in the brain have very high hydroxymethyl-C values. Particularly important is the discovery that all brain tissues associated with higher cognitive function have high hydroxymethyl-C levels that go to about 1% of hydroxymethyl-C in the genetic material. Other researchers have recently discovered that hydroxymethyl-C is formed directly from methyl-C by oxidation with the help of TET enzymes. These TET enzymes are 2-ketoglutarat dependent enzymes that are able to oxidize the methyl group to give the hydroxymethyl group. It was speculated that further enzymes might be involved that oxidize the hydroxymethyl group to form a carbocylic acid group followed by de-

carboxylation. Synthesis of the formyl-C and carboxyl-C derivatives in isotopically labeled form and quantitative HPLC mass analysis provided no support for the hypothesis.

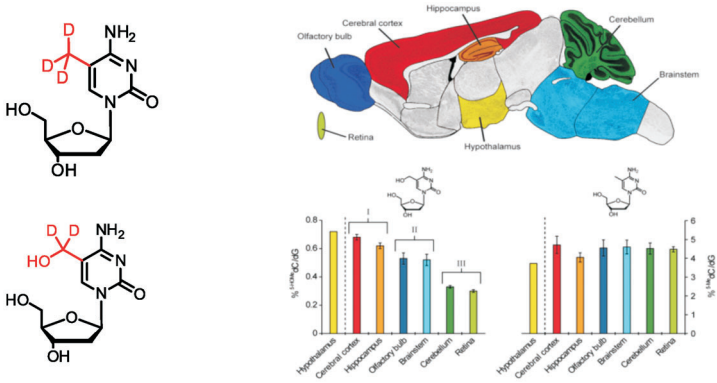

FIG. 5
Depiction of the isotopically labelled methyl-C and hydroxymethyl-C compounds and their levels in brain tissues

In summary in the Šorm-lecture I will cover three aspects of modified DNA bases. We will discuss the repair issue and here we use UV induced DNA lesions and their repair by photolyases as an example. I am going to discuss lesion tolerance processes and here I will focus on bulky DNA adducts and polymerase η as an example. Finally I am showing that DNA lesions are not the only unusual bases present in DNA but that recently hydroxymethyl-C was discovered as a new sixth base in genetic material. We will discuss new methods to quantify in sequence hydroxymethyl-C in genetic material.

We thank Bayer-Schering Pharma AG, the Excellence Cluster CiPSM, and SFB 749 for generous financial support. Further support from the Fonds of the German Chemical Industry is gratefully acknowledged.

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CLICK DNA AND RNA LIGATION FOR NEW BIOCOMPATIBLE NUCLEIC ACID BACKBONE MIMICS

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We have used the CuAAC reaction (click chemistry) to construct cyclic DNA strands, DNA catenanes, and artificial DNA backbones that can be read through by DNA polymerases. We have also constructed chemically-modified ribozymes up to 100 bases in length that are biochemically active.

INTRODUCTION

Click chemistry[1] provides a simple and convenient method to join together organic molecules in high yield under mild conditions in the presence of a diverse range of functional groups. The best example of click chemistry is the Cu^{I}catalysed (3+2) azide-alkyne cycloaddition (CuAAC) reaction[2,3]. It is a useful and convenient reaction for DNA strand ligation (Fig. 1) and other biological applications for the following reasons:

• Azides and alkynes are small, non-polar and neither acidic nor basic. They can be appended to nucleic acids without disturbing their biophysical properties.

• Azides and unactivated alkynes are almost entirely unreactive towards the functional groups normally encountered in nature; they react only with each other.

• The [3+2] azide-alkyne cycloaddition (AAC) reaction involving unactivated alkynes is extremely slow but can be speeded up enormously by Cu^{I} catalysis and by holding the reactants in close proximity. Both accelerating factors are easy to incorporate into the click DNA ligation process and provide a switch to turn the reaction on.

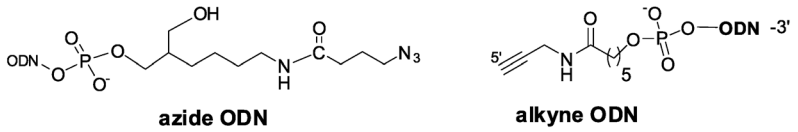
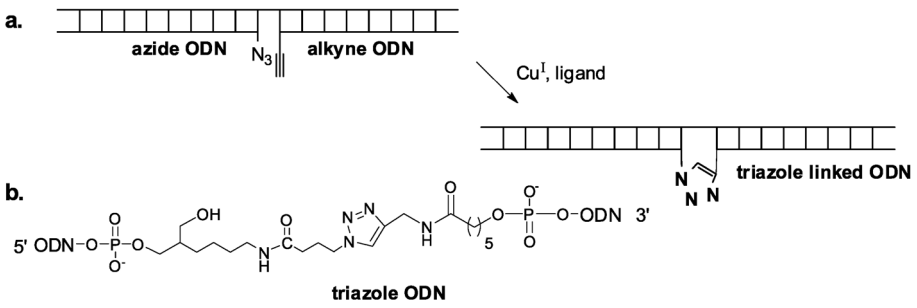


FIG. 1
Azide and alkyne oligonucleotides for DNA strand ligation

• The templated CuAAC reaction on DNA is simple to carry out in aqueous media.
• The resultant triazole unit is extraordinarily stable and is not toxic.

RESULTS AND DISCUSSION

Initially we used the CuAAC reaction for the template-mediated chemical ligation of two oligonucleotide strands, one with a 5′-alkyne and the other with a 3′-azide (Scheme 1) to produce a DNA strand with a continuous 5′- to 3′- backbone and a lengthy triazole linkage at the ligation point[4]. To achieve this, convenient syntheses of the relevant alkyne and azide-modified oligonucleotides were required (Fig. 1). Azides are not stable in the presence of P^{III} so they cannot be converted to phosphoramidite monomers and used in oligonucleotide synthesis. However, the active esters of organic azides can be added to amino-derivatised oligonucleotides in high yield after solid-phase synthesis (post-synthetic modification). Using this strategy, azide-ODNswere prepared by reacting 3′-amino-C7-modified ODNs with 4-azidobutyric acid NHS ester in bicarbonate buffer at pH 8.75.



SCHEME 1
Template-mediated click-ligation of two oligonucleotides. a. Schematic. b. Chemical structure at ligation point

Alkyne ODNs were prepared by adding the relevant alkyne phosphoramidite monomer in automated solid-phase oligonucleotide synthesis. This alkyne is mildly activated for the CuAAC reaction by the neighboring amide moiety. The Cu^{I} click catalyst was prepared *in situ* from aqueous copper sulfate and sodium ascorbate, and all ligation reactions were carried out in 0.2 M NaCl to ensure duplex formation with the complementary template (splint). The reaction of azide ODNs with alkyne ODNs proceeded efficiently in the presence of the water-soluble tris-hydroxypropyltriazolyl-

amineCu^{I}-binding ligand (HPTA)[5] to give ligated triazole ODNs (Scheme 1). At concentrations below 2.0 μM the ligation reaction did not proceed at a significant rate in the absence of the template oligonucleotide. This is important when contemplating simultaneous template-mediated ligation of multiple alkyne/azide-labelled DNA strands. It suggests that such an objective is achievable provided that the DNA concentration is sufficiently low to prevent undesirable non-templated reactions from occurring. The requirement for Cu^{I} catalysis is advantageous in most contexts; it means that oligonucleotide duplexes can be mixed and annealed to give the correct alignment of DNA strands before the click ligation reaction is switched on by addition of Cu^{I}.

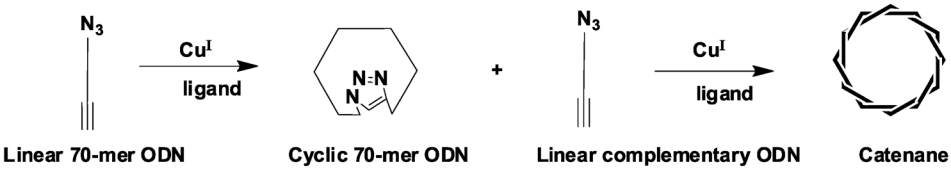
OLIGONUCLEOTIDE CYCLISATION

We have applied click chemistry to the assembly of single-stranded cyclic DNA constructs using oligonucleotides labelled with both 5′-alkyne and 3′-azide. Such ODNs are straightforward to synthesise because 5′-alkyne oligonucleotides containing 3′-amino functions can be efficiently labelled at the amine with azidoalkyl carboxylic acid NHS ester derivatives. Undesirable cycloaddition reactions do not occur between the 5′-alkyne ODN and the azide labelling reagent because the AAC reaction is extremely slow in the absence of Cu^{I}. After oligonucleotide purification, non-templated click ligation was used to cyclise single stranded oligonucleotides up to 72 bases in length[4]. Quantitative cyclisation was achieved for the shorter constructs and the cyclic nature of the oligonucleotides was confirmed by HPLC, gel electrophoresis, mass spectrometry and restriction enzyme digestion of duplexes.

FORMATION OF AN OLIGONUCLEOTIDE CATENANE

Next we applied the above approach to the assembly of a double-stranded DNA catenane. To construct the catenane, two complementary oligonucleotides were labelled with a 3′-azide and 5′-alkyne as described above. Tandem T.T mismatches were placed after every 10 base pairs of Watson–Crick duplex to produce points of flexibility. Without these “hinges” such a short duplex would be too rigid and would be incapable of bending to form a complete circle. To form the catenane, the first ssDNA closed circle was prepared by an intramolecular Cu^{I}-catalysed click ligation reaction. Template-mediated formationof a double stranded DNA construct was then carried out by mixing the purified circular single stranded DNA with its

linear complement (Scheme 2). After the click ligation reaction, a new retarded band appeared on the denaturing polyacrylamide gel owing to formation of the covalently closed double-stranded catenane[4]. Modelling studies showed that a structure in which the strands are entwined six times can be formed, as well as constructs in which the single-strands have fewer crossovers.



SCHEME 2
Formation of double-stranded DNA catenane from single-stranded cyclic template ODN and linear complementary strand

CYCLIC MINI-DUPLEXES

At the opposite end of the size scale, the CuAAC reaction was also used to construct very stable cyclic DNA duplexes with as few as two base pairs[6]. The precursors to the cyclic duplexes were hairpin oligonucleotides with a 5′-terminal alkyne, a 3′-azide and a loop region consisting of one or more hexaethylene glycol units. Two different alkynes were used, the first was based on 6-propargylamidohexyl and the other, which is shorter by four atoms, was based on the 5-hexynyl moiety. The corresponding phosphoramidite monomers were incorporated at the 5′-end of hairpin oligonucleotides which were then labelled with 3′-azides and used in intramolecular self-templated click ligation reactions. The very high efficiency of the cyclisation reactions was confirmed by polyacrylamide gel electrophoresis, reversed-phase HPLC, capillary electrophoresis and mass spectrometry. The CD spectra of the cyclic constructs showed the presence of helically stacked nucleotides, consistent with the B-family of conformations. In general these mini-duplexes are remarkably similar to normal double-stranded DNA. The one exception is the very high thermodynamic stability of the base pairs which makes them excellent model systems for detailed studies on DNA[6]. This high stability should be particularly useful in cases when it is necessary to carry out high resolution investigations on modified DNA, and conventional duplexes are too unstable to permit this. For the very stable cyclic GC dimer, ^{1}H NMR studies in H_2O clearly showed H-bond-mediated base pairing. A dinucleotide duplex is the shortest in which base

stacking is possible. Its stability is due a combination of electrostatic and aromatic interactions, plus inter-base hydrogen bonding. Not surprisingly, a cyclic construct with a single isolated GC base pair did not show any evidence of base pairing, presumably because there is no possibility of stabilisation by base stacking.

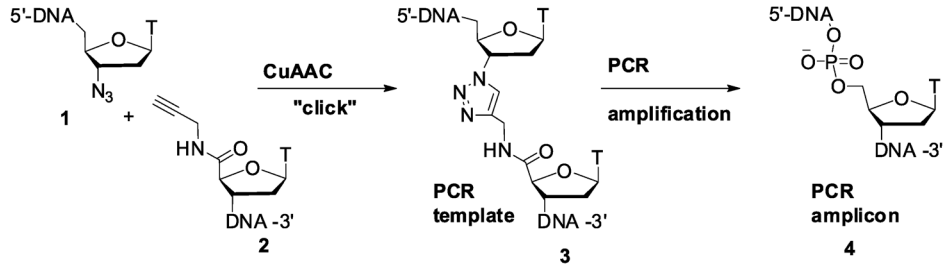
The interaction of the cyclic mini-duplexes with DNA-binding drugs is sequence-dependent in a manner that would be predicted from equivalent linear DNA duplexes, confirming that they are essentially "normal" DNA duplexes. For example, a cyclic AT-rich heptamer containing the AAT binding site for distamycin-A interacted strongly with this drug, and a cyclic GC dinucleotide with the GpC binding site for 7-aminoactinomycin D was greatly stabilised by the intercalator. In a subsequent study on DNA drug binding, the mode of action of a novel threading intercalator was elucidated using an end-sealed duplex that was cyclised using the CuAAC reaction[7].

In order to assess the stability of cyclic duplexes in biological media, another series of hairpin oligonucleotides was cyclised using the CuAAC reaction. The resultant cyclic constructs were designed as decoys for targeting the DNA binding site of the TCF/LEF and GLI transcription factors[8]. Incubation of a 20 base pair fluorescein-labelled cyclic oligonucleotide and its hairpin counterpart in fetal calf serum showed that the cyclic construct has significantly greater stability to enzymatic degradation. Cell uptake studies on the cyclic construct using wild-type HEK-293 cells in the presence of lipofectamine 2000 transfection agent demonstrated that the ODN is taken up by the cells, and localizes to the nucleus. Intranuclear fluorescence persisted for up to 24 h.

PCR AMPLIFICATION OF TRIAZOLE LINKAGES IN DNA

There are currently no reliablemethods of chemically ligating DNA strands that work with high efficiency on a short timescale. Moreover, enzymatic methods that utilise ligases have several limitations, not least of which is the necessity to work on a small scale. If fast and efficient chemical ligation methods can be developed, the size range of synthetic oligonucleotide analogues available for gene synthesis and related applications will be greatly extended, provided that the resultant modified DNA backbone is a good substrate for enzymes used to replicate DNA. Clearly, for PCR to be successful on such modified backbones, the triazole linkage should be a good mimic of normal DNA. In an attempt to produce such a biologically compatible chemical linkage, we have synthesised a ligated DNA strand con-

taining an unnatural T-triazole-T linkage by click ligation between an oligonucleotide with 3′-AZT (**1**) and another with 5′-propargylamido dT (**2**) (Scheme 3). The reaction was assisted by a complementary splint oligonucleotide. The resulting click-ligated DNA strand (**3**) was used as a template in PCR, and amplification was successful with several different thermostable polymerases. Interestingly, DNA sequencing of the PCR amplicons and clones revealed the presence of a single thymine at the ligation site (**4**) instead of the two thymine bases that were present in the original template.



SCHEME 3
Synthesis and PCR amplification of an unnatural triazole-based DNA backbone

A possible explanation for the T-deletion mutation in artificial DNA backbone is the presence of a rigid amide bond which could cause the triazole T in the template to turn away from the growing DNA strand during replication. The lack of a 3′-oxygen atom and a 5′-methylene group as enzyme recognition sites might also be causing this backbone to be a sub-optimal substrate for polymerases. With these considerations in mind a second generation backbone was designed without an amide linkage (Fig. 2) but with 5′-methylene and 3′-oxygen, like a natural phosphodiester. Conveniently this new backbone is constructed from oligonucleotides made entirely by the solid-phase phosphoramidite method, one bearing a 5′-azide and the other a 3′-alkyne. Importantly, the bases around this triazole backbone are correctly copied by thermostable polymerases.

The remarkable ability of thermostable polymerases to copy DNA templates containing such an unnatural backbone opens up intriguing possibilities in gene synthesis, genetic analysis, biology and nanotechnology. In order to fully exploit this discovery it will be necessary to develop robust methods to synthesise long oligonucleotides bearing a suitable alkyne at one terminus and an azide at the other. This might enable the simulta-

neous template-mediated ligation of several ODNs to construct functional genes.

The above triazole linkage (Fig. 2a) has also been used in RNA synthesis to construct chemically-modified ribozymes up to 100 nucleotides in length. Moreover, this triazole linkage supports cleavage activity when incorporated into a ribozyme at the cleaving site[9].

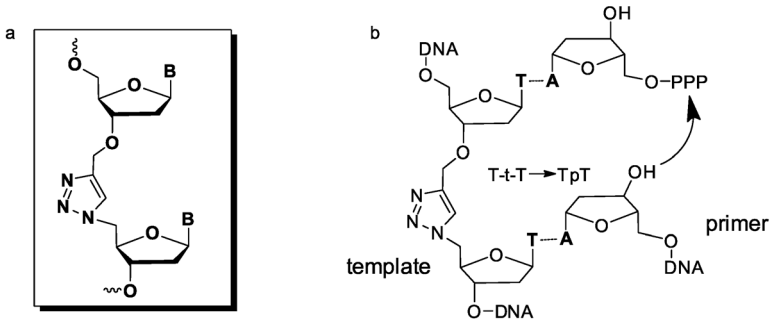


FIG. 2
(a) Second generation triazole linker. (b) Accurate read-through by DNA polymerases

CONCLUSIONS

We have demonstrated the utility of click ligation in the synthesis of cyclic DNA, DNA catenanes, for the ligation of DNA strands to produce templates that can be read through by PCR, and in the synthesis of biologically active RNA constructs. We are continuing to explore this and related chemistry for the synthesis of other biocompatible DNA constructs and novel nucleic acid-based nanoconstructs.

The research leading to these results has received funding from the European Community's Seventh Framework Programme [FP7/2007-2013] under grant agreement number [HEALTH-F4-2008-201418] entitled READNA.

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EXPLORING UNIQUE PROPERTIES OF UNLOCKED NUCLEIC ACID

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Unlocked nucleic acid (UNA) is a flexible nucleic acid analog that has recently been shown to be useful in a number of applications. A facile, three-step synthesis of UNA phosphoramidites has been optimized in our laboratory and these phosphoramidites have been used to incorporate UNA monomers into oligonucleotides. Studies of the hybridization properties of UNA containing duplexes have revealed a destabilizing effect of UNA. Depending on the position of incorporation, UNA can also either increase or decrease the discrimination of a mismatched sequence by an oligonucleotide. These properties are important in a variety of applications and UNA has been shown to be beneficial to reducing off-target effects and increasing potency of siRNAs. This is an important development towards the use of siRNAs *in vivo* to treat a multitude of diseases and could also be applied to the use of siRNAs in molecular biology research and high-throughput target validation studies. Another promising class of therapeutic oligonucleotides is aptamers. UNA has been shown to increase thermal stability and binding affinity of a known thrombin aptamer and continues to be developed for use in other aptamer contexts.

INTRODUCTION

Oligonucleotides have been shown to be useful in a number of different applications such as degrading targeted RNA sequences, detecting minute amounts of ligands in solution, inhibiting transcription of RNA from DNA, and interfering with innate cellular processes like microRNA regulation of gene expression. By chemically modifying the structures of DNA or RNA in these oligonucleotides, their physical and chemical properties can be improved in several ways. Chemical modification of natural nucleic acids can increase their resistance towards endogenous nucleases, modulate their binding affinity with a target sequence, append a second functional moiety (such as a fluorescent label, other oligonucleotide or cargo drug) or increase the cellular uptake of the oligonucleotide[1–3]. These are all important properties to consider when applying oligonucleotides for therapeutic or molecular biological uses.

Over the past few decades, a large number of chemical modifications have been made to the phosphate backbone, sugar ring and nucleobases of DNA and RNA. Research in our group has focused on the chemistry of locking and unlocking the sugar ring to modulate the properties of oligonucleotides. Locked nucleic acid (LNA) has a rigid 2′,4′-*O*-methylene bridge that

locks the sugar in an RNA-like C3′-endo configuration[4]. More recently we have synthesized and studied an unlocked nucleic acid (UNA) analog, also termed 2′,3′-*seco*-RNA, which is an extremely flexible RNA mimic shown in Fig. 1 [5]. The flexibility of this modified nucleotide can be beneficial in certain applications[6].

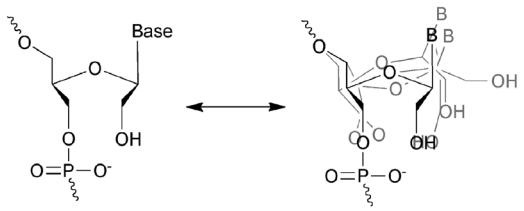
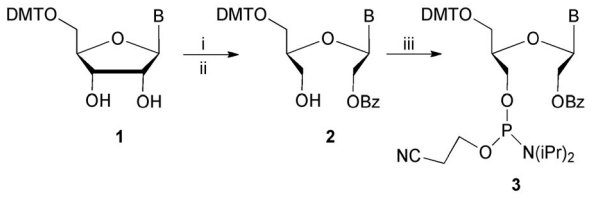


FIG. 1
The highly flexible structure of UNA

UNA SYNTHESIS

Although UNA was originally synthesized and incorporated into oligonucleotides more than a decade ago, it was not well characterized or widely used[7–9]. More recently, the synthesis of UNA phorphoramidites has been optimized in our laboratory to give high yields and increased step-wise coupling yields in automated oligonucleotide synthesis (Scheme 1)[5]. O5′-dimethoxytrityl protected ribonucleosides **1** are the starting material in UNA phosphoramidite synthesis. The C2′–C3′ bond is cleaved by treatment with sodium periodate followed by reduction with sodium borohydride. This highly efficient cleavage reaction is then followed by selective benzoylation of the 2′-hydroxy function to give **2**, followed by standard O3′-phosphitylation. This method has been used to prepare large quantities of UNA phosphoramidites **3** and they are now commercially available



SCHEME 1
Reagents and conditions (i) (a) $NaIO_4$, 1,4-dioxane/water; (b) $NaBH_4$, 1,4-dioxane/water; (ii) BzCl, 2 equiv. DBU, DCM, –70 °C; (iii) 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, *N*,*N*-diisopropylethylamine, acetonitrile

(www.ribotask.com). UNA phosphoramidites can be coupled into DNA or RNA oligonucleotides with 98–99% stepwise yield when standard oligonucleotide synthesis reagents and conditions are used.

A couple additions to the basic UNA structure have also been synthesized (Fig. 2). The synthesis of 2′-linked UNA is parallel to regular (3′-linked) UNA synthesis but the resulting monomer is slightly less destabilizing when incorporated into a duplex[8,10]. In addition, UNA has been derivitized at the 2′-position with a piperazino group[11], which has the potential to be functionalized with a variety of useful ligands, including a terpyridine ligand for metal chelation[11]. When terpyridine-modified UNA is incorporated into complementary strands of DNA in a "-1 zipper" arrangement, the duplex is greatly stabilized by addition of divalent metal ions[11]. UNA remains amenable to diverse chemical modifications, which are only beginning to be explored.

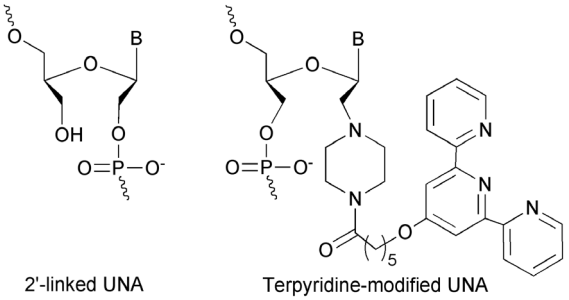


FIG. 2
Derivatives of UNA

UNA HYBRIDIZATION

UNA has universally been shown to decrease the thermal stability of a full-matched, UNA modified duplex. Incorporation of one UNA into the center of a DNA/DNA duplex can decrease the melting temperature (T_m) by 7–10 °C [8]. An RNA/RNA duplex is also affected by UNA incorporation and the decrease in T_m value can be 5–10 °C per UNA modification[5,10,12]. The loss of thermal stability seen with UNA modification is dependent on the position of the UNA within the duplex. Modification near (1-3 bases) the end of a duplex may cause only a 1–3 °C drop in the T_m value[8]. The change in T_m value is also decreased if UNA is incorporated into a longer duplex. Other acyclic nucleotides have shown a similar decrease in duplex stability to UNA and it is believed that this is because of greater flexibility of the

phosphodiester backbone[8,13–16]. An initial study of the thermodynamics of RNA/RNA duplexes containing UNA shows the effects of UNA modification to be additive on the ΔΔG value[12]. The decrease in thermal stability of UNA modified duplexes makes UNA-modified oligonucleotides inconvenient for use in single-stranded antisense oligonucleotides, but as described below, UNA modification can actually be beneficial in some other oligonucleotide applications.

Another property that is important to oligonucleotides is their recognition of fully matched versus mismatched sequences. UNA modification can modulate the mismatch discrimination of an oligonucleotide. A large decrease in mismatch discrimination is seen if UNA is incorporated directly opposite to a mismatched base[5,12]. Better discrimination is seen with mismatch sites neighboring a UNA incorporation, though still less efficient than discrimination of mismatches in unmodified duplexes. Alternatively, when UNA is incorporated distal to the site of the mismatch, an improvement in discrimination between matched and mismatched sequences is seen[5]. Therefore with distally UNA modified duplexes, the mismatch is less stable than the matched duplex but also less stable than the unmodified, mismatched duplex. The ability of UNA to either increase or decrease mismatch discrimination can be applied to different nucleotide applications depending of their requirement for specificity.

UNA-MODIFIED siRNAs

UNA was recently identified in a large screen of siRNA modifications, to be one of the most potent and least toxic nucleotide modifications tested. UNA was one of 21 different modified nucleotides tested in a combination of 2160 siRNA duplexes[17]. It was found that UNA could be introduced effectively into both the sense and antisense strands of the siRNA and increase the silencing effect while remaining non-toxic to HeLa cells. These findings lead to a number of additional studies into the positional effects of UNA in siRNAs and how the modification affected processing and loading into the RISC complex. Detailed studies of the optimal position for UNA modification were carried out by several groups[18–20]. A significant reduction of off-target effects was seen with siRNAs modified with UNA in the antisense strand. UNA at position 7 of the antisense strand was particularly effective at reducing off-target effects[21]. Another group studied the effects of UNA modified siRNAs (UsiRNAs) by microarray analysis on >47,000 mRNAs from HepG2 cells. This study showed that UsiRNAs could reduce off-target effects by 11-fold over unmodified siRNAs[22]. UNA could be reducing off-target

effects by destabilizing the seed region of the antisense strand (positions 2-8), which is responsible for binding to other sequences and causing a microRNA-type of silencing. UsiRNAs have also been applied to inhibit coxsackievirus B3 in cell[19]. Overall, UNA has been shown to be a very promising modification for future siRNA development.

UNA MODIFICATION OF APTAMERS

Another major focus in oligonucleotide research has been on aptamers. These short, single-stranded DNA or RNA molecules are able to form unique tertiary structures that can bind to a variety of ligands with very high affinity. Aptamers have been developed to recognize small molecules, peptides, entire proteins, other nucleic acids or extracellular receptors with nanomolar binding constants. These tight binding nucleic acids are discovered through the use of cyclic amplification of the best binding sequences. This procedure has been termed Systematic Evolution of Ligands by EXponential enrichment (SELEX)[23,24].

By modifying some of the nucleotides in a selected aptamer sequence, we can enhance binding, nuclease stability and/or cellular uptake of the aptamer. The flexibility of UNA was hypothesized to be useful in relieving strain in tight loop structures of aptamers. Recently, UNA was incorporated into loops of the quadruplex thrombin binding aptamer and shown to increase binding affinity, as well as increase clotting time in human blood samples[25]. This illustrates the usefulness of UNA for improving aptamer characteristics. UNA has also been shown to stabilize i-motif formation when introduced into the loops of the quadruplex[26].

While UNA has proven to be useful for modifying known aptamer sequences, being able to select aptamers with UNA already incorporated, could lead to new, tighter binding aptamer structures. In order to adapt SELEX to include UNA nuleotides, a DNA polymerase that can read UNA strands and a polymerase that can incorporate UNA nucleotides needed to be found. Previous studies in our laboratory had found that this was possible with LNA through the use of Phusion HF, KOD and 9°N DNA polymerases[27–29] and other laboratories have incorporated a variety of modified nucleotides using DNA polymerases[30–32].

Towards this goal, we have studied the reading of a UNA-containing template and incorporation of UNA nucleotides using a variety of DNA polymerases. A polymerase's ability to read a UNA-containing template was assessed using primer extension assays. A radiolabeled primer was annealed to a template containing a single UNA modified base, then a mix of deoxy-

nucleotide triphopsphates (dNTPs) and DNA polymerase were added and the products of the polymerase reaction were analyzed by polyacrylamide gel electrophoresis. The reading of templates containing single UNA bases was assessed for a number of DNA polymerases. Fig. 3 shows gel analysis of reading of four templates containing one of the four UNA bases by KOD XL DNA polymerase. In each case, the polymerase stops upon encountering the UNA base and no difference is seen between lanes where dCTP was added to the reaction (+) or dCTP was left out of the reaction (–). A variety of other DNA polymerases were also tested for reading of a single UNA modification, including other B-family polymerases (Phusion HF, Therminator, Deep Vent(-exo) and 9°N), A-family polymerases (Klenow fragment, T4 DNA pol, HIV-RT), a Y-family polymerase (Dpo4) and an X-family polymerase (Pol β), and none of them showed significant reading past the site of the UNA base.

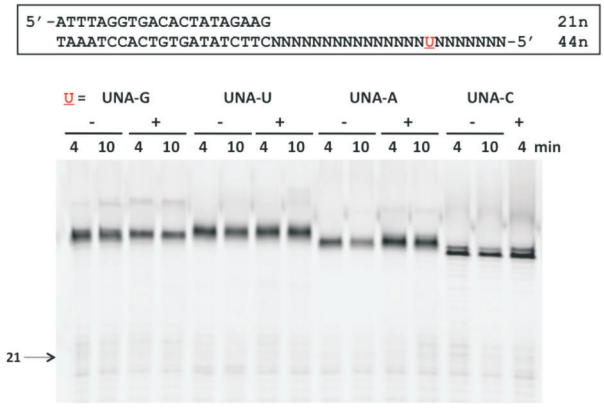


FIG. 3
Reading of a UNA containing template by KOD XL DNA polymerase. Primer and template for primer extension reaction shown above; 13% polyacrylamide gel electrophoresis of ^{32}P-labeled primer in primer extension reactions either with (+) or without (–) the dNTP complimentary to the UNA found in the template; no extension is seen upon addition of all four dNTPs

We also searched for a DNA polymerase that could use UNA triphosphates to incorporate UNA modifications into a DNA strand. In these primer extension experiments, the templates were normal DNA with a single site for each of the four UNA nucleotides to be incorporated opposite it. Shown in Fig. 4 is the reaction with KOD XL DNA polymerase and each of the four UNA triphosphates. We found that UNA was not able to be incor-

porated by KOD XL DNA polymerase, as there was little to no full length product present in the lanes where UNA triphosphate was substituted for the corresponding DNA triphosphate in the reaction mixture (U). The inability of DNA polymerases to recognize UNA modifications is most likely due to the flexible nature of its structure. DNA polymerases need to be highly selective of their substrates, so as not to propagate mutations in genomic DNA and this means that their binding pockets are highly selective for DNA nucleotides in both the template and incoming nucleotide triphosphate. The flexible nature of UNA has proven incompatible with known DNA polymerases, but it may still be possible to evolve a DNA polymerase that could recognize UNA. Until such a polymerase is found, UNA will have to continue to be incorporated into aptamers after their SELEX-based selection.

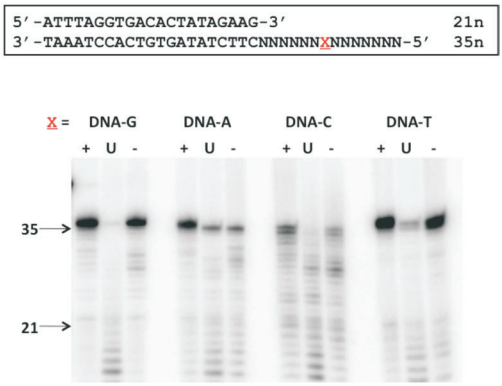


FIG. 4
Incorporation of UNA nucleotides by KOD XL DNA polymerase. Primer and template for primer extension reaction shown above; 13% polyacrylamide gel electrophoresis of ^{32}P-labeled primer in primer extension reactions either with (+) or without (–) the dNTP complimentary to the DNA nucleotide at position X in the template, or with the UNA triphosphate (U) complimentary to position X; the UNA triphosphates are not utilized by the polymerases in the primer extension reaction (U lanes)

CONCLUSION

In the continued development of oligonucleotides for therapeutic use, UNA could play an important role in optimizing oligonucleotide chemistry for use in cell culture or *in vivo*. The relatively simple synthetic route to UNA phosphoramidites and their highly efficient incorporation into oligonucleotides have made UNA a commercially viable modified nucleotide. UNA modifications have been shown to modulate duplex stability and re-

sist degradation by exonucleases. Modification of siRNA duplexes with UNA at position 7 of the antisense strand has proven to significantly reduce off-target effects while maintaining silencing potency. This feature could make UNA-modified siRNAs the number one choice for therapeutic and target validation purposes. In addition, UNA has been shown to be promising for modification of known aptamers. By incorporating UNA into loop structures of aptamers, the melting temperature and binding affinity of the aptamer can be improved. Thus far, UNA has not been found to be recognized by DNA polymerases, so incorporation of UNA into the SELEX process for aptamer evolution remains a challenge. Further chemical modification of UNA could be useful for a variety of applications including increased uptake of oligonucleotides by cells, targeting specific cells with oligonucleotides and detection of DNA or RNA sequences.

The Danish National Research Foundation and The Danish National Advanced Technology Foundation are thanked for generous financial support.

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LOST IN REPLICATION: DNA POLYMERASES ENCOUNTERING NON-INSTRUCTIVE DNA LESIONS

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Abasic sites represent the most frequent DNA lesions in the genome. These lesions have high mutagenic potential and lead to mutations that are commonly found in human cancers. Although these lesions are devoid of the genetic information, adenine is most efficiently inserted when abasic sites are bypassed by DNA polymerases, a behavior that is termed "A-rule". Intrinsic properties of purines like superior stacking ability of the incoming purine nucleotide to the nucleobase π-system at the primer end were proposed as being the driving forces for preferential purine selection. Here we present functional and structural data of DNA polymerases caught while incorporating a nucleotide opposite an abasic site. We found that a protein side chain directs for nucleotide incorporation rather than DNA in the absence of a nucleobase. It fills the vacant space of the absent template nucleobase and thereby mimics a pyrimidine nucleobase directing for preferential purine incorporation opposite abasic residues due to enhanced geometric fit to the enzyme active site. This side chain is highly conserved throughout evolution from bacteria to humans indicating a general mechanism for how non-instructive lesions are bypassed by DNA polymerases.

INTRODUCTION

The most frequent DNA damage observed under physiological conditions are abasic sites resulting from spontaneous hydrolysis of the bond that connects the sugar and the nucleobase in DNA[1]. It has been estimated that approximately 10,000 abasic sites are formed in a human cell per day[1–3]. Guanine and adenine nucleobase residues are cleaved most efficiently resulting in the abasic sugar moiety (AP, Fig. 1a) with the loss of the genetic information stored in the nucleobase[2]. Since these lesions are devoid of the genetic information they are potentially mutagenic. The bulk of this damage is removed by DNA repair systems, which use the sister strand to guide incorporation of the right nucleotide in places of the lesion. However, undetected lesions or those which are formed during S-phase pose a challenge to DNA polymerases. Indeed, abasic sites are strong blocks for bypass DNA synthesis catalysed by DNA polymerases[4,5]. Additionally, several studies indicated the mutagenic potential of these lesions in translesion synthesis which is more pronounced in animal compared to bacterial cells, presumably due to higher translesion synthesis in eukaryotes[6–8]. Although abasic sites are devoid of any information stored in the nucleobase residues and

are considered being non-instructive, *in vitro* and *in vivo* studies of abasic sites or the stabilized tetrahydrofurane analogue F (Fig. 1b) have shown that adenine, and to a lesser extent guanine, is most frequently incorporated opposite the lesion.

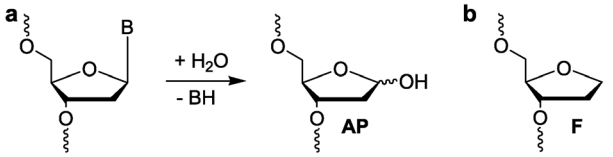

FIG. 1
Formation of abasic sites. (a) Hydrolysis of the glycosidic bond leading to nucleobase loss and formation of abasic site AP. B = Nucleobase. (b) Structure of the abasic site analogue F

This preference of DNA polymerase for adenine incorporation has been termed the "A-rule" and is mainly found for DNA polymerases from family A (including human DNA polymerases γ and θ) and B (including human DNA polymerases α, ε and δ)[6,7,9]. Intrinsic properties of purines like superior base stacking ability of the incoming purine nucleotide to the nucleobase π-system at the primer end were proposed as being the driving forces for preferential purine selection[10,11]. This apparent selectivity for incorporation of purines ultimately results in transversion mutations commonly found in human cancers[12].

What are the structural determinants of enzymatic specificity for purines? Up to recently structural insights into mechanisms of abasic site bypass by DNA polymerases according to the A-rule were missing. Here, we present functional and structural details of DNA polymerases incorporating a nucleotide opposite an abasic site that provide insights into the mechanistic origin for purine selectivity[13–16].

RESULTS AND DISCUSSION

Significant mechanistic insights of nucleotide incorporation during DNA polymerization were derived from high-resolution structures of *KlenTaq*, an *N*-terminally truncated form of *Thermus aquaticus* (*Taq*) DNA polymerase[17,18]. *KlenTaq* is a member of family A DNA polymerases that play a role in prokaryotic and eukaryotic repair of DNA lesions as well as in somatic hypermutation[5,19]. To reveal the structural basis for the ability of DNA polymerases to promote abasic site bypass according to the A-rule we crystallised *KlenTaq* in complex with DNA containing an abasic site in the template and an incoming 2′-deoxynucleotide-5′-triphosphate (dNTP). Using

the optimized *KlenTaq*, we were able to obtain several crystals and could solve their structures. The structure of a ternary complex of *KlenTaq* bound to an abasic site containing an 11 nucleotide primer strand, a 16 nucleotide template, and an incoming 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP) opposite the lesion was solved. The structure provides a snapshot of nucleotide incorporation opposite an abasic site F containing complex.

KlenTaq in complex with ddATP opposite an abasic site template adopted an overall conformation very similar to the one in a ternary complex of the enzyme bound to non-damaged DNA and ddATP. However, there are several structural changes of the O helix, near the primer terminus and the dNTP binding site. In the *KlenTaq* structure containing the undamaged substrates (Fig. 2) the O helix packs against the templating nucleobase and incoming ddATP and thereby "closes" the active site[17]. In the F containing *KlenTaq* structure, the O helix is in a conformation that leaves the active site more solvent exposed ("opened") compared to the structure containing the undamaged primer template complex.

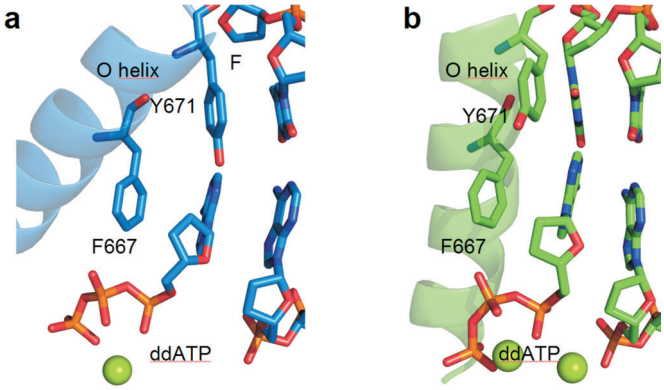

FIG. 2
Close-up views of the enzymes active site processing abasic site containing template. Highlighted and labeled are residues that interact with the incoming triphosphate. (a) Structure of *KlenTaq* active site processing a ddATP opposite template F. (b) Structure of *KlenTaq* active site processing a ddATP opposite template T

One significant difference in the structures containing undamaged and damaged DNA comprises orientations of amino acid side chains in the O-helix. In the closed ternary *KlenTaq* structure, Tyr671 is located on the template side and undergoes nucleobase-stacking interactions with the nascent nucleobase pair (Fig. 2b). However, in the ternary structure obtained with the abasic site substrate opposite incoming ddATP the phenol ring of

Tyr671 is in a different orientation. It directly interacts with the nucleobase residues of the incoming ddATP (Fig. 2a). Thereby, the aromatic side chain of Tyr671 fills the vacant space that has been left by the missing nucleobase and may act as a positioning device to substitute for the missing nucleobase. These interactions place the incoming ddATP opposite the abasic site in a position where stacking interactions to the nucleobase π-system at the primer end, which are found in the non-damaged complexes, are lost (Fig. 3a). The Tyr671 on the other hand is positioned in a way that it nicely stacks to the template nucleobase positioned 3′ of the abasic site (Fig. 3a). These findings indicate that Tyr671 almost perfectly mimics the shape and size of a six-membered pyrimidine nucleobase in the template strand (Figs 2, 3a). These active site constraints might determine the preferential incorporation of adenine and guanine by a close geometric fit to the enzyme active site.

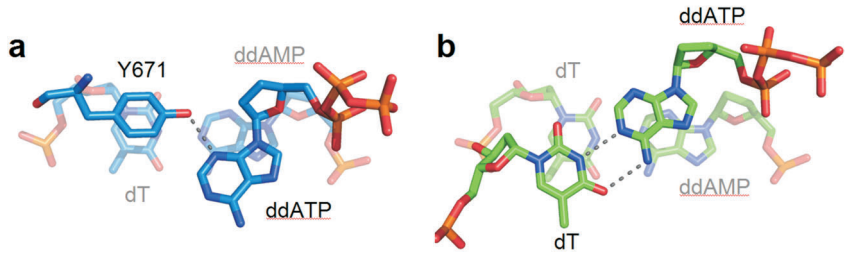

FIG. 3
(a) Top view to the nascent base pair opposite F. In the front the incoming ddATP opposite Y671 is depicted. The hydrogen bond between the hydroxyl group of Y671 and N3 of adenine is indicated as dashed line. In the back the nucleobase pair of the primer template terminus is shown. (b) Top view of the nascent base pair in *KlenTaq* containing an undamaged template. The hydrogen bonding between the incoming ddATP and the templating dT is shown in dashed lines. The primer template terminus is illustrated in the back

To study the functional role of Tyr671 in abasic site lesion bypass we constructed mutants at this position of *KlenTaq*. The most interesting, the mutation Tyr671Trp was constructed since it will transform the six-membered phenol ring of Tyr671 into a bicyclic indole consisting of a six-membered ring fused to a five-membered ring. This mutation results in an amino acid side chain with the approximate size of a purine. If a molecular mimicry based mechanism in which the amino acid side chain takes over the role of the absent nucleobase is indeed at work, the smaller pyrimidine nucleotides thymidine and cytidine should be more preferentially incorporated opposite an abasic site. Indeed, this was observed in primer extension studies

and quantified by measurement of transient kinetics using quench flow techniques[13,14,20–22]. Although the wild type enzyme incorporates dTMP and dCMP with 183- and 261-fold reduced efficiency opposite the abasic site compared to dAMP, respectively, the contrary is observed for Tyr671Trp. In the Tyr671Trp mutant enzyme the preference for dATP vanished and instead the pyrimidines dTMP and dCMP are about five-fold more efficiently incorporated than dAMP.

CONCLUSIONS

Our study reveals a molecular mimicry-based mechanism of error-prone processing of the most abundant mutagenic DNA lesion that is formed under physiological conditions and provides a general mechanism for how non-instructive lesions are bypassed by DNA polymerases. The depicted mimicry of nucleobase shape and size as well as the role of Tyr671 have not been reported previously for translesion synthesis. Unlike other DNA lesions abasic sites are devoid of any structural information that is at least residual in damaged nucleobases. Residual information is used by DNA polymerases to catalyse translesion synthesis through aberrant hydrogen bonding patterns of the incoming dNTP to the damaged template nucleobase. Bypass of DNA lesions such as thymidine dimers, 8-oxidized guanosine residues, cisplatin or nucleobase adducts is promoted in this fashion[23]. In contrast, the purine specificity of abasic site bypass in DNA polymerases from the A-family stems from specific interactions of the incoming dNTP with the protein side chain that mimics the size and shape of the absent nucleobase rather than the DNA.

This work was supported by the DFG.

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STEREOSELECTIVE SYNTHESIS OF 3-METHYL-*cyclo*SAL-NUCLEOTIDES

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*Cyclo*Sal-nucleosyl-phosphate triester are a known class of highly effective nucleotide prodrugs (pronucleotides) of antivirally active nucleoside analogues. The originally developed synthesis for these compounds led always to diastereomeric mixtures. Now, convergent routes for the stereoselective and the stereospecific synthesis of *cyclo*Sal-triesters were discovered to give isomerically pure *cyclo*Sal-prodrugs. The new methods involved the use of chiral auxiliaries to yield these pronucleotides stereospecifically. A thiazolidin-2-thione derived from valinol fulfilled all three requirements to act as a suitable chiral moiety allowing a: i) strong chirality transfer, ii) the formation of separable diastereomeric intermediates and iii) to be a suitable leaving group that allows the introduction of the nucleoside analogue (e.g. d4T) in the final step under mild reaction conditions. Moreover, *N*-cyanimino-oxazolidines derived from phenylalaninol or norephedrine were used for the stereospecific synthesis of 3-methyl-*cyclo*Sal-pronucleotides. The title compounds were obtained with very high diastereomeric excesses (≥95 *d.e.*)

INTRODUCTION

Chiral phosphorus compounds are important target structures. It has been proven that the configuration at the phosphorus atom has an influence on the biological activity while interacting with biomolecules. Therefore, the stereoselective synthesis of such compounds is an important aim to achieve. One application of chiral phosphorus compounds are pronucleotides that act as lipophilic nucleotide precursors e.g. in antiviral chemotherapy. Since the discovery of a new disease in 1981 called AIDS which is caused by the Human Immunodeficiency Virus (HIV), many efforts have been made to inhibit its replication in infected cells. The use of nucleoside analogues such as 3′-azido-3′- deoxythymidine (AZT) **1** and 3′-deoxy-2′,3′-didehydrothymidine (d4T) **2** for the inhibition of the virus-encoded reverse transcriptase (RT) proved to be efficient for the treatment of AIDS (Fig. 1). For their antiviral activity the intracellular activation into the 5′-mono-, 5′-di- and the ultimately biological active 5′-triphosphates by cellular nucleoside kinases or 5′-nucleotidases is necessary after cell penetration of the nucleoside analogues. However, these phosphorylation steps

represent a critical point because the first phosphorylation step of d4T **2** into d4TMP catalyzed by thymidine kinase (TK) is the rate-limiting step in human cells and the conversion of AZTMP into AZTDP mediated by thymidylate kinase is the metabolic bottleneck in the case of AZT. In principle, the direct administration of the monophosphorylated form of the nucleosides should bypass the limiting step in the thymidine kinase-mediated anabolism. However, nucleotides are very polar molecules and do not passively pass cellular membranes. Therefore, strategies have been developed in order to mask the charged phosphate using (bio)degradable lipophilic carrier groups which release the 5'-nucleotides after cellular uptake (pronucleotide-approach).

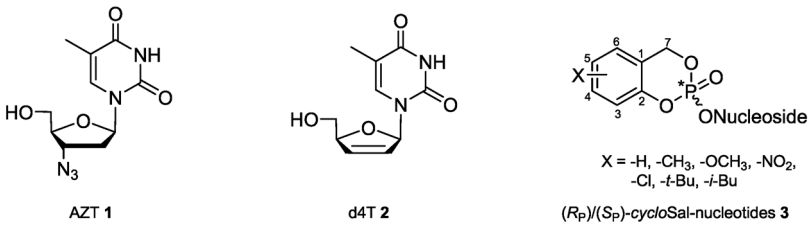


FIG. 1
Nucleosides **1**, **2** and the general formula of $(R_P)/(S_P)$-*cyclo*Sal-triesters **3**

Consequently, pronucleotides are membrane-permeable nucleotide precursors. Several pronucleotide strategies have been reported for nucleotide delivery, e.g. the mixed-SATE compounds[1], the HepDirect technique[2], the phosphoramidates[3], the *cyclo*Sal-phosphate triesters **3** (Fig. 1)[4], and recently a first approach for the intracellular delivery of nucleoside diphosphates[5]. Due to their synthesis, all P-chiral pronucleotides are obtained as 1:1 mixtures of diastereomers with respect to the configuration at the phosphorus center[3,4]. The mixtures of diastereomers can be separated in only rare cases. However, it has been shown that the individual diastereomers of the pronucleotides showed significantly different antiviral activity, toxicity and hydrolysis stabilities, e.g. in the case of the *cyclo*Sal-compounds **3** [4].

Here, we report on a stereoselective synthesis routes leading to (R_P)- or (S_P)-*cyclo*Sal-pronucleotides **3** with very high diastereoselectivities based on the chiral auxiliary strategy.

RESULTS AND DISCUSSION

Early attempts using thiophosphoramidates as reactive intermediates in combination with prolinesters, prolinolethers and Evans-type oxazolidin-

ones led not to the formation of the *cyclo*Sal-phosphateesters. However, these studies gave first important insights into the reaction mechanism and showed the proof-of-principle that the use of such auxiliaries may be successful for the new reaction pathway. Particularly, the prolinolethers gave strong chirality transfer in the reaction of a dichlorothiophosphoramidate with salicylalcohol (up to 90% *d.e.*)[6]. The proposed mechanism of the reaction is shown in Fig. 2. Finally a modification of the chiral auxiliary in order to increase its leaving group properties was performed which was finally successful. In a report, thiazolidin-2- thiones as **4** (Scheme 4) were used as leaving groups in carboxylic acids derivatives in which they can be removed under mild reaction conditions[7].

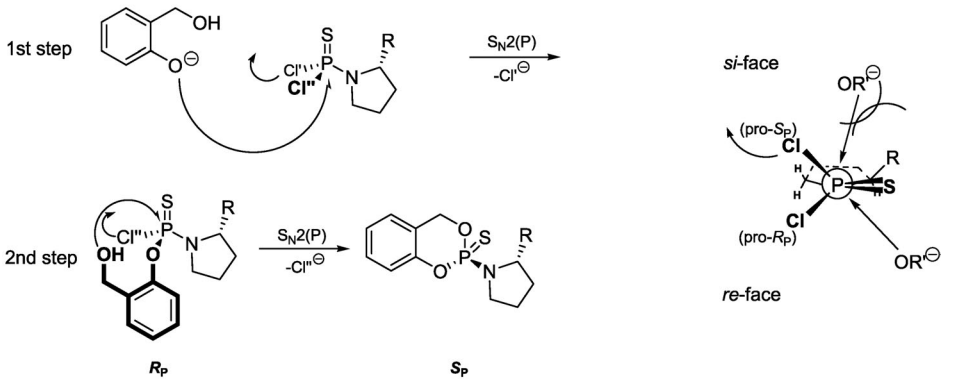
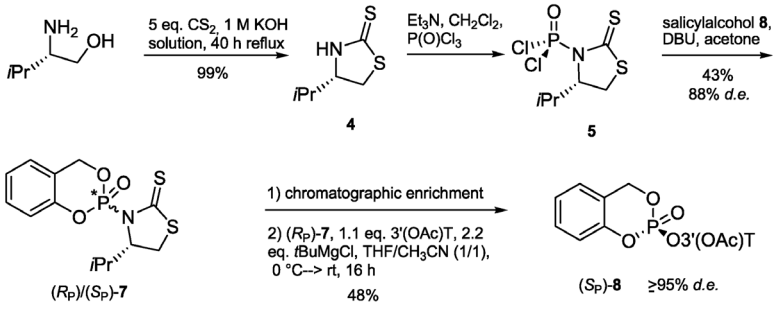


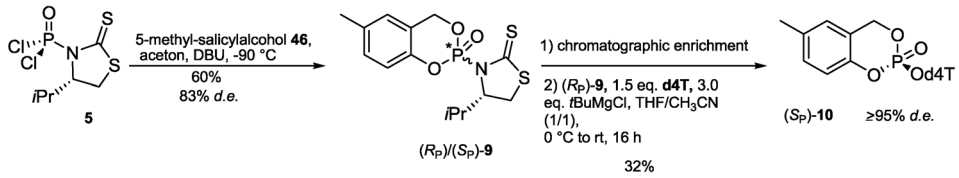
FIG. 2
Proposed mechanism of the cyclisation and the possible transition state



SCHEME 1
Diastereoselective synthesis of the imides (R_P)/(S_P)-**7** and the conversion of the isomerically pure (R_P)-**7** to the triester (S_P)-**8**

Based on this, (*S*)-4-*iso*propyl-2-mercapto-2-thiazoline **4** was used as chiral auxiliary. The synthesis of **4** was conducted as reported by Zhang and Delaunay (99%, Scheme 1)[8,9].

The dichloro intermediate **5** could not be purified by means of chromatography due to its instability. The ^{31}P-NMR spectrum of the crude mixture showed almost quantitative formation of compound **5**. Next the reaction of **5** with saligenol **6** led to the mixed imides (R_P)/(S_P)-**7**. This reaction was always carried out as a one-pot-reaction. Many attempts have been made in order to obtain not only a high yield but also a high diastereoselectivity. The combination of DBU and acetone at –90 °C gave the best result, with other bases either the yield or the diastereomeric excess was lower. Interestingly, the diastereomers (S_P)-**7** and (R_P)-**7** could be separated easily by column chromatography. The (S_P)-configuration of the "minor"-diastereomers was confirmed by several X-ray crystal structure analyses. The isomerically pure diastereomer (R_P)-**7** was then first reacted with 3'-(*O*-acetyl)thymidine (3'(OAc)T) as model for nucleoside analogues (Scheme 1). The best results were obtained by using *tert*-butylmagnesium chloride as base and a 1:1 mixture of THF/CH_3CN as solvent. The reaction proceeded stereospecifically and the product **8** was obtained with a diastereomeric excess of ≥95% *d.e.* in 48% yield. Assuming that the reaction took place with inversion of configuration, compound **8** should have (S_P)-configuration[10].



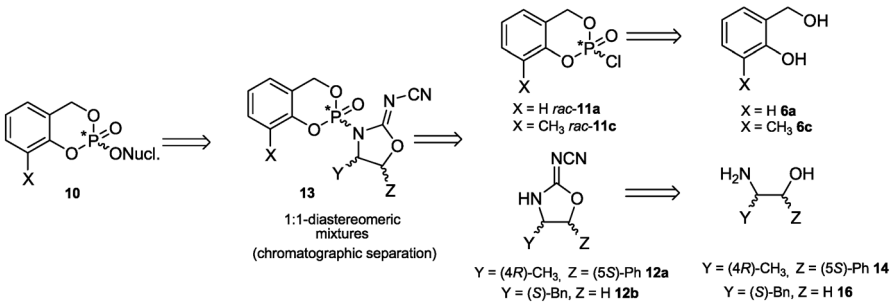
SCHEME 2
Stereoselective synthesis of (S_P)-*cyclo*Sal-d4TMP **10**

In order to prove the general applicability of the novel synthetic route 5-methylsalicylalcohol **6b** and d4T **1** as an antivirally active nucleoside analogue were used (Scheme 2). The optimized reaction conditions for the cyclisation were then applied. As above, this reaction led via the phosphoroamidate **9** to the corresponding 5-methyl-*cyclo*Sal-triester **10** in 32% yield and in very high diastereomeric excess (≥95% *d.e.*).

Surprisingly, the synthesis of the corresponding 3-methyl-*cyclo*Sal-derivative failed using this reaction sequence because unexpectedly the second step did not lead to the chiral *cyclo*Sal-phosphoramidate **9**. Presumably the substituent in the 3-position caused unfavorable steric interactions in

this reaction and after some unsuccessful variations of the reaction conditions it became clear that (*S*)-4-*iso*propyl-2-mercapto-2-thiazoline **4** is not suitable for the synthesis of the 3-substituted *cyclo*Sal-counterparts. However, because particularly 3-substituted *cyclo*Sal-pronucleotides of nucleoside analogues showed very interesting antiviral activities by intracellular nucleotide delivery, enzymeinhibition and hydrolysis properties a different synthetic strategy was developed that allows the access to these important compounds[11].

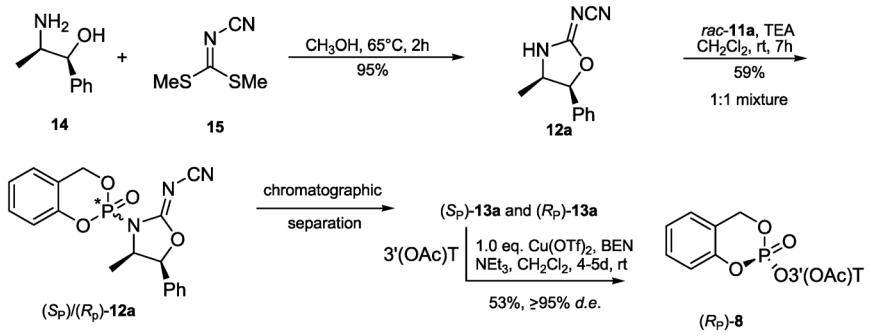
The following concept was envisaged: 3-Methylsaligenol **6c** was first reacted with $P(O)Cl_3$ to give *cyclo*Sal-phosphorochlorides *rac*-**11c**. The reaction of the chiral *N*-cyaniminooxazolidines **12a**,**12b** with the *cyclo*Sal-derivative *rac*-**11** should lead to a diastereomeric mixture of the *cyclo*Sal-amidates **13**. This mixture should to be easily separable into the individual diastereomers. Finally, the nucleoside will be introduced to yield the products, e.g. **10c**. The retrosynthesis for the preparation of isomerically pure 3-alkylated *cyclo*Sal-pronucleotides is depicted in Scheme 3.



SCHEME 3
Retrosynthesis of the route to the *cyclo*Sal-phosphotriesters **10**

3-Methyl-salicylic acid was reduced with $LiAlH_4$ leading to the corresponding salicylalcohol **6c** in 80%. Compounds **11a**, **11c** were obtained as racemic mixtures after esterification of the salicylalcohols **6a**, **6c** with $P(O)Cl_3$ in 61 and 76% yield. The chiral group **12a** was prepared by the reaction of D-norephedrine **14** with dimethylcyanodithioiminocarbonate **15**. Interestingly, (4*R*,5*S*)-configurated compound **12a** precipitated during the reaction and the pure product was isolated by simple filtration in 95% yield. The following coupling of **12a** with the *cyclo*Sal-phosphorchloridates *rac*-**11a** led to the formation of the corresponding 1:1 diastereomeric mixture (S_P)/(R_P)-**13a** in 59% yield. The crude mixture displayed two baseline

separated signals in the ^{31}P-NMR spectra. The diastereomers could be easily separated by column chromatography to give the isomerically pure diastereoisomers (S_P)- and (R_P)-**13a** (Scheme 4). Taking the individual

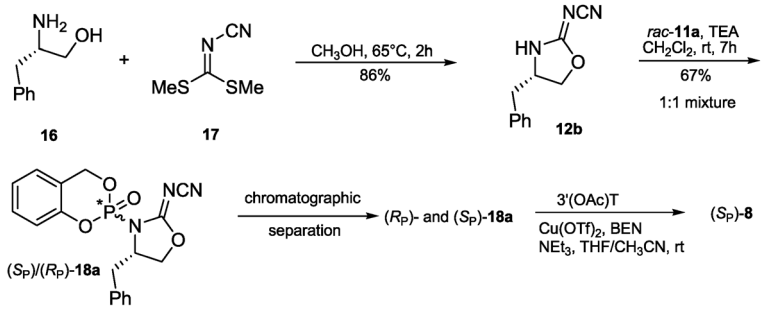


SCHEME 4
Synthesis of the chiral group **12a** and preparation of the single diastereomers (S_P)- and (R_P)-**13a**

diastereomers, in the last step the chiral leaving group was substituted by 3'(OAc)T[12]. Several attempts have been tried. Among those the addition of a strong base like alkylmagnesium halides, the reaction conditions used by Jones et al. or a combination of both were successful (Scheme 5)[12]. In our case, the combination of $Cu(OTf)_2$, BEN as well as the addition of base for the deprotonation of the nucleoside led to the formation of (R_P)-*cyclo*Sal-(3'OAc)dTMP **8** in 53% yield and ≥95% *d.e.* Since (R_P)-*cyclo*Sal-triester **8** could not be crystallized, no X-ray data were available proving the absolute configuration at the phosphorus atom. However, assuming that the reaction took place with inversion of configuration, compound **8** must have (R_P)-configuration because the great majority of the substitution reactions at the phosphate proceed following an addition-elimination mechanism with inversion of configuration (S_N2-type reaction). Several bases, Lewis acid and solvent combinations were studied. To our surprise in most of the reactions we observed that the stereochemical purity of the product decreased significantly. According to these results, the $[Cu(BEN)](OTf)_2$ complex in combination with NEt_3 was found to be optimal for promoting the diastereoselective synthesis to yield (R_P)-*cyclo*Sal-phosphotriester **8** [13].

To synthesize (S_P)-*cyclo*Sal-(3'OAc)TMP **8**, (R_P)-**13a** was treated under identical conditions as described above for (S_P)-**13a**. Surprisingly, the reaction gave very low yields of 6% and a diastereoselectivity of 93% *d.e.* and in addition decomposition of the starting material was observed. Therefore,

another chiral leaving group with inverted stereogenic centers was needed that forms after coupling with the *cyclo*Sal-phosphorochloridates *rac*-**11** chromatographically separable diastereoisomers. In addition to this, the more stable diastereoisomer should lead to the desired (S_P)-*cyclo*Sal-triester **8**. Finally, the chiral compound **12b** that was synthesized from L-phenylalaninol **16** and dimethylcyanodithioiminocarbonate **17** in 86% yield proved to be suitable to achieve the goal (Scheme 5). As observed with compound **12a**, the chiral product **12b** precipitated during the reaction and was isolated by simple filtration. Compound **12b** was then coupled with *cyclo*Sal-phosphorochloridate *rac*-**11a** to the corresponding diastereomer mixture (S_P)/(R_P)-**18a** (Scheme 5). The formed diastereomers (S_P)- and (R_P)-**18a** were separated using silica gel column chromatography. After reaction of the more stable (R_P)-isomer with 3'OAcT, the (S_P)-*cyclo*Sal-triester **8** was obtained (Scheme 5). Triethylamine was used as base in all reactions. Best diastereomeric excess was obtained using 1.0 equivalent of the promoter and a 1:1 mixture of THF/CH_3CN as solvent. Having identified chiral groups as well as a suitable promoter system, the synthesis of *cyclo*Sal-nucleotides of the antivirally active nucleoside analogues AZT **1** and d4T **2** was started.

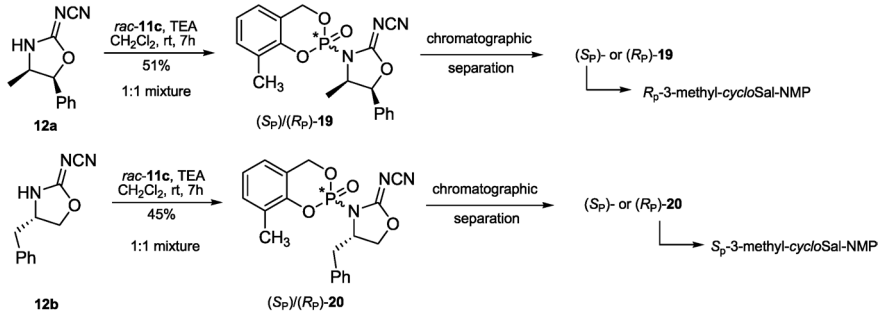


SCHEME 5
Syntheses of the chiral group **12b**, (S_P)- and (R_P)-**18a** and (S_P)-*cyclo*Sal-phosphotriester **8**

For this purpose both chiral leaving groups **12** were coupled with the *cyclo*Sal-phosphorochloridate *rac*-**11c** to give the corresponding diastereomeric mixtures (S_P)/(R_P)-**19** and (S_P)/(R_P)-**20** (Scheme 6). Both mixtures were easily separated into the individual diastereomers using silica gel column chromatography and petroleum ether/ethyl acetate (1:2) as eluent[13].

Each stable isomer (S_P)-**19** or (R_P)-**20** was reacted with AZT **1** to give the corresponding (R_P)- and (S_P)-3-methyl-*cyclo*Sal-AZTMP in 30 and 21% yield,

respectively. The observed diastereomeric excess for both compounds (S_P)-**6** and (R_P)-**6** was ≥95% *d.e.* Next, (R_P)- or (S_P)-3-methyl-*cyclo*Sal-d4TMPs were prepared using the same chiral entities and the same reaction conditions. (S_P)-**19** and (R_P)-**20** were reacted with d4T **2** to give the corresponding (R_P)- or (S_P)-3-methyl-*cyclo*Sal-d4TMP in 33% (94% *d.e.*) and 28% (≥95 *d.e.*) yield, respectively. Analogous reactions using 3,5-dimethyl-salicylalcohol led by the same route to the corresponding diastereomers of (S_P)/(R_P)-3,5-dimethyl-*cyclo*Sal-d4TMP.



SCHEME 6
Preparation of the single diastereomers (S_P)- or (R_P)-**19** and (S_P)- or (R_P)-**20**

3-Methyl-*cyclo*Sal-nucleotides were evaluated for their antiviral potencies against HIV-1 and HIV-2 infected CEM/0 and HIV-2 infected CEM/TK$^-$ cells. A significant difference has been observed for the two *cyclo*Sal-d4TMP derivatives. In both HIV-infected CEM/0 and CEM/TK$^-$ assays, the (R_P)-diastereomer was found to be 5- to 12-fold more active as compared to the (S_P)-diastereomer. Moreover, both diastereomers of the *cyclo*Sal-d4TMP triesters fully kept antiviral activity in the HIV-2-infected CEM/TK$^-$ cell cultures compared to CEM/0 cells. In conclusion, these data perfectly agrees to our earlier observation that the (R_P)-diastereomers always showed higher antiviral activity as compared to the (S_P)-isomer. The work presented here led for the first time to the synthesis of stereochemically pure *cyclo*Sal-pronucleotides. By using two different approaches using chiral auxiliaries all possible substitution pattern within the *cyclo*Sal-aromatic system can be used for the synthesis. The antiviral evaluation proved again the significance of a stereoselective access of the *cyclo*Sal-derivatives.

Recently we have shown that the same chiral auxiliary-approach as described here is also suitable for the synthesis of phosphoramidate pronucleotides[14].

C. M. thanks for support by the Deutsche Forschungsgemeinschaft (DFG; ME 1161/9-1) and the University of Hamburg and J. B. received a grant from the K.U. Leuven (GOA 10/14).

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ARTIFICIAL RESTRICTION DNA CUTTERS TO MANIPULATE HUGE GENOMES

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Manipulation of genomes is one of the most important and attractive targets in chemistry, biochemistry, and molecular biology. However, the current technique using naturally occurring restriction enzymes cannot be applied to this task, due to too poor site-specificity of these enzymes. In order to pave the way to genome manipulation and open a possibility to variety of applications, we recently prepared completely chemistry-based artificial restriction DNA cutter (ARCUT) which can selectively cut huge genomes at desired site. This cutter is composed of Ce(IV)/EDTA complex (molecular scissors) and two strands of pseudo-complementary peptide nucleic acid (pcPNA; sequence-recognizing molecule). Its scission site is determined simply by Watson–Crick rule so that ARCUT for desired site-selective scission of genomes is easily and straightforwardly designed and synthesized. In fact, we succeeded in site-selective scission of the whole human genome at one target site (e.g., one site in a gene in X-chromosome). This cutter recognized subtle difference in DNA sequence so precisely that analogous sites having similar sequences were never cut by this cutter. Furthermore, the DNA scission induced by this cutter was satisfactorily recognized by the repair system in human cells and promoted homologous recombination at the scission site.

INTRODUCTION

In current DNA manipulations, plasmid DNA is cut at one predetermined site by naturally occurring restriction enzyme(s), and the scission fragments are connected with other fragments using ligase. However, this technique cannot be directly employed to manipulate genomes, mainly because the site-specificity of scission by these restriction enzymes is too low to cut genomes site-selectively. Recently, we have developed a completely chemistry-based DNA cutter (artificial restriction DNA cutter; ARCUT) which can selectively cut genomes at predetermined site[1–3]. In this paper, we present the molecular design of ARCUT and its application to genome manipulation.

MOLECULAR DESIGN OF ARTIFICIAL RESTRICTION DNA CUTTER

The artificial restriction DNA cutter (ARCUT) cuts double-stranded DNA by hydrolyzing target phosphodiester linkages. As shown in Fig. 1, they are composed of Ce(IV)/EDTA complex and two strands of pseudo-complementary peptide nucleic acid (pcPNA)[3]. In pcPNA, modified nucleobases 2,6-di-

aminopurine (D) and 2-thiouracil (U) are used, in place of conventional A and T bases, in order to promote the formation of double-duplex invasion complex with double-stranded DNA. In the first step of the scission, two pcPNA strands invade double-stranded DNA substrate at the target site, where each of them forms a duplex with the corresponding DNA strand according to Watson–Crick rule. The binding sites of these two pcPNA strands are laterally shifted one another by several nucleobases, so that only the predetermined sites in the double-stranded DNA become single-stranded (the black lines in Fig. 1). In the second step, these single-stranded portions are preferentially hydrolyzed by Ce(IV)/EDTA, since this complex hydrolyzes only single-stranded DNA and hardly hydrolyzes double-stranded DNA. Importantly, the efficiency of site-selective scission by ARCUT can be further promoted by attaching phosphoserine residues to the pcPNAs and placing the monophosphate groups at the target site. These groups interact with Ce(IV)/EDTA and recruit this complex to the scission site to increase its effective concentration for the site-selective scission.

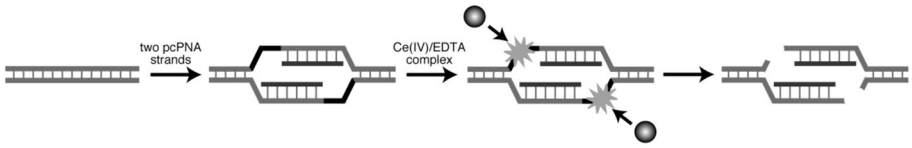

FIG. 1
Site-selective scission of double-stranded DNA by ARCUT at predetermined position. By adding two pcPNA strands to double-stranded DNA, single-stranded portions are formed within the invasion complex. These single-stranded portions are selectively hydrolyzed by Ce(IV)/EDTA

One of the most significant advantages of ARCUT is the fact that its scission site is a priori determined by Watson–Crick rule. Thus, both the scission site and scission specificity can be tuned simply by changing the sequences of two pcPNA strands. Accordingly, the cutters required for aimed experiments (even for selective scission of one site in huge DNA such as human genome) are straightforwardly designed and synthesized. The DNA scission proceeds via the hydrolysis of the targeted phosphodiester linkages (as naturally occurring nucleases do) so that the fragments obtained by the ARCUT scission can be easily connected with other DNA fragments using ligase to prepare recombinant DNA[4]. Other enzymatic transformations are also plausible.

MISMATCH-RECOGNITION BY ARCUT

In huge genomes, there are many analogous sequences which are different from the target site by only one or two base-pairs. In order to cut only one target site in genomes, ARCUT must strictly differentiate these subtle differences. Otherwise, off-target scissions (the scissions at the sites other than the target site) should concurrently occur and induce critical effects on therapy and other applications. We addressed this important issue by carrying out systematic studies in which one base-pair at various positions in the DNA substrate was changed to another base-pair to introduce the corresponding mismatches to the two pcPNA strands. The effects of these mismatches on the scission activity were quantitatively analyzed[5].

The conclusions obtained by these studies are as follows. (1) The mismatch-recognition of ARCUT is almost perfect in the middle portion where both of the pcPNA strands form the duplexes with the DNA strands (see the structure of DNA-ARCUT invasion complex in Fig. 1). In other words, if only one DNA base-pair in this region is altered to another base-pair, the scission by the ARCUT completely disappears. (2) The mismatch-recognition is less strict in the franking regions where only one pcPNA binds with either of the DNA strands. In typical ARCUT presented in Fig. 1, 16 base-pairs are completely recognized (the scission does not occur if only one base-pair in the DNA sequence is different from the base-pair in the target site). This means that the site-specificity of this ARCUT statistically appears only at every 4^{16} ($>4 \times 10^9$) DNA base-pairs. This number is larger than the number of total base-pairs in human genome (3×10^9) so that the site-specificity of this ARCUT is high enough to cut the human genome at one target site. If the site-specificity of scission is insufficient due to some reason, the lengths of the pcPNA strands should be increased by one nucleobase.

SITE-SELECTIVE SCISSION OF HUMAN GENOME AT ONE TARGET SITE BY ARCUT

As discussed above, the site-specificity of ARCUT can be freely modulated and increased as high as we desire. As an example, the whole human genome (3×10^9 bp) was successfully cut by ARCUT at one target site in Fig. 2 [6]. The target site chosen here is located in FMR1 (Fragile X Mental Retardation 1) gene in X chromosome of human beings (the choice of this site is arbitrary). This gene is highly conserved and involves CGG trinucleotide repeats in its 5′ untranslated region. If the number of this repeat exceeds about 200 and abnormal hypermethylation occurs, the transcription of FMRP protein is silenced to cause fragile X syndrome. In the

two pcPNA strands used for the site-selective scission at this FMR1 site, pcPNA1 is complementary to A146801423-C146801473 in the upper DNA strand of the target site, whereas pcPNA2 is complementary to G146801428-A146801442 in the lower strand (Fig. 2a). In the double-duplex invasion complex, A146801438-T146801442 and T146801423-C146801427 in these two DNA strands are kept single-stranded and thus are susceptible to the catalysis of Ce(IV)/EDTA.

In the scission experiments, human cells were first cultured, and the whole genome was isolated from them (Fig. 2b). Then, the whole genome was treated with the ARCUT at pH 7.0 and 50 °C. The fact that the ARCUT

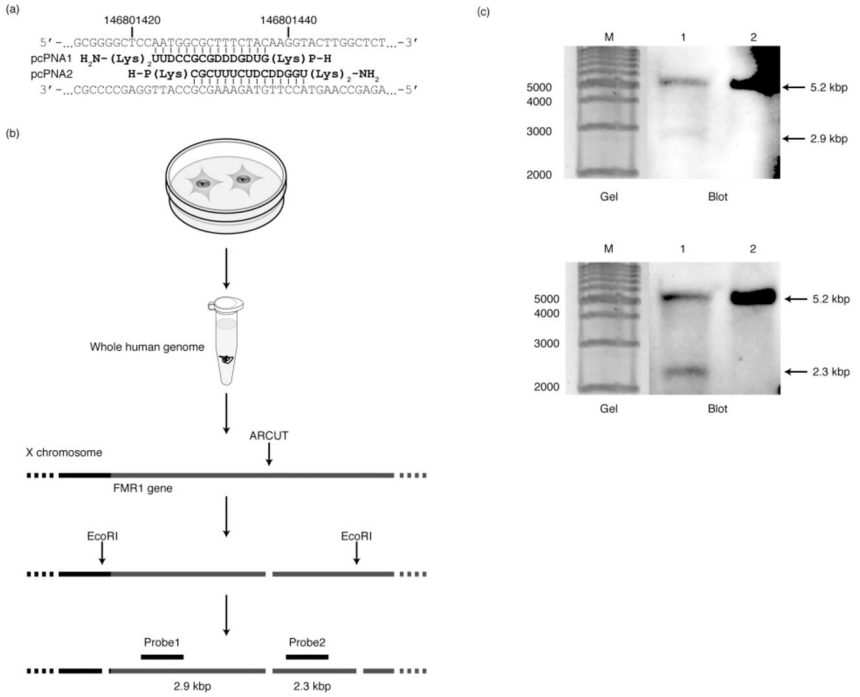

FIG. 2

(a) The sequences of target site (one site in FMR1 gene) in human genome and the pcPNA strands employed in ARCUT for the site-selective scission. The term P at the end of the pcPNAs is a phosphoserine residue used to promote the scission (see text). (b) Outline of the procedure for the site-selective scission by the ARCUT. (c) Southern blotting using two different probes to confirm the site-selective scission by the ARCUT. In lane 1, the scission products by the ARCUT were further digested by a restriction enzyme *Eco*RI, and then analyzed by the Southern blotting. In lanes 2, human genome was directly digested by *Eco*RI without ARCUT scission (lanes M, 1 kbp DNA ladder)

really achieved the site-selective scission at the target site was confirmed as follows. First, the reaction mixture of the ARCUT scission was further digested by a restriction enzyme *Eco*RI, and then the products of this ARCUT/*Eco*RI double-scission were analyzed by Southern blotting using the corresponding probes (Fig. 2b). As expected from the targeted site-selective scission by the ARCUT, only two fragments of 2.9 kbp (in the upstream side from the ARCUT scission site) and 2.3 kbp (the downstream side from the site) were formed (see Fig. 2c). When the ARCUT product was digested with other restriction enzymes and the product was analyzed by Southern blotting in the same way as described above, the corresponding double-scission products were formed. The site-selective scission of human genome by the ARCUT was further substantiated.

It is noteworthy that the site-specificity of the present scission was quite strict and other analogous sequences in the human genome were kept intact in the reactions. In the chromosome 7 of human beings, for example, there exists an analogous sequence in which 15 continuous nucleotides are identical to those of the target site in FMR1 and only adjacent base-pairs are different. However, no scission occurred at this site by this ARCUT, as also confirmed by Southern blotting using the corresponding probe. Even this small difference between the target site and off-target site was precisely differentiated. Successful applicability of ARCUT to manipulation of human genome has been absolutely shown.

PROMOTION OF HOMOLOGOUS RECOMBINATION IN HUMAN CELLS BY ARCUT

Recently, homologous recombination (genetic recombination between two double-stranded DNAs of analogous sequences) has been attracting much interest as a means for gene manipulation. In this biological process, a sequence in one double-stranded DNA is altered to the corresponding sequence of another double-stranded DNA through DNA polymerase reaction using the latter as the template. Accordingly, by using this process, we can convert desired site in genome to another sequence in cells. Insertion of a gene to target site is also plausible. Importantly, homologous recombination is greatly accelerated when both strands in a chromosome are cleaved at close distance. Furthermore, even short DNA fragment (e.g., 200 bp) can be used as the template for the polymerase reaction. Thus, it was expected that targeted homologous recombination can be triggered by combining (i) artificial restriction DNA cutter (ARCUT) which cuts the target site in the genome and (ii) short DNA fragments used as the template.

It was found that the DNA scission by ARCUT in fact greatly promotes targeted homologous recombination in human cells and ARCUT is very useful to enhance this biological recombination[7]. Apparently, the scission by the ARCUT is satisfactorily recognized by the repair systems in human cells. A typical example of ARCUT-promoted homologous recombination is presented in Fig. 3. As shown in (a), the gene of blue fluorescent protein

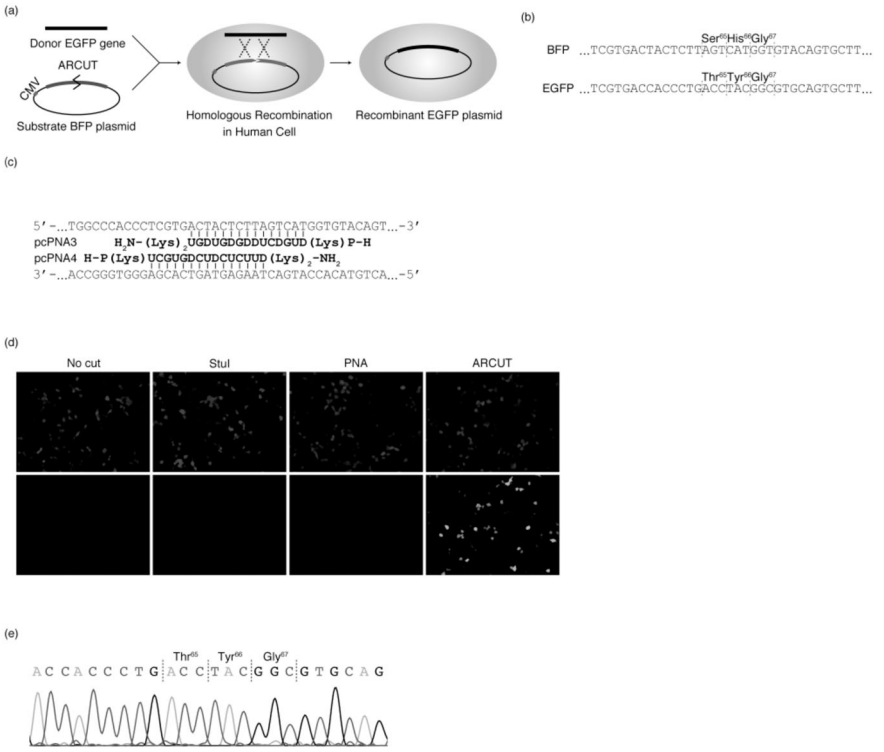

FIG. 3

(a) Outline of the ARCUT-promoted homologous recombination in human cells. The scission induced by the ARCUT in the chromophore site of BFP is repaired in human cells by the DNA polymerase reaction using the EGFP donor as the template, and thus this part is converted to the sequence of EGFP. (b) The DNA sequences around the chromophores of BFP and EGFP (the corresponding amino acid sequences are also shown). (c) The sequences of the target scission site and the pcPNA strands used for the ARCUT. (d) Fluorescence microscopy images of the 293T cells cultured for 48 h after the transfection with both the ARCUT-treated BFP and the donor EGFP fragment. The upper and the lower panels show blue channels (Ex: 360 nm, Em: 470 nm) and green channels (Ex: 480 nm, Em: 520 nm), respectively. (e) Sequencing analysis of the recombinant DNA formed by the homologous recombination

(BFP), placed in a plasmid, was cut by ARCUT at the chromophore site. Independently, a donor DNA (742 bp) containing the gene of enhanced green fluorescent protein (EGFP) was prepared. The BFP protein and the EGFP protein are similar and different only in the three amino acids in their chromophore regions (Ser65, His66, and Gly67 for BFP vs. Thr65, Tyr66, and Gly67 for EGFP; Fig. 3b). If homologous recombination between them successfully occurs in human cells, the chromophore site of the BFP gene should be converted to that of EGFP, and thus the resultant protein, produced in the human cells, should emit green fluorescence. By ARCUT using pcPNA3 and pcPNA4, the chromophore site of BFP gene in the plasmid was cut (Fig. 3c). Then, the ARCUT-treated BFP gene was introduced into human cells together with the donor EGFP fragment. In 48 h after the transfection, the human cells efficiently emitted green fluorescence as expected from the targeted homologous recombination (the far right-hand lower panel in Fig. 3d). The sequencing results were completely consistent with the arguments (Fig. 3e). Essential roles of the ARCUT scission were verified by various control experiments (the other three lower images in Fig. 3d). The ARCUT-stimulated homologous recombination in human cells was also successful when BFP gene was incorporated into adenovirus vector (35 kbp).

By using the ARCUT-promoted homologous recombination, any targeted gene can be altered in human cells to predetermined form (sequence change, insertion, deletion, and others). Thus, this method should be highly useful for therapy of genetic diseases, knock-out of unfavorable gene, knock-in of useful gene, and many other purposes[8]. As described above, newly developed artificial DNA cutter ARCUT is straightforwardly designed by Watson–Crick rule so that the site-selective DNA cutter having required properties (desired scission site and site-specificity) is easily obtained. These features are certainly big advantages for versatile applications. In order to further widen the scope of applications of ARCUT, we have already attempted to directly cut a target site of human genome in human cells and achieve homologous recombination at this site. Our preliminary experiments are very promising and the corresponding homologous recombination in human cells (conversion of a gene in human genome to another gene) is notably promoted. Artificial tools to cut telomeres, located at the end of chromosomes, have been also developed[9].

At present, further chemical modifications of ARCUT for still more versatile applications, as well as the optimization of the conditions for the homologous recombination in human cells, are the most important projects

for our group. These points will be also discussed more in detail in the conference.

This work was partially supported by Grants-in-Aid for Specially Promoted Research from the Ministry of Education, Science, Sports, Culture and Technology, Japan (18001001 and 22000007). Support by the Global COE Program for Chemistry Innovation is also acknowledged.

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SHAPING NUCLEIC ACIDS FOR APPLICATIONS IN THERAPY: THE TRICYCLO-DNA STORY – AN UPDATE

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In this mini-review an update on the chemical, biophysical and biological properties of the bicyclo- and tricyclo-DNA molecular platform is given.

INTRODUCTION

Since the late seventies of the last century there has been a growing interest in nucleic acid based therapeutics. Targeting RNA by oligonucleotides is particularly promising for various reasons, first and foremost because of the unique design principle of inhibitors for specific RNA sequences based on the Watson–Crick pairing rules. While in the beginning the focus was set to the antisense principle as the biological mode of action[1,2], this has been expanded to other biological mechanisms in the recent past such as RNA interference[3,4], and miRNA regulation[5]. As most basic developments in medicinal chemistry these experience various highs and lows as the understanding and knowledge about their biological properties is increasing with time. Even three decades later a rigorous proof of concept is still lacking despite the fact that there is one oligonucleotide drug, Vitravene®, on the market. Although it became clear over the years that the claim of the universality of the approach needs to be put into perspective, certain applications such as antisense oligonucleotides for splice interference in genetically based (neuro)muscular diseases are very promising. This can be inferred from the very recent entry into clinical phase III trials of 2′-OMe-PS-RNA-oligonucleotides (www.prosensa.eu) and the late clinical phase II trials of a phosphorodiamidate morpholino oligomer (PMO) (www.mdex.org.uk) for the treatment of Duchenne muscular dystrophy[6].

While oligonucleotide chemistry in the past was mostly concerned with increasing target affinity and biostability by semi-rational and rational design, the focus has shifted recently to questions of improving cellular uptake, toxicity and biodistribution as well as pharmacokinetics and formulation. These are the largely unsolved problems in the field and is to some extent also the consequence of observations that there is not always a clear correlation of target affinity with biological activity.

Over the years a selected number of oligonucleotide molecular platforms have been investigated in detail, amongst them the families of the phosphorodiamidate morpholino oligomers (PMO)[7], the 2′-*O*-alkyl-RNA, the locked nucleic acids (LNA)[8] and the hexitol nucleic acids (HNA)[9].

Over the last decade we developed in our laboratory the bicyclo-DNA family with tc-DNA as its most promising member so far in terms of therapeutic applications. These oligonucleotides were designed to improve RNA affinity by entropic means via constraining the flexibility of their sugar-phosphate backbone.

STRUCTURE/RNA AFFINITY PROFILE OF THE BYCYCLO-DNA FAMILY

Over the recent years we have synthesized and characterized a series of oligonucleotides containing the bc-DNA modifications shown in Fig. 1, starting with the lead, bc-DNA[10], which showed equal RNA affinity as DNA. Initially we interpreted this lack of enhanced affinity by misaligning torsion angle γ (C(4′)–C(5′) bond) relative to natural DNA, due to the fact that O(5′) prefers a pseudo-equatorial instead of a pseudo-axial arrangement on the carbocyclic ring. To further understand the influence of this torsion angle we investigated the bc-analogue with inverted configuration at the

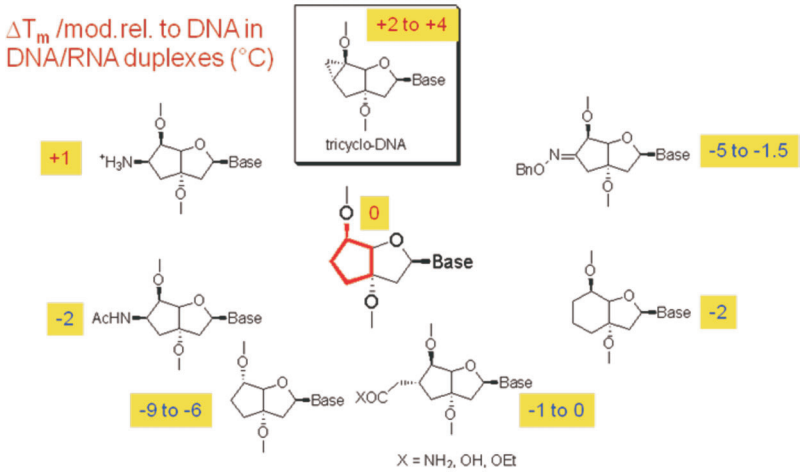

FIG. 1
Memebers of the bc-DNA family syntheiszed and characterized so far. Center: bc-DNA. From top center clockwise: tc-DNA, bc^{ox}-DNA, $bc^{4,3}$-DNA, bc^{alk}-DNA, 5′epi-bc-DNA, amido-bc-DNA, amino-bc-DNA

5′-carbon (5′-epi-bc-DNA). It came as no surprise that locking this torsion angle in an unnatural geometry was associated with a substantial drop in RNA affinity as determined by T_m measurements and thus confirmed the importance of this particular backbone torsion angle on complement affinity.

Given the extra carbocyclic ring in bc-DNA it was obvious to produce derivatives with variations in this particular structural subunit. In this context we investigated the ring enlarged variant bc^{4,3}-DNA, reasoning that a six-membered ring constrains the backbone flexibility even more. But also this change did not improve RNA affinity, suggesting that an increased number of carbon atoms leads to steric crowding of the backbone structure and that no correction of torsion angle γ takes place[11]. Another measure to improve affinity is to reduce the negative charge of the backbone. This can be achieved when introducing amino-bc-DNA units into the oligonucleotide. Each residue neutralizes one phosphate unit in the backbone. Indeed this lead to higher affinity by 1 °C/mod. That this increase in affinity is due to charge compensation is supported by the fact that the corresponding amido-bc-DNA does not show enhanced affinity. Much along the lines of improving cell uptake properties we started investigating bc-analogues which show a more hydrophilic backbone. Analogues such as bc^{ox}-DNA, and bc^{alk}-DNA are such family members. While bc^{ox}-DNA, equipped with a hydrophobic benzyl group, indeed showed uptake into HeLa cells in the absence of transfection agents, which was not the case for unmodified control oligonucleotides, synthetic difficulties and decreased affinity to RNA were unavoidable drawbacks[12]. A related approach was tested with bc^{alk}-DNA. While the monomeric building block is equipped with an ester function this could be converted into amide functions or into the free carboxylate or left unchanged, depending on the deprotection conditions, in a parallel manner[13,14]. Given that these modifications were mostly T_m-neutral there exists considerable potential to further improve cell uptake properties in the future by varying the side chains.

TC-DNA: SYNTHETIC CONSIDERATIONS

With its improved RNA affinity by 2–4 °C/mod, tc-DNA is by far the most interesting member of the bc-DNA family. In our original work we started the synthesis of the tc-sugar from an achiral building block[15,16]. Soon we realized that this synthesis is not efficient enough to be able to produce quantities necessary for extensive biophysical and biological testing. We therefore redesigned the synthesis to start with a compound from the chiral

pool[17]. Although this protocol is very robust an can be executed on a multi-mole scale, it is still lengthy with its 19 steps. While we have already optimized tc-nucleoside and oligonucleotide synthesis[18], we are just completing now a novel tc-sugar synthesis, in which we cut the steps down from 19 to 10, starting from a commercially available glucose derivative (unpublished work). Although scale-up has yet to occur, we are already now in the position to produce tc-oligonucleotides on multi-milligram quantities for in vivo testing. Perhaps a peculiarity of tc-DNA synthesis is the chemical unstability of 5′-unprotected oligonucleotides. During deprotection the 5′-terminal nucleoside suffers from a typical Simmons–Smith rearrangement of the cyclopropanol subunit followed by β-elimination leaving a 5′-phosphorylated oligonucleotide behind that is one unit shorter but protected from further degradation (Fig. 2)[19]. Therefore we typically synthesize n+1 oligonucleotides in the trityl-off mode and isolate quantitatively 5′-phosphorylated n-oligonucleotides.

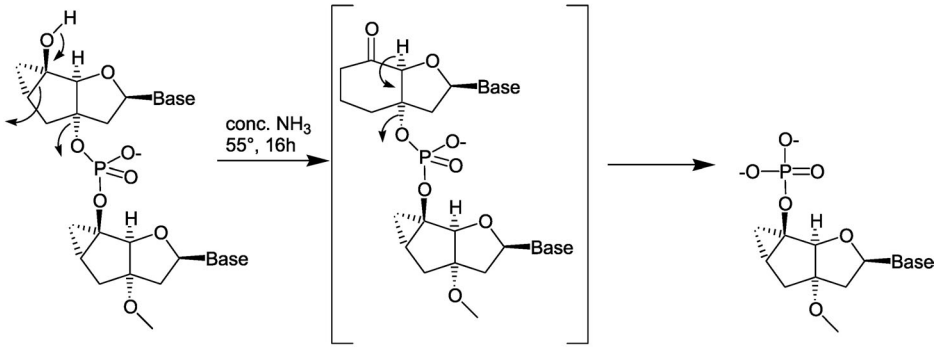

FIG. 2
Mechanism of 5′-terminal, unprotected, nucleoside elimination via ring expansion and β-elimination

TC-DNA: STRUCTURAL CONSIDERATIONS

One of the important questions to be addressed was of course that of the origin of the improved RNA affinity compared to bc-DNA. Early CD-spectroscopic investigations together with molecular modeling revealed that tc-DNA prefers an overall RNA-like A-duplex conformation irrespective whether the complement is DNA or RNA[20]. A more detailed analysis of the relevant backbone torsion angles showed that the role of the cyclopropane ring is not to bring back torsion angle γ in its natural conformation, but to force the adjacent torsion angle β- from *anti* to *syn* conformation. These

two changes are compensatory in nature and bring the backbone back into register with that of RNA. Recently we obtained structural proof for this by X-ray crystallography of a tc-modified oligodeoxynucleotide (Fig. 3)[21].

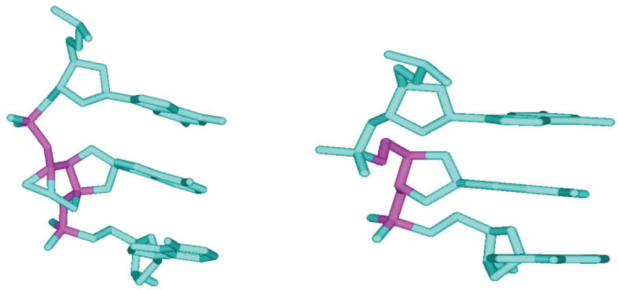

FIG. 3
Exerpts of the X-ray structure of a tc-DNA duplex (left) compared to that of an unmodified duplex (right) highlighting the compensatory nature of the changes in the backbone bonds (magenta)[21]

TC-DNA: BIOSTABILITY

Tc-DNA turns out to be highly biostable, not only towards enzymatic degradation by exonucleases but also in cell extracts and human serum[18,22]. While many other nucleic acid modifications, the structure of which is closer to that of DNA and RNA, need additional stability enhancing measures such as phosphorothioate groups instead of phosphate groups, this is not necessary in the case of tc-DNA. A rationalization of this increased stability beyond the mere fact that primary and secondary hydroxyls have been changed to tertiary hydroxyl groups is difficult. Given again the changes in the backbone angles β and γ, it can be hypothesized that either binding to the nucleases is inhibited because of steric constraints imposed by the enzyme binding pocket or that the chemical step of bond breaking is inhibited due to unfavorable alignment of the incoming nucleophile with the P–O-bond to be hydrolyzed.

TC-DNA IN ANTISENSE APPLICATIONS

Given the fact that fully modified tc-oligonucleotides do not elicit RNaseH activity when in complex with RNA, a fact that can be understood from the structural point of view (tc-DNA is structurally an RNA analogue), it seemed logic to look for antisense mechanisms that are RNaseH independent. Typically these are steric block mechanisms in which an antisense oligonucleo-

tide is competing with other molecules, mostly proteins, for binding to the RNA target, or mechanisms which interfere with splicing of pre-mRNAs. From collaborative work with other groups we have evidence that tc-DNA is able to compete with the best nucleic acid modifications known so far. As steric block inhibitor of human immunodeficiency virus type 1 tat-dependent *trans*-activation, tc-oligonucleotides showed equal activity as mixed OMe/LNA oligonucleotides in suppressing *in vitro* transcription and *trans*-activation in HeLa cells when delivered by cationic lipids. In addition, a tc-DNA 16-mer showed sequence-specific inhibition of β-galactosidase expression in an anti-HIV HeLa cell reporter assay[23]. In the context of splice interference we have already shown before that incorrect splicing of β-globin as a consequence of point mutations in intron 2 of the β-globin gene can be corrected by designing antisense oligonucleotides over a 3′-cryptic alternative splice site that is activated as a consequence of the mutation[24]. The activity of a tc-18-mer was compared with that of a 2′-OMe-PS-RNA oligomer of the same sequence and was found to be higher by up to 100-fold in a HeLa cellular assay[22]. In another application we compared tc-oligonucleotides of various lengths (9–15-mers) with corresponding LNA oligonucleotides in their ability to induce exon skipping during cyclophilin pre-mRNA maturation. Although the LNA oligonucleotides undoubtedly showed higher affinity to their RNA targets as determined by T_m measurements, the activity in suppressing cyclophilin expression was higher in the case of tc-DNA on both the level of mRNA (RT-PCR) and protein (western blot).

In very recent applications we determined the activity of tc-DNA in the context of Duchenne muscular dystrophy (DMD). DMD is a X-chromosome linked genetic disease in which mutations on the very large dystrophin gene leads to non-functional protein during splicing. While some of the mutations lead to premature stop codons, others lead to incorrect splicing and with this to frameshifts in the open reading frame. It has been shown before that applying antisense oligonucleotides to exon/intron junction sites can cause exon skipping and with this produce a mutant protein which is still functional. In a mouse model (mdx mouse), a 25-mer PMO- and a 20-mer 2′-OMe-PS-RNA oligonucleotide can systemically induce restoration of functional dystrophin[25,26]. In preliminary experiments we found a tc-oligonucleotide 15-mer to be highly active when injected intramuscularly into the tibialis anterior of the mdx mouse (unpublished results). Currently we are investigating systemic delivery of tc-oligonucleotides in the mdx mouse. One of the advantages of tc-DNA may be that simi-

lar therapeutic activity compared to other chemistries can be obtained with shorter oligonucleotide lengths.

TC-DNA IN siRNA APPLICATIONS

In all antisense applications mentioned so far, fully modified tc-oligonucleotides have been used. For siRNA applications, however, fully modified duplexes (sense or antisense strands) are often inactive because they inhibit RISC processing of a targeted mRNA. Typically sense strand modifications are less sensitive to chemical modification compared to antisense strand modification for most chemistries investigated so far. In a recent assay we investigated the efficiency of siRNAs containing tc-DNA residues in various positions on the sense and antisense strands[27]. While sense strand modifications were generally well tolerated, only 3′-end and one central

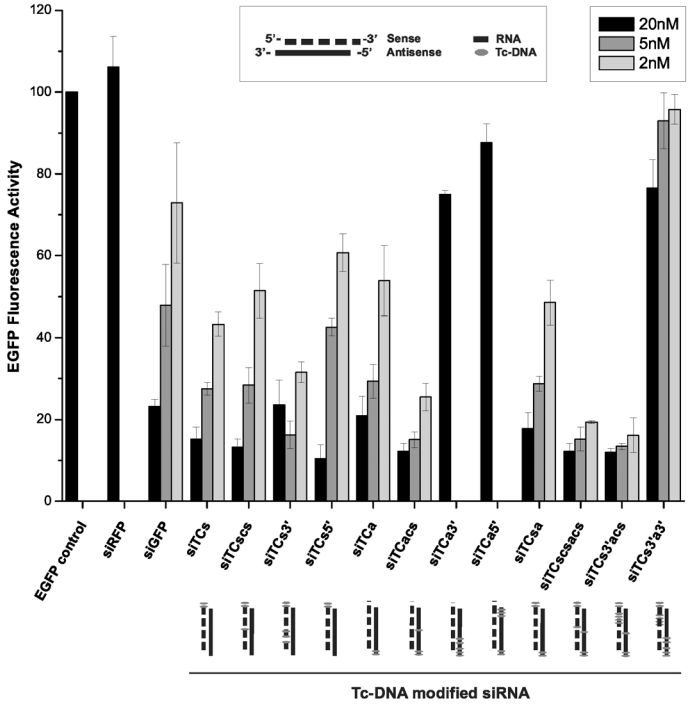

FIG. 4
EGFP silencing caused by si-RNA duplexes containing tc-DNA residues at various positions in the sense or antisense strand. siGFP: unmodified control; siRFP: nonsense control; EGFP control: without siRNA

modifications were tolerated in the antisense strand. Extensive 3′- or 5′-end modification completely abolished activity. The best modified siRNAs showed up to 4-fold higher activity than unmodified siRNAs at 2 nM concentration. The enhanced activity is likely a consequence of higher serum stability of the modified siRNAs.

During these investigations we unexpectedly found that there are subtle differences in thermal stability of tc-modified DNA-oligonucleotides as compared to tc-modified RNA oligonucleotides. While spiking tc-nucleosides into the DNA backbone leads systematically to increased affinity. Spiking RNA with tc-nucleosdies can have a destabilizing effect depending on the position of the modifications in the sequence. The destabilizing effect is stonger the more tc-DNA/RNA backbone junctions occur in the sequence. With other words discontinuous substitution within an RNA backbone leads to destabilization while continuous substitutions lead to neutral or slightly increased affinity. The rationale for this behavior as determined by molecular modeling lies most likely in the repulsive steric effects between the tc-residues and their nearest neighbor ribonucleotides[28]. While for antisense applications this would be a drawback, for siRNA applications where RNA affinity is less a driving force for activity, this can be beneficial. Given the fact that the RISC complex selects the antisense strand from a siRNA duplex on the basis of thermodynamic asymmetry of the duplex ends[29,30], this opens ways to predetermine antisense strand selection by judicious modification of the ends of a sense strand with tc-DNA residues (continuous vs discontinuous arrangement of tc-residues).

CONCLUSIONS

The bc-DNA platform, in contrast to many other oligonucleotide chemistries, provides unique structural features for further chemical modification with the presence of the carbocyclic ring. One of the most successful modifications of this family so far is tc-DNA, the antisense properties of which compare with the best chemical modifications known to date. While we are currently investigating systemic delivery of tc-DNA in order to push this analogue slowly but surely to the clinic we are also dedicated to improve cellular uptake without compromising RNA affinity by further modification of the 5-membered carbocyclic ring of bc-DNA or the cyclopropane ring of tc-DNA. All these efforts go along with improvements in the synthesis of the bicyclo-sugar and monomeric nucleoside building blocks, without which this endeavor would be difficult. Oligonucleotide based therapies as many other molecular based therapies have their pros and cons. The fact

that there are not yet more oligonucleotide drugs on the market despite some 30 years of effort does not put into question the whole approach; it just indicates that not all problems have been solved yet. There is no reason, whatsoever, to believe that these obstacles cannot be solved.

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NANOPORE DETECTION OF DNA DAMAGE IN SINGLE MOLECULES

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Current methods for detection of DNA damage typically do not provide information about the surrounding sequence or the existence of multiple sites of damage within the same strand. Recently, our laboratory has been investigating the use of protein ion channels supported on a glass nanopore membrane bilayer for single-molecule detection of 8-oxo-7,8-dihydroguanosine or other base lesions. Challenges of this approach include problems of signal-to-noise discrimination of A, T, C, G and the base lesion to be detected, as well as temporal resolution of individual nucleotides translocating through the nanopore. Approaches to these challenges are described.

INTRODUCTION

Genomic and mitochondrial DNAs undergo continuous base modifications as a result of both natural processes such as the introduction of epigenetic markers or exposure to endogenous and exogenous sources of damage. The latter includes reactive oxygen species, alkylating agents, UV radiation, and various toxicants introduced into the cell either by drug administration or environmental exposure. DNA damage can lead to mutations when polymerases make errors in replicating past the sites of damage. DNA sequencing of mutated strands provides information about the location of mutations, but does not permit identification of the original chemical structure of the base lesion that caused the mutation. In the present work, we examine a single-molecule approach to the analysis of DNA damage.

Recent interest in the use of solid-state and protein nanopores for DNA sequencing has mushroomed. Among the protein nanopores, alpha-hemolysin (α-HL), a bacterial ion channel-forming protein, has shown considerable promise for DNA sequencing because its transmembrane channel is sufficiently large for either individual nucleotides or single-stranded DNA to translocate electrophoretically from one side of a lipid membrane to the other (Fig. 1)[1]. The ability to fabricate extremely robust lipid bilayers supported in a glass or quartz nanopore of diameter ~500 nm has aided this field considerably[2]. Nanopore ion channels can be constructed in which a single α-HL ion channel is inserted into a lipid bilayer at the end of glass capillary. Application of an electrical potential, typically 120 mV, across the bilayer permits the observation of electrolyte current flow through the

channel. When a DNA strand translocates through the channel, the current is blocked temporarily until the entire strand exits on the other side. Ideally, changes in the current during translocation provide information about the identity of the base as an individual DNA strand passes through the narrowest constriction (~1.4 nm) of the pore.

At present, there are two major challenges to the implementation of protein nanopore sequencing: (1) the change in the current blockage is very small for A vs G vs C vs T, and (2) translocation through the pore is very fast, about 1 microsecond per nucleotide, requiring extremely fast data acquisition rates[3]. These issues also persist for the detection of damaged bases, although in some cases DNA damage may be in the form of a larger, more pore-blocking adduct, thus ameliorating the first of the challenges. As a first step to solving these problems and permitting detection of DNA damage, we investigated the effects of size and shape of DNA adducts on the electrical signals generated when lesions were trapped in the constriction zone of the α-HL ion channel. As a case in point, we describe studies with the common product of oxidative stress, 8-oxo-7,8-dihydro-2′-deoxyguanosine (OG).

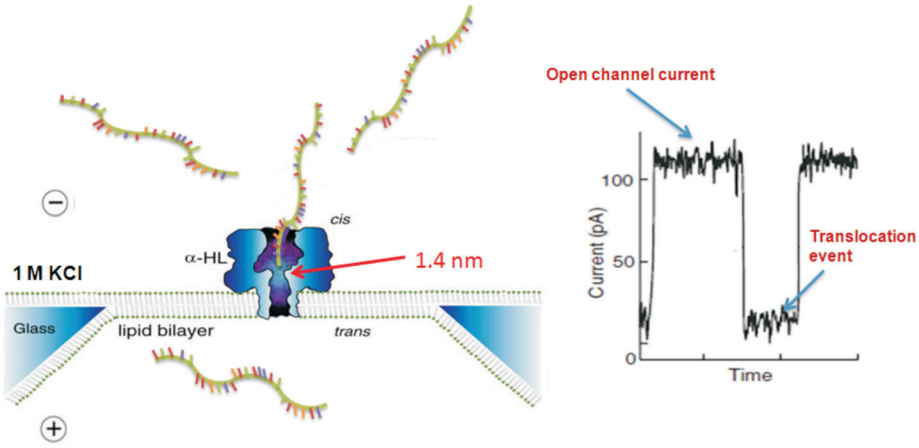

FIG. 1
Single molecule sequencing of DNA is under investigation using the α-hemolysin protein ion channel in a lipid bilayer suspended across a glass nanopore. Electrical measurements indicate current blockage when individual strands of DNA translocate through the pore

RESULTS AND DISCUSSION

OG differs from its parent base by the addition of an oxygen atom at C8 (and tautomerization) making its size, shape and polarity similar to G. In contrast, OG is much more sensitive to oxidation than G, and we capitalized on this feature to form oxidized adducts specifically to OG, so that the steric bulk of the adduct added to the OG lesion would create a noticeable change in the current blockage level. Treatment of an OG-containing strand of DNA with mild one-electron oxidants, such as Na_2IrCl_6 or $K_3Fe(CN)_6$ produces an electron-deficient intermediate that can be trapped by nucleophilic primary amines in high yield (Fig. 2)[4,5]. The 5-alkylamino-8-oxoguanosine product is unstable with respect to rearrangement to form a spirocyclic dihydantoin structure (Sp-NR) when the reaction is conducted at pH 8, 45 °C. The identity of the R group was varied from short bulky groups (glucosamine) to long, narrow side chains (spermine and spermidine). The N-terminus of a short peptide could also be employed; various examples of primary amines used to form oxidized adducts to OG are shown later in this work.

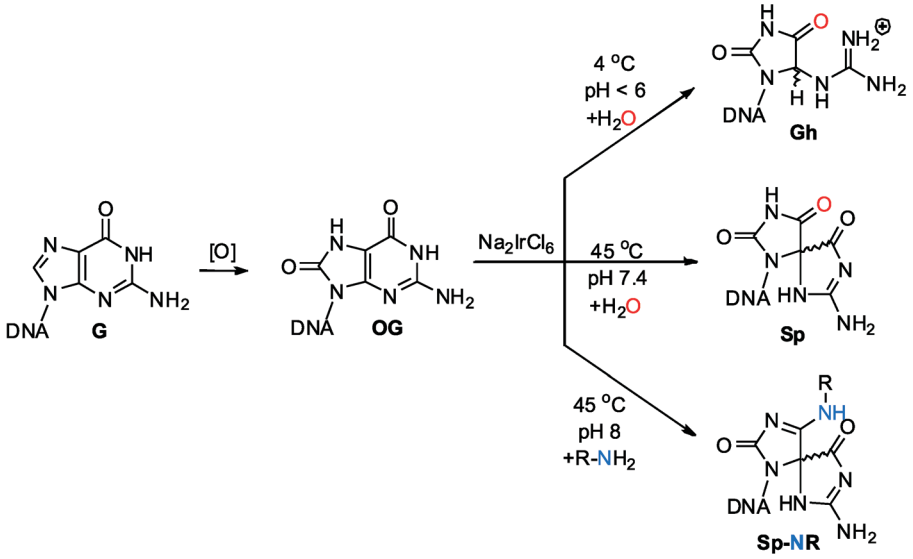

FIG. 2
Oxidative stress leads to the common DNA damage product OG. Further oxidation with Ir(IV) leads to hydantoin products Gh and Sp, or to adducted Sp products, Sp-NR, in the presence of a primary amine RNH_2

To investigate the electrical signatures of OG-containing DNA strands, a synthetic oligodeoxynucleotide containing a single OG lesion at a defined position was converted to a series of adducts following the protocol above. Adducts as large as a tetrapeptide, GPRP, appeared to translocate through the pore, but larger ones, such as an adduct formed from amino-β-cyclodextrin, did not. For those that were capable of translocation, the transit time τ was longer for larger adducts. However, it was still not possible to measure the degree of current blockage because the residence time of the adduct in the constriction zone of the α-HL pore was too short. We therefore turned to studies of immobilized strands in the nanopore in experiments that permitted accurate measurement of the residual current levels for various adducts (Fig. 3). To do this, we employed the method of the Schmidt and Bayley laboratories in which biotin-tethered oligomers are bound to streptavidin, and the uncomplexed end of the DNA is electrophoretically driven into the nanopore until the streptavidin plugs the pore[6,7]. This immobilizes the DNA, permitting current data collection for

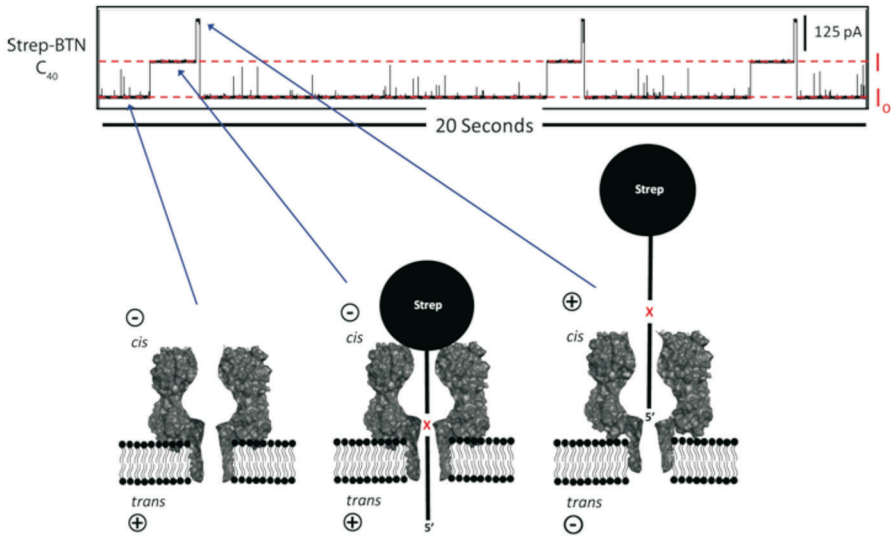

FIG. 3
Top: Current vs time trace for 20 s of ion channel recordings. Biotinylation of the 3′ end of a DNA strand (bottom) and addition of streptavidin allows the strand to be captured in the ion channel and immobilized, permitting time to signal average the current blockage level of a particular nucleotide X as it resides in the constriction zone (center). Reversal of the current (right) releases the DNA strand allowing capture of another

an indefinite period of time until the potential is reversed, releasing the strand, as shown in Fig. 3.

Biotin was appended to the 3′ terminus of the oligodeoxynucleotide via standard linkage chemistry. The streptavidin-complexed oligomer was then captured and suspended in the α-HL channel such that the nucleotide situated 14 residues from the 3′ terminus was located in the constricted transmembrane β-barrel portion of the pore; various normal bases and OG adducts were then studied at this ω14 nucleotide position[8]. For example, when a C_{40} oligomer is immobilized in the ion channel, a specific residual current, I, is measured. When the ω14 nucleotide is G, T, or A, the current level is slightly higher (less blocking), depending upon how much electrolyte flow is blocked when different nucleotides reside in the narrow constriction zone of the ion channel. After averaging the current levels from hundreds of measurements, we obtain histograms of the percent change in current from the poly-dC standard, as shown in Fig. 4. In the poly-dC sequence background, G in the ω14 site is the least current blocking while C is the most blocking.

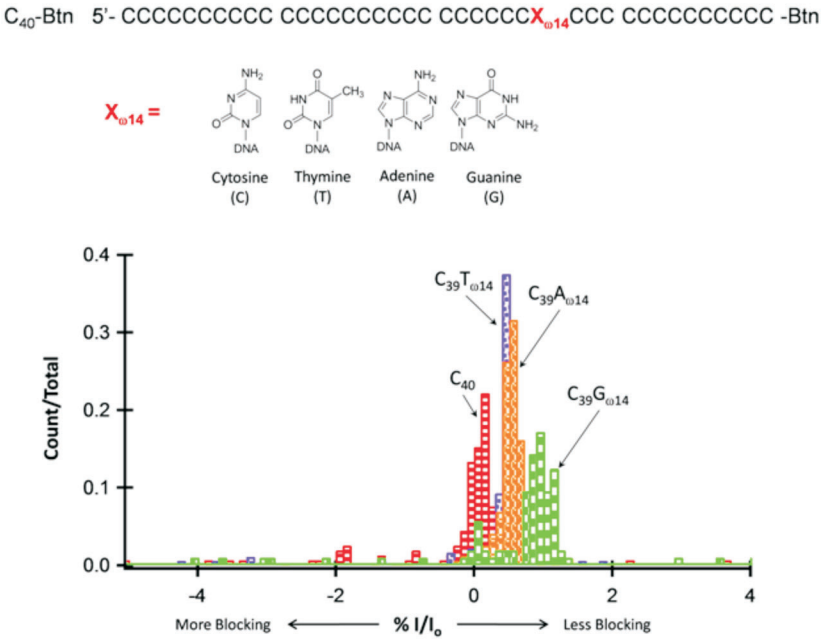

FIG. 4
Histograms of residual current levels of C, T, A or G at the ω14 position of a dC_{40} homopolymer obtained from immobilization experiments. (Adapted from ref.[8])

When the ω14 position is occupied by an OG nucleotide, the current blocking level is essentially the same as G. However, oxidation of the OG to form adducts with primary amines leads to progressively larger changes in current blockage as shown in Fig. 5. In these experiments, spirocyclic adducts formed from small amines, such as benzyl amine, lysine and glucosamine, were about 2% more blocking than C (3% relative to OG), but larger and longer amine adducts, such as spermine and the tetrapeptide GPRP, led to changes in current blockage levels of nearly 10% compared to OG. One potential drawback to the present method of analysis is the observation of two peaks in the histograms of certain adducts; we tentatively assign these to the formation of two diastereomeric products because the spirocyclization creates a new chiral center. Further optimization of the design of covalent adducts is underway. At the next stage, these adducts need to be reinvestigated in translocation experiments to determine if the single base changes can be observed during translocation of a DNA strand through the nanopore.

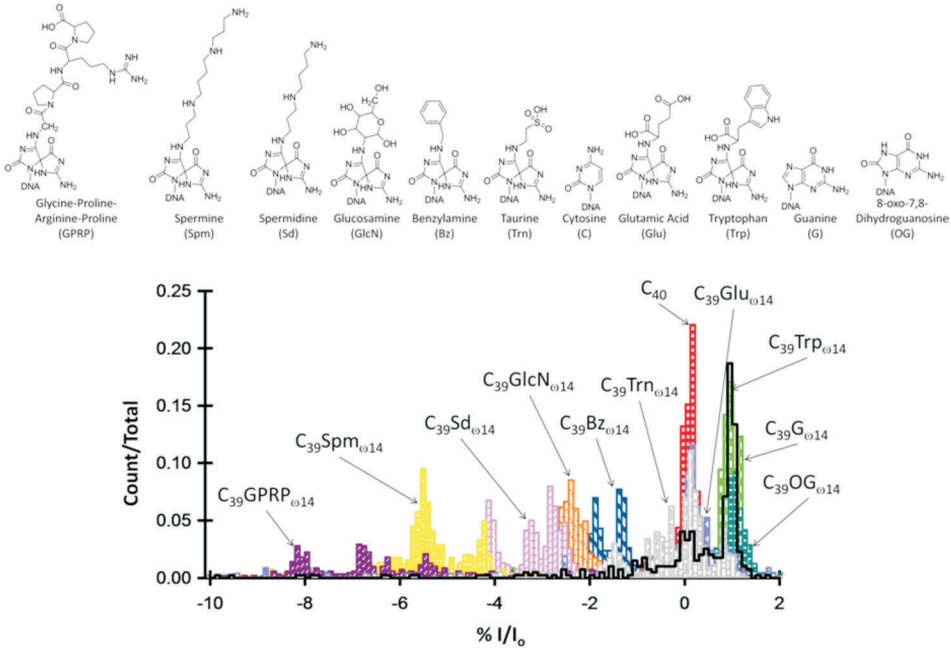

FIG. 5
Histograms of percent changes in residual current levels (bottom), relative to poly-dC$_{40}$, when an OG nucleotide at position ω14 is converted to a spirocyclic adduct (top) in the presence of a primary amine

In order to test the general strategy outlined above in a more realistic setting, an oligodeoxynucleotide was synthesized that contained a portion of the k-ras gene surrounding codon 12, a hotspot for G-to-T mutations implicated in certain human lung cancers. OG is a potential player in the mutagenesis, and thus it was incorporated into the strand, again at position ω14, to test the ability of the α-HL nanopore to detect the single base change. Results are shown in Fig. 6. Curiously, in this sequence background, current blockage signals for G were identical to those of poly-dC_{40}, and OG was less blocking that G, but only by less than 1%. The hydantoin lesions Sp and Gh were also distinct in their electrical signatures, but most dramatically, the spermine adduct (Spm) was again about 8% more blocking than the G nucleotide from which it was derived. This shows future promise for chemical modification of DNA damage to enhance the signal changes observed during nanopore sequencing.

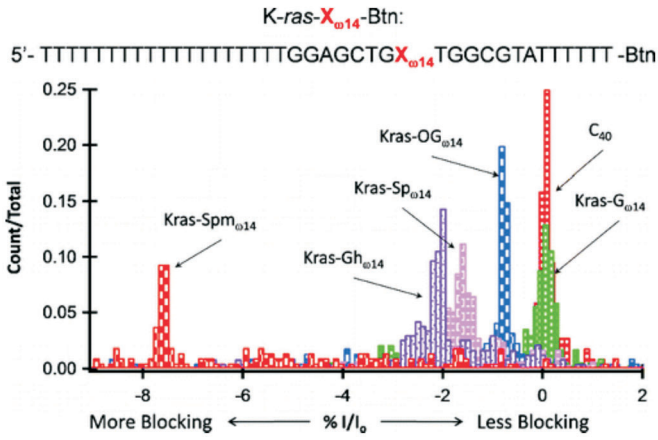

FIG. 6
Histograms of percent changes in residual current for various oxidized G derivatives at position ω14 of the k-ras sequence surrounding codon 12. See Figs 2 and 5 for structures. (Adapted from ref.[8])

CONCLUSIONS

Nanopore sequencing of DNA and RNA at the single molecule level opens the door to obtaining sequence information about DNA damage or chemical modifications to DNA and RNA, including epigenetic sequencing, without the need for amplification. The early results shown here demonstrate that the electrical signatures of individual bases in the constriction zone of the α-hemolysin ion channel can be modulated by simple chemospecific re-

actions on DNA bases. Both the translocation time and the current blockage levels can be significantly altered in a predictable fashion, paving the way for single-molecule sequencing of DNA damage.

This work was supported by the National Institutes of Health (HG005095) and the US Department of Defense (DARPA). The authors thank Prof. Henry S. White, Dr. Anna Schibel, Ms. Qian Jin (University of Utah) and Dr. Geoffrey Barrall and colleagues at Electronic Bio Sciences (San Diego and Salt Lake City) for fruitful collaborations on this topic.

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STRUCTURAL MIMETICS OF A NUCLEOTIDE Ca^{2+}-MOBILISING SECOND MESSENGER: SYNTHESIS AND CHEMICAL BIOLOGY

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Chemically synthesised mimetics of the cellular second messenger cyclic adenosine 5′-diphosphate ribose (cADPR) have a key role to play in helping to unravel the biology of this still enigmatic Ca^{2+}-mobilising cyclic nucleotide. Structural modifications can be envisaged in the adenine base, in both "northern" and "southern" ribose moieties and in the pyrophosphate linkage, with analogues synthesised by total chemical synthesis or *via* a chemoenzymatic route. The value of such compounds as receptor agonists, antagonists and enzyme inhibitors is only just beginning to be appreciated. Contributions to the developing structure-activity relationships of cADPR are reviewed and analogues synthesised by both routes presented, using the chemoenzymatic route in particular to effect base modifications, including the established biological tool 7-deaza-8-bromo-cADPR, hydroxyl group deletion and *N*6-amino group replacement. The cyclic inosine-5′-diphosphate ribose template is of particular interest to provide non-hydrolysable analogues and, while accessible by chemoenzymatic synthesis, a newly-developed total synthetic approach is presented. The chemistry and pharmacology of examples and their application to the exploration of ligand-protein complexes by X-ray crystallography is discussed, illustrating the potential for structure-based design of *e.g.* novel CD38 inhibitors. A targeted electronic change in the adenine ring can produce an NAD^+ analogue with structural biology applications. 2-Fluoro NAD^+ co-crystallised with *Aplysia* ADP-ribosyl cyclase revealed an active site folded ADPR entity with the nicotinamide partially dissociated.

INTRODUCTION

Ca^{2+} is a ubiquitous intracellular messenger that regulates diverse cell functions such as fertilisation, gene transcription, muscle contraction, cell proliferation and the secretion of bioactive compounds. Cyclic ADP-ribose (cADPR, Fig. 1) is one of the principal second messenger molecules that mobilise intracellular Ca^{2+} in various types of cell in a different way to the now well established D-*myo*-inositol 1,4,5-trisphosphate, $Ins(1,4,5)P_3$. cADPR Is a cyclic dinucleotide metabolite of nicotinamide adenine dinucleotide (NAD^+), produced enzymatically by ADP-ribosyl cyclases. Reviews dealing with the biology and chemistry of cADPR and the cADPR/Ca^{2+} signalling system have appeared and several groups have addressed the challenge of

structural modification[1–4]. The cADPR/Ca^{2+} signalling system is active in diverse cellular systems such as animal cells *e.g.* smooth, skeletal and cardiac muscle, acinar cells, protozoa, and plant cells[5]. Over some years, we have provided evidence for an important role of the cADPR/Ca^{2+}-signalling system in the sustained Ca^{2+} response during the process of T cell activation[6] and pharmacological studies indicate that ryanodine receptors (RyR) are the intracellular Ca^{2+} channels involved in cADPR-induced Ca^{2+} release[7]; however, it is still unclear whether cADPR elicits Ca^{2+} release by direct binding of RyR or *via* a separate binding protein[1–3].

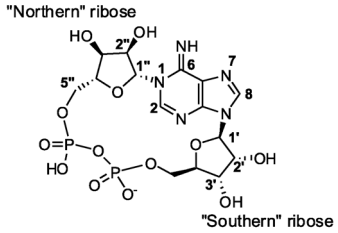


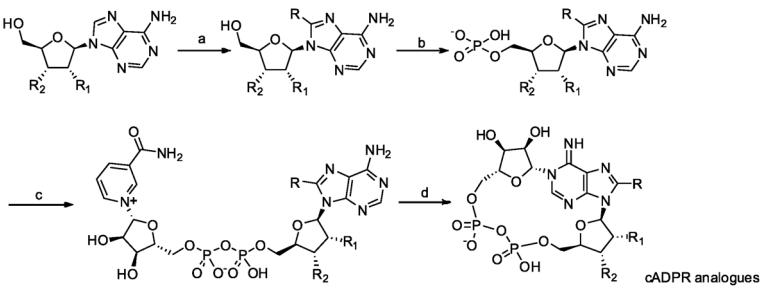
FIG. 1
cADPR structure and numbering system

RESULTS AND DISCUSSION

cADPR is readily hydrolysed at the labile *N*1 linkage to give the linear ADPR in both neutral aqueous solution and under physiological conditions[8,9] thus rendering chemical synthesis of stable analogues important. To date, their generation has been a difficult process. The main choice has been between total synthetic[10] and chemo-enzymatic approaches[11,12] the latter modelled on the biosynthesis of cADPR from NAD^+. Both strategies suffer from different drawbacks, *e.g.* multi-step chemical synthesis and also structure limitations relying upon an enzyme to a cyclise a synthetic precursor respectively. Numerous NAD^+ and cADPR analogues have, however, been synthesised (Scheme 1) and their Ca^{2+} release activities investigated in several systems.

A broad survey of the currently known structure-activity relationships (SAR) at the cADPR "receptor" is shown in Fig. 2. These differ in some detail depending upon actual biological system[13]. Analogues modified at the adenine 8-position (8-Br, 8-NH_2, 8-N_3, etc.) are generally antagonists of cADPR-induced Ca^{2+}-release[6,14,15]. Further adenine modification in 8-Br-cADPR has shown that removal of nitrogen *N*7 results in a hydrolysis resistant, cell permeant competitive antagonist[16–18]. This compound, 7-deaza-

8-Br-cADPR, was employed to unravel the role of cADPR in T cell activation[6] and supported proof of a role for cADPR in long term synaptic depression in the hippocampus[19,20].



SCHEME 1
Key steps for the general chemo-enzymatic synthesis of cADPR analogues: a) Modifications of the nucleobase and/or ribose unit(s); b) Phosphorylation at 5′-OH; c) Pyrophosphate bond formation *via* phosphoramidate activation or carbonyldiimidazole coupling; d) Enzymatic cyclisation with *Aplysia* ADPRC

Modifying the "southern" ribose ring provides information about agonist SAR. A phosphate group at the 3′-position is not tolerated for activity in Jurkat T cells (JTC), whilst it is at the 2′-position[14], contrary to the SAR in sea urchin egg homogenates (SUH). Cyclic aristeromycin diphosphoribose (cArisDPR), a carbocyclic "southern" ribose analogue[21], is a potent agonist[9]

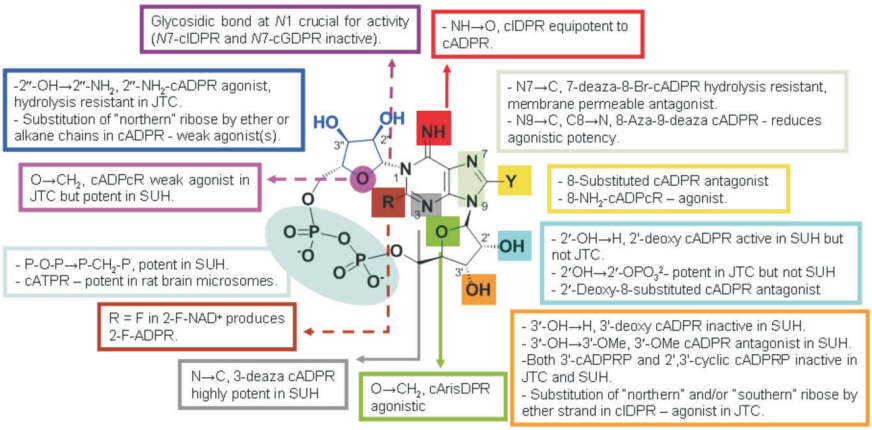
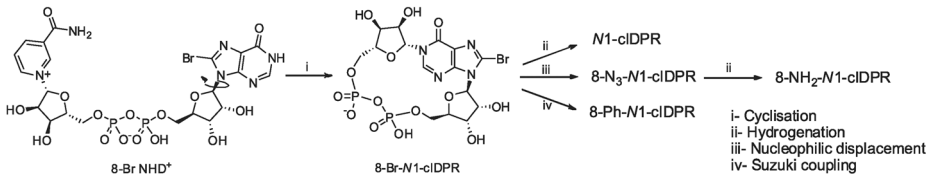


FIG. 2
SAR in Jurkat T cell (JTC) and sea urchin egg homogenate (SUH) for cADPR analogues

while resistant to enzymatic hydrolysis. The Ca^{2+} release activity of 2′′-NH_2-cADPR showed that substitution of 2′′-OH in the "northern" ribose for an amine retains activity[9].

The carbocyclic "northern" ribose analogue, cADPcR (cyclic adenosine diphosphocarbocyclic ribose), is also an obvious target; it is a weak agonist in JTC[9] but potent in SUH [10]. In an attempt to discover antagonists other than the C8-modified compounds, analogues of cADPR were prepared by total synthesis bearing an ether or alkane bridge instead of the northern ribose, while keeping the adenine base intact. All compounds are weak agonists of cADPR-induced Ca^{2+} release[22]. We demonstrated that the ribose-modified 3′-methoxy cADPR has antagonist properties in SUH[8]. Also, a purine-simplified nucleobase cADPR analogue exhibits Ca^{2+} release in intact T cells[23].

We recently pioneered a new synthetic strategy, based upon chemoenzymatic synthesis, that affords highly stable (both chemically and biologically) cADPR analogues related to the *N*1-cyclic inosine 5′-diphosphate ribose (cIDPR) structure (Scheme 2). This centred around the key derivative 8-Br-*N*1-cIDPR (cyclic 8-Br-IDP-ribose), prepared enzymatically from synthetic nicotinamide 8-bromo-hypoxanthine dinucleotide (8-Br-NHD^+)[24,25], despite the reduced nucleophilicity at the *N*1 position of the hypoxanthine base, and which proved to be a novel agonist for Ca^{2+}-release in intact cells[26]. The opposed bioactivities of the antagonist 8-Br-cADPR and agonist 8-Br-cIDPR thus show marked effects for even the smallest of structural changes (*i.e.* $NH_2 \rightarrow$ =O at C6). Furthermore, the enhanced stability of this template allows for the *direct* modification of this cyclic dinucleotide at the 8-position (Scheme 2), providing cIDPR analogues such as the parent *N*1-cIDPR, which is equipotent to cADPR itself in permeabilised T cells[25].



SCHEME 2
Chemical transformations of 8-bromo *N*1-cIDPR

Similar targets had previously only been achievable by complex multistep synthetic routes[27] that could not yield such a close analogue of cADPR in the crucial and little-explored "northern" ribose. We recently synthesised a series of NHD^+ analogues to investigate the requirements for *N*1/*N*7

cyclisation by ADP-ribosyl cyclase[28]. Others used a total synthetic approach to cIDPR analogues, typically inaccessible by enzymatic methods, with the "northern" ribose replaced by a carbocycle (cIDPcR)[29] or, more radically, one or two ether strands (cIDPRE and cIDPDE respectively) to obtain compounds resistant to both chemical and enzymatic hydrolysis. These cIDPR mimics are partial agonists in permeabilised JTC and induced local and global Ca^{2+}-signalling in intact JTC[30,31]. These compounds, including cIDPR, are stable to hydrolysis when incubated with CD38 hydrolase, whilst cADPR is degraded to ADPR[32]. We demonstrated also that 8-halo cIDPRs degrade into the 8-halo-*N*1-IMP derivatives *via* the 8-halo-*N*1-IDPR intermediates, the reverse of what occurs with cADPR[33]. An unexpected result is the strong inhibitory effect of *N*1-cIDPR on Jurkat T-lymphoma cell proliferation[32], that appears to be specific for tumour cells, as opposed to primary T cells driven to proliferation by antigen. cIDPR Derivatives are thus emerging as important biological tools to study cADPR-mediated Ca^{2+} signalling in T cells and are worthy of further investigation. The excellent stability of such compounds makes them ideal for co-crystallisation studies with cADPR binding proteins. We recently obtained X-ray crystal structures of ligands complexed with CD38, the first time that the native enzyme has been co-crystallised with a complete cyclic cADPR analogue, leading to the most complete structural elucidation of the pathway of cADPR hydrolysis[34] and opening up the possibility of structure-based ligand design for this protein using this template.

In an attempt to derive a non-cyclisable NAD^+-ADPRC complex for X-ray studies we designed the adenine-modified 2-fluoro NAD^+ (2-F-NAD^+)[35] with

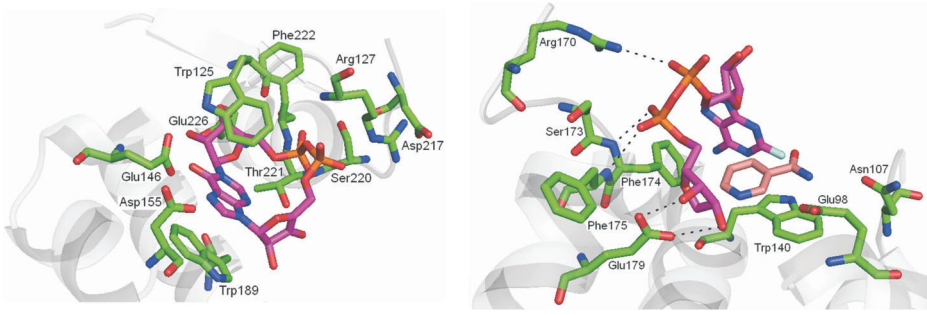
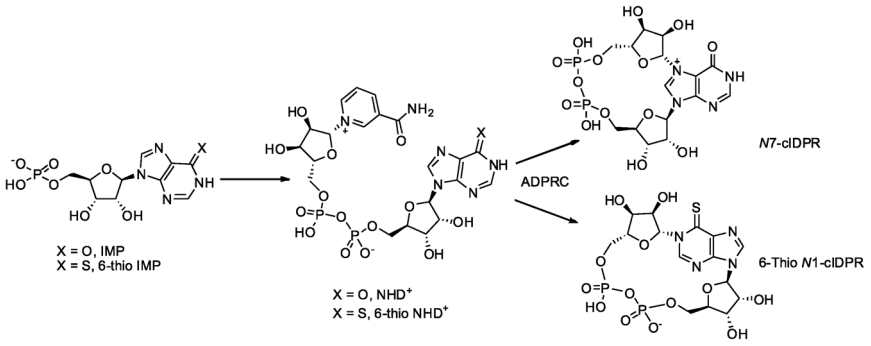


FIG. 3
X-ray structure of *N*1-cIDPR with wild-type CD38 (adapted from pdb. 3PGJ, left panel), Folded and partially dissociated complex of 2-F-NAD^+ co-crystallised with wild-type ADPRC (adapted from pdb. 3I9J, right panel). The dissociated nicotinamide is shown in light pink

the fluorine atom to electronically deactivate the adenine *N*1 atom for cyclisation to cADPR. Interestingly, 2-F-NAD^+ did not cyclise but was hydrolysed slowly to the linear 2-F-ADPR. Examination of a co-crystallised complex of 2-F-NAD^+ and ADPRC revealed intriguingly and for the first time a folded complex with the nicotinamide partially dissociated[36]. C-6 modifications represent a particular structural challenge. The only modifications explored to date are NH_2 and C=O, with major changes in biological activity. It has long been known that this modification leads to ADPRC-mediated cyclization at *N*7 and not *N*1, but this is little understood. Also, 8-Br-*N*1-cIDPR is an agonist, whilst 8-Br-cADPR is an antagonist and cIDPR analogues are very stable to chemical hydrolysis compared to their cADPR counterparts. We anticipated that the 6-thio derivative would be of significant interest, since large activity changes are invariably seen more widely on transformation of C=O to C=S. Chemoenzymatic synthesis relies on the substrate specificity of the *Aplysia* cyclase, which does not always produce the *N*1-cyclic product from all NAD^+ analogues. Thus, NHD^+, NGD^+ and etheno NAD^+ give the *N*7 cyclised dinucleotide (Scheme 3). Since there is minimal structural difference between NHD^+ and 6-thio

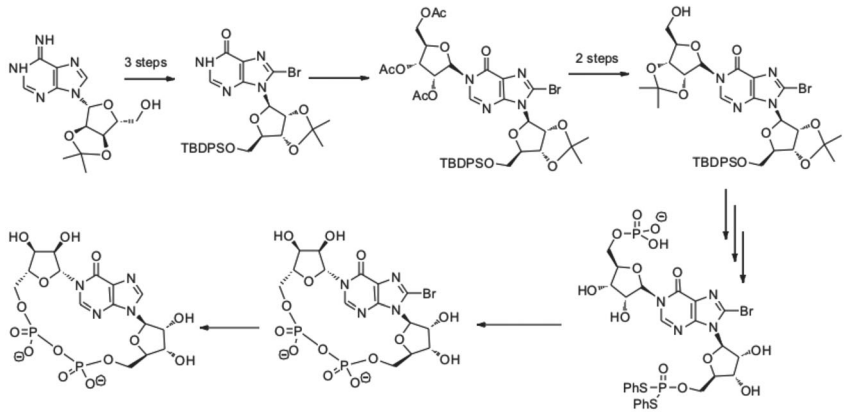


SCHEME 3
Differential *N*1 vs *N*7 cyclisation observed in structurally related precursors

NHD^+ (replacement of the oxygen atom by a sulfur at C-6), it was expected that the sulfur-containing compound would cyclise also through the *N*7-nitrogen. 6-Thio NHD^+ was prepared *via* 6-thio IMP (Scheme 3) in 5 steps from 2′,3′-isopropylidene 6-thioinosine and surprisingly, when incubated with *Aplysia* cyclase, it cyclises at *N*1 providing 6-thio *N*1-cIDPR (Scheme 3). Interestingly, 6-thio *N*1-cIDPR also proved to be the first C-6 modified cADPR analogue acting as an antagonist in JTC [37].

In addition to the chemo-enzymatic approach, total chemical synthesis of cIDPR analogues remains essential, particularly to provide inaccessible structural variations. So far the total synthesis of cADPR analogues has been achieved broadly following one approach relying on the replacement of the "northern" ribose with a carbocyclic surrogate[10,29], an alkyl chain[38] or an ether bridge[39] to yield stable mimics of cADPR. This method is however limited in its scope as it does not grant access to cyclic dinucleotides with an intact "northern" ribose, the site of enzyme activity *inter alia*. The "northern" ribose seems an area of great sensitivity and new chemical approaches are necessary.

We have developed a novel total chemical synthesis of *N*1-cIDPR starting from 2′,3′-*O*-isopropylideneadenosine (Scheme 4). The key step is generation of the sensitive *N*1 glycosidic link between the base and northern ribose, formation of which is required to be both regio- and stereospecific. Other methods have generated mixtures of O6 and *N*1 products, with a mixture of α and β ribose configurations[40]. Following introduction of the 8-bromo substituent, to direct later ring closure, the inosine core is coupled with 1,3,4,5-tetra-*O*-acetyl-β-D-ribose using Vorbrüggen glycosylation conditions. Only the *N*1-β-ribosylinosine product is formed. Sequential introduction of a 5′-*O*-phosphate and 5″-*O*-phosphorothiolate is followed by phosphate deprotection and intramolecular pyrophosphate closure to 8-Br-cIDPR, from which the bromine can be removed[25] to generate cIDPR. With this strategy we aim to pursue more challenging cyclic dinucleotides modified at both the "southern" and/or "northern" ribose(s).



SCHEME 4
Total synthesis of *N*1-cIDPR

In summary, while over the last decade in particular synthetic methodology has been put in place to enable targeting of almost any structural motif of cADPR, there is much still not yet understood regarding the biology of this signalling pathway and the pharmacology of cADPR analogues. This leaves considerable unexploited potential for chemical design and intervention to better elucidate and modulate this new cell signalling mechanism.

This work was supported by the Wellcome Trust (grants 084068 and 082837). We thank Dr M. P. Thomas for compiling Fig. 3.

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RECOGNITION OF DNA SECONDARY STRUCTURES: FROM STRUCTURE TO FLUORESCENT PROBES

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DNA can exist in a variety of secondary structures that have profound effects on several biological processes such as gene regulation and disease. Targeting DNA secondary structures by small molecules is challenging but holds a great potential for future therapeutic and biochemical applications. Herein we give examples of diverse chemical scaffolds and strategies developed for targeting mismatched DNA and G-quadruplexes.

INTRODUCTION

Many diseases with different form of appearance such as cancer, neurodegenerative diseases (Huntington, Fragile X-syndrome), cystic fibrosis, diabetes *etc*. have genetic determinants. Understanding genetic components may thus give clues to biological mechanisms, help predicting disease or susceptibilities and allow individualizing treatments. In particular, DNA sequences containing repeats of bases are highly susceptible to aberrant replication and perturbation of various DNA-related processes such as recombination and transcription[1]. These dysfunctions may lead ultimately to modifications of the genetic material (*i.e.* mutations, genomic instability) which may be of importance to explain mechanisms linked to cancer development or more largely be involved in pathogenic rearrangements genome-wide. At the molecular level the peculiar behavior of repeat sequences is attributed to the formation of secondary structures *via* intrastrand Watson–Crick base pairing (*e.g.* folded back hairpins) or inter- or intra-strand non-canonical base-assemblies (*e.g.* mismatched base-pairs, base-triplets or guanine quartets). Therefore single-stranded domains when containing repeats are predicted to form unusual non-B structures like hairpins, triplexes and quadruplexes. Consequently the development of new chemical tools to target these particular structural features of nucleic acids is of great interest for understanding their biological role, but also for drug design and diagnostics.

Over the past few years, we have developed a number of molecular architectures with the capability to recognize mismatched DNA bases and G-quadruplex structures (Fig. 1). Our approaches rely both on structure-

based design and on the development of medium to high-throughput assays for screening chemical libraries.

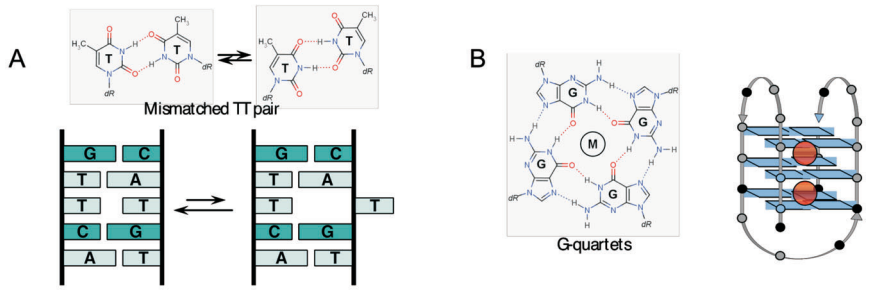

FIG. 1
Targeted DNA structures and the corresponding base associations A) Mismatched DNA, B) G-quadruplex DNA (metallic cation in red)

Mismatched Ligands

Studies aimed at a deeper understanding of the recognition of mismatches by repair enzymes have raised continued attention for more than a decade. Several models have been proposed to rationalize the mechanisms of mismatch recognition, but these are still poorly understood[2]. Given the complexity of these processes the task is highly challenging and requires several approaches, such as genetic, biochemical and chemical ones. In particular, a chemical tool for studying mismatch recognition is represented by small molecules that, similar to the mismatch-recognizing enzymes, can bind base mispairs with a high selectivity over fully paired DNA. Such mismatch-binding ligands (*mismatch binders*) may eventually interfere with the repair systems with negative or positive consequences, leading to inhibition or promotion of repair, and thus display high biochemical and pharmacological potential.

Structure probes: For several years we developed original cyclophane compounds of cyclobisintercalator-type (CBIs) which specifically insert at pairing defects in DNA mismatched sites[3,4]. The prototype of this series is the bisacridine compound (BisA) that was shown to selectively bind to thymine-containing mismatched sites by a unique mode involving ejection of a thymine residue into an extrahelical position (*base flipping*). Subsequently it was shown that the bisnaphtahlene analogue (2,6 BisNP) exhibits a higher binding affinity than the BisA model while retaining a high selectivity[4].

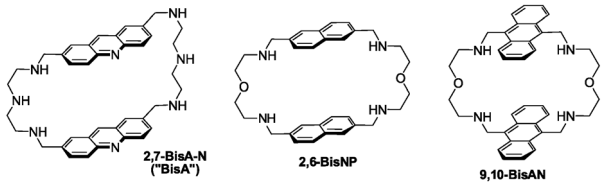

FIG. 2
Schematic representation of BisA, BisNP and BisAN macrocycles

To gain deeper understanding of the interaction of the BisNP compounds with a TX mismatched duplexes, a structure-activity study was undertaken. A series of compounds differing by the connectivity of the diamino spacers have been synthesized. These compounds adopt different topology and therefore are termed topomers (Fig. 3A) UV-vis Tm measurement with 17-mer duplexes containing a TX mismatched site in the center have shown that the 2,7 BisNP compound is the most selective ligand as no binding to the control fully paired duplex TA is observed (Fig 3B).

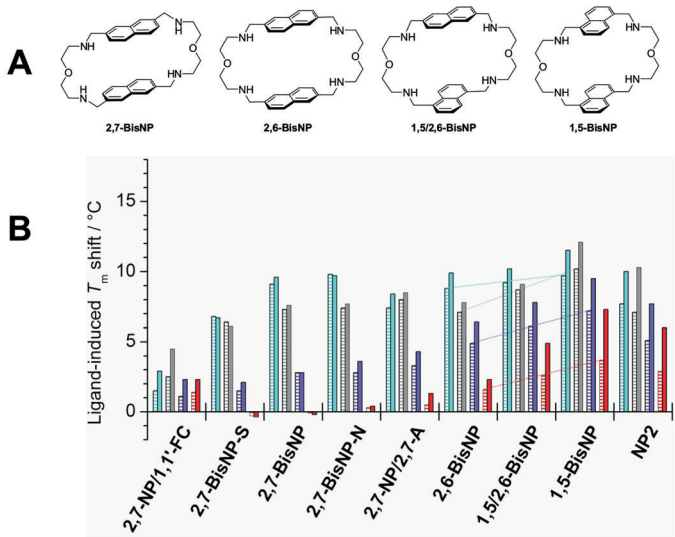

FIG. 3
A) Topomers of the BisNP series. B) Results of thermal denaturation experiments with duplexes TX (c = 6 μm in cacodylate buffer, pH 6.0, $[Na^+]$ = 20 mM) and ligand-to-duplex ratios q = 1 and q = 2. TX (5′-CCAG TTC GTA GTA ACCC-3′/5′-GGGT TAC TXC GAA CTGG-3′), containing either a matched (TA) or a mispaired (X = G, C, T) thymine residue (TT blue, TC grey, TG purple, TA red, q = 1 stripped, q = 2 plain)

Molecular dynamics calculations indicated that the selectivity of this compound steps on a restrained internal molecular flexibility as well as on a particular 3D conformation[5]. An NMR study aiming at determining the structure of the complex formed between the most active bisnaphtahlene derivative BisNP and a TT mismatched duplex is currently under progress.

Fluorescent probes: Although the two macrocyclic series described above contain fluorescent moeities (acridine, naphthalene) they exhibit rather low fluorescence quantum yield ($\Phi_F < 10^{-2}$). We therefore speculated that the derivative (BisAN) that possesses highly emissive anthracene units, would give a fluorescence signal that can be modulated upon binding to mismatched DNA. Although this compound is less selective than the two former series and bind both fully paired and mismatched duplexes, drastic changes are observed in its fluorescence upon binding. In aqueous solutions the free macrocycle displays a yellow-green emission with a broad band ($\lambda \approx 480$ nm) which corresponds to the intramolecular excimer emission of the two anthracene chromophores hold in a close proximity (Fig. 4a, red curves). Upon addition of the matched duplex TA, the excimer emission was reduced and a new structured band with two maxima ($\lambda = 419$ and 440 nm) was detected (Fig. 4a). This blue-shifted emission band, may

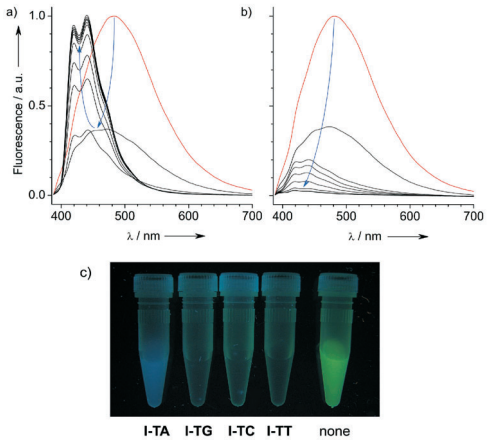

FIG. 4

Spectrofluorimetric titrations of duplexes (a) I-TA and (b) I-TC (0–6 μm) to solutions of macrocycle 2 (5 μm in sodium cacodylate buffer, excitation wavelength $\lambda_{ex} = 385$ nm). The red curves correspond to the fluorescence spectrum of 2 in the absence of DNA; the arrows represent the changes in the spectra in the course of titrations. (c) Image of solutions of 2 in the presence of indicated duplexes (5 μm each) and without DNA under UV illumination ($\lambda = 312$ nm). Sequence of the duplexes same as in Fig. 3

be attributed to the fluorescence of the monomeric anthracene unit. This may suggest that one anthracene moiety of the ligand intercalates into the base stack of DNA, that reduces its interaction with the second moiety. However, when the mismatch-containing duplex TC was added to BisAN, the emission of the excimer form was strongly quenched (about 80-fold) without simultaneous increase of the monomer fluorescence (Fig. 4b). The same behavior, was observed in the case of mismatch-containing duplexes TT and TG. Most remarkably, this drastic difference in fluorescence properties of the BisAN macrocycle allows a "naked-eye" discrimination between fully matched and TX-mismatch-containing duplexes (Fig. 4c).

Although the use of the anthracene fluorophore in the design of base-discriminating oligonucleotide probes has been described[6], the BisAN macrocycle is one of the first examples of a non-covalently bound probe which allows a simple "mix-and-measure" method for detection of mismatched base pairs in DNA[7].

In summary the novel macrocyclic compounds which are highly water-soluble hold great potential for use as modulators of mismatch repair pathways or as molecular diagnostic tools.

Design of G-Quadruplex Binders

Over the past decade, the occurrence of G-quadruplex structures in DNA and RNA has attracted considerable research effort. A wealth of knowledge has accumulated concerning G-quadruplex motifs and their potential role in various DNA-and RNA-related processes such as recombination, telomere maintenance, oncogene transcription, and more recently splicing[8]. These structures can be considered as particular receptors for small molecules, their recognition allowing in turn targeting of a given G-rich sequence. The quadruplex field is now expanding at a fast rate but even if significant progresses have been made recently, it is clear that, for a fuller understanding of G-quadruplexes, better analytical tools must be developed. One major issue resided in the poor selectivity of the first ligands generations towards quadruplex, which seriously limited their application. To make further advances in the current context, new generations of chemical tools are imperatively required that would exhibit selectivity for a given quadruplex structure and/or have interesting biological functions.

Structure probes: In this goal, our research has led to the emergence of several chemical series of high affinity and selectivity for G-quadruplex DNA[9]. using both rational design and HTS assays such as FRET-melting and G4-FID [10]. In term of structure, these molecular tools rank from classical

planar aromatics, to metal complexes and non-planar macrocyclic architectures. Recently our efforts were more particularly focused along three lines i) design of on/off fluorescent probes, ii) design of cross-linking agents using metal complexes, iii) interference of ligands with quadruplex forming sequences (QFS) in cellular contexts. In particular the Bisquinolinum compounds (Fig. 5) rank amongst the best ligands displaying nanomolar affinity for various quadruplex structures and poor binding to duplex DNA.

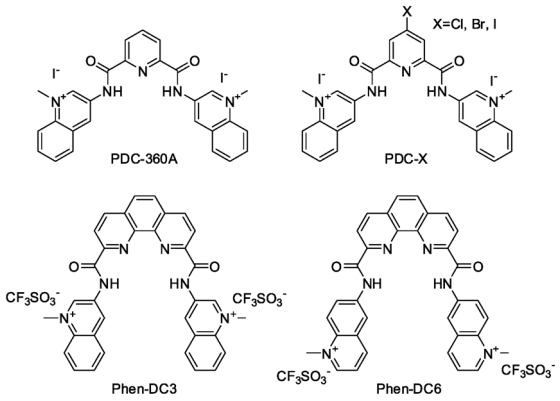

FIG. 5
Structure of the most active bisquinolinum compounds

Although these compounds are currently used as tools to probe the formation of quadruplex in cell-based assays[11,12], their water-solubility and cellular uptake are moderate and require improvement.

We recently observed that the presence of halogen atoms on the central pyridine ring significantly enhances the binding affinity as seen from stability measurements (Fig. 6) and also considerably improves the capacity of the molecule for passing through the cell membrane.

Fluorescent probes: While reports dealing with selective G-quadruplex ligands or DNA fluorescent probes are frequent in the literature, examples combining these two characteristics in a single molecule are still scarce[13].

In this regard our group was the first to propose the synthesis of hybrid fluorescent molecules based on a merging a G4 binder and the thiazole orange motif. Previously we have shown that the well-known fluorescent DNA probe Thiazole orange (TO) exhibits a high affinity for quadruplex but is not able to distinguish between various forms of DNA. Taking advantage of the presence of a quinolinium unit in both the highly selective quadruplex ligand PDC and Thiazole Orange (TO) we have assembled these

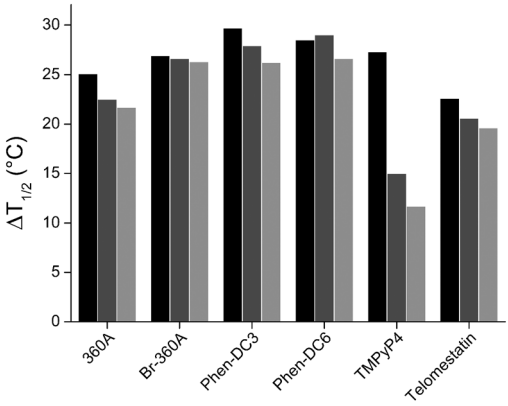

FIG. 6
FRET-melting data ($\Delta T_{1/2}$/°C) for Bisquinolinium compounds and reference molecules (TMPyP4, Telomestatin), ODN = F21T: sequence *FAM*-$G_3[T_2AG_3]_3$-*Tamra*, *fam fluorescein*, *tamra rhodamine*, concentration 0.2 µM, ligand 1 µM (black bar). Same experiment in presence of a 26bp duplex competitor (1 and 10 µM, dark grey and light grey bars). Conditions: lithium cacodylate buffer 10 mM, 10 mM KCl, 90 mM LiCl, pH 7.2

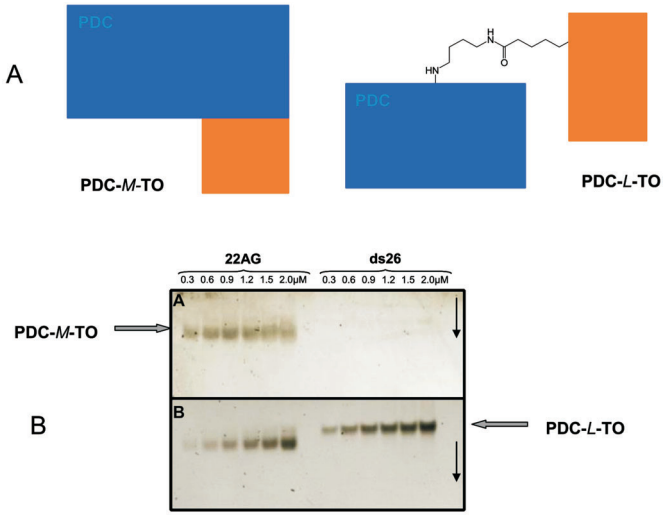

FIG. 7
A) Structure of the two fluorescent compounds. B) Gel electrophoresis of G-quadruplex forming ODN (22AG) and duplex ODN (ds26) after staining by the probes

two motifs in a single scaffold to obtain the merged conjugate PDC-*M*-TO (Fig. 7).

Using fluorescence spectroscopy and FRET-melting assay we showed that PDC-*M*-TO fulfills the requirements of a selective on/off probe *i.e.* a low fluorescence in the free state and a pronounced fluorescence enhancement when bound to DNA, combined with a robust ability to discern telomeric quadruplex- from duplex-DNA (Fig. 7B). In contrast the control compound PDC-L-TO which features the TO moiety directly linked to the PDC motif has completely lost quadruplex selectivity, thereby labelling both duplex and quadruplex-containing tracks (Fig. 7B).

Although the fusion of the two scaffolds comes at the cost of a decreased quantum yield of PDC-M-TO as compared to PDC-L-TO, this novel series provides nonetheless a proof of concept demonstrating that analogues of TO, when properly engineered, could be valuable probes for signalling of G-quadruplex structures[14].

CONCLUSIONS

The objective of developing small molecules with specificity for unique nucleic acid structures represents a highly demanding challenge in view of the many competitor structures present in a biological context. However, although this field is in its infancy, we have shown herein that high binding selectivity can be reached by using a proper structure-based design approach combined with screening methods. Some of these molecules are currently used to probe the presence of secondary structures in cell-based assays in combination with genetic approaches. Therefore our structure and fluorescent probes represent useful tools for sensing and mapping secondary structures of nucleic acids and evidencing their biological roles.

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ORTHOGONAL RIBOSWITCHES AS TOOLS FOR CONTROLLING GENE EXPRESSION IN BACTERIA

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Recently we developed a new method for controlling gene expression in cells using synthetic small molecules. The novel strategy builds on the discovery that certain cellular metabolites can turn genes on or off by triggering switches present within mRNA. We have succeeded in re-engineering (or rewiring) these so called 'riboswitches' so that they are no longer triggered by the natural metabolites, but instead can be controlled by the addition of various synthetic molecules[1].

INTRODUCTION

A number of small-molecule inducible expression systems have been developed for bacteria. Notably, systems based on the lactose (*lac*), arabinose (*ara*) and tetracycline (*tet*) operons have been extensively utilized for production of recombinant proteins and are also widely used for *in vivo* gene functional analysis. Similarly, inducible expression systems have been used to control antisense RNA-mediated downregulation of critical genes in pathogenic bacteria[2], allowing for the development of more sensitive screens for new antimicrobial agents[3]. Inducible expression systems have also become indispensable tools in synthetic biology and have been used to control genetic circuits that can function as sensors, switches and oscillators[4]. The engineered expression of novel metabolic pathways, under the control of inducible promoters, has been employed for the production of fine chemicals as well as natural products, including drug precursors and fatty acids for biofuels[5]. Despite many notable successes, the commonly used regulatory systems do have limitations. For example, some of these systems exhibit cross-talk, which limits their application when it is desirable to control the simultaneous and differential expression of multiple genes using distinct inducers. A number of the promoter based systems also exhibit all-or-none expression, where a subpopulation of cells are fully induced, whilst others remain uninduced. Also, some systems are limited by undesirable side-effects of the small molecule inducer.

In light of this, alternative methods have been sought to control gene expression using small molecules. For example, RNA aptamers generated by *in vitro* selection (e.g. SELEX), have been inserted into the 5′-untranslated regions (5′-UTRs) of bacterial mRNAs to control translational in response to selected ligands[6]. Whilst these aptamers have significant potential for small molecule modulation of gene expression, there are currently very few aptamers available that are selective for ligands with desirable physicochemical and pharmacokinetic properties[7]. However, nature has already evolved its own RNA aptamers which can provide exquisite control over gene expression through binding to a wide range of small molecule metabolites, including amino acids, nucleobases, carbohydrates and co-enzymes[8–9]. These so-called "riboswitches" are found in all domains of life[10], but occur with the highest frequency in bacteria, where they typically regulate genes involved in the biosynthesis, catabolism or transport of metabolites[11]. Bacterial riboswitches typically function through the binding of a specific metabolite to an aptamer domain within the 5′-UTR of mRNA, which affects the conformation of an adjacent expression platform, modulating either the termination of transcription, initiation of translation or mRNA self-cleavage[11–13]. This is a fundamentally different paradigm from protein-based regulatory systems. Riboswitches therefore offer an ideal platform from which to develop new small-molecule controllable expression systems that address many of the limitations of the classical systems (e.g. the *lac*, *ara* and *tet* promoters). However, the applicability of riboswitches as generic genetic control elements is limited by the fact that natural riboswitches have evolved to bind to primary metabolites that are common within cells. Recently, we provided a solution to this problem by re-engineering the first orthogonally-selective riboswitches which no longer respond to natural metabolites present in the cell, but instead can be controlled by the addition of various synthetic molecules.

RESULTS AND DISCUSSION

At the outset we focused on re-engineering the thiamine pyrophosphate (TPP) riboswitch from the *thiM* gene of *E. coli*, which upon TPP binding sequesters the ribosome-binding site (RBS), blocking the initiation of translation. Mutant *thiM* riboswitches were generated by targeted mutagenesis and gene synthesis, then cloned into an expression plasmid upstream of the *sacB* gene, which is toxic to *E. coli* in the presence of sucrose. Transformants where then grown in sucrose-containing media, along with a TPP analogue from a library of *ca*. 30 compounds which we had designed and synthe-

sized. Although clones were selected, further investigation failed to reveal any mutant riboswitch-ligand pairs that elicited significant repression of reporter gene expression. Subsequent ITC and equilibrium dialysis showed that some of the TPP analogues did bind weakly with the mutant *thiM* riboswitch RNAs *in vitro*. We suggest that the lack of *in vivo* activity could stem from the fact that the analogues are not pyrophosphorylated *in vivo*, which is essential for the high affinity binding of TPP to the parental riboswitch. Whilst pyrophosphorylated analogues could be synthesized, these will not penetrate the cell membrane. In light of this, we turned our attention to re-engineering the adenine-responsive riboswitch (addA) from the *add* gene of *Vibrio vulnificus*, which upon ligand binding releases the RBS to allow translation (Fig. 1A)[12,14].

The advantages of this system were soon apparent. Firstly, there are very many purine analogues and related heterocyclic compounds commercially

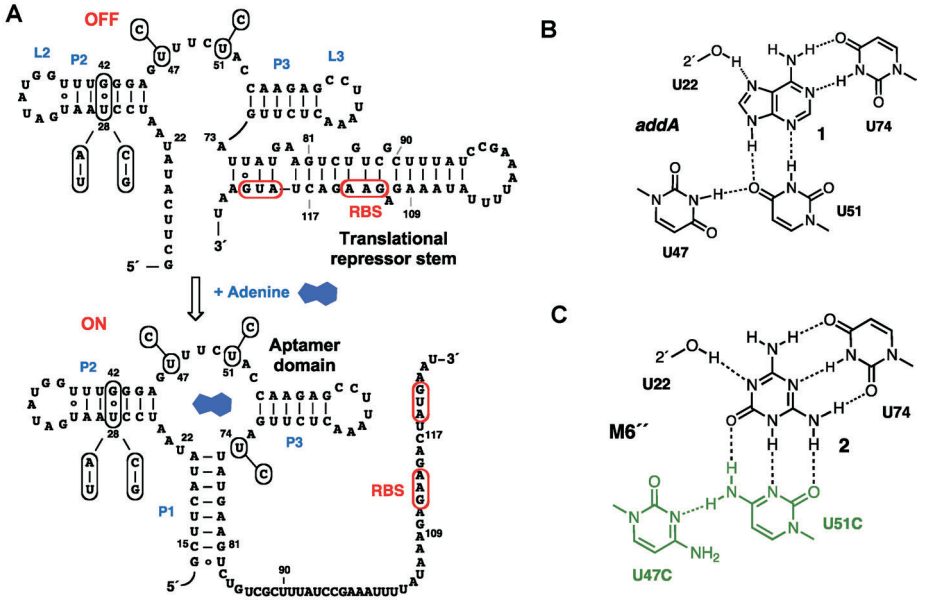

FIG. 1

(A) Secondary structure model of the parental *add* A-riboswitch. In the OFF-state, the RBS is sequestered in a repressor stem-loop which blocks translation. When present at sufficient concentrations, adenine binds to the aptamer domain to stabilize the P1 stem, releasing the RBS. (B) The H-bonding contacts between adenine **1** and the parental *add* A-riboswitch. (C) Model of the H-bonding interactions between ammeline **2** and the mutant M6″ aptamer (U28G, G42C, U47C, U51C)

available, or easily synthesized, which have excellent drug-like physicochemical and pharmacokinetic properties. Also, it is much easier to select for gene activation (gain of function) as opposed to repression (loss of function). Accordingly, the three residues (U47, U51 and U74) that form the adenine-binding site in the addA aptamer domain (Fig. 1B) were subjected to random mutagenesis. The addA mutants were then cloned upstream of the chloramphenicol resistance gene (*cat*) in the pMOD3 expression plasmid[1], which was used to transform *E. coli* (Top10F′). The resulting transformants were screened for chloramphenicol resistance in the presence and absence of individual small molecules from a library of 80 heterocyclic analogues. Mutant riboswitch-ligand pairs which displayed significant differential growth rates that were dependent on small molecules from the library, but which showed no change upon the addition of adenine, were selected for further characterization. From this we identified a mutant, M6 (U47C, U51C), which was able up-regulate eGFP expression upon the addition of ammeline **2**, with an induction factor of 6.5 (The induction factor is defined as the ratio of the maximum ligand-induced protein expression level over the basal level). In comparison, the parental riboswitch was shown to exhibit an induction factor of 11 with adenine **1**. Another mutant was selected, M6C (U47C, U51C, U74C), which was activated by 5-azacytosine **3** with an induction factor of 4. Neither mutant M6 nor M6C showed any response to adenine or other natural purines typically present in the cell[1].

The maximum eGFP expression level for both mutants was only 30% of that observed for the wild-type riboswitch. Secondary structural calculations (mFold) suggested that the U47C, U51C double mutation could give rise to a subpopulation of misfolded riboswitches, with no P2 stem and an alternative stem-loop within which C51 is hybridized to G42. We therefore mutated the G42-U28 wobble pairing in the P2 stem-loop to C42-G28, in order to stabilize the P2 stem-loop and thereby prevent misfolding. This turned out to be successful, as the resulting mutants M6″ (U28G, G42C, U47C, U51C) and M6C″ (U28G, G42C, U47C, U51C, U74C) both induced higher eGFP expression levels than the wild-type *add* A-riboswitch in *E. coli*. The aptamer domains of the mutant and wild-type riboswitches were also produced by *in vitro* transcription and subjected to SHAPE analysis. SHAPE is a method that allows RNA secondary structure to be mapped in solution, through selective 2′-hydroxyl acylation followed by primer extension[15]. From this we were able to show that the M6-**2** and M6C-**3** complexes clearly form multiple structures in solution. On the other hand, M6″-**2** and M6C″-**3** exhibit 2′-acylation patterns that are very similar to addA-**1**. In

addition, mutant M6″ and M6C″ were shown to be completely orthogonal to the parental *add* A-riboswitch *in vivo*. Neither mutant responds to adenine or other purines including 2-aminopurine, 2,6-diaminopurine, guanine or hypoxanthine; nor does addA respond to either ammeline **2** or 5-azacytosine **3**. Both mutant riboswitches also show excellent dose-dependent control of gene expression[1].

To confirm the orthogonality of M6″ and M6C″, ITC experiments were performed to explore the thermodynamics of ligand binding to aptamer domains. From this we established that the K_d values for M6″-**2** and M6C″-**3** were 1.0 and 1.2 μM, respectively, whilst adenine and other purines showed no affinity for either aptamer domain. In comparison, addA exhibited a K_d of 0.021 μM with adenine, and does not bind ammeline **2** or 5-azacytosine **3**. A structural model for the M6″-**2** complex can be proposed based on the published addA-**1** crystal structure [1Y26.pdb] (Fig. 1B and C). However, it was not immediately apparent how **3** could bind to the M6C″ aptamer based on this structure. Therefore the M6C″ aptamer was co-crystallized with **3** and a 1.7 Å X-ray crystal structure was determined [3LA5.pdb] (Fig. 2A and B). This shows that the mutations result in a reor-

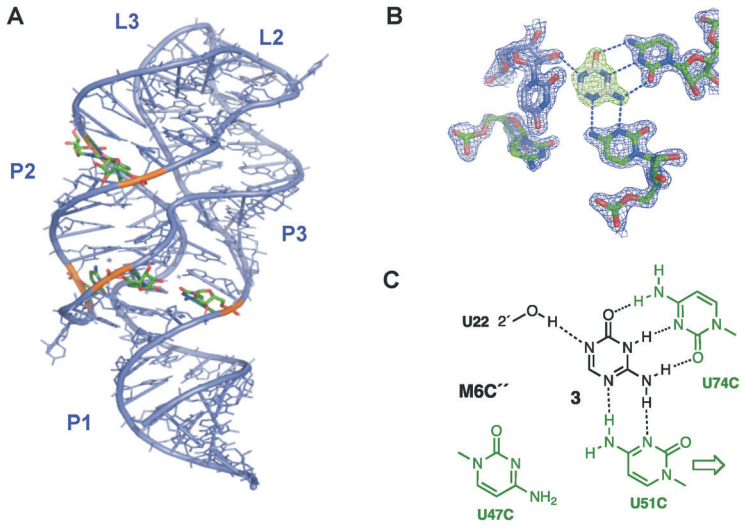

FIG. 2
(A) 1.7 Å X-ray crystal structure of the mutant M6C″ (U28G, G42C, U47C, U51C, U74C) aptamer domain in complex with 5-azacytosine **3**. (B) Electron density contour map showing contacts between M6C″ and 5-azacytosine **3**, with H-bonding patterns shown below (C). Note the C51 translocation relative to the addA structure

ganization of the ligand binding site, with the mutated residue C51 undergoing a 1.8 Å lateral translation away from C47, compared with the wild type structure (Fig. 2C). In addition, Mg^{2+} coordination was shown to induce a kink in the phosphodiester backbone between U22 and A23, enabling the 2′-OH of U22 to engage with the N7 lone pair of **3** (Fig. 2B and C)[1].

Subsequently we have gone on to show that the orthogonal riboswitches M6″ and M6C″ provide very tight control of gene expression in *E. coli*, using a variety of reporter systems including LacZ, luciferase, eGFP and DsRed (red fluorescent protein). Notably, the orthogonal riboswitches provide very low basal levels of expression, which are *ca.* 20-fold lower than control experiments with the *lac* system in an *E. coli* strain with the LacI repressor overproduced (Top10F′). However, for our orthogonal riboswitches, the maximum levels of ligand-induced eGFP expression were 3-fold lower than the IPTG-induced *lac* system, so there is still scope for improvement. We have also shown how the mutually orthogonal riboswitches can be used for the differential and simultaneous control of two genes in the same *E. coli* cell. For example, we generated a plasmid containing both M6″-*DsRed* and addA-*eGFP* under the transcriptional control of individual *lac* promoters and separated by a transcriptional terminator (Fig. 3A). The addition of ammeline to *E. coli* cells transformed with this plasmid results in the selective expression of DsRed, whilst the adenine analogue 2-aminopurine

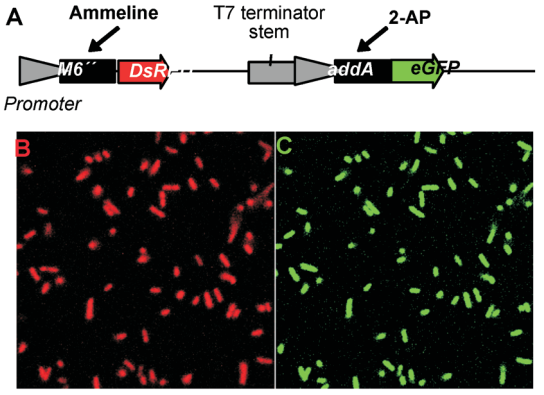

FIG. 3
(A) Dual promoter construct with *DsRed* and *eGFP* under control of the M6″ and *add* A-riboswitches respectively. (B and C) Confocal microscopy of *E. coli* transformed with the dual promoter system. Cells fluoresce red (B) with ammeline (250 μM) and green (C) with the adenine analogue 2-AP (250 μM)

(2-AP) selectively activates eGFP expression. When both ligands are added, cells produce both red and green fluorescent proteins. Individual *E. coli* transformants were subsequently observed by confocal microscopy (Fig. 3B and C). From this it was clear that ammeline results in red fluorescent cells, whilst 2-AP induces green fluorescence. When both ligands are added the confocal images of individual *E. coli* cells show fluorescence at both the eGFP and DsRed emission wavelengths. These studies also indicated that fluorescence is evenly distributed across all cells, with no apparent all-or-none expression response which characterizes some inducible promoter systems.

A second plasmid was also constructed comprising an operon of the M6''-*DsRed* and addA-*eGFP* genes, with one lac promoter driving transcription of a single mRNA. This system also allows differential control of both genes. This is notable as the first demonstration that naturally-derived riboswitches can function between coding regions within a single mRNA. All of the natural prokaryotic riboswitches characterized to date have been found in the 5'-UTRs of single gene or operon transcripts. However, of more significance is the remarkable 30-fold induction of the downstream *eGFP* gene when both 2-AP and ammeline are added, roughly three times greater than with the single addA-*eGFP* construct. This may be due to ribosomal read-through resulting in a more efficient initiation of translation at the *eGFP* RBS. In addition, whilst it has yet to be experimentally verified, it has been suggested that riboswitches which control gene expression through changing the accessibility of the RBS (like addA), might also rely on Rho-dependent termination of transcription[9]. In the case of the M6''-*DsRed*-addA-*eGFP* operon when both riboswitches are activated, ribosomes will be recruited at both sites and will occupy the full length of the transcript thereby preventing Rho binding. Consequently, the levels of full-length mRNA transcripts in the cell will be higher. However, if there are no ligands, or if only ammeline is added to activate expression of the first gene (*DsRed*), then the downstream mRNA will have no ribosomes associated, allowing Rho to terminate transcription prematurely. To test this we have carried out QRT-PCR experiments to determine if mRNA levels are affected by ligands binding to the riboswitches *in vivo*. As a model system we studied expression of addA-*eGFP* under control of the *lac* promoter. QRT-PCR clearly shows that addA-*eGFP* mRNA levels increase 5-fold when IPTG is added, and 10-fold when both IPTG and 2-AP are added. This demonstrates that binding of 2-AP to addA, and the subsequent recruitment of ribosomes, results in higher mRNA levels. To test if this effect is a consequence of Rho-dependent termination we are currently repeating these experi-

ments with bicyclomycin, a known inhibitor of Rho in *E. coli* [16]. Understanding how orthogonal riboswitches can control expression of operons is potentially of considerable value for synthetic biology and metabolic engineering applications. Moreover, the significant amplification of gene induction achieved when two riboswitches are present in the same mRNA, could be exploited in the development of more effective tandem riboswitches for digital control of gene expression.

In addition, to the orthogonal *add* A-riboswitches which activate translation in response to specific ligands, we have also engineered orthogonal $preQ_1$ riboswitches that can repress gene expression in response to synthetic ligands. The $preQ_1$ riboswitch, from the *queC* gene of *Bacillus subtilis*, forms an antiterminator which is disrupted upon binding of 7-aminomethyl-7-deazaguanine ($preQ_1$), resulting in a terminator stem-loop that causes the RNA polymerase to dissociate from the DNA template. The $preQ_1$ riboswitch is particularly attractive for re-engineering because it is remarkably simple, possessing one of the smallest aptamer domains (34 nt) discovered to date. Accordingly, we have mutated the $preQ_1$ riboswitch aptamer domain and identified various orthogonal riboswitches which no longer respond to the endogenous $preQ_1$, but can repress gene expression in response to synthetic $preQ_1$ analogues. Orthogonal riboswitches that can repress gene expression in response to small molecule effectors are particularly attractive for establishing conditional mutants in bacteria and for other applications. For example, in generating conditional mutants, it is desirable for the essential gene of interest to be expressed at first, to allow growth to be established, and then downregulated at the desired time point upon the addition of an effector molecule.

In summary, we have successfully re-engineered the first orthogonal riboswitches, which no longer respond to the natural cellular ligands, and which can be controlled in a precise dose-dependent fashion by a variety of synthetic ligands not present naturally within the cells. Mutually orthogonal riboswitches have been developed that can activate or repress gene expression, and tandem arrangements of orthogonal riboswitches can be used to affect the differential and simultaneous control of expression for multiple genes within the same cell. As such, these new genetic switches could be used in gene functional analysis, protein expression, synthetic biology, metabolic engineering and many other applications. Moreover, the novel approach we have developed could be applied to re-engineer the many other riboswitches, which can be found across all domains of life.

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FUNCTIONALIZED DNA ARCHITECTURES: FLUOROPHORE ASSEMBLIES AND NANOSTRUCTURES

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Nucleic acids have been emerging as a supramolecular structural scaffold for the helical organization of chromophores in the creation of functional nanomaterials mainly because of the unique structural features and the synthetic accessibility. A large number of chromophores have been successfully incorporated into DNA or RNA as base modifications, as base surrogates or as modified sugars using the solid phase phosphoramidite chemistry. Moreover, multiple incorporations yield the helical organization of the chromophores inside or outside the DNA or RNA double helix depending upon the conjugation of the chromophores. Significant photophysical interactions are observed in the chromophore stacks resulting in unique optical properties that are significantly different from the monomer properties. Carbon nanotubes and upconversion nanoparticles represent additional tools to create self-assembled DNA architectures with optical functionalization.

The central problem for advances in bio- and nanotechnology is the miniaturization that drives the search for application of molecular wires and devices on the nanometer scale. In principle, organic nanomaterials can be realized by using the so-called bottom-up approach in which the system is composed of small synthetic building blocks with recognition, structuring, and most importantly, self-assembling properties. A clear structural scaffold is required for such molecular functional π-systems since the properties depend on the relative orientation and the resulting photophysical interactions of each of the molecular components to the others. For this approach, oligonucleotides play an especially important role since the use of duplex DNA-like architectures provide a structural scaffold with a unique combination of properties[1,2]:

(i) Self-assembly: Two oligonucleotides spontaneously organize themselves into a duplex structure as encoded by the DNA base sequence.

(ii) Regular helical structure as a predictable topology. In B-DNA, the base-pair distance along the helical axis is 3.4 Å providing the ideal basis for photophysical interactions.

(iii) Synthetic accessibility: Automated oligonucleotide chemistry makes DNA available in any desired base sequence and also in sufficient quantities.

(iv) Functional bioorganic π-system: A building block strategy can be applied for synthetic functionalisation on the way to new DNA-inspired structures.

(v) Molecular recognition Based on the DNA-helix as a regular secondary structure, sequence-specific recognition by DNA-binding proteins or other molecules is possible.

(vi) Hierarchically structured organisation: Even complex tertiary structures can be envisioned, e.g. four-way junctions or complex 2D and 3D structures.

The generation of defined molecular structures based on functionalized nucleic acids is a research topic of increasing interest with important applications in nanobiotechnology

The helical organization of at least three chromophores along the DNA backbone which are covalently attached to the nucleic acid can be achieved by 1) as base surrogates, 2) as modified base or 3) as modified sugar moieties. The subsequent self-assembly induces modulation of the optical properties as a result of the electronic interactions within the stacked chromophore arrays.

1) The first alternative to create applicable optical properties is to attach organic chromophores covalently to DNA bases (Fig. 1). Over the last years we attached synthetically several organic chromophores benzpyrene[3], BODIPY [4], ethynyl nile red[5], ethynylpyrene[6], phenothiazine[7], pyrene[8] and to the natural DNA bases.

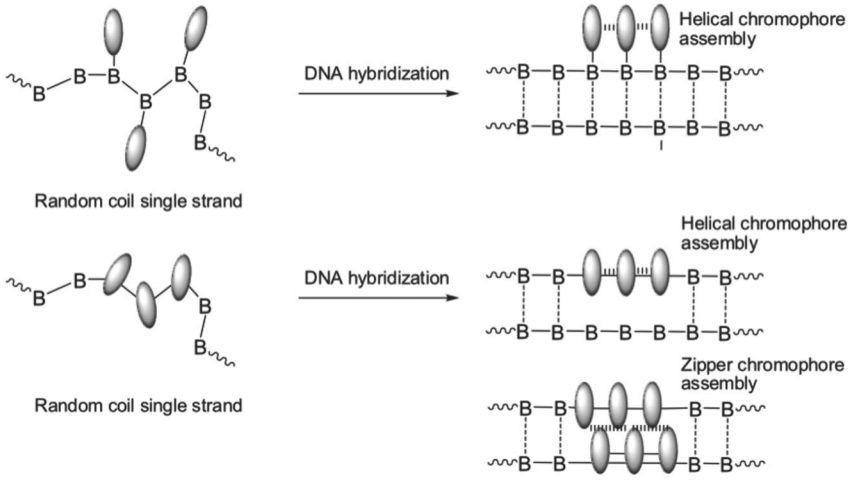

FIG. 1
Fluorophores assembled as DNA base modifications (top) or DNA base substitutions (bottom)

Non-covalent assembly of ethynyl nile red modified 2-deoxyuridine results in the formation of optically active vesicles in non-polar media through left-handed helically twisted packing of the dye whereas the stacking of several ethnynyl nile red-modified uridines as DNA bases covalently incorporated into oligonucleotides yield a right-handed helically twisted structure[9]. The potentiality of DNA scaffold to assemble a suitable donor-acceptor couple on DNA backbone became obvious by combination of ethynylpyrene and ethynyl nile red as base modifications yielding a white light emitting DNA[10]. Moreover, the temperature controlled self-assembled association and dissociation of two oligonucleotides into the double strand and back into the single strands yields fully reversible emission color change by modulating the FRET efficiencies. Molecular beacons with two fluorophores derived from ethynyl pyrene and ethynyl nile red exhibit red emission in the hairpin that changes to blue through white upon binding to the target sequence with a dramatic shift of ca. 225 nm [11]. The chromophores pyrene and bordipyrromethenylbenzene directly linked to the 5-position of uridine are tolerated and recognized as thymine derivatives by DNA polymerases in primer extension experiments[12] KF- is also able to bypass the modification site during further elongation.

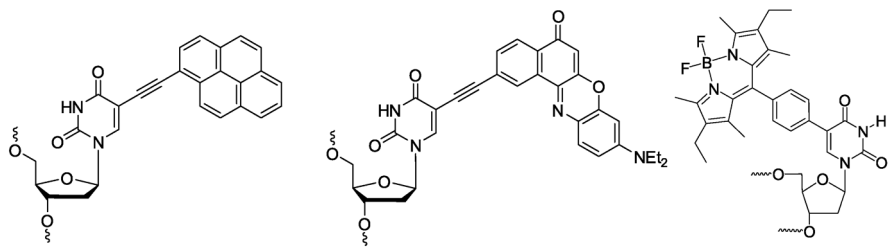

FIG. 2
Examples of DNA base substitutions

2) Alternatively, fluorophores can be incorporated as artificial DNA bases substituting natural bases. This approach has the advantage that the organic chromophore is intercalated in the DNA base stack which is a requirement for e.g. an efficient electronic coupling during photoinduced DNA-mediated charge transfer processes. We applied this modification strategy for the incorporation of ethidium[13], indole[14], perylene bismide[15], phenothiazine[16], spiropyrane[17], thiazole orange and thiazole red[18]. The synthetic protocols for this kind of DNA modifications do not follow a principle strategy which can be applied in a versatile fashion, as it is the case for the DNA base modifications. It is important to point out, that in all cases it

turned out to be useful to replace the 2′-deoxyribose moiety by acyclic linker systems. Avoiding the labile glycosidic bond, the 1-amino-2,3-propandiole linker provides the necessary chemical stability for the preparation of chromophore-DNA conjugates via automated phosphoramidite chemistry and for biophysical and bioanalytical applications in aqueous buffers.

The perylene bisimide dye can aggregate in an interstrand fashion inside the duplex to form zipper motivs[19] and in and intrastrand fashion to detect single base mismatches by fluorescence and absorption readout[20]. The chromophores of thiazole orange (TO) and its derivative TO3 were incorporated synthetically as base surrogates into oligonucleotides using automated phosphoramidite chemistry[18]. The photophysical interaction of two TO chromophores as artificial DNA bases alter the the optical properties significantly[21]. The interstrand TO dimers exhibit strong excitonic interactions and red-shifted excimer-type emission. Thus, the interstrand TO dimer could be regarded as a hydrophobically and diagonally interacting base pair that stabilizes the duplex and shows a clear fluorescence readout signal for DNA hybridization. The large "Stokes'-shift" of this TO pairs of nearly 100 nm together with a brightness that is comparable to a single TO label in DNA make the TO pair a powerful fluorescent label for for imaging.

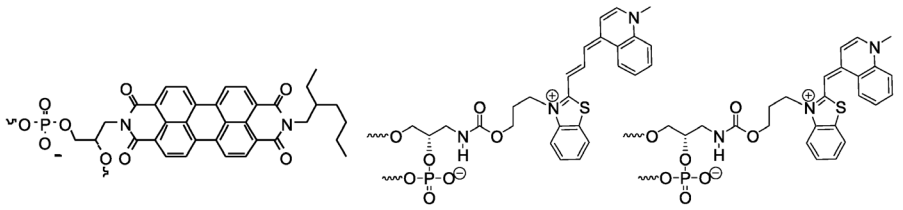

FIG. 3
Examples of DNA base substitutions

Interstrand TO dimers in RNA show a yellow colored emission that can be distinguished from the green TO monomer emission by confocal microscopy inside CHO cells[22]. In comparison to TO, the TO3 chromophore contains an extended carbomethine bridge that shifts the absorption and emission significantly to the red. TO and TO3 as fluorescent DNA base substitutions show a brightness that is sufficient for bioanalytic and imaging applications. Moreover, TO and TO3 can be combined to an interstrand chromophore pair and a DNA hybridization-dependent energy transfer process can be obtained between the modifications[18]. As a result, the emission is shifted from the TO-typical value of 530 nm to 670 nm. This concept can be applied for fluorescence cell imaging.

Finally, the photochromic spirobenzopyran was incorporated as an internal modification into oligonucleotides by two different synthetic strategies, which are phosphoramidite chemistry and postsynthetic "click"-type ligation[17]. However, photoinduced ring opening of the chromophore could not be achieved in duplex DNA.

3) Alternatively to the above mentioned strategies, chromophore and upconversion nanoparticles can be attached to the 2′-position via postsynthetic click chemistry[23]. Especially fluorescent coumarin dyes with large Stokes shifts can be combined to FRET pairs that shift the emission by ca. 250 nm into the far-red/near-infrared regime[24]. Synthetic GFP-like chromophores were incorporated by "click"-type chemistry into oligunucleotides[25]. It became evident that DNA is able to provide an artificial environment for the GFP chromophore that controls the photophysical property in such a way that nearly solely the ESPT-driven, red-shifted fluorescence is occurring. The apparent Stokes' shift is more than 200 nm. The combination of ESPT-controlled fluorescence with large apparent Stokes' shifts and increase of intensity by restricting of internal conversion, provides an important concept for the design of fluorescent labels for DNA and RNA.

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NEW FLUORESCENT NUCLEOSIDES

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Nucleic acids experience diverse perturbations while performing their cellular functions. Because the native nucleobases are practically non-emissive, such events are spectroscopically transparent and cannot be explored by sensitive fluorescence-based techniques. When judiciously designed and incorporated, synthetic emissive nucleosides analogues, which are responsive to changes in their local environment, can become powerful tools for interrogating nucleic acid structure, dynamics, recognition and damage. We have articulated and implemented several design criteria for "ideal" fluorescent nucleoside analogues. These include: (a) structural similarity to the native common nucleobases to closely mimic their size and shape, as well as hybridization and recognition properties, (b) red shifted absorption maxima, (ideally >300 nm) to minimize overlap with the absorption band of the natural bases, (c) red shifted emission maxima (preferably >400 nm), (d) practically useful emission quantum yields, and, importantly, (e) responsiveness of their photophysical parameters (λ_{em} and/or ϕ_F, τ) to changes in the microenvironment. The first generation of emissive analogues relied on conjugating aromatic five-membered rings to the 5-position of the pyrimidines. These nucleosides were incorporated into oligonucleotides enzymatically or through standard solid phase protocols. Despite their modest quantum yields, these nucleoside analogues display visible fluorescence, relatively large Stokes shifts and are highly responsive. Newer emissive and responsive nucleosides rely on fused pyrimidines and quinazoline based nucleobases. Their design, synthesis, properties and utility in biophysical and discovery assays are described.

INTRODUCTION

Nucleic acids play diverse and critical roles in cellular pathways and are valuable targets for therapeutic intervention. Low MW ligands, which specifically bind unique RNA or DNA sites and prevent the formation of functional folds or nucleic acid-protein complexes, can alter cell functions and dictate cells' fate. Such ligands can also turn out to be lead structures for future therapeutics. Nucleic acids are also subjected to environmental and cellular assaults, which can generate transient or permanent damage. To interrogate such recognition and detrimental events, effective assays need to be developed. Fluorescence spectroscopy, due to its high sensitivity and relatively simple instrumentation, is a natural choice for implementation in such biophysical and discovery assays. The poor emission characteristics of the native nucleosides, however, present a major obstacle.

The common building blocks of our genetic material contain aromatic nucleobases capable of efficiently dissipating their electronic excitation energy. Cytosine (C), thymine (T), uracil (U), Adenine (A), and guanine (G), all possess excited state lifetimes on the picoseconds time scale[1]. Being capable of such efficient non-radiative decay pathways, these native nucleobases possess extremely low fluorescence quantum yields of 0.03% or less[1,2]. This renders these nucleosides non-emissive for any practical purpose. The major challenge in this field is, therefore, to design nucleoside analogs with "optimal" photophysical characteristics while maintaining high structural homology to the native bases[2].

The following represents our criteria for the ideal features of fluorescent nucleoside analogues: (a) high structural similarity to the native nucleobases to closely mimic their size and shape, retain their H-bonding face and minimally perturb their hybridization and recognition properties, (b) red shifted absorption maxima (ideally >300 nm) to minimize overlap with the absorption of the natural bases ($\lambda_{abs} \approx 260$ nm), (c) red shifted emission maxima (preferably >400 nm), (d) practically useful emission quantum yields (>0.02), and, importantly, (e) responsiveness of the photophysical parameters (λ_{em} and/or ϕ_F, τ) to changes in the microenvironment. Several motifs that satisfy these criteria have been explored over the past several years[2]. Their design, properties, and utility in diverse biophysical and discovery assays are described.

DESIGN OF EMISSIVE ISOMORPHIC PYRIMIDINES

To preserve the H-bonding face of the natural pyrimidines and to best maintain the overall dimensions of the base pair they may form, three primary scaffolds for emissive pyrimidines were explored. Motif I relies on conjugating five-membered aromatic heterocycles to position 5 in the pyrimidines[3]. Motif II is based on fusing related heterocycles along the

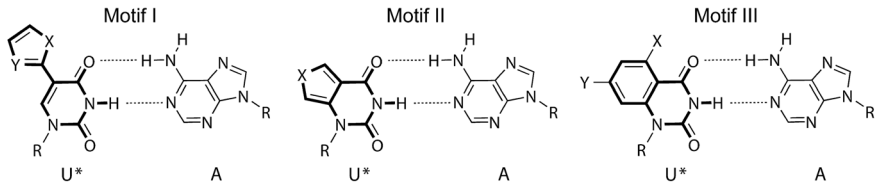

FIG. 1
Watson–Crick base pairing of modified uridines (generically labeled as U*) and A, showing the three different motifs (X = O, S; Y = CH, N). R stands for D-ribose and 2′-deoxy-D-ribose

5,6 bond and motif III is based on substituted quinazoline-2,4(1H,3H)-diones (Fig. 1). Figure 2 shows selected examples.

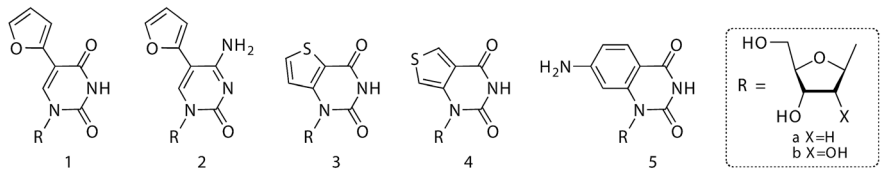

FIG. 2
Selected emissive nucleosides

Inspiration for the 5-modified and the 5,6-fused pyrimidines (motifs I and II, respectively) comes from the favorable photophysical properties of the parent conjugated heterocycles, where 2-phenylfuran and benzofuran are known to be much more emissive than their individual aromatic constituents[4,5]. The design of the quinazoline-based motif III has been inspired by emissive proteins, which contain highly polarizable chromophores. The firefly luciferase-mediated bioluminescence represents a well-established example, where a single substrate, luciferin, is responsible for different emission wavelengths, depending on the structure and composition of the surrounding protein fold (Fig. 3)[6]. While the exact nature of the excited state is still being investigated, it is evident that the high polarizability of this conjugated system contributes to its complex photophysical behavior and multi-color emission[6–8]. Nature exploits related molecular approaches in other luminescent systems, such as the various fluorescent proteins (FPs), where an intramolecular condensation reaction of three neighboring amino acids leads to highly delocalized charge transfer chromophores (Fig. 3)[9,10].

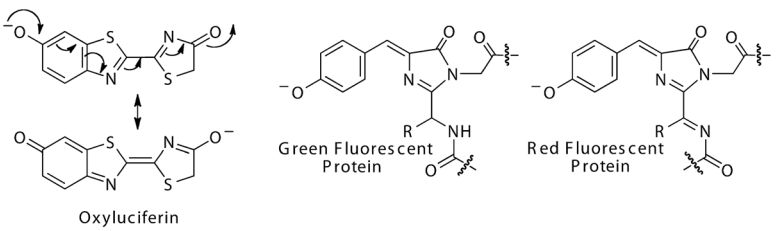

FIG. 3
Highly emissive and environmentally-sensitive naturally occurring chromophores. Shown are oxyluciferin, the emissive chromophore in luciferase-mediated bioluminescence, and the conjugated chromophores in GFP and related proteins

Enhancing the polarization of the pyrimidine nucleus can, in principle, be achieved by substitution with electron donating functionalities as shown for **6** (Fig. 4). Such nucleosides are not, however, effective fluorophores. Conjugating an additional aromatic ring as shown for **7** and **8** can extend the polarization axis. Substitution with electron donating substituents can give rise to charge transfer transitions (Fig. 4). Such derivatives are emissive and responsive[11].

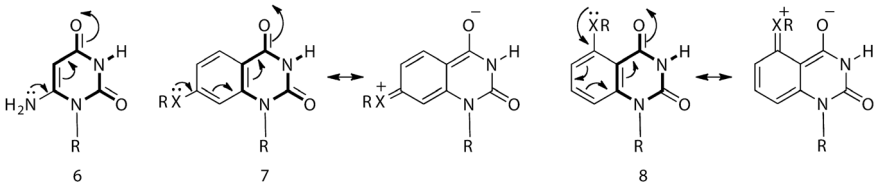

FIG. 4
Enhancing the polarizability of a pyrimidine (**6**) and quinazoline-based chromophores **7** and **8**. R as in Fig. 2

PHOTOPHYSICAL CHARACTERISTICS OF EMISSIVE PYRIMIDINES

To evaluate the promise of new emissive nucleosides as responsive probes, the impact of solvent polarity on their absorption and emission spectra is determined[4,12]. For most nucleosides illustrated above solvent polarity has little influence on the absorption maxima, while the emission maxima and intensity are significantly affected. Figure 5A shows the correlation between Stokes shifts and $E_T(30)$, a microscopic solvent polarity parameter, for

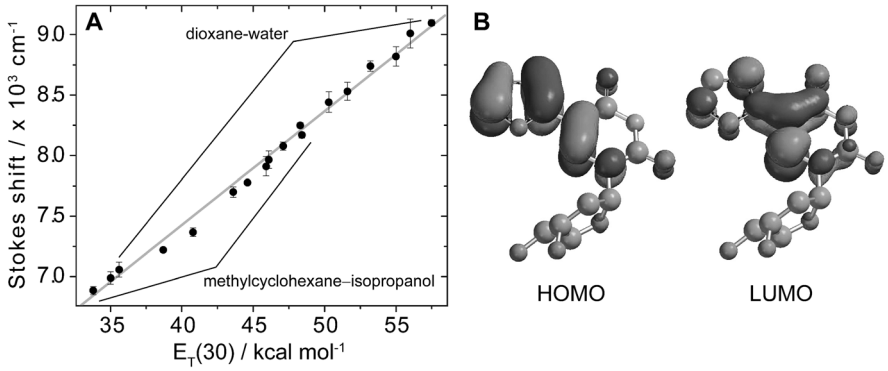

FIG. 5
A) Correlating changes in Stokes shift of **1a** vs $E_T(30)$, a microscopic solvent polarity scale, as determined in two different solvent mixtures. B) HOMO and LUMO representations of **1a**

nucleoside **1a** (correlations with dielectric constants, representing bulk parameters, tend to be much poorer)[13]. The relatively large Stokes shift in polar environment (9×10^3 cm^{-1} in water) suggests a higher charge-transfer character of the excited state compared to the ground state. This is reproduced by a simple calculation of the ground and excited states (Fig. 5B). Although different nucleosides exhibit rather distinct Stokes shifts, most display similar sensitivity with significant red shifts upon increasing solvent polarity, as illustrated in Fig. 6 for **2a**, **4b** and **5b**, emissive nucleosides that represent the three motifs.

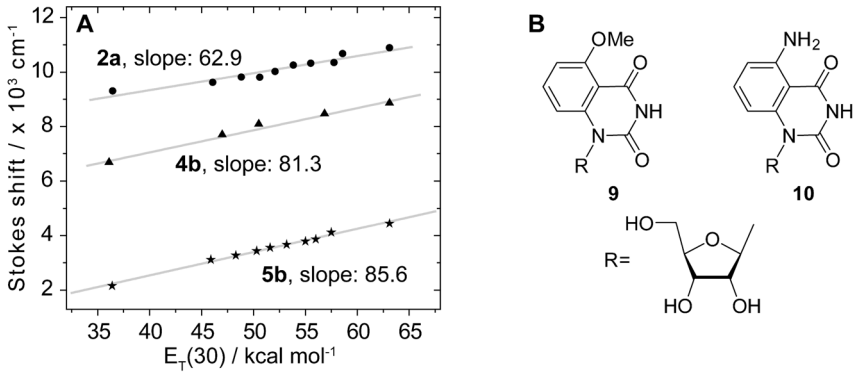

FIG. 6
A) Stokes shift responsiveness of **2a**, **4b**, and **5b** to solvent polarity. B) Examples of quinazoline derivatives **9** and **10** that were used in FRET-based assays

APPLICATIONS OF EMISSIVE PYRIMIDINES

The development of fluorescent nucleosides is propelled by the need for tools to probe nucleic acid structure and dynamics and as well as rapid and sensitive assays for nucleic acids damage and recognition. Such assays have academic utility where they facilitate, simplify and accelerate the accumulation of data by providing a window into otherwise spectroscopically-silent events and by generating data in real time. In the pharmaceutical industry, fluorescence-based assays are essential for high throughput screening protocols. The nucleosides developed in our laboratory have been primarily implemented in biophysical assays, including:

a) Nucleoside **1a** (λ_{em} = 431 nm, Φ_F = 0.03), when incorporated into a reporter oligonucleotide, positively signals the presence of abasic sites in DNA upon hybridization[3].

b) The corresponding ribonucleoside **1b** (λ_{em} = 440 nm, Φ_F = 0.035) can be used in antibiotic discovery assays[14].

c) A minimally disruptive fluorescent dC analogue **2a** (λ_{em} = 443 nm, Φ_F = 0.02) provides a rapid and non-destructive method for in vitro detection of G, 8-oxoG (a marker for cellular oxidative stress) and T, the downstream transverse mutation product of G oxidation[15].

d) RNA constructs, which contain the highly emissive fused pyrimidine **4b** (λ_{em} = 412 nm, Φ_F = 0.48) and are complementary to the α-sarcin/ricin RNA loop, signal the depurination by ribosome inactivating proteins (RIP, e.g., ricin and saporin) with enhanced emission intensity[16]. This technique facilitates the detection of RIP activity, and could be useful in identifying RIP inhibitors.

e) Nucleoside **9** (λ_{em} = 395 nm, Φ_F = 0.16) serves as a donor in a FRET-based system for the analysis and discovery of antibiotics targeting the bacterial A-site (Fig. 7)[11]. This assay has been extended to provide a three-fluorophore FRET assembly that allows us to determine the selectivity of

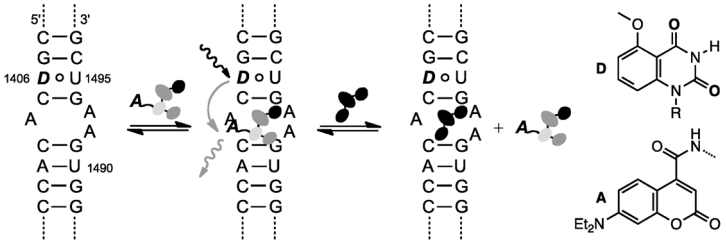

FIG. 7
By replacing U1406 in the A-site with an isosteric emissive donor (D = **9**) and tagging an antibiotic with an acceptor (A), binding and displacement events can be monitored using FRET

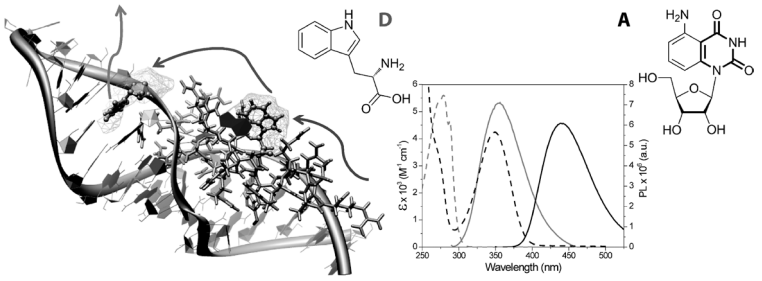

FIG. 8
A fluorescent ribonucleoside analogue (A) acts as a FRET acceptor for tryptophan (D) and displays visible emission (440 nm). Shown is the binding of a Rev peptide to the labeled HIV-1 RRE (left)

new antibiotics toward prokaryotic and eukaryotic ribosomal RNA in a single experiment[17].

f) Nucleoside **10** (λ_{em} = 440 nm, Φ_F = 0.42), containing 5-aminoquinazoline-2,4(1H,3H)-dione, acts as a FRET energy transfer acceptor for tryptophan and displays visible emission centered at 440 nm. As Trp is frequently found at or near the recognition domains of RNA binding proteins, this FRET pair facilitates, for the first time, the study of RNA binding to native (unlabeled) proteins and peptides (Fig. 8)[18].

This work was supported by the National Institutes of Health (GM 069773).

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DNA AS SCAFFOLD FOR NEW BIO-INSPIRED CATALYTIC SYSTEMS

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DNA-based catalysis is a powerful new concept in catalysis. It involves binding an achiral transition metal complex to the DNA and using the chiral environment provided by the DNA to catalyze a reaction in an enantioselective fashion. A mechanistic investigation has revealed that in case of the most enantioselective catalyst, based on the ligand 4,4′-dimethyl-2,2′-bipyridine, the role of the DNA two-fold: it provides the chirality for the reaction and it participates actively, resulting in rate accelerations up to two orders of magnitude. Recently, this novel concept was extended to the first catalytic enantioselective *syn*-hydration of enones; a reaction for which there is no equivalent in conventional asymmetric catalysis.

INTRODUCTION

DNA has emerged as an attractive scaffold for new bio-inspired catalytic systems due to its characteristic chiral double helix structure and the simple rules of Watson–Crick base pairing, which allows the programmable construction of almost any desired molecular architecture. Catalytic DNA, in the form of Deoxyribozymes (DNAzymes), has been known for >15 years and has found many applications, particularly in the area of sensing[1]. Applications in organic synthesis, however, are virtually inexistent, which is the result of the fact that the available catalytic scope is almost completely restricted to nucleotide chemistry. For this reason, our research has focused on DNA structures containing an extended catalytic functionality, such as a transition metal complex[2]. By placing this catalytic moiety in close proximity of the chiral DNA, a catalyzed reaction can be directed towards selective formation of one of the mirror image forms of a product.

Two general approaches to the construction of such "hybrid" catalysts[3] are available, which differ by the mode of attachment of the catalytic moiety to the DNA. The supramolecular approach relies on the ability of small molecules to bind to DNA via intercalation and/or groove binding. Hence, a catalytically active transition metal complex containing such a DNA-binding moiety in its ligand structure, will spontaneously bind to the DNA.

Alternatively, the catalytic moiety can be linked to the DNA via a covalent linkage. The advantage is that this allows for precise control over het positioning of the catalyst in the DNA. However, this approach is synthe-

tically more involved and thus complicates the design and optimization process.

RESULTS AND DISCUSSION

Two generations of supramolecular DNA-based catalysts have been developed to date. These differ in the general structure of the ligand for the catalytically active transition metal ion, which to date has generally been Cu(II). In the first generation of ligands the metal binding domain is attached to the DNA-binding domain via a small spacer. The aminomethyl pyridine is used for metal binding and 9-aminoacridine, which is a well known intercalator, is the DNA binding moiety[4]. The second generation ligands are based on the 2,2′-bipyridine structure, which combines the DNA and metal binding moieties in one structural unit[5]. Representative members of both classes are shown in Fig. 1.

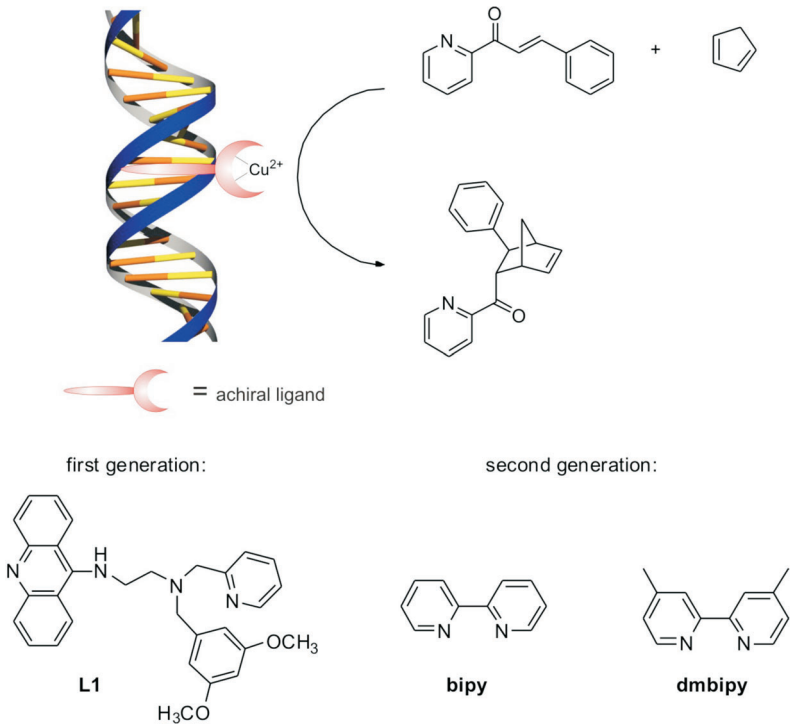

FIG. 1
DNA-based catalytic enantioselective Diels–Alder reaction of azachalcone with cyclopentadiene and representative structures of first and second generation ligands

The DNA-based catalysts is self-assembled by combining the corresponding Cu(II) complexes of these ligands with DNA, usually salmon testes DNA (st-DNA).

The Diels–Alder reaction of azachalcone with cyclopentadiene has been used as the benchmark model reaction for the study of DNA-based asymmetric catalysis. This reaction gives rise to the formation of both the *endo* and *exo* isomers of the Diels–Alder product. Since the endo/exo ratio generally exceeds 90:10, only the results for the endo isomer will be discussed.

Using the first generation ligands, up to 49% enantiomeric excess (*ee*) was observed for the Diels–Alder product[4]. Interestingly, by changing the design of the ligand, for example by changing the spacer length, the opposite enantiomer of the product was obtained in excess. This is an attractive feature of this system, since natural DNA is available in one enantiomeric form only.

With the second generation ligands, the *ee* was improved to 90% in case of 2,2′-bipyridine (bipy) and an impressive 99% *ee* with 4,4′-dimethyl-2,2′-bipyridine (dmbipy)[5]. These results were quite surprising since the binding affinity of Cu(II)-dmbipy to DNA is only moderate ($K_b \sim 10^4$ M^{-1}). The result is that at the concentrations of the catalysis experiments, not all of the Cu(II) complex is bound to DNA; 5–10% is free in solution and, hence, can catalyze the reaction in a racemic fashion. Still 99% *ee* is obtained. Moreover, Cu(II)-dmbipy does not display sequence selectivity in its binding to DNA, which means that "the DNA-based catalyst" is actually a heterogeneous mixture of Cu(II) complexes that bind at different locations of the DNA and, hence, provide a different microenvironment for the reaction.

For this reason, a kinetic and DNA sequence dependence study of DNA-based asymmetric catalysis was initiated[6,7]. The key results of the kinetic study are summarized in Table I. Using the first generation ligands, a slight deceleration of the Diels–Alder was observed in the presence of DNA. Using bipy as the ligand, a small increase in the apparent second order rate constant (k_{app}) was found. Surpringly, with dmbipy a 58 fold rate acceleration was observed in the presence of DNA. Thus, in this case the DNA is not only the chiral scaffold, but also participates actively in the reaction, resulting in a large rate acceleration. Therefore this reaction was studied in more detail. The mechanism of this Diels–Alder reaction involves reversible binding of the azachalcone to the Cu(II) ion, followed by the irreversible Diels–Alder reaction and, finally, dissociation of the product. The binding of the azachalcone to the DNA was not affected by the presence of DNA: similar K_a values were found in the absence and presence of DNA. Instead, it is

the k_{cat} of the reaction that is increased by two orders of magnitude. Hence, the effect of DNA is truly kinetic in nature.

The effect of DNA sequence was studied by using a variety of synthetic self-complementary oligonucleotides as scaffold. From the results in Table II it is clear that catalysis of both the first and second generation cata-

TABLE I
Kinetic parameters of the catalyzed Diels–Alder reaction in the presence and absence of DNA

	Cu(II)-L1[a]	Cu(II)-bipy[b]	Cu(II)-dmbipy[b]
k_{app} w/o DNA (M^{-1} s^{-1})	0.031	0.0092	0.0069
k_{app} with DNA (M^{-1} s^{-1})	0.022	0.026	0.40
K_a w/o DNA (M^{-1})	1.2×10^4		4.0×10^2
K_a with DNA (M^{-1})	8.2×10^3		5.0×10^2
k_{cat} w/o DNA (M^{-1} s^{-1})	0.11		4.5×10^{-2}
k_{cat} with DNA (M^{-1} s^{-1})	0.10		3.8

[a] Data taken from ref.[7]; [b] Data taken from ref.[6]

TABLE II
DNA sequence dependence of the *ee* and kinetics of the DNA-based catalytic Diels–Alder reaction

DNA	Cu(II)-L1[a]	Cu(II)-dmbipy[b]	
	ee, %	ee, %	k_{app}, M^{-1} s^{-1}
no DNA	–	–	5.1×10^{-3}
st-DNA	37 (+)	98.5 (+)	2.4×10^{-1}
poly(dA-dT)	6 (+)	15 (–)	n.d.
poly(dG-dC)	62 (+)	78 (+)	n.d.
$d(GCGCGCGCGCGC)_2$	54 (+)	95 (+)	6.5×10^{-2}
d(GCGCGCGC)	27 (+)	86 (+)	3.0×10^{-2}
$d(GACTGACTAGTCAGTC)_2$	34 (+)	78 (+)	4.4×10^{-2}
$d(TCGGGTACCCGA)_2$	16 (+)	98.6 (+)	2.9×10^{-1}
$d(TCAGGGCCCTGA)_2$	10 (+)	99.4 (+)	5.0×10^{-1}

[a] Data taken from ref.[7]; [b] Data taken from ref.[6]

lysts is very dependent on DNA sequence. Moreover, both classes of catalysts also have different DNA sequence requirements.

The highest *ee*'s with the first generation catalysts were obtained with DNA containing alternating GC nucleotides, such as poly d(GC). In contrast, only low *ee*'s were obtained with AT rich sequences.

Also in the case of Cu(II)-dmbipy, AT rich sequences give significantly lower *ee*'s. However, also DNA sequences containing alternating GC nucleotides gave significantly lower *ee*'s compared to st-DNA. Instead, the highest enantioselectivities were obtained with oligonucleotides containing tracts of 3 G's: up to >99% *ee*. These results raised an important question: since Cu(II)-dmbipy does not display a sequence selectivity in DNA binding, the results obtained with st-DNA cannot simply be the average all contributing DNA sequences. For this reason the kinetics of the Diels–Alder reaction was determined with all the synthetic oligonucleotides. Indeed, it was found that also the kinetics of the catalyzed reaction was depended on DNA sequence. Moreover, by plotting the k_{app} as a function of the *ee* of the reaction, it was found that there is a clear trend, with the DNA sequences that are responsible for the highest enantioselectivities also giving rise to the largest rate acceleration. When plotting this graph in a different fashion,

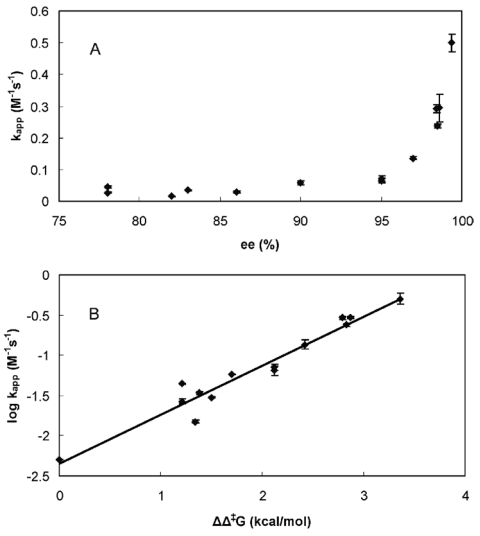

FIG. 2

(A) Relation between the k_{app} and the *ee* obtained with different oligonucleotides. (B) Relation between log k_{app} and the $\Delta\Delta G^{\ddagger}$. Reprinted with permission from ref.[6], Copyright American Chemical Society.

i.e. the log k_{app} against the difference in activation energy $\Delta\Delta G^{\ddagger}$ going to either enantiomer of the product, a linear correlation was observed.

These results combined explain why DNA-based asymmetric catalysis using Cu(II)-dmbipy/st-DNA gives rise to such excellent enantioselectivities: the low binding affinity of Cu(II)-dmbipy is no problem since the contribution of unbound Cu(II)-dmbipy to the overall reaction is negligible since the reaction is DNA accelerated and the heterogeneous nature of the catalyst is no problem since the Cu(II) complex that are bound to the DNA sequence that gives the highest *ee*, also cause the largest rate accelerations and, hence, dominate the overall outcome of the reaction.

This raises the question what is so special about these G tracts sequences. A CD spectroscopic study of several of the duplexes tested revealed some interesting trends. The sequences giving the highest *ee* gave rise to a CD spectrum that is associated with a DNA structured that is distorted from B-DNA; the structure is intermediate between B and A DNA. Apparently, in these duplexes the structure of the DNA grooves provides the optimal microenvironment for the reaction, resulting in high *ee*'s and rate accelerations.

The structure of the microenvironment in which the catalyzed reaction occurs is also proposed to be the reason for the observed differences between the first and second generation ligands. In the first generation ligands, the DNA binding moiety is connected to the metal binding moiety via a spacer. As a result the catalysis most likely takes place at the edge of the DNA structure, thus more resembling the catalytic reaction free in solution. Thus, the role of DNA in this case is strictly that of chiral scaffold and the chirality is most likely transferred to the catalyzed reaction in two steps: the DNA forces a chiral structure on the Cu(II) complex, which in turn is translated in preferential formation of one of the enantiomers of the product. In contrast, with the second generation DNA-based catalysts, the reaction is proposed to occur within the DNA groove, thereby taking full advantage of the second coordination sphere interactions, e.g. hydrogen bonding, hydrophobic effects, that the DNA has to offer. Hence the DNA participates actively, either by stabilizing the transition state of the reaction or destabilizing the ground state, resulting in high enantioselectivities and rate accelerations.

The Cu(II)-dmbipy/DNA catalysts has since been employed successfully a variety of catalytic enantioselective reactions, including Michael addition[8], Friedel–Crafts alkylation[9] and electrophilic fluorination reactions[10]. In all cases good to excellent *ee*'s have been obtained with multiple substrates.

Only recently a reaction was discovered in which the first generation catalysts gives superior results compared to the second generation, *i.e.* the

enantioselective *syn*-hydration of enones[11]. This reaction involves the conjugate addition of H_2O to an α,β-unsaturated 2-acyl(1-methyl)imidazole substrate, resulting in the corresponding β-hydroxyketone product with up to 72% *ee,* which could be further improved to 82% by performing the reaction in D_2O and using a specific DNA sequence. Moreover, by deuterium labeling the *syn*-diastereospecificity of the reaction was demonstrated using NMR spectroscopy. This is a unique reaction: there is no equivalent in conventional asymmetric catalysis. This is mainly due to the reversibility of the reaction and the fact that H_2O at neutral pH is a poor nucleophile. It is proposed that the DNA-based catalysts, analogous to hydratase enzymes, directs the H_2O nucleophile to one prochiral face of the enone substrate by using the second coordination sphere interactions provided by the biomolecular scaffold. The current hypothesis is that the spine of hydration in the DNA groove, which is proposed to be relatively structured, plays a key role in the asymmetric hydration process.

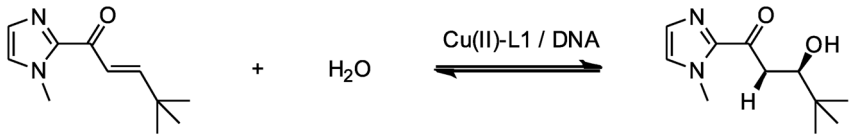

FIG. 3
DNA-based catalytic enantioselective *syn*-hydration of enones

In conclusion, DNA-based asymmetric catalysis has emerged as a powerful new concept in catalysis. Current research is directed at novel catalytic asymmetric reactions for which there is no alternative in conventional asymmetric catalysis, in particular using H_2O as nucleophile, thereby taking full advantage of the second coordination sphere interactions provided by the DNA.

This work was supported by the Netherlands Research School Combination Catalysis (NRSC-C) and the Netherlands Organisation for Scientific Research (NWO).

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OLD BUT NEW ARTIFICIAL NUCLEIC ACIDS FROM ACYCLIC THREONINOL (*a*TNA) AND SERINOL (SNA)

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A key feature of natural DNA is its spontaneous hybridization of two strands that are complementary with each other in an antiparallel fashion. Although nature uses ribose or deoxyribose as a scaffold for nucleic acids to carry genetic codes, investigation of DNA hybridization has revealed that deoxyribose is not a prerequisite for this supramolecular property. Here, we propose two *old* but *new* artificial nucleic acids, *a*cyclic *t*hreoninol *n*ucleic *a*cid (*a*TNA) and *s*erinol *n*ucleic *a*cid (SNA) tethering four natural nucleobases via 2-amino-1,3-butanediol (threoninol) and 2-amino-1,3-propanediol (serinol) scaffolds. The ***a***TNA oligomer formed an exceptionally stable duplex with its complementary *a*TNA in an antiparallel manner that is far more stable than corresponding DNA or RNA duplexes, even though single-stranded ***a***TNA was rather flexible and thus did not take a preorganized structure. Unlike peptide nucleic acid that also possesses acyclic scaffold, this *a*TNA oligomer could not cross-hybridize with native DNA or RNA. However interestingly, the SNA that lacks methyl group of *a*TNA cross-hybridized with DNA and RNA with sufficient stability. Although the melting temperature of SNA/SNA duplex was lower than the corresponding *a*TNA duplex, it was still far higher than DNA or RNA duplex. In addition, SNA had unique stereochemical property based on its achiral scaffold: chirality of the SNA oligomer is controllable by sequence design so that the chirality of SNA can be inverted by reversing its sequence. We strongly believe that these unique properties of *a*TNA and SNA carrying acyclic scaffold might provide an insight into why D-ribose was selected as a scaffold for nucleic acids.

INTRODUCTION

Construction of an artificial double helix that mimics natural DNA or RNA has been one of the most challenging themes in chemistry[1–5]. Recently, Meggers demonstrated that the glycol nucleic acid (GNA: see Fig. 1a) bearing phosphodiester linkages comprised of artificial oligonucleotides synthesized from an acyclic propylene glycol forms a more stable duplex in an antiparallel fashion than does natural DNA[6]. This result proved that simple acyclic diols can be an excellent scaffold for new "foldamers" that can spontaneously be folded into a double-helical structure in a programmed manner[7]. Over the last decade we have developed unique base surrogates based on an acyclic compound with three carbons in its main chain

(D-threoninol, 2-amino-1,3-butanediol) to covalently incorporate various functional dyes into natural DNA[8]. These base surrogates derived from D-threoninol are compatible with natural DNA such that wedge-type insertion of the surrogates does not destabilize but fairly stabilizes the duplex. Furthermore, zipper-like stacked dyes intermolecularly bound to the D-threoninols in the double-stranded DNA remarkably stabilized the duplex as the number of surrogates increased[9,10]. High compatibility of D-threoninol with natural DNA as well as the stable GNA duplex prompted us to design a new foldamer with this more flexible acyclic scaffold.

Herein we show two artificial nucleic acids, from acyclic threoninol nucleic acid (*a*TNA) and serinol nucleic acid (SNA), prepared with threoninol and serinol as a building block that is tethered to each of the genetic nucleobases A, G, C and T, respectively (see Fig. 1)[11]. As we all know, there have been several reports of modified DNA involving one or two "thymidine" *a*TNAs or SNAs[12–15]. In this sense, *a*TNA and SNA are *old-fashioned* artificial nucleic acids. However, no one has tried to synthesize a fully changed *a*TNA or SNA oligomer[16,17]. Interestingly, we found that these *new a*TNA and SNA oligomers form an exceptionally stable duplex in an antiparallel fashion with its fully complementary strand[16,17]. In addition, SNA oligomer that has unique stereochemical property cross-hybridized with native DNA and RNA whereas *a*TNA oligomer did not[17].

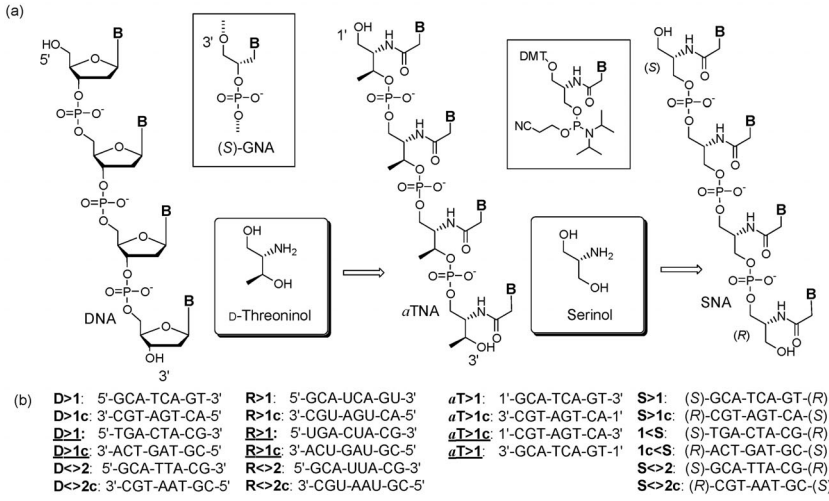

FIG. 1
Chemical structures of XNA synthesized in this study

RESULTS AND DISCUSSION

All of the *a*TNA and SNA oligomers were synthesized from the corresponding phosphoramidite monomers[16,17]. The melting profiles of *a*TNAs were first examined by analyzing the change of absorbance at 260 nm. As summarized in Table I, the 1:1 mixture of 8 mer ***a*T>1** and ***a*T>1c**, both of which are complementary to each other in an antiparallel fashion, exhibited a typical sigmoidal curve. The melting temperature (T_m) was determined to be as high as 62.7 °C, which was remarkably higher than that measured for the corresponding natural DNA (**D>1/D>1c**, 29.0 °C) and RNA duplex (**R>1/R>1c**, 38.9 °C). In contrast, neither ***a*T>1** nor ***a*T>1c** in a single-stranded state showed such a sigmoidal curve above 20 °C. Furthermore, a parallel combination of complementary ***a*T>1** and **<u>*a*T>1c</u>** also did not display sigmoidal melting. These results demonstrate that two complementary *a*TNAs could form a remarkably stable duplex in an antiparallel fashion. Similarly, **<u>*a*T>1c</u>** displayed a sigmoidal curve only with an antiparallel counterstrand of **<u>*a*T>1</u>**, and not with parallel ***a*T>1**. Introduction of mismatch lowered the T_m, indicating that canonical Watson–Crick base-pairing dominates the stable association of *a*TNAs.

TABLE I
Melting temperatures of *a*TNA, SNA, DNA, and RNA homo-duplexes

Sequence X = D, R, *a*T and S	Direction of Duplex	T_m, °C[a]			
		*a*TNA	SNA	DNA	RNA
X>1/X>1c	antiparallel	62.7	51.1	29.0	38.9
<u>X>1/X>1c</u>	antiparallel	58.1	51.2	23.1	37.9
X>1/<u>X>1c</u>	parallel	<10	15.9	<10	<10
<u>X>1</u>/X>1c	parallel	<10	15.9	<10	<10
X<>2/X<>2c	antiparallel	–[b]	47.0	28.1	34.3

[a] [SNA] = [DNA] = [RNA] = 2.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer). [b] Not measured.

Next, the *a*TNA duplex was analyzed by circular dichroism (CD). As shown in Fig. 2a, hybridization of ***a*T>1** and ***a*T>1c** below its T_m induced a symmetrical positive-negative Cotton effect similar to that seen for the typical B-type **D>1/D>1c** duplex (compare Fig. 2b with 2a), demonstrating that hybridization of *a*TNA allowed formation of a right-handed helix. The

induced CD decreased with increasing temperature, however, and at 80 °C where the duplex was completely dissociated, it became very weak (see dotted line in Fig. 2a). CD analysis of the single-stranded ***a*T>1** and ***a*T>1c** revealed that the shapes of their CD spectra in the 200–300 nm region were entirely different from that of the duplex. Although they are chiral oligomers, the CD signal of each individual strand was very small at any temperature examined here, indicating that single-stranded *a*TNA did not take a particularly pre-organized structure but existed as a random coil[16]. Nevertheless, hybridization of these strands induced formation of a right-handed double-helical structure. We also examined cross-pairing of *a*TNA with DNA or RNA. But we could not observe clear sigmoidal curves attributable to hybridization. We hypothesize that the threoninol scaffold is still not flexible enough to form duplex with natural DNA or RNA. Accordingly, we synthesized serinol nucleic acid (SNA, see Fig. 1a), that has a 2-amino-1,3-propanediol (serinol) scaffold which is even more flexible than threoninol.

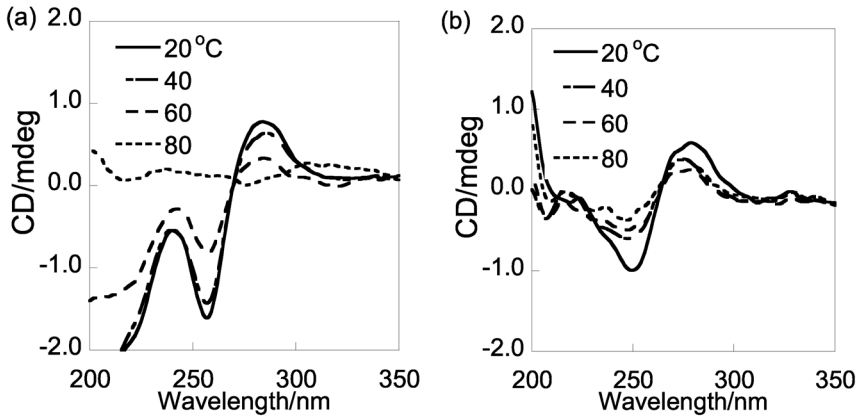

FIG. 2
Temperature dependence of the CD spectra of (a) ***a*T>1/*a*T>1c** and (b) native **D>1/D>1c** duplexes. Conditions: [oligonucleotide] = 5 μM, [NaCl] = 100 mM, 10 mM phosphate buffer (pH 7.0)

The only structural difference between SNA and *a*TNA is the lack of a methyl group next to the amino group. However, this small change affords the SNA oligomer a very unique stereochemical property. Since this methyl group on D-threoninol (2-amino-1,3-buthanediol) provides chirality, its absence makes the scaffold achiral as well as flexible. Accordingly, chirality of the "pure" SNA oligomer (or helicity of its duplex) fully synthesized from

four SNA monomers depends only on its sequence (*vide infra*). It should be noted that this unique property is specific to the SNA oligomer only and not to DNA, RNA, or *a*TNA which all have chirality (or helicity) that is inherently determined by the chirality of the scaffold. Serinol (2-amino-1,3-propanediol), which has the same three carbon atoms on its backbone as DNA, is originally an achiral diol. However, modification of two hydroxyl groups with different functional groups results in a chiral monomer. We synthesized four chiral SNA phosphoramidite monomers by attaching four nucleobases via the amide bond from L-serine to avoid racemization. When these optically pure monomers are conjugated, the obtained SNA oligomers show unique stereochemical properties, as depicted in Fig. 3a and 3b. For example, when a symmetric oligomer is synthesized such as T → T (Fig. 3b), its mirror image is identical to the original dimer, demonstrating that the SNA oligomer of symmetric sequence is achiral (i.e., meso compound). On the other hand, when an A → T dimer is synthesized from monomers (Fig. 3a), its mirror image (i.e., enantiomer) does not coincide with the original one because the unsymmetric A → T is chiral. However, interestingly, its enantiomer is identical to a dimer of reversed sequence T → A. More specifically, the chirality of the oligomer can be exactly inversed by revers-

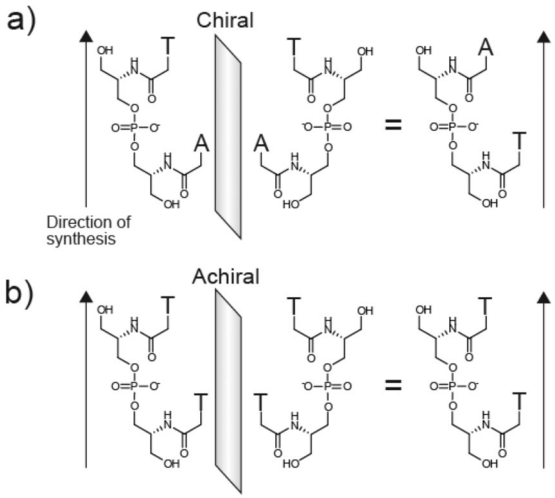

FIG. 3
a) Mirror image of SNA with an asymmetric sequence ((*S*)-AT-(*R*)) is identical to SNA with reversed sequence ((*S*)-TA-(*R*)). b) Mirror image of SNA with a symmetric sequence ((*S*)-TT-(*R*)) is identical to itself

ing sequences with the same chiral SNA monomers: two enantiomers can be synthesized from the same chiral monomers by programming the sequences but not from their enantiomeric monomers[17].

Sequences of SNA oligomers are shown in Fig. 1b. *R* and *S* termini are named after the chirality of the terminal residues: a terminal residue of the first step of oligomer synthesis has *R* configuration, whereas the other terminal residue incorporated at the last step of synthesis has *S*. **S>1** is a random 8-mer SNA oligomer and **S>1c** is its complementary strand in an antiparallel orientation. **1<S**, which has a reversed sequence of **S>1** (**1<S** is also complementary to **S>1a** in a parallel fashion, i.e., **S>1**), is an enantiomer of **S>1**. Similarly, **1c<S** is an enantiomer of **S>1c** (**1c<S** is also complementary to **S>1** in a parallel fashion, i.e., **S>1c**). Accordingly, the **1<S/1c<S** duplex is an enantiomer of the **S>1/S>1c** duplex and helicity of the duplex should be inversed. In addition, two enantiomeric combinations of **S>1/1c<S** and **S>1c/1<S** are parallel duplexes. On the other hand, the duplex made up of **S<>2** and **S<>2c**, both of which have symmetric sequences and are complementary to each other, is achiral. First, we measured melting temperatures (T_ms) of these duplexes. **S>1/S>1c** duplex showed a typical sigmoidal curve, whereas single-stranded **S>1** and **S>1c** did not show any transition above 20 °C. T_m was determined as 51.2 °C, which was rather lower than the corresponding *a*TNA duplex (***a*T>1/*a*T>1c**: 62.7 °C) but still remarkably higher than that of DNA (**D>1/D>1c**: 29.0 °C) and RNA (**R>1/R>1c**: 38.9 °C) duplexes. Because the **1<S/1c<S** duplex is an enantiomer of **S>1/S>1c**, T_m of **1<S/1c<S** (51.1 °C) was the same as that of **S>1/S>1c** (51.2 °C). We also found that the parallel combination of **S>1/1c<S** showed a typical sigmoid curve and we determined its T_m as 15.9 °C, although this was about 35 °C lower than that of the antiparallel **S>1/S>1c** duplex. Accordingly, the SNA oligomer recognized strongly antiparallel complementary strands in the same way that DNA and RNA do. Since introduction of mismatch into SNA duplex lowered the T_m, the bases in the SNA oligomer formed Watson–Crick base pairs and recognized complementary nucleobases in a similar manner as for *a*TNA, DNA and RNA.

To evaluate the helicity of the duplex, the CD spectra of these duplexes were measured. **S>1/S>1c** showed symmetrical positive and negative Cotton effects, indicating that this duplex formed a right-handed helix. As expected, **1<S/1c<S** (the enantiomer of **S>1/S>1c**) with its reversed sequence showed exactly inversed CD signals: negative and positive Cotton effects appeared at around 260 nm (compare solid with dotted line in Fig. 4). On the other hand, **S<>2/S<>2c**, which has symmetric sequences, showed no induced CD because the **S<>2/S<>2c** duplex is achiral. Thus, the helicity of

the SNA duplex can be modulated by the sequence. Since the symmetric SNA duplex (**S<>2/S<>2c**), which does not exhibit helicity, showed an almost comparable T_m (47.0 °C) to chiral SNA (**S>1/S>1c**: 51.1 °C), and helical preference (i.e., helical pre-organization) of the corresponding single strands did not strongly affect the stability of the SNA duplexes. Although an SNA monomer has a simple and symmetrical structure, SNA has the prerequisites for a genetic carrier: SNA oligomers can form a highly stable duplex (remarkably more stable than DNA or RNA duplexes) with antiparallel orientation and also specifically recognize the complementary SNA sequence. However, the conformation of the SNA duplex is significantly sequence-dependent, which might be a severe disadvantage for recognition by proteins and other biomolecules. We believe this is one of the reasons why ribose was selected as a scaffold for DNA and RNA.

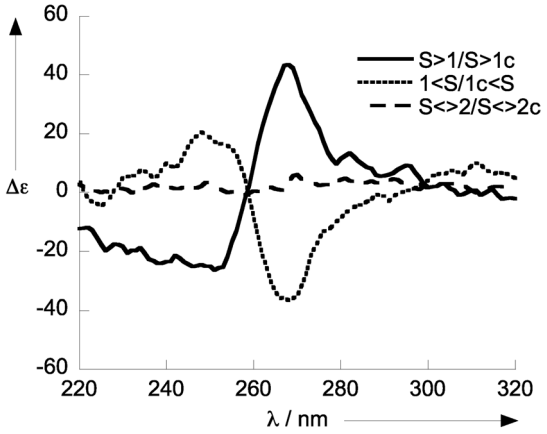

FIG. 4
CD spectra of **S>1/S>1c**, **1<S/1c<S** and **S<>2/S<>2c** duplexes at 20 °C. Δε represents molar circular dichroism per duplex (l mol^{-1} cm^{-1})

Next, we investigated the cross-hybridization ability of SNA with DNA or RNA. As described above, both GNA and *a*TNA could not hybridize with DNA or RNA[16]. Surprisingly, however, SNA can form hetero duplex with DNA and RNA as listed in Table II: the melting curve of the **S>1/D>1c** duplex showed a sigmoidal curve that was not observed with single-stranded **S>1** or **D>1c**. In addition, the CD spectrum of **S>1/D>1c** was completely different from the sum of the spectra of single-stranded **S>1** and **D>1c**. These results demonstrate that the SNA oligomer can hybridize with DNA. T_m was determined as 23.5 °C, which is almost comparable to that of

corresponding native **D>1/D>1c** (29.0 °C). Similarly, a melting profile and CD spectrum of **S>1/R>1c** revealed that the SNA oligomer could also form a duplex with RNA: T_m of **S>1/R>1c** as 35.0 °C, which is also comparable to that of **R>1/R>1c** (38.9 °C). Note that the T_m of **S>1/R>1c** was even higher than that of **D>1/R>1c** (27.3 °C), showing the potential of SNA as an antisense agent. On the other hand, an enantiomer **1<S** with respect to **S>1** hybridized with neither **D>1c** nor **R>1c**. Based on these results, we regarded *S* and *R* termini of the SNA oligomer as 5′ and 3′ termini of natural oligonucleotides with a ribose scaffold, respectively. Interestingly, both **1<S** and **1c<S** formed a stable duplex with DNA and RNA in an "antiparallel" fashion, although the **1<S/1c<S** duplex formed a left-handed helix. In addition, achiral SNA oligomers, **S<>2** and **S<>2c**, also formed duplexes both with DNA and RNA. Accordingly, helical preference of the SNA oligomer in the single-stranded state did not much affect the recognition ability towards DNA or RNA. CD spectra of **S>1/D>1c** and **S>1/R>1c** duplexes demonstrates that these hetero duplexes form a right-handed helix. Since these CD spectra were similar to **R>1/R>1c** rather than to **D>1/D>1c**, we think that both SNA-DNA and SNA-RNA duplexes form an A-form-like structure.

TABLE II
Melting temperatures of hetero-duplexes of DNA, and RNA with SNA

Sequence of XNA	T_m, °C[a]			
	D>1	**D>1c**	**R>1**	**R>1c**
S>1	<10	23.5	<10	35.0
S>1c	21.2	<10	32.2	<10
D>1	<10	29.0	<10	27.3
R>1	<10	–[b]	<10	37.9

[a] [SNA] = [DNA] = [RNA] = 2.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer). [b] Not measured.

In conclusion, we synthesized a unique artificial oligonucleotide, the *a*TNA and SNA oligomers. The *a*TNA forms remarkably more stable duplex with each other in an anti-parallel fashion than does DNA or RNA, although it cross-hybridizes with neither DNA or RNA. The SNA that lacks methyl group of the *a*TNA and thus becomes achiral also forms highly stable duplex in an anti-parallel fashion, although the T_m of SNA duplex is rather lower than the corresponding *a*TNA duplex. In contrast with *a*TNA,

however, the SNA oligomer whose scaffold has a symmetrical structure (achiral) is able to control the helicity of the SNA duplex only by sequence design. More interestingly, the SNA oligomer sequence-specifically recognized its complementary DNA and RNA in an antiparallel fashion with sufficient thermal stability. SNA is the first example of the artificial nucleic acid composed of a fully acyclic scaffold with phosphodiester linkage that can cross-hybridize with DNA and RNA irrespective of the sequence. These *old* but *new* XNA oligomers may be applied to biological tools such as antigene/antisense agents as well as nanomaterials.

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DNA FUNCTIONALIZATION AND CROSS LINKING BY CLICK, DOUBLE CLICK, BIS-CLICK AND STEPWISE CLICK CHEMISTRY

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Herein, we report on recently developed new click chemistry protocols expanding the utility of the click reaction. The "click" reaction and the "double click" reaction were used to conjugate DNA with various reporter groups. The "bis-click" protocol has been applied to cross-link DNA strands in a single-step with bis-functional azides in a template-free manner while a "stepwise click" reaction was used to cross-link any DNA strand in a chemoselective way.

INTRODUCTION

The copper-catalyzed azide-alkyne Huisgen–Meldal–Sharpless cycloaddition "click" (CuAAC) reaction has found broad application in chemistry, chemical biology, drug development and material science[1,2]. The reaction is high yielding, robust and can be performed in aqueous medium[3]. This method is used for functionalization of DNA and RNA to introduce reporter groups such as dyes or to cross-link DNA strands to duplexes, triplexes or multistranded species[4]. Sequential click reactions were reported[5]. All four constituents of DNA have already been modified with side chains bearing terminal triple bonds which were clicked to azides or bis-azides[6–10]. Among the various nucleobases, the canonical pyrimidines were functionalized at the 5-position while 7-deazapurines (pyrrolo[2,3-*d*]pyrimidines) or 8-aza-7-deazapurines (pyrazolo[3,4-*d*]pyrimidines) were selected as purine surrogates functionalized at position-7 (purine numbering is used throughout)[4,6–10]. This modification points into the major groove of duplex DNA and gives the residues steric freedom.

Recently our laboratory has developed new click chemistry protocols expanding the utility of the click reaction. The "click" reaction and the so called "double click" reaction were used to conjugate DNA with fluorescent dyes, nitroxide spin labels or other reporter groups[6–9]. Moreover, the CuAAC has been applied to cross-link DNA strands in a single-step with bis-functional azides in a template-free or template dependent manner

("bis-click" protocol)[10]. A "stepwise click" chemistry was developed for bis-azides which allows cross linking of any DNA strand modified by an alkynyl chain with bis-azides in a chemoselective manner. This communications reports on our recent results obtained with DNA and its constituents.

RESULTS AND DISCUSSION

Various click reactions were performed with the 2′-deoxyribonucleosides **1–6** (Fig. 1). Nucleosides **1–6** were synthesized and phosphoramidites were prepared[6–9]. A series of corresponding oligonucleotides were obtained by solid-phase synthesis.

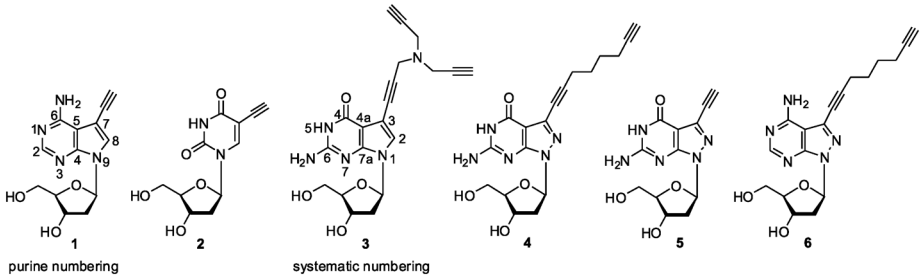


FIG. 1
Structures of nucleosides utilized for the different click protocols

7-Ethynyl-7-deaza-2′-deoxyadenosine (**1**) and 5-ethynyl-2′-deoxyuridine (**2**) were utilized for click functionalization with azido nitroxide spin labels (4-azido-2,2,6,6-tetramethylpiperidine-1-oxyl, 4-azido-TEMPO) to yield the 4-azido-TEMPO conjugates **7** and **8** (Fig. 2a)[9]. This modification allowed the

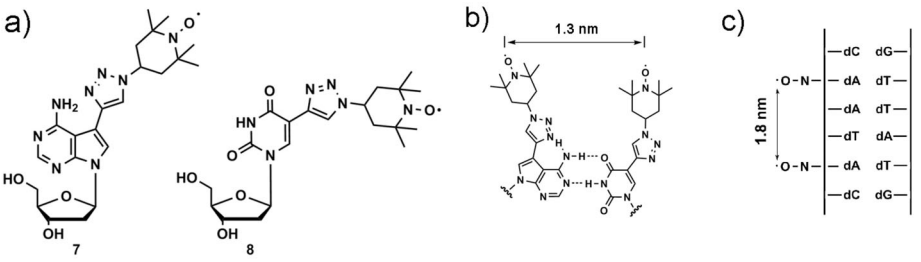
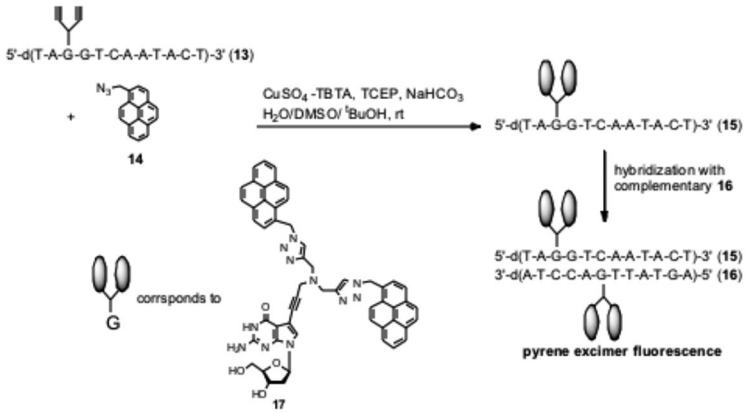


FIG. 2
a) Structures of the 4-azido-TEMPO conjugates **7** and **8**; b) modified base pair of **7** and **8** and the spin label distance in the duplex **9•10**, determined for the oxygen atoms of the nitroxides; c) spin label distance for **11•12** containing two spin labels within one of the strands, determined for the oxygen atoms of the nitroxides

application of cw and pulse EPR spectroscopy to study distance measurements of modified DNA constituents within a modified "dA-dT" base pair in the duplex 5′-d(TAG GTC **7**AT ACT) (**9**) • 3′-d(ATC CAG **8**TA TGA) (**10**) (Fig. 2b) or within one strand of the duplex 5′-d(TAG GTC **7**AT **7**CT) (**11**) • 3′-d(ATC CAG TTA TGA) (**12**) (Fig. 2c).

Also, we have introduced a branched tripropargylamine residue instead of a non-branched alkynyl side chain in various DNA constituents and in corresponding DNA fragments to increase the density of labels on the oligonucleotide chain[8]. Both terminal triple bonds of nucleoside **3** and corresponding oligonucleotides were functionalized by "double click reactions" with 1-azidomethyl pyrene **14**, decorating the side chain with two proximal pyrenes (Scheme 1).

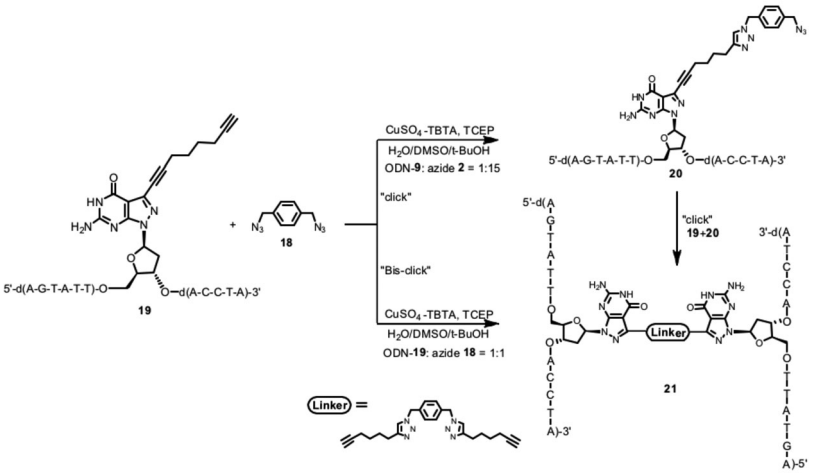


SCHEME 1
"Double click" reaction performed on oligonucleotide level and pyrene excimer fluorescence in duplex DNA

While the monomeric tripropargylamine nucleoside with two proximal pyrenes (**17**) shows strong excimer fluorescence, single-stranded oligonucleotide containing **17** do not. This was also observed for DNA duplexes when the complementary strand was unmodified. However, duplex DNA bearing pyrene residues in both strands exhibit strong excimer fluorescence when each strand contains two pyrene residues linked to the tripropargylamine moiety. This pyrene-pyrene interstrand interaction occurs when the pyrene modification sites of the duplex are separated by two base pairs which bring the fluorescent dyes in a proximal position.

The "bis-click" procedure has been applied to cross link two identical alkynylated oligonucleotides together by using bis-functional azides (→**21**,

Scheme 2)[10]. Contrary, in the "stepwise click" protocol first only one azido group was reacted to give a triazole mono-functionalized oligonucleotide conjugate (**20**) bearing still another reactive azido group. Consequently, this intermediate has the potential to be crosslinked in a second step with another strand of any type of DNA bearing an alkynyl group. The triazole mono-functionalization requires a large molar excess of the bis-azide (15:1), while cross linked oligonucleotides are formed almost exclusively in one step, when the molar excess of azide is small (1:1). Cross-linked oligonucleotides with identical chains can be hybridized with complementary strands to form four stranded DNA consisting of two duplexes ligated by a linker unit. Our protocol offers the freedom to synthesize both identical as well as non-identical crosslinked oligonucleotides.



SCHEME 2
Interstrand cross-linking of oligonucleotide **19** by "bis-click" and "stepwise click" reactions

We thank Dr. S. Budow for discussion and support and Mr. N. Q. Tran for oligonucleotide synthesis. Financial support ChemBiotech, Münster, Germany is highly appreciated.

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SYNTHESIS OF NOVEL NUCLEOSIDE DERIVATIVES CONTAINING PRECURSOR ALKYNE OR AMINO GROUPS FOR THE POST-SYNTHETIC FUNCTIONALISATION OF NUCLEIC ACIDS

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A series of novel nucleoside 5'-triphosphates and phosphoramidites containing precursor alkyne or amino groups for the post-synthetic functionalization of nucleic acids were designed and synthesized. For this aim the new 3-aminopropoxypropynyl linker group was used. It contains two alternative precursor groups: an amino group for reaction of the amino-alkynyl-modified oligonucleotides with corresponding activated esters and alkyne for the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. As was shown, a variety of methods of the new linker joining can be used to synthesize of diversity of modified pyrimidine nucleosides.

INTRODUCTION

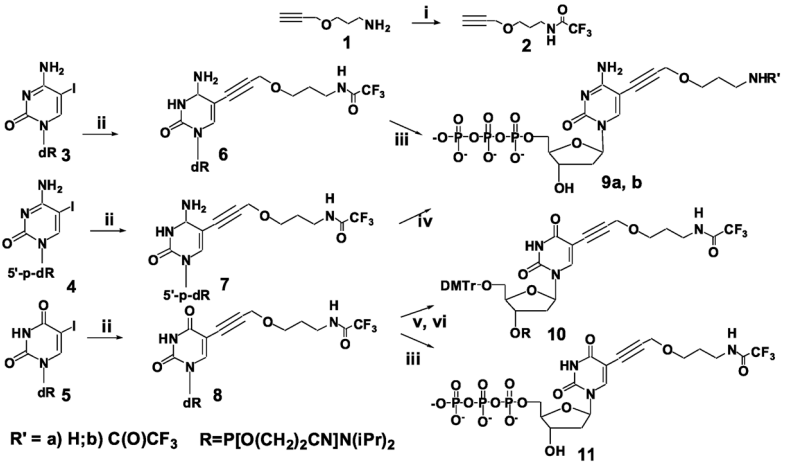
Synthesis of modified nucleoside triphosphates and phosphoroamidite nucleotide synthons both for enzymatic and chemical synthesis of modified DNA is an important field of research that permits to synthesize DNA with insertions of chemically active nucleotides. They could be then modified post-synthetically in order to construct new biologically active or labeled oligonucleotide derivatives and conjugates for research or medical applications as well as probes for diagnostics and imaging[1–3].

Usually C5-amino-modified (allylamine or propargylamine linker) nucleosides are used for the incorporation of different functional groups into DNA (or RNA)[4,5]. At the same time, the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction has already been introduced into the bioorganic chemistry arsenal and has received wide acceptance in the fictionalization of biopolymers. Therefore, we have identified a need to create and utilize a universal linker group to modify DNA (or RNA) containing an amino as well as alkyne precursor groups.

RESULTS AND DISCUSSION

In our previous work, a new 3-aminopropoxypropynyl linker group for DNA (or RNA) modification was suggested[6] (**1**, Scheme 1). This new linker group is flexible and more hydrophilic due to the presence of oxygen; it has

a more extended structure compared with propargylamine, which would probably cause less steric hindrance in the enzymatic synthesis and subsequent modifications of the amino or alkyne precursor groups. It can be introduced in the C5- and N^4-positions of pyrimidine nucleosides by Sonogashira coupling reaction[7] or by the using of the reactivity of amino group.

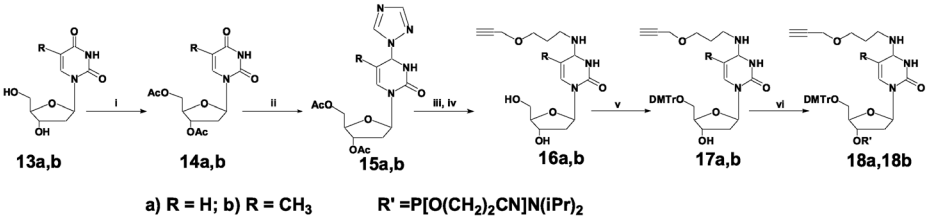


SCHEME 1

Synthesis of C5-substituted pyrimidine derivatives by Sonogashira coupling reaction: (i) $EtOC(O)CF_3$, Et_3N/CH_2Cl_2, yield 86%; (ii) $(Ph_3P)_4Pd$, CuI, DMF, Et_3N, *N*-(3-propynyloxypropyl)trifluoroacetamid **2**, argon, yield 58–65%; (iii) $POCl_3/[MeO]_3PO/N(C_4H_9)_3$, 0 °C, 10 min, $(Bu_4N)_2H_2P_2O_7$ in acetonitrile, $N(C_4H_9)_3$, 0 °C, 10 min, TEAB 0.1 M (pH = 7), 2 h, yield 54%; (iv) $N(C_4H_9)_3$ in acetonitrile, then 2,2′-dithiodipyridine/Ph_3P/*N*-Me-Im/DMF/DMSO, then $[(C_4H_9)_3N]_2H_2P_2O_7$ in acetonitrile, then NH_3/H_2O, yield 68%; (v) DMTrCl, pyridine, DMAP; (vi) $((iPr)_2N)_2PO(CH_2)_2CN$/salt Tetrazole-$NH(iPr)_2/CH_2Cl_2$, yield 70%

In our case, Sonogashira coupling was a Pd-catalysed reaction of corresponding 5-iodo nucleosides with *N*-(3-propynyloxypropyl)trifluoroacetamide (**2**, Scheme 1). For the preparation of 5-iodo nucleosides, a number of known methods were tried out[8–10]. The best yields of 5-iodo-2′-deoxycytidine **3** and 5-iodo-2′-deoxyuridine **5** were achieved in the presence of iodic acid **1** [8,9] (up to 80% compared to 40%[10]). Deoxycytidine 5′-phosphate can also be iodinated in the presence of iodic acid. Product **4** is best accomplished by chromatography, a yield of **4** up to 95% was obtained. 3-Trifluoroacetamidopropoxypropynyl was joined equally well to the C5-position of cytidine **6**, cytidine-5′-phosphate **7** and uridine **8** heterocyclic bases with 58–65% yields.

Another way of introducing the new linker into the pyrimidine nucleoside is by using amino group reactivity. In this case, we obtained modified pyrimidine nucleosides bearing the alkyne precursor group that can be used for the functionalisation of nucleic acids by CuAAC reaction. For the synthesis of the N^4-substitute 2′-deoxycytidine and 5-methyl-2′-deoxycytidine analogues the approach that utilizes triazolyl derivatives was chosen[11,12]. N^4-(3-Propynyloxypropyl)-2′-deoxycytidine **16a** and N^4-(3-propynyloxypropyl)-5-methyl-2′-deoxycytidine **16b** deoxyuridine were obtained by the reaction of 4-triazolyl derivatives **15a** and **15b** with 3-propynyloxypropylamine hydrochloride (**1**, Scheme 2) in 70–80% yields.



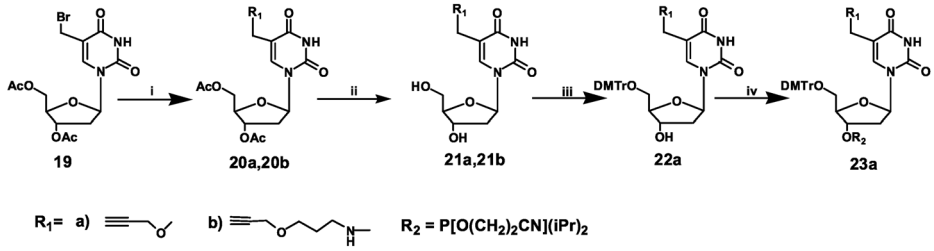
SCHEME 2
Synthesis of N^4-substituted pyrimidine derivatives bearing the alkyne precursor group: (i) Ac_2O, pyridine, yield a) 82%, b) 76%; (ii) $POCl_3$, Et_3N, 1,2,4-triazole, acetonitrile; (iii) 3-propynyloxypropylamine hydrochloride, Et_3N, acetonitrile; (iv) NH_3/H_2O, overall yield 72%; (v) DMTrCl, pyridine, DMAP; (vi) $((iPr)_2N)_2PO(CH_2)_2CN$/salt Tetrazole-$NH(iPr)_2$/CH_2Cl_2, yield 69–75%

For the synthesis of pyrimidine derivatives bearing the linker with alkyne precursor group at the C5-position, we chose a convenient procedure recommended by related literature[13]. The original method involves the reaction between the protected 5-bromomethyl-2′-deoxyuridine and alcohol. The starting material, 3′,5′-*O*-diacetyl-5-bromomethyldeoxyuridine (**19**, Scheme 3), was synthesized according to the referenced method[14].

The treatment of **19** with propargyl alcohol in dry DMF afforded 3′,5′-*O*-diacetyl-5-propargyloxymethyl-2′-deoxyuridine **20a**. After removing the protective groups, 5-propargyloxymethyl-2′-deoxyuridine **21a** was obtained in 60% yield. Analogous treatment of **19** with 3-propynyloxypropylamine hydrochloride in the presence of triethylamine afforded 3′,5′-*O*-diacetyl-5-(3-propynyloxypropyl)aminomethyl-2′-deoxyuridine **20b**. After deprotection 5-(3-propynyloxypropyl)aminomethyl-2′-deoxyuridine **21b** was obtained in a 43% yield.

Thus, four N^4- and C5-modified pyrimidine nucleosides bearing the alkyne precursor group (**16a**, **16b**, **21a**, and **21b**, Schemes 2, 3) were ob-

tained along with C5-alkynyl-modified uridine, cytidine and 5′-phosphocytidine derivatives bearing protected precursor amino group (**6**, **7**, and **8**, Scheme 1). Any one of these modified nucleosides may be converted into general-purpose triphosphate or a phosphoramidite derivative. As an attachment of the amino linker group to the C5-position through the triple bond in compounds **6**, **7**, and **8** guarantees the preservation of the substrate specificity of its triphosphate to a number of polymerase, we converted these compounds to the corresponding triphosphates and obtained phosphoramidites from other nucleosides (**16a**, **16b**, and **21a**). We obtained a phosphoramidite derivative of **8** also. All derivatives were characterized by ^{1}H, ^{31}P NMR and mass spectroscopy.



SCHEME 3

Synthesis of C5-substituted pyrimidine derivatives bearing the alkyne precursor group: (i) a) Propargyl alcohol, b) 3-propynyloxypropylamine hydrochloride, Et_3N, DMF; (ii) NH_3/H_2O, overall yield 72–43%; (iii) DMTrCl, pyridine, DMAP; (iv) $((iPr)_2N)_2PO(CH_2)_2CN$/salt Tetrazole-$NH(iPr)_2$/CH_2Cl_2, yield 69–75%

Oligonucleotide conjugates containing fluorescein residues inside of strand were synthesized by using phosphoramidites **18a** and **23a** by CuAAC reaction with fluorescein-5-carbomoyl-(11-azidoundecanoyl). Phosphoramidite reagent **10** was used for the post-synthetic fictionalization with corresponding activated esters of dyes.

The work was supported by the Interdisciplinary Integration Project of the Presidium of SBRAS No88.

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SiRNAS WITH PHOSPHORODITHIOATE MODIFICATION

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Phosphorodithioate (PS2) backbone modified RNA is a RNA mimic, where two non-bridging oxygen atoms are replaced by sulfurs. This analog is achiral, isosteric and isopolar with the normal phosphate backbone, inert toward nucleases. In this communicate we present preliminary data on the synthesis and physico-chemical and biological properties of siRNA duplexes containing strategically placed PS2 linkages. Such siRNA duplexes exhibit a slight thermal destabilization while maintaining an A-type helix structure similar to the unmodified siRNA. Their silencing activity is basically similar to their non-modified precursors. The optimized PS2-RNA duplexes may be valuable for developing stable siRNAs for biological and therapeutic applications.

INTRODUCTION

Short interfering RNAs (siRNAs) are widely used to identify gene functions and hold great potential in providing a new class of therapeutics. Although unmodified siRNAs are used with success for a gene silencing, chemical modifications of one or both strands are desired for pharmaceutical applications to enhance a nuclease stability, potency and to improve pharmacokinetic properties of duplexes. A number of chemical modifications within sugar moiety, nucleobase, and internucleotide linkage have been already evaluated for their effects on siRNA activity[1,2]. Modification of the internucleotide bond involving substitution of a single non-bridging phosphate oxygen atom with either a sulfur atom or a borane group leads to phosphoro(mono)thioate[3] and boranophosphate[4] derivatives of RNA, respectively. This renders the internucleotide linkage nuclease more resistant, but unlike natural RNA, the modified phosphorus center is chiral, which leads to a mixture of unresolvable diastereomeric oligomers possibly having variable biochemical, biophysical, and biological properties.

The substitution of both non-bridging phosphate oxygen atoms with sulfurs gives rise to a phosphorodithioate (PS2) internucleotide linkage which, like natural RNA, is achiral at phosphorus (Fig. 1)[5]. The PS2 linkage is a very attractive RNA analog because it is isosteric and isopolar with the normal phosphodiester and should have other biochemical and biophysical proper-

ties similar to natural RNA. Over the past twenty years, only a few examples of PS2-RNA chemistries were reported, like synthesis of dimer containing a single PS2 linkage[6], the solid-phase syntheses of a 12-mer containing a single PS2 linkage[7] and a 15-mer oligoribouridylate phosphorodithioate bearing exclusively PS2 linkages[8]. We report here the synthesis of the 21-22-nt RNA molecules containing the PS2 linkages, and their use as components of the novel type of the PS2-siRNA duplexes. To our knowledge these are the first studies evaluating physicochemical and biological properties of siRNAs containing the PS2 modification.

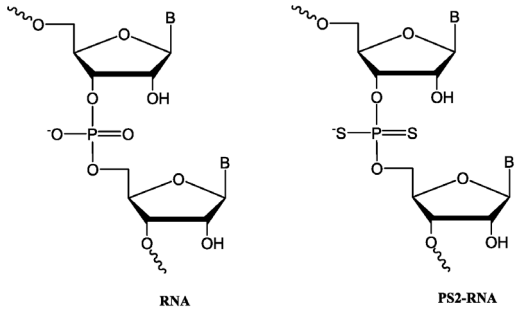
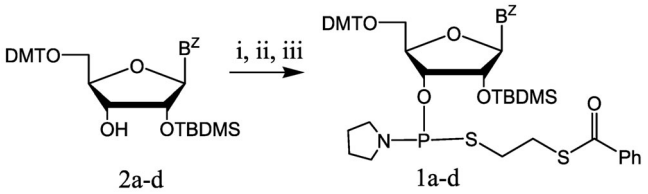


FIG. 1
Structures of natural RNA (left) and PS2 modified RNA (right); B = Ade, Cyt, Gua, and Ura

RESULTS AND DISCUSSION

Protected ribonucleoside-3′-yl *S*-[β-(benzoylmercapto)ethyl]pyrrolidinothiophosphoramidite monomers (**1a–1d**, Fig. 2) were devised based on a sulfur-linked, base-labile β-(benzoylmercapto)ethyl protecting group used in the Caruthers laboratory for the solid-phase synthesis of PS2-DNA[9]. Solid phase PS2 linkage synthesis with the thiophosphoramidites consists of a sequence of four consecutive reaction steps: deblocking, condensation, sulfurization, and capping. Condensation reaction of the thiophosphoramidite with a free 5′-hydroxyl group of the following nucleoside or oligonucleotide chain to form a thiophosphite triester intermediate was performed with commercially available 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole forming the internucleotide thiophosphite triester in about 95% yield. This step was immediately followed with an effective sulfurizing reagent of 3-ethoxy-1,2,4-dithiazolidine-5-one (EDITH)[10]. Dimers were synthesized for each of the four thiophosphoramidites[11]. The structure of all the dimers was confirmed by ^{31}P-NMR and ESI-MS (SI). Phosphate linkages were synthesized

by using standard 2′-*O*-TBDMS RNA phosphoramidites following by iodine oxidation. The modified RNAs containing PS2 linkages were deprotected and isolated using slightly modified protocols[12]. In our previous studies, we have identified siRNA sequences that target the β-secretase (BACE1) and enhanced green fluorescent protein (EGFP) mRNA and validated their silencing activity in cellular models[13,14]. Here, we chose these sequences for tests of PS2-siRNA molecules.



2a-d 1a-d

FIG. 2
Synthesis of thiophosphoramidites **1a–1d**: (i) tris(pyrrolidino)phosphine, 1*H*-tetrazole; (ii) 1-(trimethylsilyl)imidazole; (iii) ethanedithiol monobenzoate, 1*H*-tetrazole. Abbreviations: **a**: B^Z = Ade^{Bz}; **b**: Cyt^{Ac}; **c**: Gua^{Ac}; **d**: Ura; DMT = dimethoxytrityl; Ph = phenyl; TBDMS = *tert*-butyldimethylsilyl

SiRNAs were assembled in PBS buffer and their duplex structure was confirmed on a 4% agarose gel electrophoresis. The UV-monitored thermal dissociation studies have shown that the duplexes with up to five PS2 linkages exhibit transition curves essentially identical in shape to the unmodified siRNA. In most cases of the studied siRNAs, we did not observe remarkable fluctuations in *Tm* values in comparison to the unmodified reference duplex. CD spectra collected for PS2-siRNA duplexes are similar to the spectra of the unmodified duplex, and are consistent with the typical A-type structure of double-stranded RNA.

Gene silencing activity of the PS2-siRNAs was tested using developed previously a dual fluorescence reporter system[14,15], based on measurement of the relative fluorescence intensity of the enhanced green fluorescent protein (EGFP) expressed from a BACE1-EGFP fusion/or EGFP plasmid, versus red fluorescent protein (RFP) expressed from a non-target plasmid serving as a transfection control. Although CD spectra indicate that PS2-siRNA is globally similar in structure to unmodified siRNA, the substitution of two non-bridging oxygen atoms with sulfur atoms might have influence on ability of duplexes to serve as substrates for enzymes involved in RNAi mechanism. The gene silencing experiments performed on several siRNA duplexes containing the PS2 linkages either in the sense or antisense strand indicate that the extent of silencing effect depends on not only the number

of the PS2 modifications but also the strand and/or position within an siRNA duplex (Fig. 3). Interestingly, increased silencing activity of siRNA duplexes containing the PS2 modifications at the 3′-end of the sense strand was observed. This effect may originate from increased thermodynamic asymmetry of the duplex and thus proper selection of the guide strand into RISC [14].

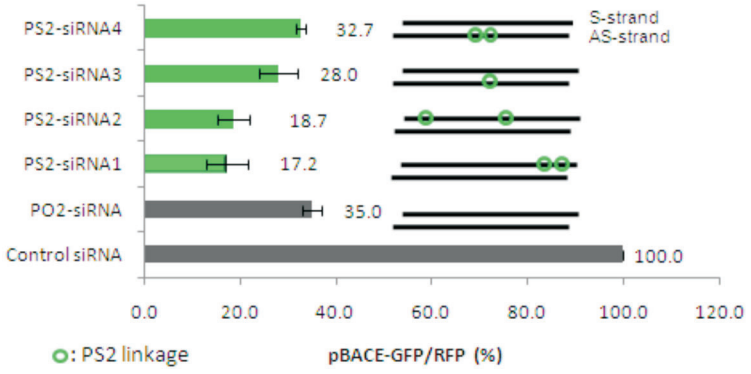


FIG. 3
Comparative analysis of PO2-siRNA and PS2-siRNA BACE1 gene silencing activity (SI) in HeLa cells

The enhanced gene knockdown activity observed here indicates that PS2-siRNAs can improve gene silencing *in vivo*. Given the straightforward synthesis of PS2 linkages, as well as their known nuclease resistance and lack of chirality, PS2 modifications are promising candidates for siRNA in research and therapeutic applications.

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DNA/LNA AND PNA CONJUGATES AS GENE MODIFYING AGENTS

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Triplex forming oligonucleotides (TFO) have been extensively studied as DNA modifying agents due to their propensity to bind double stranded DNA (dsDNA) at physiologically relevant conditions. In addition, there has been a plethora of work using TFO-psoralen conjugates that upon UV light activation induce crosslinking site-specifically at a given DNA target. Here we describe TFO's that are designed to selectively photocleave dsDNA upon light activation at the visible to near-IR region. One system is based on a DNA/LNA mixmer that is modified at its 5'-end with L-Trp (or Gly) followed by a cyanine dye (either thiazole orange (TO) or thiazole red (TR)). These TFO conjugates are shown to nick and linearize plasmid DNA (containing the target sequence) upon light activation. A scrambled DNA/LNA TFO has no apparent DNA photocleavage activity, highlighting the sequence specificity of this system. In addition, analogous PNA constructs containing a cell penetrating peptide (8 D-Lysines) on the C-terminus and a Trp-cyanine dye on the N-terminus of the PNA were prepared and characterized. These PNA conjugates are currently being evaluated as DNA photo-cleavage agents.

INTRODUCTION

Several strategies have been developed in the past decades to target dsDNA as means of up- or down-regulating gene expression.

These include zinc finger nucleases (ZFN)[1] polyamides[2] and TFOs[3]. In addition, the use of DNA/RNA mimics (e.g. PNA) to target the promoter region of a given gene, has been successfully implemented to specifically inhibit gene expression in intact cells at both mRNA and protein levels[4,5].

The TFO strategy has been also utilized for light-activated gene up- and down-regulation by attaching to these TFO's a psoralen molecule[6].

Photo-activation of triplex (TFO bound to dsDNA) with UV light then leads to site-specific DNA modulation.

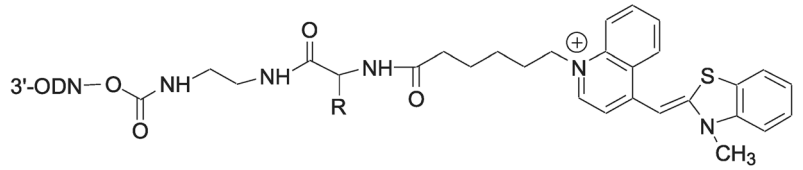
Here we present a novel approach for achieving site-specific DNA photocleavage by DNA/LNA and PNA molecules. The data suggests that such molecules may be utilized for visible light-activated gene knock-down by site-specific DNA cleavage.

RESULTS AND DISCUSSION

1. Design of DNA/LNA and PNA Conjugates

A 14-mer homopurine sequence was used for both DNA/LNA[7] and PNA constructs. This sequence targets the mdm2 gene; a negative regulator of p53 that has an important role in cancer progression[8].

The following scheme (Scheme 1) presents the chemical structures of these conjugates.



Conjugate 1a: DNA/LNA = 3'-AAAGGAAAGGGAAA-5', R = CH2-indole (TO) => WT-Trp-TO
Conjugate 1b: DNA/LNA = 3'-AAAGGAAAGGGAAA-5', R = H (TO) => WT-Gly-TO
Conjugate 1c: DNA/LNA = 3'-AAGGAAGAGAGAAA-5', R = CH2-indole (TO) => Scr-Trp-TO

Underlined bases = LNA

SCHEME 1
Chemical structure of DNA/LNA conjugates

An amino acid is introduced between the oligomer to the photo-active moiety (cyanine dye). The indole ring on L-Trp is utilized to generate a stable peroxide radical formed on this ring after photo-activation of the cyanine dye to form singlet oxygen. This radical has been show to cause a frank strand break to DNA[9]. Alternatively, when Gly is placed at this position, only singlet oxygen is formed after photo-activation. This reactive oxygen species (ROS) primarily oxidizes guanines on DNA and is therefore not expected to be as active (in breaking DNA) when compared to the peroxide radical.

Finally, to types of cyanine dyes were examined, namely, thiazole orange (TO, λ_{exc} = 501 nm) and thiazole red (TR, λ_{exc} = 635 nm).

2. Synthesis of DNA/LNA and PNA Conjugates

DNA/LNA conjugates were synthesized on an automated DNA synthesizer (1 μmol scale, ABI 3400) and the DMT group on the 5′-end was removed. The resin was then transferred to a reaction vessel and was extended with ethylene diamine by activating the 5′-OH group with carbonyl di-imidazole

(CDI). Next, NPS-protected amino acid (Gly or Trp) was coupled to the free amine. Finally, deprotection of NPS and coupling with carboxylic acid functionalized cyanine dye (TO or TR) afforded the desired DNA/LNA conjugate. After treating the CPG resin with a 33% NH_4OH solution for 12 h at 60 °C, the solution was evaporated and the DNA/LNA conjugates were purified by HPLC (phenomenex C18 clarity column). The identity of all DNA/LNA conjugates was determined by Maldi-TOF MS.

PNA conjugates were synthesized manually on a NovaSyn TGA resin (Merck) on a 20 μmol scale. Eight D-Lysines were introduced onto the resin followed by the PNA sequence, the amino acid and the cyanine dye. After cleavage of PNA conjugates from resin and deprotection, these were purified by HPLC (phenomenex C18 jupiter column) and analyzed by Maldi-TOF MS.

3. DNA Photocleavage by DNA/LNA and PNA Conjugates

Prior to irradiation, DNA/LNA conjugates (**1a–1c**) were incubated for 16 h with the plasmid DNA at 37 °C. Only a 2-fold access was used as such TFO's have been shown to readily from a triplex at such molar ratios. The photoactivity of these set of conjugates is shown in Fig. 1. Conjugate **1a** is shown to cause substantial linearization of the mdm2 plasmid after a short irradiation period of 10 min (lane 2). No significant change in the amount of nicked and linearized plasmid is observed at longer periods of irradiation (lanes 3–5). The scrambled LNA/DNA conjugate (**1c**) has no apparent photoactivity, whereas the conjugate with Glycine (**1b**) leads only to plasmid DNA relaxation (form II).

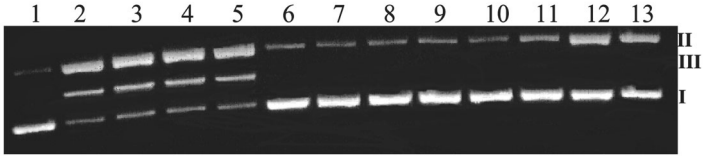


FIG. 1
Photocleavage of PCMV-MDM2 plasmid by TFO containing LNA conjugates **1a–1c** (with TO) in phosphate buffer (10 mM NaH_2PO_4, 50 mM NaCl, pH = 7.4), as determined by agarose gel electrophoresis. Lane 1: plasmid irradiated for 30 min (DNA only); lanes 2–6: plasmid + conjugate **1a** (with L-Tryptophan) after 10, 15, 20 and 30 min irradiation and in the dark, respectively; lanes 7–9: plasmid + conjugate **1c** after 15 and 30 min irradiation and in dark, respectively; lanes 10–13: plasmid + TFO conjugate **1b** in the dark and after 10, 15 and 30 min irradiation, respectively

We have now started to examine the PNA conjugates as sequence-specific DNA photo-cleavage agents. We find that the PNA conjugate with the fully complementary sequence to the plasmid DNA target causes a decrease in the intensity of the band as corroborated by agarose gel electrophoresis (data not shown). The scrambled sequence has no apparent photo-activity towards the plasmid DNA suggesting that the photo-cleavage reaction is sequence-dependent.

E.Y. acknowledges the Grass Center for Drug Design and Synthesis of Novel Therapeutics for financial support. J.K. and V.B. acknowledge the Grant Agency of the Academy of Sciences of the Czech Republic (IAA400040803).

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DESIGN AND FACILE SYNTHESIS OF NOVEL 2′,4′-BRIDGED NUCLEIC ACID

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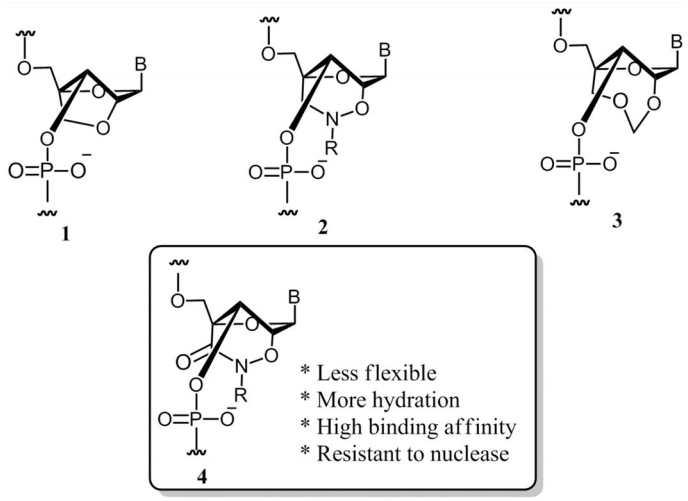
Novel 2′,4′-bridged nucleic acid was designed and synthesized by introducing carbonyl function in the bridge of 2′,4′-BNA^{NC}, resulting a perhydro 1,2-oxazinone ring system. The modification was designed to restrict the flexibility of the sugar conformation through the trigonal planarity of the carbonyl group. The synthesized monomers were incorporated into oligonucleotides, and their properties were investigated.

INTRODUCTION

Since its first synthesis[1,2], 2′,4′-bridged nucleic acid (2′,4′-BNA)[3] also called locked nucleic acid (LNA)[4] has received much attention due to its unprecedented hybridizing affinity for complimentary strands (RNA and DNA), aqueous solubility, sequence selectivity, and nuclease resistance compared with those of natural oligonucleotides[5,6]. In 2′,4′-BNA/LNA the sugar conformation is locked into *N*-conformation via bridging which significantly decreases the entropy loss during hybridization with ssRNA or dsDNA. Moreover, it is considered to be one of the most promising modifications that has potential to satisfy the requirements of an ideal nucleic acid to be used in antisense/antigene technology[7]. Many interesting modifications of 2′,4′-BNA/LNA have been carried out to search the most appropriate modification that could exhibit high binding affinity and sequence selectivity towards ssRNA and/or dsDNA target, resistant to nuclease degradation, and efficient preparation on DNA synthesizer[8–10]. Recently, a highly interesting modification of 2′,4′-BNA/LNA has been reported, termed as 2′,4′-BNA^{NC} (2′-*O*,4′-*C*-aminomethylene bridged nucleic acid)[11] which contain six-membered bridged structure with N–O linkage. The nucleic acid was reported to exhibit stronger and sequence selective duplex- and triplex-forming ability along with high nuclease resistance. Some analogs like 2′-*O*,4′-*C*-methyleneoxymethylene-bridged nucleic acid, or 2′,4′-BNA$^{COC\ 12}$, where seven-membered bridge is present, has exhibited excellent nuclease resistance. The binding affinity of 2′,4′-BNA^{COC}, though higher than the natural oligonucleotide, is slightly lower than that of 2′,4′-BNA. Therefore, it has been concluded that increasing the size of the bridge between 2′- and

4′-position of BNA will increase the nuclease resistance, but it will cost the hybridizing affinity. Considering these facts, our current research is targeted to develop a novel analog of 2′,4′-BNA having six-membered bridge with N–O linkage and less flexibility. To achieve the goal, we have introduced a carbonyl function in the bridge of 2′,4′-BNA^{NC} that will restrict the flexibility of the structure via trigonal planarity of the function. The novel design will assist in increasing the hydration of the nucleic acid which is very important to address the issue of molecular crowding inside the cellular environment, and the rigid structure of the design will increase the duplex- and triplex-forming ability.



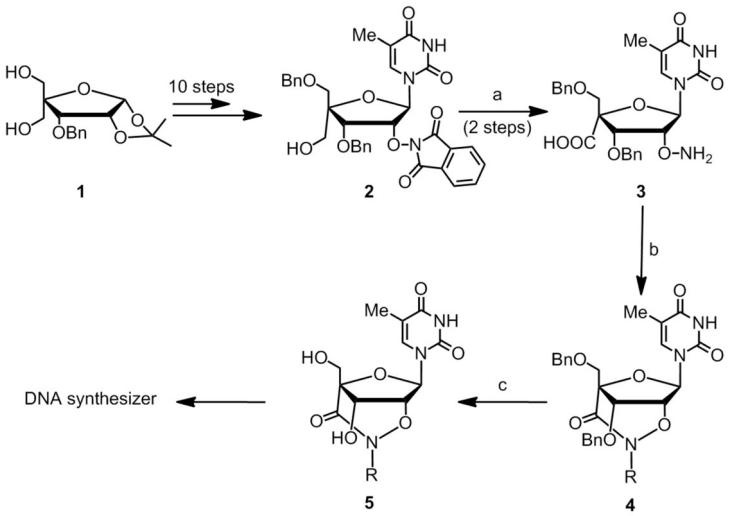
SCHEME 1
Structures of various 2′,4′-bridged nucleic acids. 1) 2′,4′-BNA/LNA, 2) 2′,4′-BNA^{NC}, 3) 2′,4′-BNA^{COC}, 4) Novel 2′,4′-BNA

RESULTS AND DISCUSSION

Starting from the common precursor of 2′,4′-BNA/LNA i.e. 3-*O*-benzyl-4-*C*-hydroxymethyl-1,2-*O*-isopropylidene-α-D-ribofuranose **1**, our target bridged nucleosides **5** were achieved after 14 to 16 steps. In our study, we have preserved the labile N–O moiety of 2′,4′-BNA^{NC} due to the interesting results exhibited by the nucleic acid, which is attributed to the moiety. The moiety was introduced into the nucleoside by the reaction with *N*-hydroxyphthalimide in presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). Considering the labile nature of the N–O moiety, the cyclization step for our target **5** was carried out at the late stage of the synthesis. The cyclization of

the bridge was accomplished by the coupling reaction between carboxylic moiety and amino moiety of **3**, employing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI.HCl) in DMF as the coupling agent and 1-hydroxybenzotriazole monohydrate (HOBt.H_2O) as a condensation additive. The debenzylation of **4** was accomplished with reductive hydrogenolysis by employing Pearlman's catalyst (20% $Pd(OH)_2$ on C) under hydrogen atmosphere. It is noteworthy that reductive hydrogenolysis was not possible in case of 2′,4′-BNA^{NC} as bond cleavage occurred at N–O moiety[11d].

The methylated derivative of the monomer **5** was achieved by reductive alkylation of **3** and following cyclization and debenzylation, similar to the procedures described earlier for the [NH] derivative of **5**.



SCHEME 2
Synthesis of the novel 2′,4′-BNA monomers

The synthesized novel BNA monomers were incorporated into oligonucleotides. For the purpose, the primary hydroxyl of the nucleoside **5** was selectively protected with 4,4′-dimethoxytrityl group, and the secondary hydroxyl was then phosphitylated with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite to yield the novel 2′,4′-BNA-thymine phosphoroamidite. The phosphoroamidite was incorporated into oligonucleotides by automated DNA synthesizer and their properties were studied.

A part of this work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

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OLIGONUCLEOTIDE GLYCO-CENTERED GALACTOSYL CLUSTER CONJUGATES SYNTHESIZED BY MULTI-CLICK AND PHOSPHORAMIDITE CHEMISTRIES AND THEIR AFFINITY FOR PSEUDOMONAS AERUGINOSA LECTIN 1

François MORVAN[a,*], Béatrice GERLAND[a], Gwladys POURCEAU[a], Alice GOUDOT[c], Albert MEYER[a], Sébastien VIDAL[b], Yann CHEVOLOT[c], Jean-Pierre PRALY[b], Eliane SOUTEYRAND[c] and Jean-Jacques VASSEUR[a]

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Oligonucleotide glycoconjugates with a mannose core bearing four, height or sixteen galactose residues introduced by phosphoramidite chemistry and multi-click chemistry have been synthesized. Five different linkers between the galactose moiety and the mannose core were introduced to study their impact on the affinity for PA-IL using a DNA glycoarray. Then, the best galactose linker was introduced on galactose, glucose and lactose core for a second screening.

INTRODUCTION

Carbohydrates are involved in many important biological events like cell-cell communications, inflammation or infections by pathogens[1]. However, since monovalent carbohydrate interaction with carbohydrate-binding protein (lectins) is generally weak, it is necessary to have multivalency for a cluster effect to gain tight binding[2]. For this purpose, many glycoclusters prepared from multiple scaffolds have been reported in the literature[3]. Among these glycoclusters, only few have been prepared from oligonucleotide scaffolds. We present herein, the synthesis of new carbohydrate-centered glycomimetics[4] prepared by phosphoramidite chemistry[5] in combination with Cu(I) catalyzed 1,3-dipolar cycloaddition between azide and alkyne[6,7] conjugated with an oligonucleotide[8]. Then, in order to study the interaction of the glycomimetics with lectin I of *Pseudomonoas aeruginosa* (PA-IL)[9], they were immobilized on DNA chip by means of DNA directed immobilization (DDI)[10] leading to a glycoarray[11] and IC_{50} values were determined[12,13].

RESULTS AND DISCUSSION

These glycomimetics were efficiently synthesized starting from an azide solid support **1** [14] which reacted with 1-*O*-propargyl-α-D-mannose leading to a mannose solid support **3** (Fig. 1). Then four alkyne functions were coupled on the four hydroxyls of the mannose by means of very reactive pent-4-ynyl phosphoramidite derivative[14] according to well established

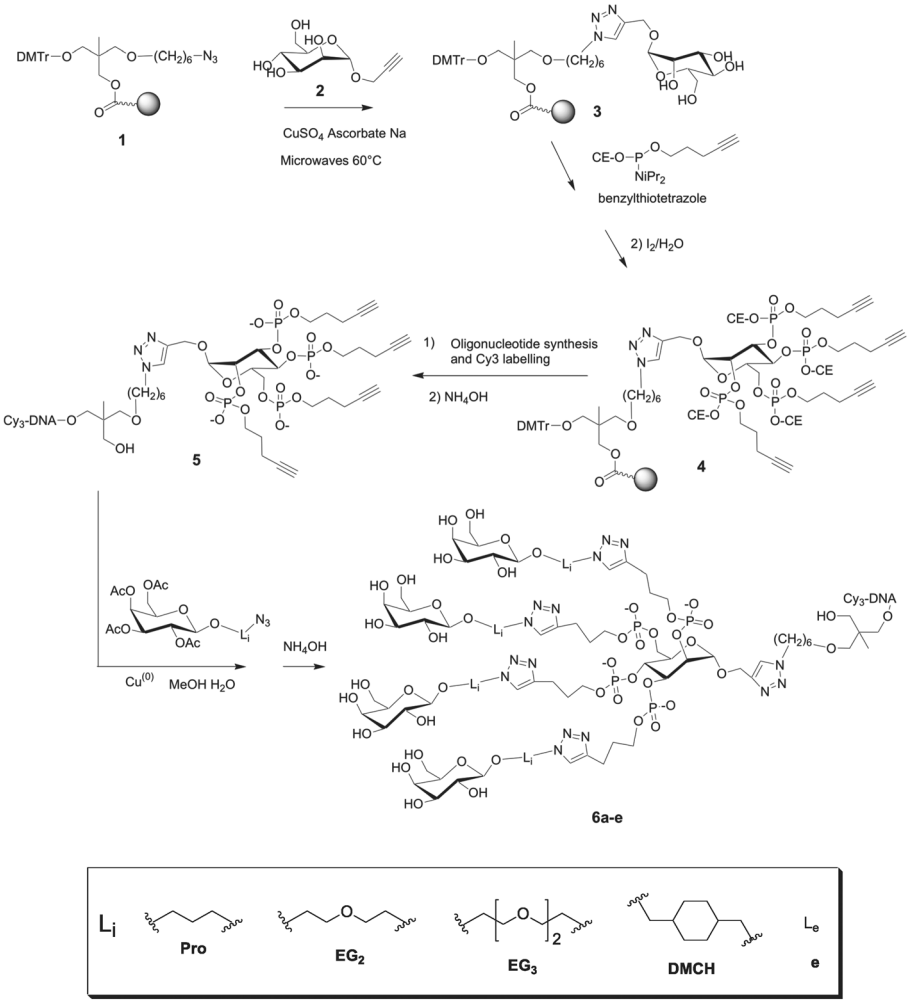

FIG. 1
Structure of the mannose-centered tetragalactose oligonucleotide conjugates

phosphoramidite method[5] to give a tetra pentynylphosphotriester mannose core **4**. The oligonucleotide was synthesized and labeled using standard method and deprotected by ammonia affording the 5′-Cy3-oligonucleotide conjugated at its 3′ end with the tetra-pentynylphosphodiester mannose core **5**. Finally, it was engaged, in parallel synthesis, with five different galactose linker (L_i) azide derivatives affording a small library of five glycoconjugates. After their immobilization by DDI on a glycoarray, PA-IL labeled with Alexa-647 was incubated and after wash, the fluorescence was read at 665 nm allowing visualizing the relative affinity of the glycomimetics for PA-IL by direct semi-quantitative fluorescence scanning (Fig. 2)[11].

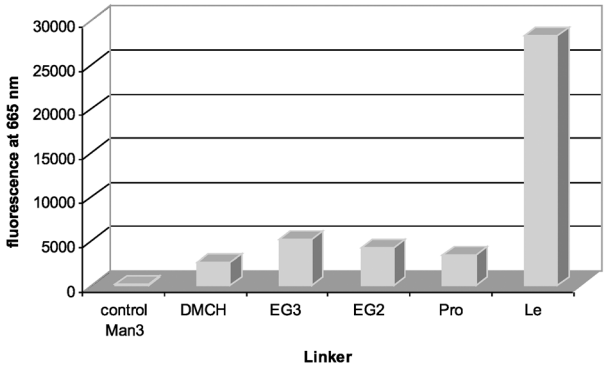

FIG. 2
Mean fluorescence intensities at 635 nm (a.u.) of Alexa 647-labeled PA-IL after incubation with immobilized glycomimetics

We found that the glycomimetic with the L_e linker displayed a very high avidity for PA-IL. Then, the determination of the concentration of lactose to displace 50% of the binding of this glycomimetic in respect of the monogalactose bearing the L_e linker confirmed its high avidity for PA-IL. A SAR study using other core like galactose, glucose and lactose is in progress.

CONCLUSION

The combination of phosphoramidite and click chemistries is a straightforward strategy to prepare oligonucleotide glycomimetics. Thus, the main part of the synthesis is performed on solid support allowing a rapid, efficient and automated synthesis of the oligonucleotide with a polyalkyne scaffold and finally by a divergent strategy the conjugates are obtained by click chemistry with high efficiency. The study of their avidity for lectins

using a DNA microarray[11,12,15] requires only minute amounts of these conjugates offering the perspective of high throughput analysis. The strong avidity of our glycomimetic with L_e linker prompts us to evaluate the potency of the glycomimetic as anti-adhesive compound of *Pseudomonas aeruginosa* bacteria on whole cells.

This work was financially supported by the CNRS interdisciplinary program "Interface Physique Chimie Biologie: Soutien à la prise de risque", ANR-08-BLAN-0114-01, Lyon Biopole, and Vaincre la Mucoviscidose. G.P. thanks the CNRS and the Région Languedoc-Roussillon for the award of a research studentship.

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POLYMERASE CONSTRUCTION OF BASE-MODIFIED DNA FOR CHEMICAL BIOLOGY

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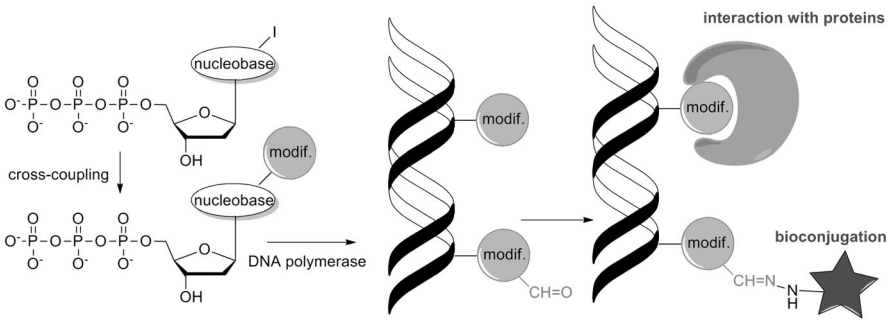
An efficient two-step methodology for construction of base-modified DNA was developed based on aqueous-phase cross-coupling reactions of halogenated nucleoside triphosphates (dNTPs) followed by polymerase incorporation. A number of diverse chemical modifications have been successfully incorporated in this way for the use in chemical biology (modulation of cleavage by restriction enzymes, interactions with diverse DNA-binding proteins and bioconjugations).

INTRODUCTION

Base-functionalized nucleic acids are frequently used in different areas such as bioanalysis, nanotechnology and chemical biology. Apart from chemical synthesis by phosphoramidite method on solid support, they can be easily prepared by polymerase incorporations of modified dNTPs [1].

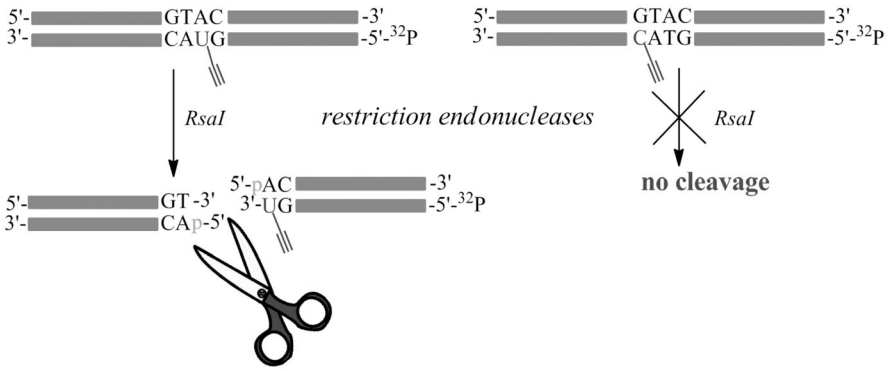
RESULTS AND DISCUSSION

An efficient and general two-step approach for the construction of base-modified DNA was developed[2]. The first step consists of a Pd-catalyzed aqueous-phase cross-coupling reactions of halogenated dNTPs with arylboronic acids (the Suzuki–Miyaura reaction) or terminal acetylenes (the Sonogashira reaction). 5-Substituted pyrimidine dNTPs and 7-substituted 7-deazapurine dNTPs usually are good substrates for at least some DNA polymerases and can be incorporated to DNA by primer extension (PEX) or PCR. PEX is typically used for synthesis of short DNAs with a few modifications in one strand, whereas PCR is suitable for construction of long DNA duplexes covered by high-density of modifications at both strands. Apart from redox labelling of DNA[3], the modifications include diverse alkynyl or aryl groups[4], amino acids[5], steroids[6], bipyridine ligands, fluorescent labels, additional nucleobases etc. Reactive formylthienyl group was also successfully incorporated[7] in this way and used for conjugations via hydrazone formation.



SCHEME 1
Construction of base-modified DNA and possible uses in chemical biology

Cleavage of base modified DNA by diverse restriction endonucleases (RE) was also studied[4]. The results show that some RE tolerate certain modifications (depending on the size) at position 7 of 7-deazaadenine or at position 5 of uracil and still cleave the corresponding sequences but none of them tolerates any modification at position 5 of cytosine. A new protocol for protection of DNA against the cleavage by RE was developed.



SCHEME 2
Cleavage of base-modified DNA by restriction endonucleases

This work was supported by the Academy of Sciences of the Czech Republic (Z4 055 0506), the Ministry of Education, Youth and Sports of the Czech Republic (LC512), Grant Agency of the Academy of Sciences of the Czech Republic (IAA400040901), the Czech Science Foundation (203/09/0317), and Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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REDOX LABELLING OF NUCLEIC ACIDS FOR ANALYZING NUCLEOTIDE SEQUENCES AND MONITORING DNA-PROTEIN INTERACTIONS

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Nucleobase labelling of DNA for electrochemical sensing was attained through chemical modification of thymine bases with osmium tetroxide in the presence of nitrogenous ligands, or via enzymatic incorporation of nucleotide conjugates with redox-active moieties using labelled deoxynucleoside triphosphates. DNA hybridization, primer extension and PCR techniques were used for sequence-specific DNA assays. Tail-labelled DNA substrates were applied to monitor DNA binding by tumour suppressor p53 protein.

INTRODUCTION

DNA is electrochemically active owing to electrochemical oxidizability or reducibility of nucleobase residues[1]. A number of electrochemical biosensors and bioassays using no external DNA labelling have been proposed. The label-free approaches proved to be particularly useful in structure-sensitive DNA sensing[2] and/or for the detection of DNA damage[3]. On the other hand, these approaches exhibit certain limitations related to electrode material of choice (structure-sensitive DNA signals can be measured only at mercury based electrodes) and to insufficient selectivity in some types of sequence-specific DNA assays. For example, discrimination among DNA fragments differing in their nucleotide sequence but not in abundance of individual electrochemically active bases is difficult on the basis of measurements of the intrinsic DNA responses. Covalently bound electroactive labels are thus developed and applied in the electrochemical DNA sensing to reach unambiguous discrimination between a specific DNA sequence and other, non specific DNA elements.

Incorporation of redox active moieties into DNA (oligonucleotide, ON) molecules can be attained through different ways and at different levels. Apart from solid-phase phosphoramidite ON synthesis, more versatile techniques suitable for on-demand labelling in any biochemical laboratory have

been introduced. Natural DNA molecules as well as ONs composed of natural nucleotides can easily be redox labelled using osmium tetroxide in the presence of nitrogenous ligands, giving rise to stable adducts with thymine residues[4]. Techniques that use enzymatic incorporation of labelled nucleotides (if available as deoxynucleoside triphosphates, dNTPs) are in principle even more versatile because they allow one to combine various labels with different nucleotide sequences (represented by unmodified and thus inexpensive ONs used as primers for primer extension or terminal nucleotide transfer)[5].

RESULTS AND DISCUSSION

Techniques for ON probe tail-labelling with osmium tetroxide in the presence of 2,2′-bipyridine (Os,bpy) were developed. This approach, conferring accumulation of multiple redox tags in a single-stranded ON tail attached to a specific sequence[6], to retain the probe capability of forming duplex with complementary target sequence, chemical modification nucleobases within the recognition stretch must be avoided. A general protocol of the labelling reporter probes with Os,bpy in oligo(dT) tails, applicable for any probe/target sequence, involves application of "protector strands" i.e., ONs complementary to the RP recognition sequence[4]. The modified hybridization probe should then be separated from the protective oligonucleotide prior to using it in a hybridization assay. For this purpose, the protector ON was terminally modified with biotin for capture at streptavidin-coated magnetic beads and separation of the labelled probe strand by thermal denaturation. Similar approach was used for the preparation of osmium tail-labelled double-stranded DNA substrates for the monitoring of DNA-protein interactions (in this case, no strand separation was needed). Using magnetic immunoprecipitation at magnetic beads and the osmium-labelled indicator substrates, competitive assays for the evaluation of structure- or sequence-specific DNA binding by tumour suppressor p53 protein were designed[7].

Long labelled tails were also attached to ON probes by means of terminal deoxynucleotidyl transferase (TdT), an enzyme attaching nucleotides at the 3′-OH terminus of DNA using dNTPs as substrates. For this study, we chose dNTP of 7-deazaG substituted with 2-nitrophenyl at the position 7 (**dG^{PhNO2}TP**) with respect to facile TdT incorporation to form long tails, and to favourable electrochemical properties of the nitro group giving 4-electron electrochemical reduction at mercury- or carbon based electrodes[8]. Compared to the Os,L modification, the TdT technique is simpler because it does not require any protection of nucleobases during the label-

ling reaction. ONs tail-labelled with G^{PhNO2} were used in DNA hybridization experiments as well as in DNA-protein binding experiments.

Techniques based on polymerase incorporations of labelled nucleobases are suited for more direct assays, compared to hybridization with prelabelled signalling probes. In primer extension (PEX) and PCR the sequence specificity is conferred by hybridization between primer ON and target DNA serving as a template for the polymerase. Analysis of the PEX or PCR product for the presence of a labelled nucleotide is then sufficient to detect the target template in the sample. Moreover, relative signal intensity of a tag assigned to a specific nucleobase indicates abundance of the given nucleobase in the extended DNA stretch. Site-specific incorporation of a specifically labelled single nucleotide represents an efficient way to electrochemical SNP typing. Besides nucleobases bearing external conjugate groups, such as ferrocene[9], nitro- or aminophenyl[10], $[Os(bpy)_3]^{2+}$ complex[11] etc., 7-deazapurines themselves were also applied as electroactive tags for PCR-amplified DNA owing to considerably lower potentials (by 200–300 mV) of their oxidation compared to the respective natural purines. Particularly 7-deazaG (G*) is oxidized at a lower potential than any natural DNA component, making it a potent electroactive DNA label[12]. Efficient incorporation of G* and easy electrochemical detection has been utilized to monitor electrochemically the PCR amplification of a DNA fragment and to assess partial or full substitution of G with G*.

This work was supported by the Academy of Sciences of the Czech Republic (Z4 055 0506, Z5 004 0507 and Z5 004 0702), the Ministry of Education (LC512, LC06035), Grant Agency of the Academy of Sciences of the Czech Republic (IAA400040901, IAA400040903), Czech Science Foundation (203/09/0317, P206/11/P739) and by Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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FLUORESCENTLY UNIQUE UNNATURAL BASE PAIRS FOR THE EXPANSION OF THE GENETIC ALPHABET

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Creation of an artificial extra base pair, unnatural base pair, working with the natural A-T and G-C pairs in replication, transcription, and translation, could expand the genetic alphabet and code through the genetic information flow. We recently developed an unnatural base pair between 7-(2-thienyl)-imidazo[4,5-*b*]pyridine (**Ds**) and 2-nitro-4-propynylpyrrole (**Px**) that functions in PCR amplification with high efficiency and fidelity. DNA fragments containing the **Ds-Px** pair can be amplified 10^8-fold after 40-cycles of PCR with the unnatural base pair retained in 93–97% of the amplified DNA fragments. In addition, certain modifications of the **Ds** base endow it with fluorescent properties. For example, the deoxyribonucleoside of 7-(2,2′-bithien-5-yl)imidazo[4,5-*b*]pyridine (**Dss**), which was generated by adding an extra thiophene, is strongly fluorescent (excitation: 371 nm; emission: 442 nm; quantum yield: 32% in EtOH). Furthermore, we found that the 2-nitropyrrole moiety (**Pn**) in the **Px** base functions as a fluorescence quencher. Thus, the **Dss-Px** and **Dss-Pn** pairs act as a fluorophore and quencher pair that functions in replication. Here we report the unnatural **Ds-Px**, **Dss-Px**, and **Dss-Pn** pairs in PCR amplification and their applications as sensing and diagnostic tools.

INTRODUCTION

Nucleic acids are composed of four different bases, A, G, C, and T, and the mechanism of genetic information flow relies on complementary A-T and G-C base pairings, through replication, transcription, and translation. If we artificially introduce an extra, third base pair, unnatural base pair, into DNA, the unnatural base pair system could provide a new biotechnology enabling the site-specific incorporation of extra, functional components into nucleic acids and proteins. Many unnatural base pairs have been designed and tested in a biology system[1–5].

Our research group has developed a series of unnatural base pairs[6–14], which exhibit unique specificity in *in vitro* replication, transcription and/or translation. Among them, an unnatural hydrophobic base pair between 7-(2-thienyl)-imidazo[4,5-*b*]pyridine (denoted by **Ds**) and pyrrole-2-carbaldehyde (denoted by **Pa**) functions in PCR amplification and transcription with high fidelity[14] (Fig. 1a). In the system for PCR amplification, we employ modified triphosphate substrates, γ-amidotriphosphates, in place of the usual triphosphate substrates of some bases. We found that the

γ-amidotriphosphates increase their incorporation fidelity in replication. Although the γ-amidotriphosphates are useful, the use of the modified substrates decreases the PCR amplification efficiency and restricts the range of *in vivo* applications. Now we have improved the **Ds-Pa** pair and developed a new pair between **Ds** and 2-nitro-4-propynylpyrrole (**Px**), which no longer requires γ-amidotriphosphates for efficient (Fig. 1b), and selective PCR amplification[15,16]. Here we describe the biological properties of the **Ds-Px** pair and related unnatural base pairs (Figs. 1c and 1d), as well as their applications.

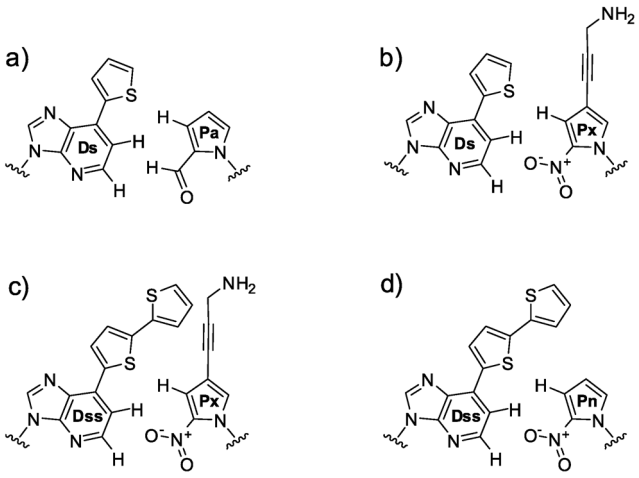

FIG. 1
The unnatural **Ds-Pa** and **Ds-Px** pairs and fluorescent unique unnatural **Dss-Px** and **Dss-Pn** pairs

RESULTS AND DISCUSSION

We developed the **Ds-Px** pair (Fig. 1b) by modifying the **Pa** base to increase the selectivity of the pairing with **Ds** in replication[16]. The shapes and sizes of **Ds** and **Px** were designed to differ from those of the natural bases, and to fit well together. In addition, **Px** efficiently excludes the pairing with A, by the electrostatic repulsion between the 2-nitro group of **Px** and the 1-nitrogen of A[15]. Furthermore, the propynyl group of **Px** increases the hydrophobicity of the unnatural base, strengthening the interaction with polymerases[16].

The selectivity of the **Ds-Px** pairing in PCR amplification is as high as 99.8–99.9% in PCR using DeepVent DNA polymerase with a 3′→5′ exonuclease. We found that the fidelity and efficiency of the **Ds-Px** pairing de-

pend on the natural base sequence contexts around the unnatural base pair site. DNA fragments containing the **Ds-Px** pair can be amplified 10^8-fold after 40-cycles of PCR with the unnatural base pair retained in 93–97% of the amplified DNA fragments[16]. In addition, the amino group of **Px** can be used for a modification site with functional groups of interest, and the modified **Px** substrates are also site-specifically incorporated into DNA by PCR [16].

The **Ds** base is not intrinsically fluorescent. However, certain modifications of the **Ds** base endow it with fluorescent properties. We modified the **Ds** base by attaching an extra thienyl group and synthesized 7-(2,2′-bithien-5-yl)imidazo[4,5-*b*]pyridine (denoted by **Dss**)[17]. The **Dss** nucleosides are strongly fluorescent, which characterized by excitation at 370 nm and emission at 442 nm with a quantum yield of 0.32 in ethanol. Since **Dss** retained the same shape complementarity with **Px** (Fig. 1c), the **Dss-Px** pair also functions in replication selectively.

Furthermore, we noticed that the fluorescence intensity of **Dss** was significantly reduced by base pairing with **Px** in a DNA duplex, and found that the 2-nitropyrrole moiety (**Pn**) has a strong quenching ability[18]. Thus, the **Dss-Px** and **Dss-Pn** pair (Figs. 1c and 1d) acts as a fluorophore and quencher base pair, as well as unnatural base pairs that function in replication. We estimated the fidelity-per-cycle of the **Dss-Px** pairing in PCR, which was more than 99.5% per PCR cycle.

We applied these fluorescently unique base pairs to a molecular beacon and in real-time PCR to detect nucleic acid targets with a specific sequence[18]. The **Dss-Pn** pair was introduced into the stem region of molecular beacons containing a 14-base single-stranded loop, which comprises the complementary sequence to a single-stranded target DNA (Fig. 2). The **Dss** fluorescence of the beacon itself is quenched by the pairing with **Pn**. Upon hybridizing the beacon with the target DNA, the fluorescence was observed with the naked eye by 375 nm excitation at room temperature.

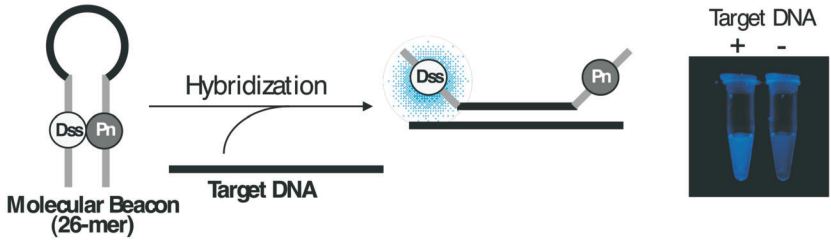

FIG. 2
Visible **Dss** fluorescence of molecular beacons by hybridization to its target DNA

For real-time quantitative PCR, we constructed a PCR primer containing an extra tag with **Dss**. In the system, real-time qPCR was achieved by monitoring the reduction of the **Dss** fluorescence, by the incorporation of the **Px** quencher substrate (d**Px**TP) opposite Dss in the primer during amplification. This qPCR system indicated the high linearity in a dynamic range from 15 to 30,000 copies of a target DNA molecule[18].

The **Ds-Px** and its derivatives, **Dss-Px** and **Dss-Pn**, can be practically used for PCR, providing a new DNA-based biotechnology including a new type of fluorescent quenching system, which can be applied to sensing and diagnostics for target DNA detection.

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NEW STRATEGIES IN SYNTHESIS OF ACYCLIC NUCLEOSIDE PHOSPHONATE PRODRUGS

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Diverse types of acyclic nucleoside phosphonate prodrugs were synthesized using activation of a phosphonic acid residue with hexafluorophosphate coupling agents (HATU, PyBOP, PyBroP). A special attention was paid to ester prodrugs of (*S*)-HPMPDAP, a potential drug candidate for the treatment of poxvirus infections. Several structurally new types of ANP prodrugs were also developed, including functionalized alkoxyalkyl esters, esters with polyethyleneglycol moiety and dioxolenone derivatives. New methodology for synthesis of tissue specific prodrugs based on enzymatic glycosylations of parent compounds is also described.

INTRODUCTION

A prodrug can be defined as a drug derivative that undergoes biotransformation enzymatically or nonenzymatically, inside the body before exhibiting its therapeutic effect. In ideal case, the prodrug is converted to the original drug as soon as the derivative reaches the site of action.

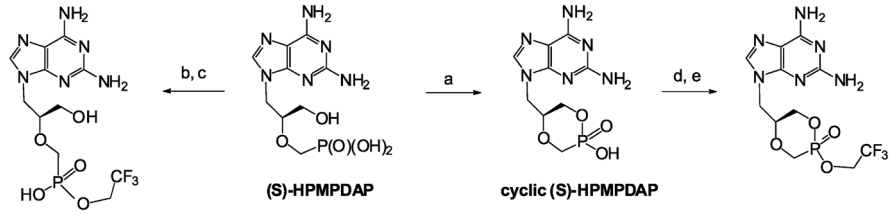
Acyclic nucleoside phosphonates (ANPs) represent a typical example of compounds which utilization in a prodrug form is highly desirable with respect to their low bioavailability caused by the high polarity of a phosphonic group[1]. ANPs lacking a free hydroxyl group in a side chain are mostly developed in a form of neutral bis(esters) or bis(amidates). Adefovir (PMEA), an approved drug for the treatment of hepatitis B is thus used in the form of its orally applicable prodrug, bis(pivaloyloxymethyl) ester (Adefovir Dipivoxil, Bis(POM)-PMEA, Hepsera^{TM})[2]. Similarly, anti-AIDS and anti-hepatitis B drug tenofovir, (*R*)-9-[2-(phosphonomethoxy)propyl]adenine is transformed to a neutral prodrug, bis(isopropoxycarbonyloxymethyl) ester (Tenofovir disoproxil, Bis(POC)-PMPA)[3,4]. Another type of diester prodrugs with a good bioavailability are bis(S-acyl-2-thioethyl) esters (SATE-esters)[5]. Cyclic 1-aryl-1,3-propanyl esters are designed specifically to be activated within the liver to treat liver-based diseases (HepDirect prodrugs). One of the representatives of this group, PMEA prodrug pradefovir was advanced into the Phase II of human clinical trials[6].

In contrast to PME and PMP series, no prodrugs are commercially available in case of HPMP derivatives, compounds having a free hydroxyl group

in a side chain. Synthesis of their diesters is problematic due to a formation of corresponding cyclic phosphonates. So far, the most promising prodrugs seem alkoxyalkyl monoesters[7]. One representative of this group, hexadecyloxypropyl ester of cidofovir (CMX001) is currently developed as antipox virus agent in clinical Phase II.

RESULTS AND DISCUSSION

One of the main tasks of our prodrug research was synthesis of prodrugs in HPMP series. A special attention was paid to 9-(*S*)-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine (HPMPDAP), a compound selected as a drug candidate against poxvirus infections[8]. Besides known procedures based on alkylation of tetrabutylammonium salt of the cyclic phosphonate[8,9], we focused to development of new approaches based on activation of a phosphonic acid residue with hexafluorophosphate coupling agents – PyBOP, PyBroP or HATU. Such reagents are currently used as coupling agents especially in peptide chemistry. These approaches were successful e.g. in preparation of 2,2,2-(trifluoro)ethyl esters, so far known only in PME series, esters with alkyl salicylates or prodrugs based on peptidomimetics (Scheme 1, Fig. 1).



(a) DCC, *N,N*,-dicyclohexyl-4-morpholinecarboxamidine, DMF, 100 °C; (b) PyBroP, DMF, ethyldiisopropyl amine, r.t.; (c) CF_3CH_2OH, r.t. → 90 °C in 3 h; (d) HATU, ethyldiisopropyl amine, r.t.; (e) CF_3CH_2OH, r.t. → 90 °C , 3 h.

SCHEME 1
Transformation of (*S*)-HPMPDAP and its cyclic form to corresponding 2,2,2-(trifluoro)ethyl esters

Promising antiviral activities of all newly prepared HPMPDAP prodrugs not only against poxviruses (vaccinia virus) but also against other DNA viruses (HSV-1, HSV-2, VZV, HCMV, HHV-6) gave the impetus for further detailed investigation of these compounds.

An important part of our research is also development of structurally new types of ester prodrugs. An idea to overcome some undesired properties of alkoxyalkyl esters (e.g. problems with solubility) lead to development of

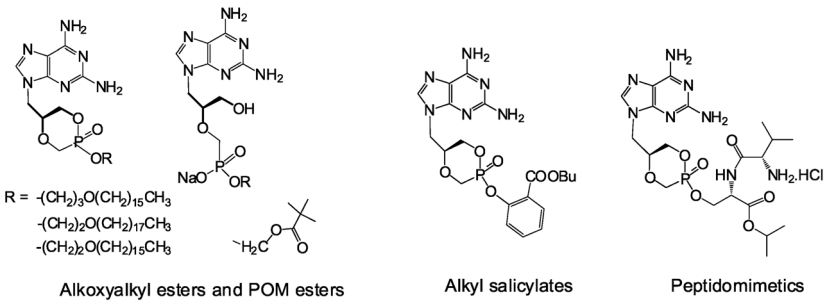
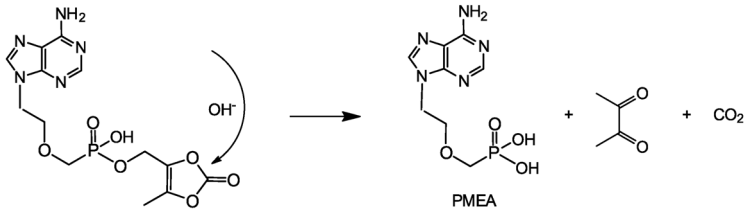


FIG. 1
Structures of new (*S*)-HPMPDAP prodrugs

prodrugs with an amphiphilic moiety consisting of decyl or decyloxyethyl chain bearing hydroxyl function(s), hexaethyleneglycol or a (5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl residue. These compounds were prepared starting from tetrabutylammonium salt of the phosphonate drug and an appropriate alkyl bromide or tosylate. The experiments were performed mostly with adefovir (PMEA); two esters bearing a hexaethyleneglycol moiety were prepared also from cidofovir (HPMPC). Most promising *in vitro* antiviral activity data were found in case of a dioxolenone derivative which has been prepared as aless toxic analogue of Adefovir Dipivoxil. The prodrug is expected to be cleaved in serum yielding non-toxic CO_2 and diacetyl as by-products (Scheme 2).



SCHEME 2
The proposed mechanism of cleavage of 5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl ester of PMEA

A completely different approach to prodrug design is transformation of parent compounds to glycosides. In our team we worked out methodology of enzymatic glycosylations of ANPs and/or nucleoside analogues. Enzymatic synthesis of glycosides has some advantages in low costs, simplicity, stereo- and regioselectivity. The most useful property of glycosides is their potency to act as tissue specific prodrugs. Glycoside prodrugs can be pre-

pared in reasonable yields via enzymatic galactosylation using β-galactosidase C 2-2 from *E. coli*. This reaction proceeds at room temperature in aqueous buffered lactose solutions. Utilization of α-glucosidase leads also to production of desired products, but generally in lower yields. An example of structure of glycosidic prodrugs of ANPs is shown in Fig. 2.

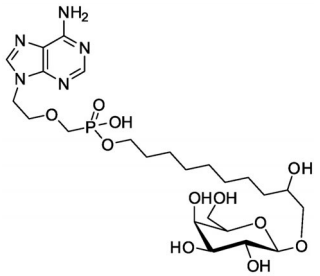


FIG. 2
An example of combined glycosidic prodrug of PMEA. The compound was prepared from an appropriate PMEA ester using the method of enzymatic galactosylation

This work was performed as a part of the Research program of IOCB 0Z40550506. It was supported by the Center for New Antivirals and Antineoplastics 1M0508 by the Ministry of Education, Youth and Sports of the Czech Republic, NIH (grant 1UC1 AI062540-01) and Gilead Sciences & IOCB Research Centre. Antiviral activity of compounds was determined by the team of Prof. Jan Balzarini (Rega Institute for Medical Research, KU Leuven, Belgium).

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CYCLIC AND ACYCLIC PHOSPHONATE TYROSINE ESTER PRODRUGS OF ACYCLIC NUCLEOSIDE PHOSPHONATES

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A series of tyrosine P-O ester derivatives of (*S*)-HPMPA, (*S*)-HPMPC, PMEA and (*R*)-PMPDAP was synthesized as potential antiviral prodrugs. The (*S*)-HPMPC and (*S*)-HPMPA derivatives were prepared by direct PyBOP coupling of a tyrosine promoiety to the cyclic form of the drug followed by hydrolytic cleavage of the internal P–O bond to generate acyclic analogues. Prodrugs of PMEA and (*R*)-PMPDAP, acyclic nucleotide phosphonates (ANPs) lacking the hydroxymethylene functionality, were obtained by an alternative approach, synthesis of a mixed phosphonate monoalkyl diester and selective BTMS-mediated dealkylation.

INTRODUCTION

Acyclic nucleoside phosphonates (ANPs) have proved to be the most effective broad-spectrum antiviral agents to date[1]. The prototype member, (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine ((*S*)-HPMPA, **1**)[2], was followed by several generations of newer ANPs, some of which are clinically significant, such as cidofovir[3], ((*S*)-HPMPC, **2**), adefovir[2] and tenofovir[4]. Ionization of the phosphonic acid group in ANPs at physiological pH greatly decreases the oral bioavailability of the unmodified drug, thus limiting its administration to intravenous injection. Various prodrug approaches have been devised to circumvent this drawback[5,6]. One under investigation in our laboratory involves conjugation of ANPs to benign water soluble promoieties, such as single amino acids or dipeptides, via esterification of the phosphonic acid group with an alcoholic amino acid (Ser, Thr or Tyr)[7]. The presence of multiple functionalizable sites (e.g. NH_2, CO_2H, α-C) at the promoieties allows tuning of the pharmacokinetic properties of the resulting prodrugs. Certain tyrosine-based derivatives of (*S*)-HPMPA and (*S*)-HPMPC have shown promise and have *in vitro* antiviral activity against pox and herpes viruses[8]. Recently, we have extended this approach to two other antiviral drugs: PMEA (**3**) and PMP-DAP (**4**). This communication reports on general synthetic routes and methods to cyclic (**5**) and acyclic (**6**) tyrosine-based derivatives of ANPs.

RESULTS AND DISCUSSION

According to their antiviral activity spectrum, the ANPs can be classified to two categories: the "HPMP" derivatives, which are active against a broad variety of DNA viruses (ANPs **1** and **2**), and the "PME" and "PMP" derivatives (ANPs **3** and **4**), which are primarily active against hepadna- and retroviruses[1]. From the structural point of view, the major difference between these two groups of ANPs consists in the presence of a hydroxymethylene function in compounds belonging to the first group, which allows their conversion into the corresponding cyclic derivatives followed by the attachment of an esterifying promoiety (general structure **5**, Fig. 1). For the compounds in the second group, formation of only acyclic derivatives **6** is possible (Fig. 1).

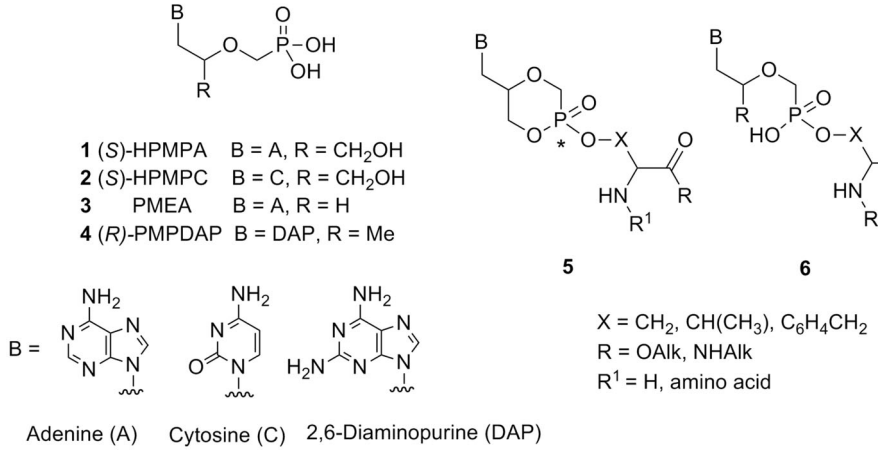
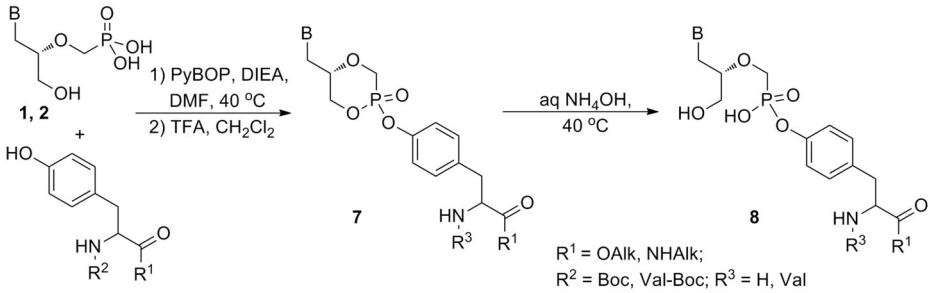


FIG. 1
Structures of ANPs **1**–**4** and their amino acid/dipeptide ester prodrugs **5**, **6**

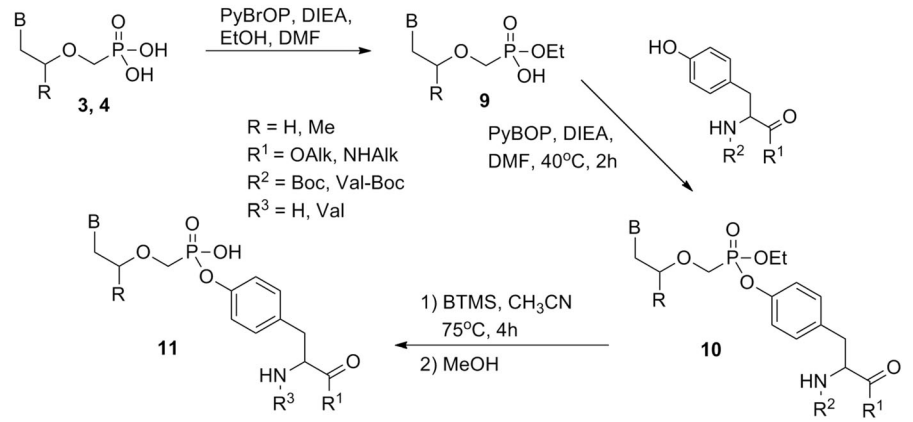
The cyclic tyrosine-based prodrugs of (*S*)-HPMPA and (*S*)-HPMPC (**5**) were synthesized as outlined in Scheme 1 utilizing a PyBOP coupling procedure that allows "one-pot" synthesis of *N*-Boc-protected conjugates **7** from **1** or **2** and a corresponding *N*-Boc-protected amino acid or dipeptide, avoiding isolation of cyclic HPMPC or HPMPA. Conjugation of cyclic intermediates **1** and **2** *via* a phosphoester bond to the promoiety introduces a new chiral center at the phosphorus atom, leading to two different diastereomers of the resulting prodrug, with two distinct ^{31}P NMR signals that facilitate monitoring the individual stereoisomers in the synthetic mixture. Enrichment of the diastereomeric mixture in one isomer is possible by a trans-

esterification procedure using a catalytic amount of the promoiety with Cs_2CO_3 as the base[8]. In the next step, the *tert*-butoxycarbonyl group is removed using trifluoroacetic acid (TFA) in dichloromethane (CH_2Cl_2). The acyclic derivatives **8** of (*S*)-HPMPA and (*S*)-HPMPC can be conveniently obtained in moderate yield from the respective cyclic analogues **7** by treatment with aqueous ammonia[8].



SCHEME 1
Synthesis of tyrosine-based prodrugs of **1** and **2**

In order to obtain acyclic prodrugs **11** of PMEA and PMP-DAP we initially attempted direct PyBOP-mediated coupling of the promoiety with PMEA by analogy to the (*S*)-HPMPA and (*S*)-HPMPC prodrug synthesis. However, the acyclic HOBt-intermediate of **3** was significantly more stable under the reaction conditions than the cyclic HOBt-HPMP-based intermediate from **1** or **2**, and did not react with the promoiety.



SCHEME 2
Synthesis of tyrosine-based prodrugs of **3** and **4**

Consequently, we developed an alternative method (Scheme 2). The first step involves masking one negative charge on the phosphonate group by preparation of the ethyl ester **9**, which in contrast to the tyrosine derivative is readily formed via PyBrOP-mediated coupling. Conjugation of this derivative to the Boc-protected promoiety is then performed by PyBOP coupling, giving rise to a mixed diester of **3** or **4**. Silyldealkylation by BTMS [9] in acetonitrile regioselectively removes the ethyl ester, and methanolysis produces the final prodrugs in moderate to good overall yields. Conveniently, the amino group is also deprotected in one pot under these "McKenna reaction"[10] conditions.

This work was supported by grants AI06145 and AI056864 from the National Institutes of Health and by AMVIS.

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ACYCLIC NUCLEOSIDE PHOSPHONATES AS INHIBITORS OF HYPOXANTHINE-GUANINE-XANTHINE PHOSPHORIBOSYLTRANSFERASE: NEW ANTI-MALARIAL CHEMOTHERAPY LEADS

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Hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT) is a widely recognized target for the discovery of new anti-malarial drugs. This is because the *Plasmodium* parasite, which is the causative agent for malaria, relies on HGXPRT, to make its 6-oxopurine nucleoside monophosphates. Specific acyclic nucleoside phosphonates (ANPs) inhibit HGXPRT and possess anti-plasmodial activity. Within the framework of a SAR-study, the classical ANPs (e.g. PME-, PMP- and HPMP-derivatives) as well as novel series of compounds were tested to investigate their efficiency and selectivity on the inhibition of *P. falciparum*, *P. vivax* and human enzyme. Effects on the growth of *P. falciparum* in cell culture were also studied with the suitable prodrugs of several promising inhibitors. The results provide a basis for the design of potent and selective ANP inhibitors of HGXPRT as anti-malarial drug leads.

INTRODUCTION

Malaria is one of the most widespread infectious diseases in the world with approximately 48% of the planet population living in areas of risk of transmission[1]. It is caused by protozoan parasites of the genus *Plasmodium*, of which there are four species that are principally responsible for human malaria (*falciparum*, *vivax*, *ovale* and *malariae*). An emerging problem in treating this disease is that resistance to most of the commonly used anti-malarial drugs is now occurring world-wide[2]. Thus, the discovery of new drugs is critical if we are to successfully continue to control this disease.

Since many of the metabolic pathways present in the parasite are identical to those of its host cells, the discovery of new anti-malarial drugs that are safe for the host organism is a challenge. One biochemical pathway

where humans and protozoan parasites differ significantly is in purine metabolism. Mammals are able to synthesize purine derivatives *de novo* as well as by salvage of preformed purine bases. In contrast, parasites can only produce nucleoside monophosphates (necessary for many cellular processes) from purine bases synthesized by their host. Not only do protozoan parasites lack the *de novo* pathway, but they also can be deficient in other purine salvage enzymes that provide alternative pathways to purine nucleotide synthesis[3]. 6-Oxopurine phosphoribosyltransferase is a key enzyme of the purine salvage pathway and its activity has been shown to be essential for the replication and survival of the parasite. However, human cells are not as dependent on the activity of the corresponding human enzyme. Patients with partial deficiencies of the enzyme, due to mutation, can lead normal lives even if the activity of the enzyme is reduced to 3% of its normal value[4]. This enzyme should therefore, be an excellent target for the discovery of new anti-malarial drugs[5].

The reaction catalyzed by 6-oxopurine phosphoribosyltransferases is shown in Fig. 1. While the human and *Plasmodium vivax* enzymes can only use hypoxanthine or guanine as naturally occurring purine substrates, for the *Plasmodium falciparum* enzyme xanthine is also a substrate. To date, the only known tight-binding inhibitors of 6-oxopurine PRTase are transition state analogues known as immucillins[6].

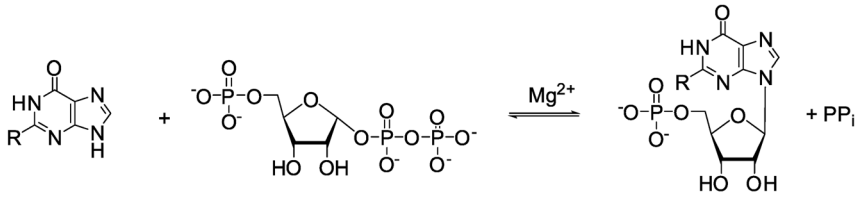

FIG. 1

RESULTS AND DISCUSSION

It was reported previously that (*S*)-HPMPG (Fig. 2) had an IC_{50} value of 4 μM for *Pf* grown in cell culture, although its mode of action was unknown at that time[7]. Recently, because of the structural similarity of acyclic nucleoside phosphonates (ANPs) to the purine nucleoside monophosphates that are the products of the reaction catalyzed by 6-oxopurine PRTases, we investigated a series of already known ANPs (some of them currently used as antiviral agents) as potential inhibitors of *Pf*HGXPRT and human HGPRT [8]. These nucleotide analogues are excellent templates for drug design because of the absence of the labile glycosidic bond and the stability of phosphonate moiety compared with the phosphate ester bond which can

be enzymatically or chemically hydrolyzed. Another structural advantage is the flexibility of the acyclic chain which enables the compounds to adopt a conformation suitable for interaction with the active site of the enzyme. The K_i values (Fig. 2) for human HGPRT and *Pf*HGXPRT, and IC_{50} values for *Plasmodium falciparum* growth in erythrocyte cell culture were determined for many of the compounds. Crystal structures of three ANPs in complex with human HPGRT have also been determined[8].

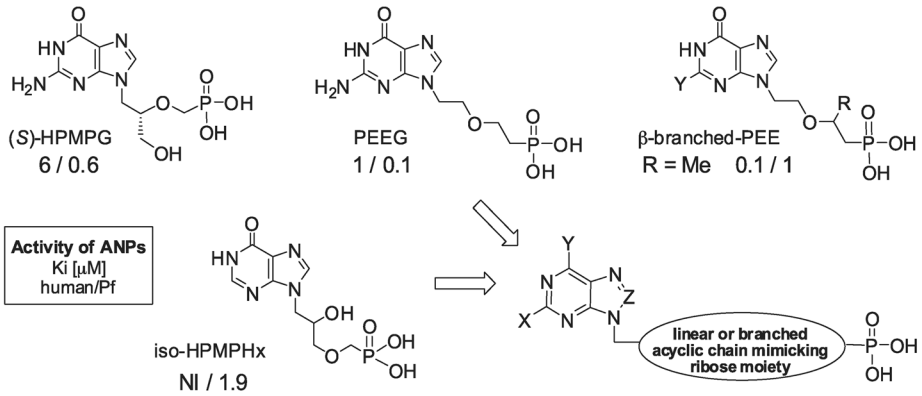

FIG. 2
Examples of ANPs tested as inhibitors of *Pf*HGXPRT and human HGPRT

Recently, we continued this extensive SAR-study by synthesizing a new series of ANPs with modified heterocyclic bases and by varying the length of the acyclic chain between the purine and phosphonate group[9]. Based on those results, a series of novel phosphonoethoxyethyl (PEE) compounds with substituents of different size added either to the carbon attached directly to phosphorus atom (α-branched derivatives) or to the adjacent carbon (β-branched derivatives) were designed. This has allowed us to study the effect of branching on activity and selectivity of inhibition[10].

More recently, recombinant *P. vivax* HGPRT has been expressed and purified to homogeneity[11]. ANPs selected in previous experiments as good inhibitors of *Pf*HGXPRT were also effective as inhibitors of the *Pv* enzyme[11].

ANPs are the first compounds that have been shown to selectively inhibit *Pf*HGXPRT and *Pv*HGPRT compared to human HGPRT. Based on a SAR-study, analysis of crystal structures (Fig. 3) and molecular modeling, lead inhibitors were designed. The synthesis of new series of ANPs as well as preparation of suitable prodrugs of selected inhibitors for *in vitro* tests in erythrocyte culture is in progress.

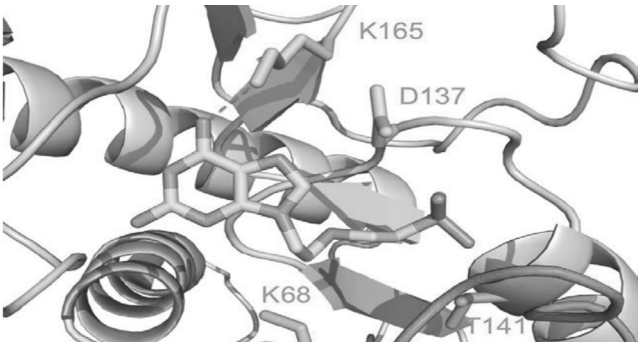

FIG. 3
PEEG bound in the active site of human HGPRT [8a]

This work is a part of the research project AVOZ40550506 of the Institute of Organic Chemistry and Biochemistry and was supported by the Grant Agency of the Czech Republic (grant No. P207/11/0108) and Centre for New Antivirals and Antineoplastics (1M0508). This study was also supported by funds from the National Health and Medical Research Council, Australia (grant No. 569703) and by Gilead Sciences (Foster City, CA, USA).

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REVERSIBLE DNA-TEMPLATED FORMATION OF A BORONATE INTERNUCLEOSIDIC LINKAGE DEPENDING ON EXTERNAL STIMULI

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5′-Borono analogues of natural 2′-deoxynucleotide monophosphates were synthesized from the respective 5′-aldehydic nucleosides through a homologation/reduction sequence followed by either borylation (dTbn, dCbn and dGbn) or cross-metathesis (dAbn). dTbn was subsequently incorporated at the 5′-end of an oligonucleotide as its phosphoramidite derivative. The reversible assembly of a borono-based helix has been accomplished through the connection of the 5′-end boronic acid oligonucleotide with a 3′-end ribonucleosidic oligonucleotide templated by their complementary DNA or RNA full-length strand. These constructions are controlled reversibly by acid/base, cyanide ions or fructose external stimuli.

INTRODUCTION

The consistency in information transfer during the biological synthesis of nucleic acids is the result of selective polymerization and correction enzymes. In contrast, in the absence of these enzymes, chemically-controlled DNA synthesis is poorly efficient and less selective. Several research groups have explored chemical systems able to achieve nonenzymatic oligonucleotide ligation[1] and diverse dynamic nucleic acids-based systems have been reported[2]. However, only a few reversible covalent reactions, providing a means for these systems to repair themselves or transform in response to their environment[3], were used.

Our goal is to create dynamic informational nucleic acid-based oligomers in which reversible internucleosidic linkages are formed in the absence of enzymatic or chemical activation. Among reversible covalent reactions, the reaction of a boronic acid and a cis-diol to form boronate esters has not been exploited in the nucleic acid field. Boronic acids bind 1,2-cis-diol-containing molecules through the reversible formation of 5-membered cyclic boronate esters without chemical activation. In water, the equilibrium between free boronic acids and boronate esters depends on diverse parameters: substitution of the diol moiety, substitution of the boronic acid, pH and temperature of the medium, presence of anions, etc. Thus, boronate es-

ters are able to dissociate and associate and reorganize their molecular components depending on the media.

In this work, we have designed and synthesized 2′-deoxy-5′-borononucleotides of thymidine[4], cytidine, guanosine and adenosine[5] (dTbn, dCbn, dGbn and dAbn; Fig. 1). Their association with uridine was studied. Finally, dTbn was incorporated at the 5′-end of an oligodeoxynucleotide to study its template-directed ligation with an oligonucleotide bearing a 3′-ribonucleotide unit.

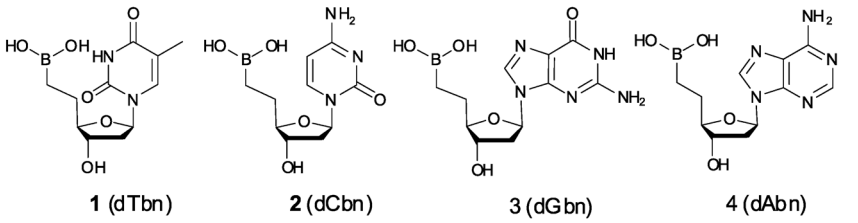

FIG. 1
2′-deoxy borononucleotides

DESIGN AND CHEMICAL SYNTHESIS OF BORONO NUCLEOTIDES

The structures of the borononucleotides fitted with natural molecules. Indeed, surface models of **1–4** with mapped electrostatic potentials showed molecules with similar electrostatic charges than natural nucleotides. Moreover, optimized structures obtained for the borononucleotides compared with their monophosphate analogues indicated no strong structural variation, especially for the C4′-oxygen of the acid function distance.

The achievement of the set of four 2′-deoxyborononucleotide analogues of natural nucleotide monophosphates, was done from the respective 5′-aldehydic nucleosides through a homologation/reduction sequence. The borononucleotides were then obtained by either borylation (dTbn, dCbn and dGbn) or cross-metathesis (dAbn)[4,5].

SPECTROSCOPIC ANALYSIS OF THE INTERACTION WITH URIDINE

With uridine, the borononucleotides form in a concentration-dependent manner, an internucleosidic linkage in which the natural phosphodiester backbone is replaced by a boronic ester[4,5]. Compared to other diols the boronate linkage is particularly stable with the cis-diol functions of ribonucleotides. The stability of these dinucleotides is dependent on a preorganized North-like ribose conformation and stacking interactions.

Indeed, ^{1}H NMR in DMSO-d_6 showed a change in the coupling constant value $^3J_{1'2'}$ of uridine upon formation of the boronate linkage showing that the North conformation of the host sugar conformation is clearly favored by the formation of the dinucleotide.

DNA- AND RNA-TEMPLATED LIGATION

The synthesis of a 5′-end boronic acid strand was performed starting from the 3′-phosphoramidite derivative of dTbn. Hybridization assays were performed with a 14mer template (**ODN3**), a 5′-boronic acid 7mer (**ODN1**) and a 7mer **ODN2** bearing a ribonucleoside at its 3′-end (Fig. 2). **ODN1** and **ODN2** display intentionally important differences in affinity for **ODN3** (Tm 12.3 °C for **ODN3**/**ODN1** and 48.5 °C for **ODN3**/**ODN2**). The ability of the template to bring the boronic acid and the 2′,3′-cis diol functions into close proximity was examined. The formation of the novel boronate-linked duplex clearly influences the lower transition (Tm 19.1 °C, ΔTm 4 °C at pH 7.5).

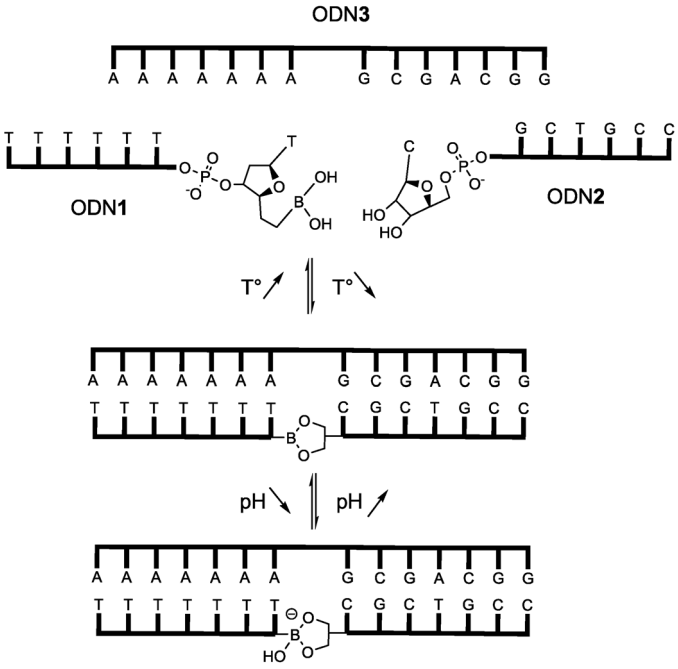

FIG. 2
Borono-based DNA-templated ligation

The duplex stability was then evaluated as a function of various stimuli. Boronate ester can be stabilized by increasing the pH due to the formation of a thermodynamically stable hydroxyboronate complex and to the release of angle tension resulting from the change of boron from sp2 to sp3. Thus at pH 9.5, the duplex displayed a stronger Tm than at pH 7.5 relative to the nicked dsDNA (Tm 26.7 °C, ΔTm 15.7 °C). It is also known that boronic acids form tight and reversible complexes with cyanide ions. Indeed, in the presence of 3 mM of NaCN at pH 7.5, the ligation proceeded efficiently (Tm 30.3 °C) showing that stable tetrahedral sp3 boronate nucleic acid complex can be formed at neutral pH. In contrast the addition of fructose at pH 9.5 prevents the DNA-templated ligation. With RNA as template, the Tm value is 14.0 °C higher than with DNA (Tm = 33.1 vs 19.1 °C at pH 7.5). The pH-dependant stabilization is however less marked than the DNA-templated system (Tm = 35.6 °C at pH 9.5). This could be due to a better accommodation of the boronate linkage inside a DNA/RNA duplex or to the fact that bononucleotides induce a favorable RNA-like North conformation on the ribonucleoside partner through the formation of the internucleosidic linkage (see before).

CONCLUSION

The goal of this work is to develop boronic ester-based internucleosidic linkages. The boronic acid-diol equilibrium allows the formation of stable nucleic acid architectures without chemical activation. This should be the first step towards the conception of an artificial self-replicating genetic system. The control of these systems by external stimuli could be important for the design of dynamic "smart" nucleic acids-based polymers.

The MENRT (A.R.M.), the University of Montpellier 2 (K.M.), the Région Languedoc-Roussillon (D.L.) and the CNRS are gratefully acknowledged for financial support. I.B. thanks the Ministery of Education, Youth and Sports of the Czech Republic (project no. MSM 0021620835).

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DEVELOPMENT OF A GENERAL AND MODULAR APPROACH TO *C*-NUCLEOSIDES

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Highly efficient and modular approach was developed for the synthesis of various types of new (het)aryl *C*-nucleosides. This protocol consists of the synthesis of haloaryl-*C*-nucleoside intermediates, followed by a functional group transformation to introduce various substituents. Using this approach protected 2′-deoxy-*C*-nucleosides bearing halogenated benzene, pyridine, thiophene, furane and pyrimidine were prepared. These intermediates were then submitted to a wide range of palladium-catalyzed reactions. The same approach was also used for preparation of *C*-nucleosides bearing ribofuranose moiety. Functional ribofuranosides bearing diverse substituted pyridine and benzene nucleobases were prepared in this way.

INTRODUCTION

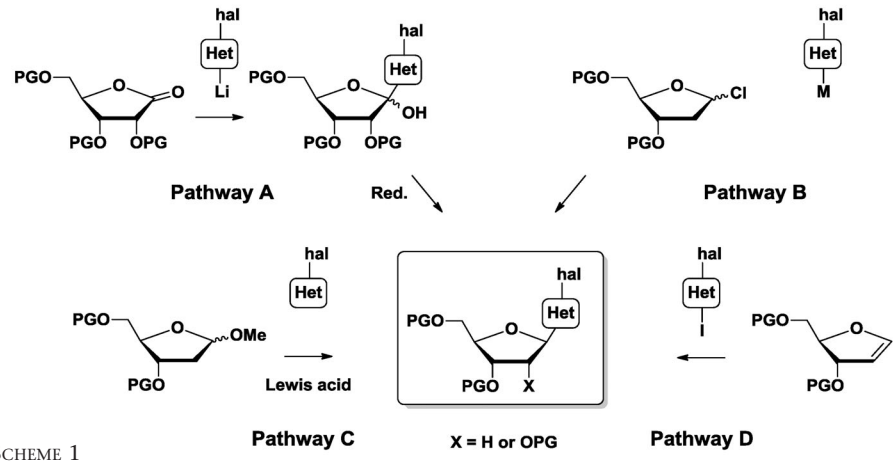
C-Nucleosides are an important class of compounds which are characterized by replacement of the labile C–N bond by a chemically and enzymatically more stable C–C bond, thus they exhibit *in vivo* stability against nucleosidase enzymes. Several *C*-nucleosides are naturally occurring compounds, e.g., pseudouridine (isolated from the yeast tRNA)[1] and showdomycin (an antibiotic). In the past few years, several Artificial Expanded Genetic Information Systems (AEGIS) were successfully developed[2]. These encouraging results gave rise to a new emerging interdisciplinary area known as synthetic biology. The ultimate targets of synthetic biology are the systems which contain a high level of behaviour of living systems which can be mimicked by artificial chemical systems[3]. These artificial aryl *C*-nucleosides could serve as new building blocks in chemical biology due to their capability of π-stacking and self pairing within DNA duplex. *C*-Nucleosides also have applications in medicinal chemistry, for one example the most important biologically active *C*-nucleosides are the inhibitors of purine nucleosides phosphorylase or IMP dehydrogenase.

RESULTS AND DISCUSSION

In our novel modular approach we have synthesized several new types of various (het)aryl *C*-nucleosides in two key steps[4]. The first step was the

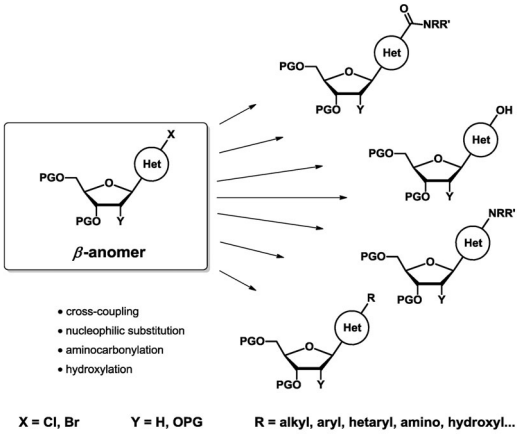
preparation of haloaryl-*C*-nucleoside intermediate which was subsequently submitted to palladium-catalyzed reactions to introduce the various types of substituents.

According to the type of the (het)arylhalogen one of the following procedures for construction of key intermediate was used[5]. The addition of the nucleophilic organometallic reagents, to ribonolactones was used for preparation of benzene and pyridine bearing *C*-ribonucleosides[10] and also for preparation of 6-substituted pyridin-2-yl *C*-nucleosides[6a] (Scheme 1, Pathway A). Coupling of the nucleophiles with halogenoses was used for construction of diverse substituted benzene *C*-nucleosides[4] (Scheme 1, Pathway B). Lewis acid mediated electrophilic substitutions of the aromatic ring by sugar part was used for preparation of thiophene and furane *C*-nucleosides[7,8] (Scheme 1, Pathway C). The Heck-type coupling reaction between anomeric carbon of the carbohydrate moiety and (het)aryl halides was used for construction of pydimidine[9] and 6-substituted pyridin-3-yl *C*-nucleosides[6b] (Scheme 1, Pathway D).



SCHEME 1

The second step in the modular synthesis was based on functional group transformations to introduce various substituents. Thus, protected key intermediates bearing halogenated (het)aryl nucleobases were submitted to a wide range of palladium-catalyzed reactions (Scheme 2). These involved simple hydrogenolysis, cross-coupling reactions with trialkylalanes, Suzuki–Miyaura cross-coupling reaction with a wide variety of boronic acids, Negishi cross-coupling reaction with benzylzinc chloride, Stille cross-coupling reaction with aryl tributyl stannanes, Hartwig–Buchwald aminations with various amines and Hartwig–Buchwald hydroxylations.



SCHEME 2

Using this highly efficient modular approach, a large series of mono functionalised 2′-deoxy-*C*-nucleosides bearing benzene substituted in different positions[4], pyridine[6], thiophene[7] and furane[8] were prepared (Chart 1). Since it is known that many dihaloheterocycles undergo regioselective or chemoselective cross-couplings or nucleophilic substitutions, we decided to extend our approach to the synthesis of hetaryl *C*-nucleosides bearing two different substituents by sequential double substitutions of dihalohetaryl intermediates. Using this aproach series of 2′-deoxy-*C*-nucleosides bearing pyrimidine ring substituted by two different substituents was prepared[9] (Chart 1).

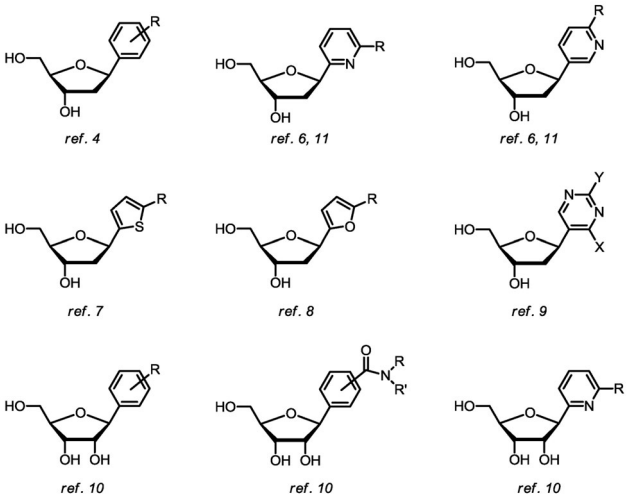


CHART 1

In contrast, *C*-ribonucleosides have not been less explored. Modified *C*-ribonucleosides could serve as building blocks for construction and modification of ribozymes or after their transformation to triphosphates as model compounds for specificity and fidelity studies of RNA polymerases and primases. For these reasons, a series of substituted pyridine and benzene *C*-ribonucleosides were also prepared[10] (Chart 1).

Some of pyridine modified *C*-nucleosides were applied in studies towards understanding on mechanisms of DNA polymerases and primases and toward the extension of the genetic alphabet[11].

This work is part of a research project from the Academy of Sciences of the Czech Republic Z4 055 0506. It was supported by the Ministry of Education, Youth and Sports of the Czech Republic (LC512), by Grant Agency of the Academy of Sciences of the Czech Republic (IAA400550902) and by Gilead Sciences, Inc.

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ON THE FEASIBILITY OF AN ESTERASE-DEPENDENT PRO-DRUG STRATEGY FOR 2-5A

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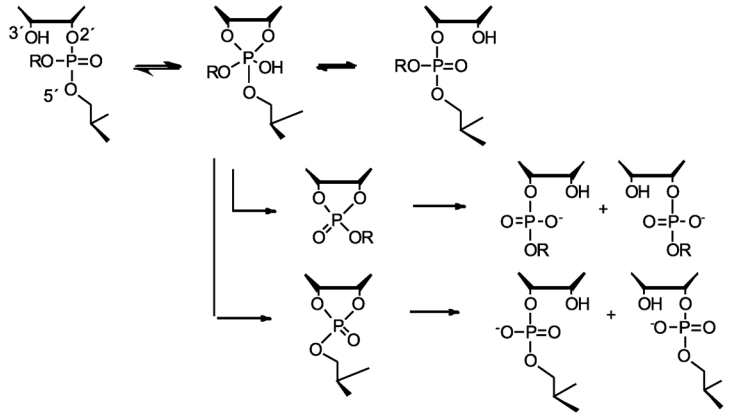
Short 2′,5′-oligoadenylates (2-5A) secreted by cells in response to viral infections could in principle be used as drugs with which to combat against viral diseases. They activate intracellular endoribonuclease RNase L that cleave viral RNA leading to apoptosis. The highly ionic character of 2-5A, however, prevents cellular uptake and, hence, the therapeutic usage. To evaluate the feasibility of an esterase-dependent pro-drug strategy for 2-5A, a fully protected 2-5A (**1**) has been prepared and the exposure of the parent compound by esterase-triggered removal of the protecting groups has been studied.

INTRODUCTION

Trimeric 2′,5′-adenylate 5′-phosphate has long been considered to show potential as an antiviral agent[1]. Main attention has been paid to structural modifications aimed at increasing the biological half-life of the compound without a marked loss of activity[2]. Less attention has been paid to conversion of 2-5A to a pro-drug that could be internalized by passive diffusion through the cell membrane. Design of such a pro-drug is actually highly

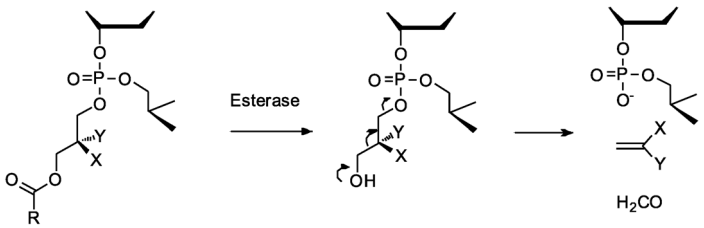


SCHEME 1
Isomerization and cleavage of internucleosidic phosphotriester linkages

demanding. Firstly, there are four anionic functionalities that have to be protected. Gradual accumulation of negative charge on the molecule upon removal of the protecting group converts the compound increasingly poor substrate for esterases. Secondly, the 3′-hydroxy groups neighboring the internucleosidic phosphodiester bond have to be protected as long as the phosphodiester bond remains protected. In other words, the order in which the protecting groups are removed is essential. Unprotected 3′-hydroxy function attacks under physiological conditions extremely fast on adjacent 2′-phosphotriester, resulting in rapid equilibration of 2′,5′- and 3′,5′-bonds and less rapid cleavage of the PO5′-bond (Scheme 1)[3]. The present survey summarizes the attempts to solve these problems.

RESULTS AND DISCUSSION

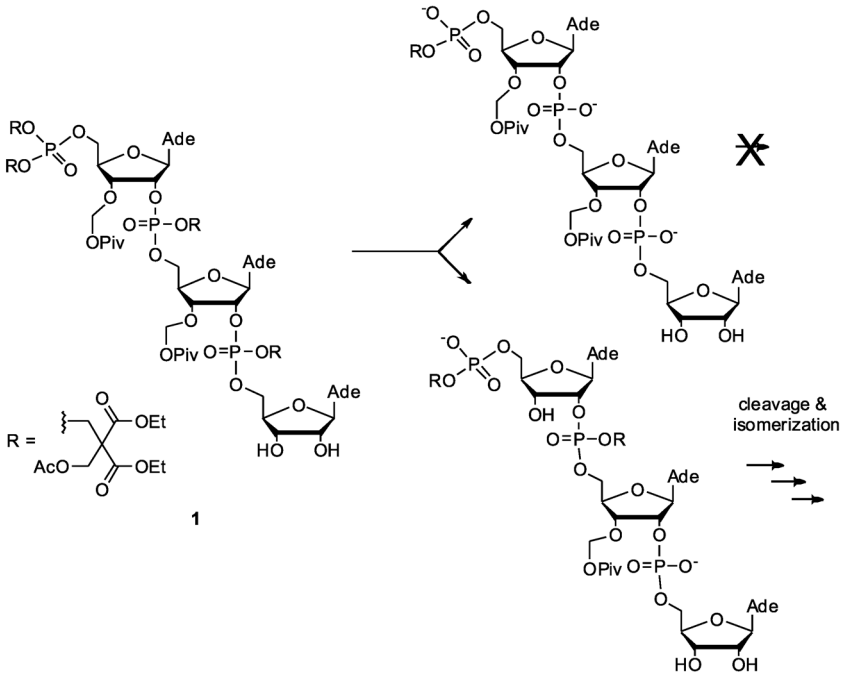
We have recently introduced 2,2-bis(substituted)-3-acyloxypropyl groups as biodegradable phosphate protecting groups[4]. They are stable under physiological conditions, but are, after enzymatic deacylation, cleaved by retro-aldol condensation and concomitant elimination (Scheme 2). An advantageous feature of these groups is that the rate of the estarease-dependent deacylation and the subsequent removal of the remnant may be tuned independently. While the enzymatic reaction is susceptible to steric properties of the acyl group, the retro-aldol condensation/elimination process may be controlled by the polar nature of the 2-substituents.



SCHEME 2
Esterase triggered removal of a 2,2-disubstituted 3-acyloxypropyl group

The first pro-drug candidate of 2-5A was based on the use of 2,2-bis-(ethoxycarbonyl)propyl group for phosphate protection and pivaloyloxymethyl group for the 3′-OH protection. Accordingly, fully protected trimer **1** was prepared[5,6]. Treatment with hog liver esterase (HLE; 2.6 units in ml) at pH 7 and 37 °C for several days exposed the internucleosidic phosphodiester bonds, but not the 5′-terminal phosphate which remained as a phosphodiester (Scheme 3). In other words, the fully phosphate deprotected

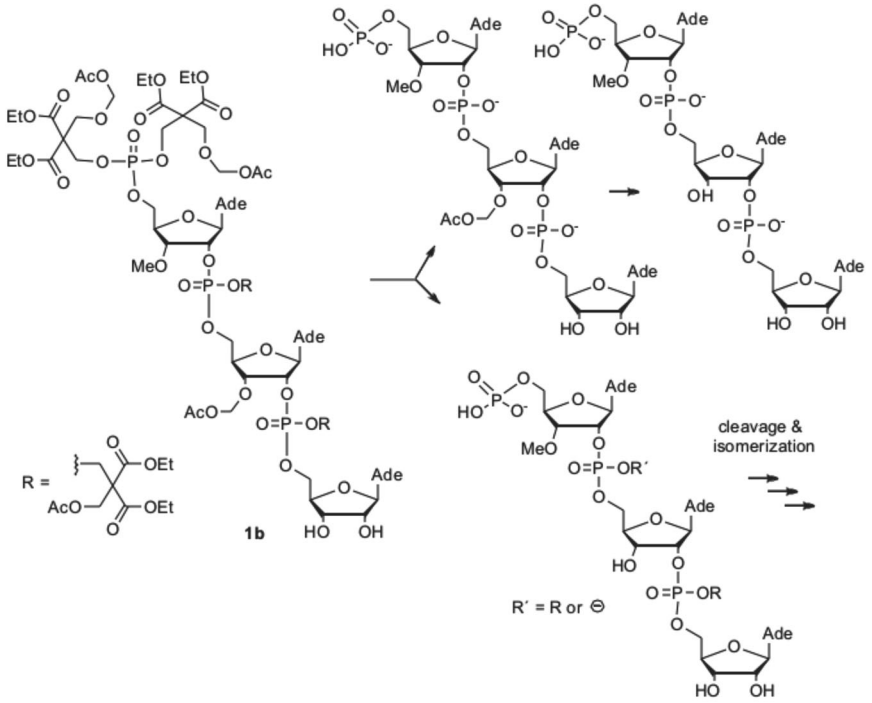
2-5A did not appear. In addition, removal of the 3′-*O*-pivaloyloxymethyl group from the 5′-terminal nucleoside competed with the deprotection of the neighboring phosphodiester linkage, which led to formation of numerous side products by the routes indicated in Scheme 1.



SCHEME 3
HLE-triggered deprotection of fully protected 2-5A trimer **1**

Since the 5′-monophosphate group was not exposed, but the deprotection stopped at the diester level, another pro-drug candidate (**2**) was prepared. The 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl groups at the 5′-terminal phosphate were replaced with more labile 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl groups. Studies on bis-protected thymidine 5′-monophosphate have shown that the latter group is removed almost two order of magnitude faster than the former one[7]. In addition, the 3′-OH of the 5′-teminal nucleotide was protected permanently as a methyl ether and the 3′-OH of the intervening nucleotide with a 3′-*O*-acetyloxymethyl group[7]. This compound (**2**), when treated with HLE under the same conditions as **1**, gave the fully deprotected trimer (Scheme 4). Unfortunately, the deprotection was not clean. Approximately half of the starting material was

converted to various side products for the reason that the 3′-*O*-acetyloxymethyl protection of the intervening nucleoside was cleaved approximately as fast as the protecting group of the neighboring phosphodiester bond, which resulted in isomerization and cleavage of the 3′-terminal phosphodiester bond. Nevertheless, the results obtained suggest that the side reaction may be largely eliminated by using a more labile protecting group for this phosphodiester linkage.



SCHEME 4
HLE-triggered deprotection of 2-5A trimer **2**

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INTRODUCTION OF ADDITIONAL NUCLEOBASES INTO THE DOUBLE HELIX

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A series of double-headed nucleosides presenting a second nucleobase on either the 2′-*C*- or the 5′-*C*-position through different linkers were synthesised and incorporated into nucleic acid sequences demonstrating the formation of thermally stable artificial secondary structures.

INTRODUCTION

In recent years, we have prepared a series of nucleosides with additional nucleobases, also termed double-headed nucleosides, with the purpose of using the recognition potential of the additional nucleobases in various nucleic acid constructs (Fig. 1)[1–5], and also other research groups have contributed to the series[6,7]. A uridine analogue with an additional thymine in the 2′-position, **1**, stabilised a three-way junction when incorporated in the core of the junction, probably due to additional stacking from the thymine.[1] A thymidine-analogue with an additional thymine in the 5′-position, **2**, formed a selective so-called (–3)-zipper-structure in the minor groove of the DNA duplex when incorporated in each of the two complementary strands with two interspacing base-pairs[2,3]. Herein we present a series of new double-headed nucleosides; (1) two analogues of **2** with other nucleobases, **3** and **4**, as well as **5**, which is a close analogue of **1** with a

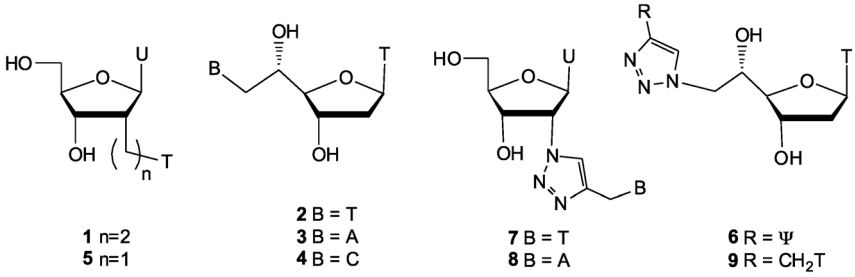

FIG. 1
Double-headed nucleosides. Ψ = uracil-5-yl

shorter linker to the additional nucleobase; (2) four analogues of these, **6–9** [4,5], in which the nucleobases are attached with triazole-linkers prepared by a Click Chemistry approach.

RESULTS AND DISCUSSION

The preparation of **3** and **4** was parallel to our former preparation of **2** [2]. A 5′-epoxide was made from thymidine[2] and reacted with *N*6-Bz-adenine or *N*4-Bz-cytosine. Standard protecting group manipulations and phosphitylation afforded the two phosphoramidites. The preparation of **5** was based on a convergent strategy starting from D-ribose[8]. In six steps, the known methyl 3,5-di-*O*-(*p*-chlorobenzyl)-2-deoxy-2-hydroxymethyl-β-D-ribose was prepared[9], and the two nucleobases were introduced by first a Mitsunobu reaction inserting *N*3-BOM-protected thymine at the 2′-position and then a stereoselective Vorbrüggen type coupling to the uracil. Hydrogenation, DMT-protection and phosphitylation afforded the amidite. The Click Chemistry based preparation of the nucleosides **6–9** and the corresponding phophoramidites have been recently presented[4,5].

All amidites were incorporated into a series of oligonucleotides (ON's) by automated solid phase synthesis. A selection of duplexes formed by these ON's is shown in Table I, and the stability of these was studied by thermal denaturation studies. Entries 1 and 2 demonstrate that the thermal stability of duplexes containing each one incorporation of **2** is decreased with ~5 °C as compared to the corresponding unmodified duplex. Other sequences have demonstrated this to be a very general thermal penalty per modification (4 to 6 °C)[2]. For the cytosine monomer **4** almost identical decreases were seen (data not shown), whereas for the adenine monomer **3** the penalty is larger but equally consistent (5 to 7 °C). The decrease is, on the other hand, significantly larger for one incorporation of **5–8** (generally 7–10 °C) but for **5** more diverse (7–15 °C, not all sequences are shown in Table I). Entry 3 shows a so-called (–3)-zipper structure, *i.e.* with two modifications in complementary strands positioned with two interspacing base-pairs. With monomer **2**, this zipper demonstrates a general and specific interaction between the two thymines across the minor groove leading to a T_m that is 6.5 °C higher than expected from just the addition of the decreases for the two single incorporations. This interaction is only found for (–3)-zippers, as validated from the large decreases in T_m seen for the (–2) and (–1)-zippers in entries 4–5. With the other 5′-modified monomers, the (–3)-zipper is in general less stabilised with only slightly positive $\Delta\Delta T_m$'s for **3**, **4** (not shown) and **6**, and a neutral $\Delta\Delta T_m$ for **9** (not shown). On the other hand,

the (–2)-zipper of **6** was relatively stable indicating a base-base interaction in the minor groove. When the (–3)-zipper was mixed between monomers **2–4**, *i.e.* with additional nucleobases paired in all combinations (data not shown), $\Delta\Delta T_m$'s were in all cases smaller than the 6.5 °C found for two thymines (entry 3). This clearly demonstrates that the base-base interaction in the minor groove is based on stacking and not base-pairing.

With the 2'-modified monomers **5**, **7** and **8** studied in the same zippers, the situation is completely different. The (–3)-zipper orientation of the two monomers **5** (entry 3) lead to complete loss of the duplex, whereas the (–2) and (–1)-zippers in entries 4–5 demonstrates substantial compensations in the T_m's ($\Delta\Delta T_m$'s of 11-12 °C) indicating favourable interactions between the additional nucleobases. For **7** and **8**, no contacts at all were indicated in

TABLE I
Thermal denaturation data

Entry (zipper)	Duplex	ΔT_m, °C[a] [$\Delta\Delta T_m$, °C][b]					
		X = 2[c]	**3**	**5**	**6**	**7**	**8**
1	5'-d(CGC ATA XTC GC) 3'-d(GCG TAT AAG CG)	–4.9	–6.0	–7.4	–7.8	–10.0	–8.5
2	5'-d(CGC ATA TTC GC) 3'-d(GCG XAT AAG CG)	–5.4	–5.9	–15.2	–7.7	–8.0	–8.5
3 (–3)	5'-d(CGC ATA XTC GC) 3'-d(GCG XAT AAG CG)	–3.8 **[+6.5]**	–7.9 **[+3.6]**	n.t.[d]	–10.7 [+4.8]	–21.0 [–3.0]	–22.0 [–5.0]
4 (–2)	5'-d(CGC ATA TXC GC) 3'-d(GCG TAX AAG CG)	–10.0 [–0.9]	–9.5 [+2.4]	–6.0 **[+12.1]**	–4.4| **[+6.6]**	–21.5 [–3.0]	–19.0 [–0.5]
5 (–1)	5'-d(CGC ATA XTC GC) 3'-d(GCG TAX AAG CG)	–10.4 [–0.8]	–12.1 [–0.6]	–3.1 **[+11.3]**	–15.2 [+2.0]	–20.0 [–2.5]	–17.0 [0.0]
6 (+1)	5'-d(CGC AXA TTC GC) 3'-d(GCG TAX AAG CG)	n.d.[e]	n.d.[e]	+0.3 **[+13.5]**	n.d.[e]	–8.0 **[+7.5]**	–5.5 **[+11.0]**
7	5'-d(GCT CAC X CTC CCA) 3'-d(CGA GTG AA GAG GGT)	–3.1/ –13.4	n.d.[e]	**+8.0**/ –2.1	–1.3/ –10.9	0.0/ –10.5	**+3.0**/ –7.5

[a] Differences in melting temperatures as compared to the unmodified duplexes (Entries 1–6: **X** = T, T_m = 45.2 °C; Entry 7: **X** = T, T_m = 43 °C/**X** = TT, T_m = 53 °C). Melting temperatures (T_m values/°C) were obtained from the maxima of the first derivatives of the melting curves (A_{260} *vs* temperature) recorded in a medium salt buffer (Na_2HPO_4/NaH_2PO_4 (7.5 mM with respect to phosphorus), NaCl (100 mM), EDTA (0.1 mM), pH 7.0) using 1.0 μM concentrations of each strand. [b] In brackets, differences in melting temperatures as compared to singly modified duplexes; $\Delta\Delta T_{m(x:y)} = \Delta T_{m(x:y)} - (\Delta T_{m(x:ref)} + \Delta T_{m(ref:y)})$. [c] Data taken from ref.[2]. [d] No transition observed. [e] Not determined.

(–3)-(–1)-zippers (entries 3–5). Due to the positioning of the additional nucleobase in the 3′-direction of these monomers, also (+1)-zippers can be possible, and as shown by entry 6, this is indeed the case with a duplex formed with two monomers **5** regaining the thermal stability of the unmodified duplex by a relative compensation of 13.5 °C and the two corresponding duplexes of **7** and **8** showing only slightly smaller relative compensations though somewhat less stable duplexes. The most obvious explanation for this observation is that the additional thymines of **5** intercalates in the duplex in the 3′-postion to the uracil. In the (+1)-zipper the two thymines are positioned opposite each other possibly forming a T-T base pair. For **7** and **8**, a similar intercalation is probably partly hampered by the triazoles.

Using a different sequence context, the ability of the double-headed nucleosides to recognise two adenosines was studied (entry 7). In other words – can the double-headed nucleotides stabilise a bulge in a duplex? Monomer **2** is not working in this context, as a decrease in melting temperature of 3.1° as compared to one natural thymidine is observed. Compared to a fully matched natural duplex (with TT against AA) the decrease in T_m is 13.4 °C. The same is valid for the other monomers except **5**, which is stabilising the A-bulge significantly as shown by the increase in T_m of 8 °C when compared to an unmodified thymidine, and **8** with an increase in T_m of 3 °C. This clearly indicates that the thymine in the 2′-position of **5** is interacting with the complementary adenine. Mis-match studies (data not shown) demonstrate a lower stability when this A is replaced with T or C but not with G. This might indicate that shape filling is more important than H-bond formation for the stabilising effect on the bulge.

MD-simulations on some of the duplexes studied supported many observations from melting stabilities. Figure 2 demonstrates that the additional uracil-triazole substituents of **6** interact in the minor groove by stacking, and

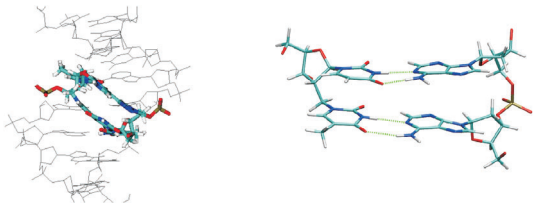

FIG. 2
Snapshots from molecular dynamics simulations of duplexes corresponding to Table I, (a) entry 4 (X = **6**) and (b) entry 7 (X = **5**)

monomer **5** forms two base-pairs with the complementary AA-sequence without violating the B-type structure.

The present results demonstrate that additional nucleobases can participate in base-base interactions both in the core of the duplex and on the duplex surface. Hereby artificial nucleic acid structures are delicately designed, and we are now investigating the scope of these in terms of both structure and function as for instance transfer of information.

This project was supported by the Nucleic Acid Center, which is funded by The Danish National Research Foundation for the studies on nucleic acid chemical biology, as well as by The Danish Research Agency's Programme for Young Researchers and The Danish Council for Independent Research | Natural Sciences.

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HETARYL DERIVATIVES OF 7-DEAZAPURINE RIBONUCLEOSIDES: POTENT CYTOSTATIC AGENTS

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A series of novel 7-deazapurine ribonucleosides substituted with aryl and hetaryl groups has been prepared. Suzuki or Stille cross-coupling reactions with 6-chloro-7-deazapurine ribonucleosides substituted with H, F of Cl atom in position 7 were used in the key step of the synthesis. Either cross-coupling of protected ribonucleoside with appropriate (het)arylboronic acid or stannane followed by deprotection, or single-step aqueous-phase Suzuki cross-coupling reaction of unprotected 7-deazapurine ribonucleoside with boronic acid provided target (het)aryl-7-deazapurine ribonucleosides. 6-Furyl- and 6-thienyl-7-deazapurine ribonucleosides showed cytostatic effect in multiple cancer cell lines in nanomolar range. Application of *cyclo*saligenyl and alanyl-ester phosphoramidate prodrugs did not improved cytostatic activity of parent nucleosides.

INTRODUCTION

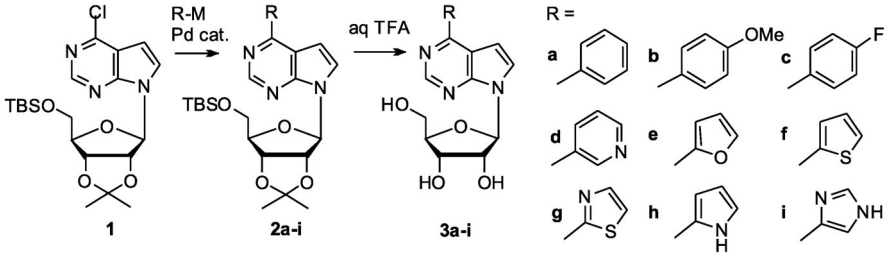
Purine nucleosides represent an important class of antiviral and antitumor drugs. Fludarabine and cladribine are examples of purine nucleoside analogues clinically used for treatment of hematological malignancies[1]. In our prior studies, micromolar cytostatic activity and anti-HCV activity of 6-hetaryl purine ribonucleosides has been discovered[2]. It has been showed that these compounds inhibit RNA synthesis by both viral and cellular RNA polymerases. Novel modifications of 6-hetaryl nucleosides were pursued with a goal either to improve the selectivity of viral RNA polymerase inhibition, or to improve cytostatic effect of these compounds. 2′-, 3′- and 5′-deoxyribonucleosides[3] as well as 2- and 8-substituted ribonucleosides[4] showed no cytostatic activity. Also 3-deazapurine ribonucleosides were inactive[5]. In this communication, we report on synthesis of 6-(het)aryl-7-deazapurine ribonucleosides substituted with H-, F- of Cl-atom in position 7, their biological activity[6]. Furthermore, synthesis and biological activity of other types of related 7-deazapurine ribonucleosides will be presented.

Application of prodrugs can improve biological activity of nucleosides. *Cyclo*saligenyl phosphates[7] and phosphoramidate ProTides[8] were used in the past to bypass the initial phosphorylation of the nucleoside and thereby caused high increase of potency of the parent nucleoside. Therefore the synthesis and evaluation of cytostatic activity of *cyclo*Sal pronucleotides[9]

and alanyl-ester phosphoramidate pronucleotides[10] of 6-(het)aryl-7-deazapurine ribonucleosides will be also covered.

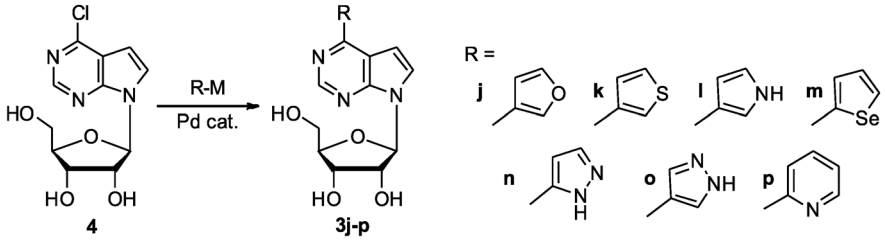
RESULTS AND DISCUSSION

Palladium catalyzed cross-coupling reactions of protected 6-chloro-7-deazapurine ribonucleoside **1** with various (het)arylboronic acids, stannanes or zinc reagents provided series of protected 6-(het)aryl-7-deazapurine ribonucleosides **2a–2i** in good yields (Scheme 1). Desired 6-(het)aryl-7-deazapurine ribonucleosides **3a–3i** were obtained using the deprotection with 90% aqueous trifluoroacetic acid.



SCHEME 1

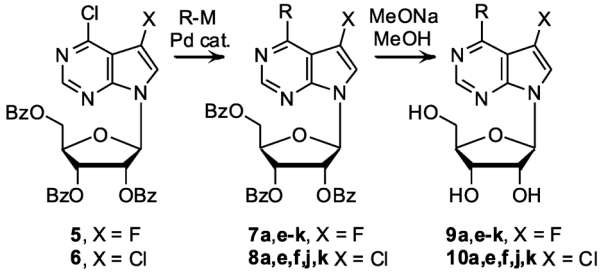
Alternatively, 6-hetaryl-7-deazapurine ribonucleosides **3j–3p** were synthesized by aqueous-phase Suzuki or Stille cross-coupling reactions of 6-chloro-7-deazapurine ribonucleoside (**4**) with the corresponding boronic acids or stannanes (Scheme 2).



SCHEME 2

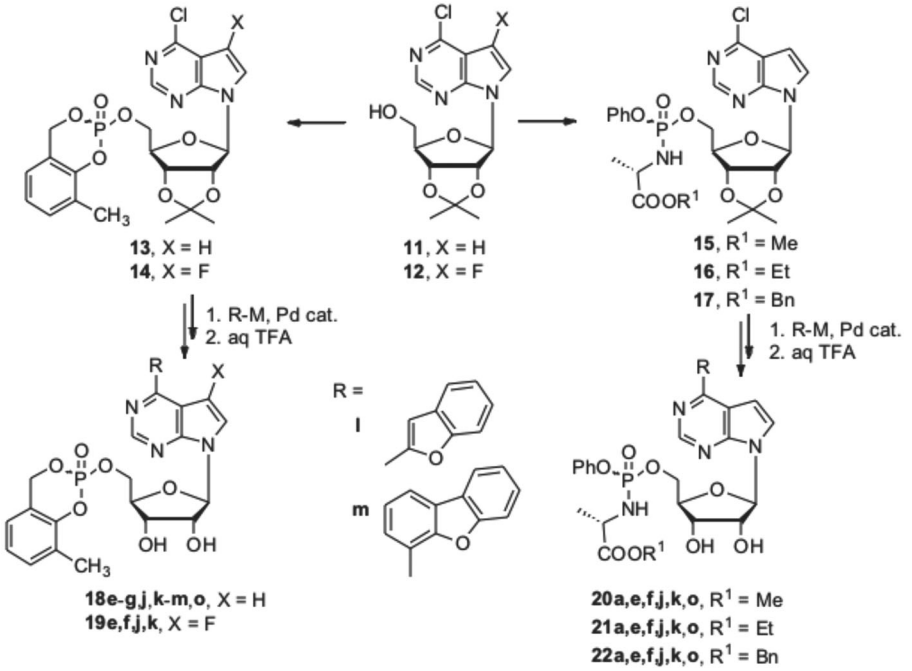
7-Fluoro- and 7-chloro-substituted 6-(het)aryl-7-deazapurine ribonucleosides were prepared under analogous conditions desribed above (Scheme 3). Per-*O*-benzoylated 6-chloro-7-deazapurine riboncleosides **5** and **6** were used as starting materials to obtain protected 6-(het)aryl-7-fluoro-7-deazapurine ribonucleosides **7a**, **7e–7k** and 6-(het)aryl-7-chloro-7-deazapurine ribonucleosides **8a**, **8e**, **8f**, **8j**, **8k**. Then, benzoyl groups were removed by reaction

with methanolic sodium methoxide to afford desired 7-fluoroderivatives **9a**, **9e–9k** and 7-chloroderivatives **10a**, **10e**, **10f**, **10j**, **10k** in high yields.



SCHEME 3

For synthesis of *cyclo*Sal-pronucleotides, protected 6-chloro-7-deazapurine ribonucleosides **11** and **12** were converted to 6-chloro-7-deazapurine pronucleotides **13** and **14** by reaction with 3-methylsalicylchlorophosphane followed by *in situ* oxidation using *t*-butylhydroperoxide. Using the same conditions for Suzuki and Stille cross-coupling reactions of 6-chloro-7-deazapurine ribonucleosides described above followed by deprotection of



SCHEME 4

isopropylidene group, a series of 6-hetaryl-7-deazapurine *cyclo*Sal-pronucleotides **18e–18g**, **18j**, **18k–18m**, **18o** and **19e**, **19f**, **19j**, **19k** was prepared in moderate yields (Scheme 4). All the *cyclo*Sal-pronucleotides were obtained as 1:1 mixtures of diastereomers as a result of non-stereoselective introduction of the centre of chirality to phosphorus atom.

The same synthetical approach was used for synthesis of alanyl-ester phosphoramidate pronucleotides – ProTides. First, 6-chloro-7-deazapurine ProTides **15–17** were obtained by reaction of isopropylidene protected 6-chloro-7-deazapurine ribonucleoside **11** with appropriate alanine-ester phosphorochloridates. The key intermediates **15–17** were used as the substrates for Suzuki and Stille cross-coupling reactions under the same conditions mentioned above. After cleavage of the isopropylidene protection group by aqueous trifluoracetic acid, three series of ProTides **20a**, **20e**, **20f**, **20j**, **20k**, **20o**, **21a**, **21e**, **21f**, **21j**, **21k**, **21o** and **22a**, **22e**, **22f**, **22j**, **22k**, **22o** were obtained as mixtures of diastereomers on phosphorus atom (Scheme 4).

All prepared compounds were tested for their cytotoxic activity in different cancer cell lines using SRB and XTT assay. 2-Furanyl and 2-thienyl derivatives were the most potent compounds among all series of derivatives, both nucleosides and pronucleotides. There was a significant drop of activity between 7-H and 7-fluoro-7-deazapurine ribonucleosides and 7-chloro-7-deazapurine ribonucleosides. Whereas 6-(2-thienyl)- and 6-(2-furyl)-7-deazapurine ribonucleosides substituted with H- or F-atom in position 7 showed cytostatic activity in nanomolar scale, 7-chloro derivatives showed only micromolar activity.

Cytostatic effect of *cyclo*saligenyl-prodrugs was similar or lower than the effect of parent nucleosides. Only 7-fluoro-7-deazapurine pronucleotides **19e**, **19f**, **19k** are slightly more potent in multiple cell lines. Application of ProTides led to significant decrease of cytostatic activity. In general, benzyl-alanyl pronucleotides were most active among ProTides that were synthesized, but even these compounds showed cytostatic activity only in micromolar scale. It was shown by cell permeability test using Caco-2 cell line that the drop of activity was probably caused by effective transport of the prodrug out of the cell.

This work is part of a research project from the Academy of Sciences of the Czech Republic Z4 055 0506. It was supported by the Ministry of Education, Youth and Sports of the Czech Republic (grant 1M0508), Czech Science Foundation (P207/11/0344), by the Programme for Targeted Research (1QS400550501) and by Gilead Sciences, Inc.

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DESIGN OF A NUCLEOSIDE INHIBITOR OF BIOTIN PROTEIN LIGASE FROM *Mycobacterium tuberculosis*

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The etiological agent of tuberculosis (TB) is *Mycobacterium tuberculosis* (*Mtb*), which is the leading cause of bacterial infectious disease mortality. We have identified biotin protein ligase encoded by the gene *birA* that globally regulates lipid metabolism as an attractive target in *Mtb*. We present the design, synthesis, biochemical, and biological evaluation of a picomolar nucleoside bisubstrate inhibitor of this enzyme that also possesses potent and selective antimicrobial activity against *M. tuberculosis* including multidrug (MDR) – and extensively resistant (XDR) – TB strains.

INTRODUCTION

The etiological agent of tuberculosis (TB) is *Mycobacterium tuberculosis* (*Mtb*) that infects over one-third of the world's population and is the leading cause of bacterial infectious disease mortality[1]. The rise of drug-resistant strains of TB precipitates the need to identify novel antibiotics and validate new molecular targets. *M. tuberculosis* encodes for more than 60 adenylating enzymes that catalyze a multitude of essential biochemical processes in protein synthesis and degradation, glycolysis, lipid metabolism, cofactor biosynthesis as well synthesis of small molecule metabolites including mycobactins and mycothiols[2]. Many of the adenylating enzymes in mycobacteria represent attractive targets based on their confirmed essentiality, lack of mammalian orthologs, and availability of three-dimensional structures. In this short communication, we will focus on biotin protein ligase encoded by *birA* in *M. tuberculosis* as a representative adenylating enzyme, which is responsible for the biotinylation of acyl CoA carboxylases and thereby globally regulates lipid metabolism[3].

Adenylating enzymes catalyze the ATP dependent activation of a carboxylic acid substrate to an intermediate acyl-adenylate (Acyl-AMP)[4]. In a second reaction, the adenylating enzyme then catalyzes transfer of the acyl group onto a nucleophilic oxygen, sulfur or nitrogen atom of an acceptor molecule leading respectively to ester, thioester or amide products. In the BirA reaction, biotin **1** is activated to the biotinyl-adenylate **2**

(Bio-AMP) that is transferred onto a conserved lysine residue of a biotin carrier protein domain (BCCP) to afford posttranslationally modified biotinylated-BCCP **3** (Fig. 1). The basis for the development of potent and selective inhibitors for this class of enzymes is the observation that intermediate acyl-adenylates bind more tightly than either substrate (carboxylic acid and ATP) owing to the large number of interactions between the acyl and AMP moieties with their respective binding pockets. Replacement of the labile acylphosphate group of the acyl-adenylate with a chemically stabile isostere provides a simple bisubstrate inhibitor. The enzyme specificity of such an acyl-adenylate mimic is derived from the acyl group. Bisubstrate adenylation inhibitors can be used as a tool compounds to chemically validate a particular target and in some cases may represent genuine leads for antibacterial drug discovery[5]. Additionally, bisubstrate adenylation inhibitors can serve as valuable chemical probes: 1) to facilitate the crystallization of challenging proteins[6], 2) to develop fluorescence polarization assays for high-throughput screening[7], 3) for systematic proteomic profiling[8], and 4) in the affinity selection process for reengineering the substrate specificity of adenylating enzymes by phage display[9]. Herein, we will describe the synthesis and evaluation of a bisubstrate adenylation inhibitor for BirA.

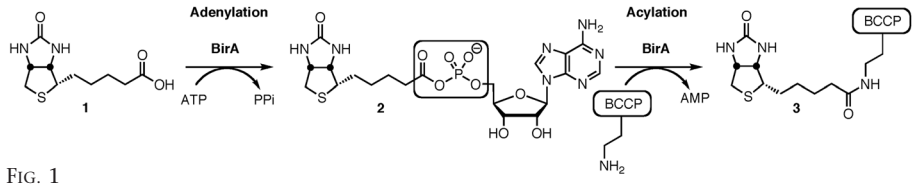

FIG. 1

RESULTS AND DISCUSSION

The acylsulfamate moiety, inspired by the natural product ascamycin, has served as the most popular surrogate for the labile acylphosphate linkage of acyl-adenylates[10]. Accordingly, we synthesized 5(-*O*-[*N*-(biotinyl)sulfamoyl]-adenosine **4** following standard procedures[11]. The pK_a of the acylsulfamate NH moiety is approximately 3–4, consequently **4** was isolated as the monotriethylammonium salt using ion-paired reverse-phase HPLC. However, extended lyophilization of **4** (12 h) led to removal of triethylamine

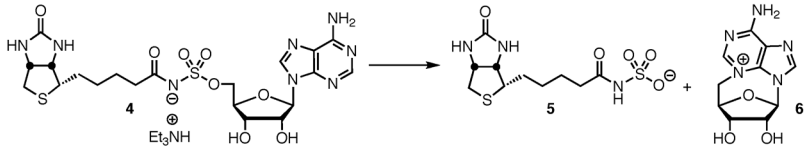

FIG. 2

and subsequent decomposition through cyclonucleoside formation to afford sulfamic acid **5** and 3,5′-cyclo-5′-deoxyadenosine **6** (Fig. 2).

Substitution of the 5′-oxygen atom in acylsulfamate **4** with a nitrogen atom provides acylsulfamide **7** (see Fig. 3) that we hypothesized would be more stabile as a result of the decreased nucleofugality of the sulfamide. The synthesis of **7** was accomplished in seven steps starting from commercially available 2′,3′-isopropylideneadenosine **8**, which was converted to 5′-azido-5′-deoxyadenosine derivative **9** through successive azidation employing diphenylphosporyl azide (DPPA)[12], Boc protection of the N-6 amino group, and hydrogenation of the azide[6a]. Treatment of **9** with sulfamoylating reagent **10**[13] provided **11** and hydrogenolysis of the Cbz group yielded sulfamide **12**. Coupling of the *N*-hydroxysuccinimidyl ester of biotin to **12** using Cs_2CO_3 followed by TFA mediated deprotection of the isopropylidene and N-6 Boc protecting groups furnished acylsulfamide **7** that we term Bio-AMS in analogy to Bio-AMP **2**. Due to its improved stability, Bio-AMS represents an excellent probe to chemically validate BirA as a target for antitubercular therapeutic development.

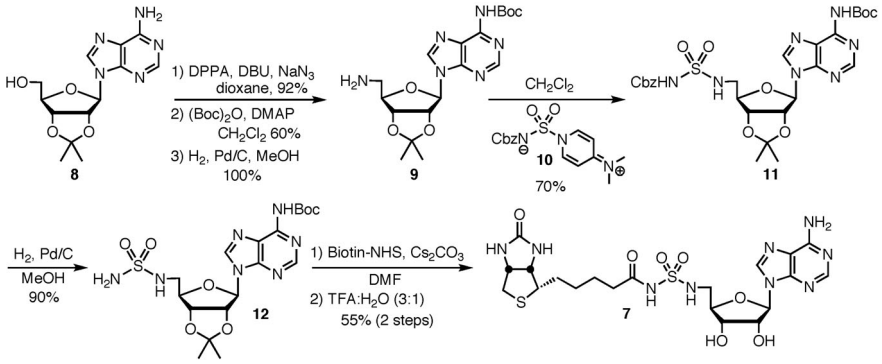

FIG. 3

We next evaluated Bio-AMS **7** in an enzymatic assay with recombinant BirA from *Mtb*. The IC_{50} value was approximately half the enzyme concentration, a characteristic of tight-binding inhibitors[14]. Attempts to determine the true inhibition constant (K_i) were complicated by the tight-binding behavior and bisubstrate nature of inhibition, which precluded assessment by traditional steady-state kinetic methods[15]. We employed displacement isothermal titration calorimetry[16] (ITC) using biotin (K_D = 0.94 μM[3]) as the competitive ligand to assess the binding affinity of **7**. ITC studies provided a K_D of 530 ± 134 pM with an *n* value of 1.12 ± 0.02 indicating one substrate site per BirA monomer. The binding was found to be solely enthalpically

driven (ΔH = –15.4 ± 0.1 kcal/mol) with an unfavorable entropic component ($T\Delta S$ = –3.0 ± 0.1 kcal/mol).

The antitubercular activity of Bio-AMS **7** was measured with *Mycobacterium tuberculosis* H37Rv, the common laboratory virulent strain (initial inocula $OD_{600} \sim 10^{-4}$). The minimum inhibitory concentration that inhibited >99% of cell growth (MIC_{99}) was 0.31 μM. Based on the promising antitubercular activity, we then tested Bio-AMS against ten phenotypically characterized multidrug- (MDR) and extensively drug-resistant (XDR) TB strains. The MIC_{99} values ranged from 0.16–0.625 μM demonstrating Bio-AMS is equally effective against susceptible and MDR- and XDR-TB strains. We also tested the ability of Bio-AMS to inhibit cell growth in other bacterial and fungal strains since BirA homologs are present in most organisms. Remarkably, Bio-AMS was inactive against a panel of Gram-negative bacteria (*A. baumanii*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*), Gram-positive bacteria (*E. faecalis* and *S. aureus*) and fungi (*C. neoformans*, *C. albicans*). Bio-AMS was also evaluated against two mammalian cell lines and exhibited very low cytotoxicity to Vero cells inhibiting 50% growth at 59 μM and no activity toward DU145 human prostate cells.

To provide evidence for the designed mechanism of action, a culture of *Mtb* H37Rv (initial inocula OD_{600} = 0.2) was treated with Bio-AMS (0.1 or 1.0 μM) along with a DMSO control. After treatment for 26 h, cells were harvested, lysed, and protein concentration were determined (note: under these much higher cell densities and short incubation times no inhibition of *Mtb* was observed). The lysates were separated by denaturing gel electrophoresis, transferred to a PVDF membrane, and biotinylated proteins were detected with a fluorescent streptavidin conjugate. When *Mtb* was incubated with 0.1 μM (1/3 × MIC_{99}), no decrease in protein biotinylation was seen. However, when 1.0 μM Bio-AMS (3 × MIC_{99}) was incubated with *Mtb*, an 80% decrease in protein biotinylation was observed. The biotinylated protein bands were confirmed by LC-MS/MS as the alpha subunits of acyl CoA carboxylases. These results clearly demonstrate that Bio-AMS decreases global biotinylation levels of proteins involved in fatty acid biosynthesis.

In conclusion, we have designed, synthesized, and evaluated a potent picomolar nucleoside bisubstrate inhibitor of the adenylate-forming enzyme BirA from *M. tuberculosis* that displays impressive antitubercular activity and an intriguing level of biological selectivity.

This work was supported by the Bill & Melinda Gates Foundation and Wellcome Trust grant for "Drugs for Treatment of Latent Tuberculosis" and the National Institutes of Health grant AI091790.

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REHAB OF NAD-DEPENDENT ENZYMES WITH NAD-BASED INHIBITORS; SYNTHESIS OF METHYLENEBIS(PHOSPHONATE) ANALOGUES OF PYRIDONE-3-CARBOXAMIDE ADENINE DINUCLEOTIDES

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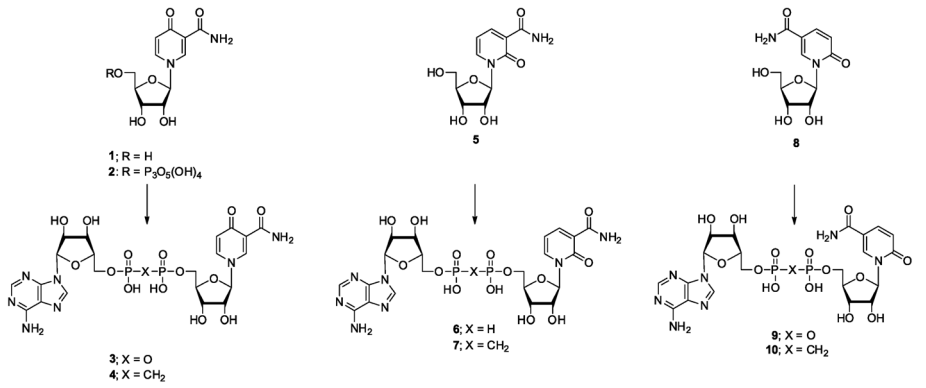
Nicotinamide adenine dinucleotide NAD and its phosphorylated form NADP are utilized as a cofactor or substrate by variety of NAD(P)-dependent (addicted?) enzymes that play an important role in biology and medicine. Since these enzymes cannot function without NAD(P), it was reasonable to assume that cutting the supply of NAD(P) or blocking the NAD(P) binding domain of these enzymes, would affect the cell behavior. A number of NAD-like molecules modified at the nicotinamide part have been reported to inhibit a targeted NAD(P)-dependent enzyme selectively. We report herein syntheses of the bis(phosphonate) analogues of 2-, 4-, and 6-pyridone-NAD. These metabolically stable compounds cannot participate in hydride transfer and are expected to inhibit enzymes that utilized them. The pyridone-NAD analogues can also be converted into affinity probes for detection or isolation of enzymes responsible for degradation of NAD(P) into the corresponding pyridone nucleosides.

INTRODUCTION

In the end of 1970's the 4-pyridone-3-carboxamide ribonucleoside (**1**, Scheme 1) has been isolated from the urine of patients with chronic myelogenous leukemia (CML) and proposed as a potential marker of progression of this disease[1].

It has been suggested that nucleoside **1** was likely formed due to oxidation of NAD^+ at the 4-position of the pyridine moiety to give the 4-pyridone



SCHEME 1

derivative of NAD **3**, which under hydrolysis and de-phosphorylation was converted into **1** [2].

Recently, the triphosphate of the 4-pyridone-3-carboxamide ribonucleoside (**2**) has been detected in erythrocytes of patients with chronic renal failure[3]. The concentration of triphosphate **2** (micromolar level) was rather high and comparable to that of ATP. Finally, 4-pyridone derivative of NADP (**3** phosphate) was reported to be formed as a result of incubation of *Mycobacterium tuberculosis* oxidoreductase FprA with NADP [4]. The crystal structure of **3** phosphate bound at the cofactor binding domain revealed *anti* conformation of the 3-carboxamido-pyridone ring and the extended conformation of the NAD analogue.

Mammalian adrenodoxin reductase, involved in steroid and vitamin D biosynthesis, belongs to the same family of oxidoreductases and also oxidized NADP to give the pyridone **3** phosphate[5,6]. However, it is not known whether or not 4-pyridone-NADP is formed in the cell and has any physiological role.

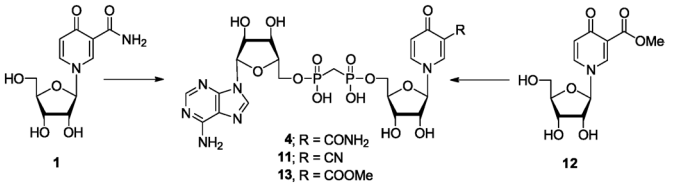
The syntheses of 2-pyridone-3-carboxamide ribonucleoside (**5**) and its anti-inflammatory activity have also been reported[7,8]. The 6-regio isomer **8** has been prepared[9] as well as the corresponding 2-, and 6-pyridone-NAD analogues (**6** and **9**, respectively)[10]. As metabolically unstable pyrophosphates these compounds, however, have not been evaluated in cellular assays.

RESULTS AND DISCUSSION

We report herein the syntheses of metabolically stable bis(phosphonate) analogues of 2-, 4-, and 6-pyridone-NAD (**4**, **7**, and **10**, respectively). All new compounds were prepared by diisopropylcarbodiimide (DIC) coupling of the corresponding 2′,3′-*O*-isopropylidene protected ribonucleosides of pyridone-3-carboxylic methyl esters (such as **12**) with 2′,3′-*O*-isopropylidene-adenosine 5′-methylenebis(phosphonate)[11] followed by ammonolysis and deprotection (Scheme 2).

Interestingly, as it is shown in Scheme 2, using the 4-pyridone-3-carboxamide nucleoside **1** in the sequence of reactions that required isopropylidenenation of **1**, followed by DIC coupling with the adenosine bis(phosphonate) derivative, and de-isopropylidenation of the product, afforded a mixture of the carboxamide derivative **4** and the undesired nitrile derivative **11**, that was inseparable (in our hands) under HPLC purification. Such DIC dehydration of carboxyamido group to the corresponding nitrile is a well known reaction. However, the similar DIC coupling of

nicotinamide-, or benzamide riboside with adenosine-5′-bis(phosphonate) did not result in the formation of the corresponding nitrile derivatives[12]. Thus, we used 4-pyridone-3-carboxylic methyl ester **12** for coupling reaction and then converted the product **13** into the desired carboxamide **4** by ammonolysis. In the similar manner the 2-, and 6-pyridone methylenebis-(phosphonates) **7** and **10** were prepared.



SCHEME 2

It is worthwhile to note that the 2-pyridone-NAD **7** adopts the same *anti* conformation as reported for 4-pyridone-NAD **4** in complex with FprA, whereas 6-pyridone-NAD **10** is fixed in the *syn* conformation. The stereoselectivity of hydride transfer depends on *syn* or *anti* conformation of nicotinamide moiety of NAD(P), i.e. A-type and B-type dehydrogenases bind the cofactor only in the required conformation (*syn* but not *anti* or vice versa). Therefore, it is interesting to investigate a potential selectivity of NAD analogues such as **7** and **10** with restricted rotation around the glycosyl bond. We evaluated our 2-, and 6-pyridone NAD analogues as potential inhibitors of human and mycobacterium NAD kinase (Magni G., unpublished results) and found that 6-pyridone **10** inhibited both human and mycobacterium enzyme equally (50% at the concentration of 0.5 mM). Interestingly, the 2-pyridone **7** was found to be *8 fold* more potent against the human enzyme (80%) then *Mycobacterium tuberculosis* NAD kinase (9%) at the same concentration.

We also found that none of our pyridone-NAD analogues showed activity against human IMP-dehydrogenase. It was recently found that 3-carboxamide-4-pyridone adenine dinucleotide phosphate **3** is not only a good ligand and a competitive inhibitor of *Mycobacterium tuberculosis* oxidoreductase FprA (K_i = 1.2 μM) but also an inhibitor (K_i = 30 μM) of *Toxoplasma gondi* FNR enzyme[6]. Thus, our synthetic bis(phosphonate) analogue **4** when converted into the 2′-monophosphate prodrug could be of therapeutic interest.

We also prepared a number of NAD analogues containing 8-bromo adenosine, using 2′,3′-*O*-isopropylidene-8-bromoadenosine-5′-methylenebis-(phosphonate) (unpublished). Thus, reaction of 8-bromo-NAD analogues

with 1,6-diaminohexane afforded 8-(6-aminohexylamino) derivatives ready for biotynylation reaction[13].

This work was supported by Center for Drug Design, University of Minnesota.

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SYNTHESIS OF CONFORMATIONALLY LOCKED CARBOCYCLIC NUCLEOSIDES WITH NORBORNANE AS PSEUDOSUGAR MOIETY

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We describe the chemical synthesis of three novel structural types of conformationally locked carbocyclic nucleosides with norbornane as sugar surrogate. The presented structures bear hydroxymethyl, nucleobase or both in the bridgehead positions of the norbornane pseudosugar and thus adopt three different conformations of the cyclopentane ring – North, South, and East.

INTRODUCTION

Nucleoside derivatives with locked conformation of sugar or pseudosugar moiety have received great attention over the last two decades. This extensive interest was motivated by two major contributions to this research field. Firstly, the frantic struggle for improvement of oligodeoxynucleotides' hybridization properties crucial for antisense technology resulted in identification of so called locked nucleic acids (LNA), the oligonucleotides with incorporated locked nucleoside monomers. These nucleic acid derivatives found wide range of applications in biomedical sciences, especially due to the great expectations connected with RNAi technology. Most of the contemporarily used locked nucleoside monomers contain bridged ribofuranose rings[1].

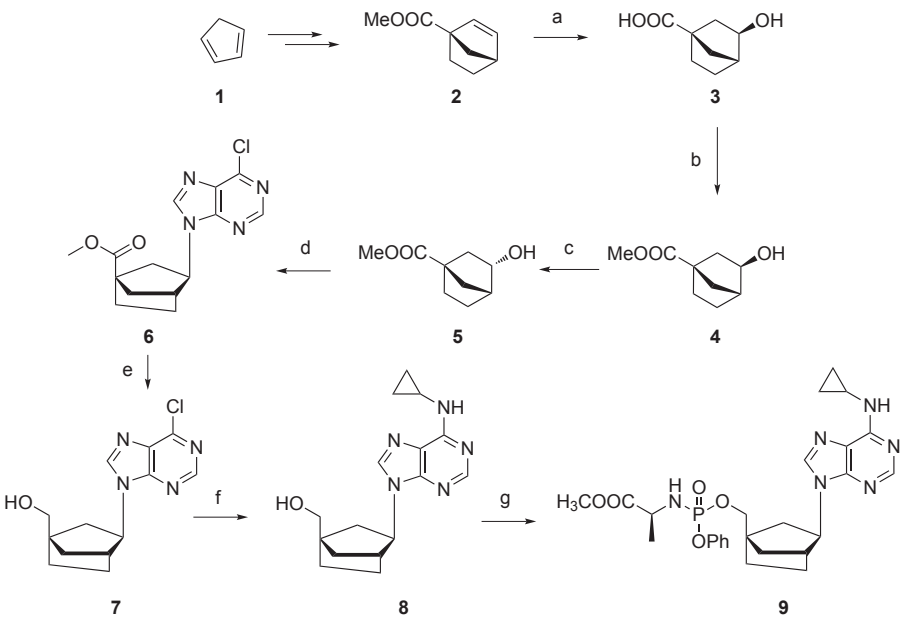
Secondly, it was the excitement for locked carbocyclic nucleosides (LCNs), with the oxygen in the furanose ring substituted by carbon, initiated by the discovery of locked methanocarba nucleosides containing bicyclo[3.1.0]hexanes as sugar surrogates. Exceptional antiviral properties of these compounds locked in North conformation were intensively studied by Marquez and coworkers[1].

In our group, we have recently been exploring synthetic approaches towards novel types of LCNs containing norbornane (bicyclo[2.2.1]heptane) as the pseudosugar moiety. This effort resulted in the discovery of a novel class of antiviral compounds against Coxsackie viruses, a group of important human pathogens[2].

RESULTS AND DISCUSSION

This communication reports on a synthesis and biological evaluation of three new types of conformationally locked carbocyclic nucleoside or nucleotide analogues bearing hydroxymethyl, base or both in bridgehead position of the norbornane skeleton. Consequently, each type adopts a different conformation – North, South, and East. Our main goal was the preparation of locked analogues resembling commercially successful carbocyclic antivirotics Abacavir and Entecavir[3].

An ester **2**, the crucial precursor for the syntheses of derivatives with both North and South conformation, was prepared from cyclopentadiene **1** in 6 steps by modified procedures known from literature[4]. These modifications were made in order to meet requirements of the multi-gram synthesis. Further introduction of a hydroxy group accompanied with hydrolysis of

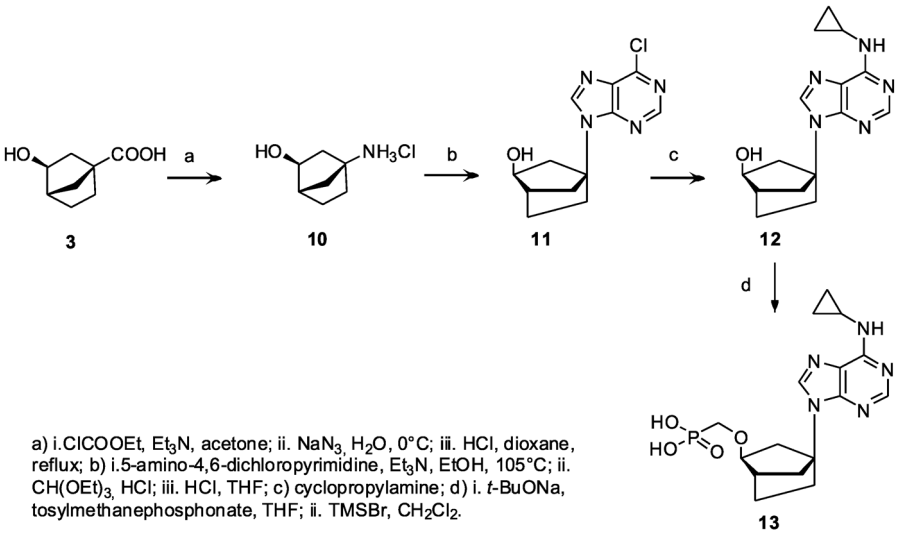


a) i. $Hg(OAc)_2$, THF, H_2O; ii. NaOH, H_2O; iii. $NaBH_4$, NaOH, H_2O; b) CH_2N_2, Et_2O; c) i. PDC, 4A m.s., CH_2Cl_2; ii. $NaBH_4$, MeOH; d) 6-chloropurine, DIAD, Ph_3P, THF; e) DIBAL-H, CH_2Cl_2, -78°C; f) cyclopropylamine; g)
t-BuMgCl, phenylmethoxyalaninyl phosphochloridate, THF.

SCHEME 1
Preparation of locked nucleosides with North conformation

the ester group was accomplished *via* one pot oxymercuration-reduction protocol. The resulting intermediate **3** was then used in the distinct pathways leading to nucleosides/tides with both conformations.

For derivatives with North conformation, the ester group was renewed using diazomethane to give compound **4**. The next step was substitution of the hydroxy group by a purine nucleobase. However, due to Walden inversion, which accompanies Mitsunobu reaction, the configuration of the hydroxy group had to be inverted in order to provide the desired *exo* derivative. Classical oxidation/reduction procedure was employed to obtain the necessary derivative **5**. After the introduction of the 6-chloropurine nucleobase, the ester functionality of the resulting intermediate **6** was smoothly reduced to the crucial hydroxymethyl group at the bridgehead position. The obtained 6-chloropurine nucleoside derivative **7** was an essential precursor for a number of further nucleobase derivatizations at the position 6 of the purine. Among others, we prepared 6-cyclopropylamino derivative **8**, which was subsequently converted into its phosphoramidate pronucleotide **9**.

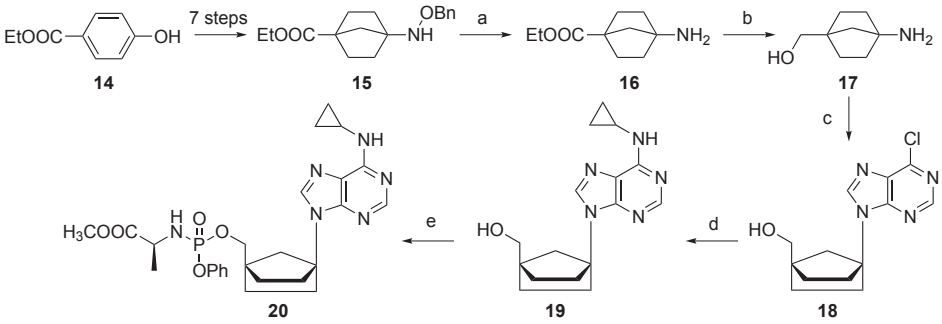


SCHEME 2
The synthetic pathway to the derivatives with South conformation

The intermediate **3** was also used in the synthesis of analogues with South conformation. In order to prepare the analogues of 5′-nornucleosides such as 5′-noraristeromycine[5], which showed interesting activity against

broad spectrum of viruses, we transformed the bridgehead carboxyl into amino group *via* Curtius rearrangement. This amine **10** was used for the introduction of the 6-chloropurine nucleobase by a traditional build-up procedure. The resulting precursor **11** served as a unique substrate for the preparation of large series of 5′-norderivatives and other nucleoside analogues, which will be further discussed. Again, 6-cyclopropylamino derivative **12**, subsequently converted into an appropriate phosphonate **13**, was one of the target compounds.

Finally, we prepared a series of locked derivatives with East conformation. The necessary starting compound **15** was prepared in 7 steps from ethyl *p*-hydroxybenzoate **14** [6]. The benzyloxy group of the derivative **15** was removed by catalytic hydrogenation to afford amine **16**, which was subsequently reduced also on the ester part to give the cardinal intermediate bearing amino and hydroxymethyl groups at the bridgehead positions of the norbornane skeleton. Once more, the 6-chloropurine nucleobase was built-up, which afforded a valuable precursor **18** used for further modifications of the nucleobase. As an example of prepared compounds the cyclopropylamino derivative **19** is given. This nuceloside analogue was also converted to its pronucleotide **20**.



a) H_2, $Pd(OH)_2$/C, MeOH; b) LAH, THF; c) i.5-amino-2,6-dichloropyrimidine, Et_3N, EtOH, 105°C; ii. $CH(OEt)_3$, HCl; iii. HCl, THF; d) cyclopropylamine; g) *t*-BuMgCl, phenylmethoxyalaninyl phosphochloridate, THF.

SCHEME 3
Synthesis of derivatives with East conformation

In conclusion, we present three new groups of locked carbocyclic nucleosides and nucleotides. Further details on the synthesis, nucleobase modifications, and biological activity of the principal compounds will be discussed in detail.

This study, a part of the research project #Z4 055 0506, was supported by the Ministry of Education of the Czech Republic (Centre for New Antivirals and Antineoplastics # 1M0508), and by Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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PHOSPHONATE SYNTHONS BEARING BIOLABILE GROUP FOR OLEFIN CROSS METATHESIS SYNTHESIS OF ACYCLONUCLEOSIDE PHOSPHONATE ANALOGS

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Bioavailability is one of the principal hamper for various polar molecules leading to a therapeutic drug. Several, *hitherto unknown*, unsaturated acyclonucleoside phosphonate prodrugs were synthesized from (acyloxymethyl)- or (hexadecyloxypropyl) allylphosphonate building blocks under olefin cross-metathesis (CM) or Mitsunobu conditions. In case of CM route, a thorough study of ruthenium-based catalyst efficiency was carried out focused on the effects of catalyst scaffold and of supporting ligands to generate the desired phosphonate prodrugs. This presented strategy is appealing for further uses in pharmaceutical and medicinal research.

INTRODUCTION

Phosphonates are prominently represented as pharmacophores in various classes of biological agents. These biological agents include for instance some antiviral and anticancer nucleotides[1] inhibitors of the biosynthesis of cholesterol[2], and bisphosphonates for the treatment of osteoporosis[3] or angiotensis-converting enzyme inhibitors[4]. However, among all synthesized phosphonate molecules with therapeutic targets, only a few led to efficient drugs. One potential reason is a lack of activity due to low bioavailability of those drugs which are in salt form at physiological pH. Thus, medicinal chemists have designed a variety of biolabile promoieties to mask phosphonate groups by derivatization of the phosphorus-coupled oxygen(s) to form neutral ester(s) which highly decrease the polarity of compound[1,5].

Once inside the targeted cell or tissue, the prodrug moiety is cleaved through a chemical or an enzymatic process to release the corresponding free acid phosphonate to achieve the desirable biological effect. One of the most commonly used prodrug type for phosphonates is the acyloxyalkyl ester [e.g., pivaloyloxymethyl (POM) or *iso*propyloxycarbonyloxymethyl (POC)][6]. This approach has been applied to numerous nucleotides as there are two antiviral phosphonates currently marketed by Gilead against hepatitis B (adefovir dipivoxil) and HIV (tenofovir disoproxil fumarate). As re-

sult, the bis(POM) nucleotide analogue LB-80380 is currently under phase 2 clinical trials as a new agent for hepatitis B[7]. More recently, numerous reports have emerged from Hostetler *et al.*[8] on lipid monoesters of nucleotide phosphonates, e.g. the octadecyloxyethyl cidofovir (ODE-CDV) (Fig. 1).

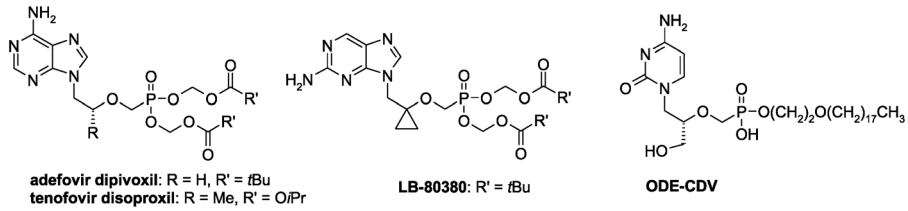


FIG. 1
Nucleoside phosphonate prodrugs bearing acyloxyalkyl or lipid esters

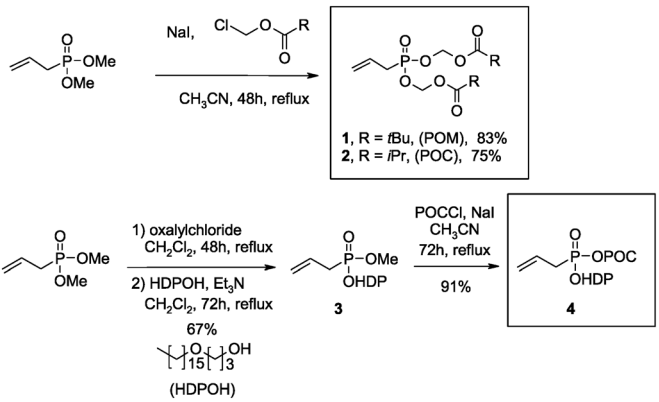
However, the synthesis of these phosphonate prodrug derivatives tends to be tedious and low-yielding. Known procedure usually proceeds in very low (less than 10%) to moderate yields (up to 30%) depending on the substrate. Thus, given the increasing interest of generating masked phosphonate derivatives as biological tools and as antiviral agents and in response to this challenge, we hypothesized that it may be possible to prepare and use *hitherto unknown* bis(acyloxyalkyl) allylphosphonate reagents (e.g., bis(POM), bis(POC) or (HDP/POC) allylphosphonates) for improved generation of phosphonate prodrugs under olefin cross-metathesis or Mitsunobu conditions.

RESULTS AND DISCUSSION

Synthesis of Phosphonate Synthons Bearing a Biolabile Group

First of all, the bis(POM) and bis(POC) allylphosphonates were synthesized from dimethyl allylphosphonate by respective reaction of chloromethyl pivalate and chloromethyl *iso*propylcarbonate in the presence of sodium iodide (Scheme 1).

To complete and diversify our acyclonucleoside phosphonate prodrug series, we became interested in the modification of the biolabile protecting group by using the lipophilic hexadecyloxypropyl (HDP) chain. We described, to the best of our knowledge, the first example of a mixed HDP/POC prodrug.

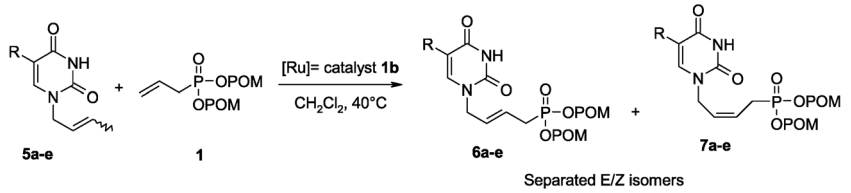


SCHEME 1
Synthesis of bis(POM)-, bis(POC)- and (POC/HPD)-allylphosphonate synthons

Synthesis of Unsaturated Acyclonucleoside Phosphonates by Olefin Cross-Metathesis

Having in hands those phosphonate synthons bearing a biolabil group, and following our previously reported method to synthesize some acid phosphonate derivatives by olefin cross-metathesis[9], we focused our efforts on the best olefin cross-metathesis catalyst system, by screening eight catalysts from the first and second generation. Among them, benzylidene complexes and boomerang Hoveyda–Grubbs type catalysts, bearing the indenylidene-framework complexes, with various *N*-heterocyclic carbenes (NHCs) ligands (IMes, IPr).

Thus, optimized conditions using IMes benzylidene catalyst **1b** at 40 °C were applied to the synthesis of unsaturated acyclonucleoside phosphonates **6a–6e** and **7a–7e**. The desired compounds were obtained in good yields (53 to 69%), with reaction times ranging from 30 min to 1 h (Scheme 2). This reaction led to the desired C^5-substituted uracil acyclo-



SCHEME 2
CM reaction between bis(POM) allylphosphonate and N^1-crotylated 5-bromouracil

nucleoside phosphonate bis-POM prodrugs as a separable mixture of *E*/*Z* isomers with the more thermodynamically stable *E* isomer as the major product (~4:1 *E*/*Z*).

Similar studies led to the synthesis of the hitherto unkown unsaturated acyclonucleoside phosphonate prodrugs **8–10** (Fig. 2).

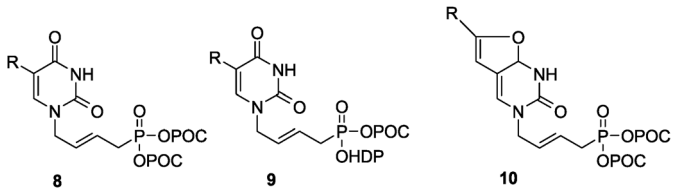
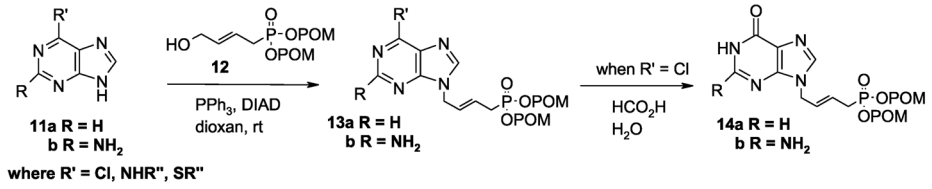


FIG. 2
Unsaturated acyclonucleoside phosphonate prodrugs obtained

Synthesis of Unsaturated Acyclonucleoside Phosphonates Under Mitsunobu Conditions

Due to the nitrogen of the purine moiety which can inhibit the [Ru] = catalyst during olefin cross-metathesis, to reach purine analogues, a Mitsunobu reaction was undertaken. Addition of the hydroxyallylphosphonate **12** to various 6-substituted purines **11a** and **11b** with PPh_3 and DIAD afforded the unsaturated purine acyclonucleoside phosphonates **13a** and **13b**, respectively, where R′ = Cl, NHR″, SR″. When R′ = Cl, **13a** and **13b** were converted to their hypoxanthine **14a** and guanine **14b** analogs (Scheme 3).



SCHEME 3
Mitsunobu route to unsaturated acyclonucleoside phosphonates

For the portion of the work performed at the University of St Andrew, funding for this project was provided by the EC through the seventh framework program (grant CP-FP 211468-2 EUMET). We thank Umicore AG for generous gift of materials.

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DNA QUADRUPLEXES FOR BIO- AND NANOTECHNOLOGIES

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Most non-canonical nucleic acid conformations are stable only under non-physiological conditions and have, therefore, been considered simple curiosities. Among these oddities, a family of nucleic acid secondary structures known as G-quadruplexes (G4) has emerged as more than a novelty. These structures can be formed by certain guanine-rich sequences and are stabilized by G-quartets. G-quadruplexes can be very stable under physiological conditions and have applications in biology, nano- and biotechnologies. We discuss here new applications as molecular beacons and original building blocks for DNA-based nanotechnologies.

INTRODUCTION

Nucleic acids are prone to structural polymorphism: in addition to the classical DNA double-helix, a number of alternative structures may be formed. Important biological processes require melting of the DNA double-helix[1] and several genetic diseases are mediated by the formation of non B-DNA structures[2]. Among these, G-quadruplexes (G4) represent an exceptional polymorphic class of higher-order nucleic acid structures in which the structural unit is formed by a planar arrangement of four Hoogsteen-bonded guanines known as *G-quartets* (Fig. 1). A vertical π-stacking arrange-

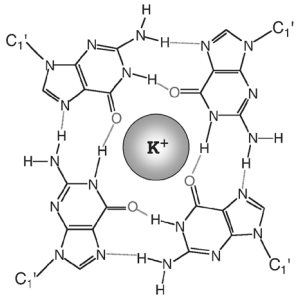

FIG. 1
Presentation of a G-quartet with four coplanar guanines

ment of several G-quartets and the presence of monovalent cations provide these structures with remarkable stabilities.

Nucleic acids are gaining in popularity and utility for creating new nanomaterials due to their ability to self-assemble[3,4]. Pairing of double-stranded DNA is being explored by a growing number of researchers to construct extremely sophisticated nanostructures and nanodevices. We believe that G4 offer interesting new possibilities for nanotechnology and biotechnology and we are currently seeking for the following properties[5–7]:

Extending the Repertoire of G-Quadruplexes

Tetramolecular G-quadruplexes result from the association of four short guanine-rich strands. Systematic modifications to the bases or the backbone of those strands have been performed pursuing two aims: improving stability of the overall structure and introducing new functionalities. Over the last years we observed that substitution of guanines by other bases may increase quadruplex stability. Furthermore we showed that modification of 5′-end guanine at position 8 generally leads to a significant increase (10-fold or more) in quadruplex formation rate. This observation indicates that one may consider 8-bromo[8], 8-amino[9] or 8-methyl[10] guanine as attractive substitutes for guanine in the context of tetramolecular quadruplexes. One should also keep in mind that not all substitutions are favored: in most cases quartets formed by oligonucleotides with modified bases exist thanks to the docking platform provided by the neighbouring G-quartets[8] without contributing to stability.

G4-Based Molecular Beacons

Molecular beacons (MB) are nucleic acid probes with hairpin shaped structures. The self complementarity of their 5′ and 3′ extremities usually rely on a Watson–Crick base pairing bringing a fluorophore and a quencher in close proximity. We previously demonstrated that a G4-based MB (MB, Fig. 2) outperforms a regular MB thanks to its differential ionic sensitivity (stability of the quadruplex stem may indeed be adjusted independently from the stability of the MB-target duplex by varying the monocation Na^+/K^+ or dication concentration)[11].

Efforts are now being pursued to take advantage of the presence of 3 loops in a intramolecular quadruplex. Provided that one may obtain a stable quadruplex involving at least two long loops, a double beacon may be designed. We have recently demonstrated that stable intramolecular G4 may

be formed with internal loops of more than seven bases[12,13]. One could envision the design of a double beacon based on an unusual quadruplex involving at least two long loops. Each individual loop would then be recognized by a complementary strand, potentially allowing an improvement in selectivity.

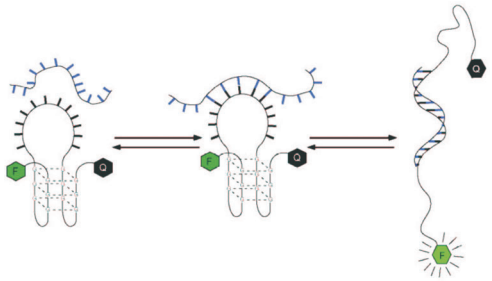

FIG. 2
A simple molecular beacon (MB) with a quadruplex stem[11]

G4-Based Nanotiles

When considering DNA-based nanotech applications, G-quadruplexes suffer from a fatal flaw: the rules of pairing, i.e. formation of a G-quartet, in which four *identical* bases are paired, prevent the controlled assembly between different strands. As a result, complex mixtures are obtained instead of well-defined objects. We propose a solution to this problem. Carefully designed parallel stranded duplexes[14] were used to direct the formation of all parallel G-quadruplex DNA from four different strands. The G-quadruplex core could thus serve as a "knot" due to its known unusual stability. The presence of multiple points of attachments allows for additions of DNA sequences that are prone to formation of desired specific structures: Watson–Crick duplexes, i-motifs, other G-quadruplexes, etc. adding to the versatility of the structure. The correct formation of the overall structure may be assessed using gel electrophoresis; the presence of four strands within the structure was demonstrated using fluorescent labels. The presence of the G-quadruplex core was demonstrated through UV-vis and fluorescent titrations with G-quadruplex specific ligands. The thermal properties of the target structure as well as its duplex components were thoroughly analyzed. The structure obtained displayed unusual stability under denaturing conditions. Attempts to extend the design to one- and two- dimensional materials are underway.

This work was supported by INSERM, Fondation pour la Recherche Málicale (FRM), University of Bordeaux Segalen, Agence Nationale de la Recherche (ANR grants G4-Toolbox & QuantADN), Institut National du Cancer (INCa) and Région Aquitaine grants. We thank all members of ARNA laboratory as well as P. Alberti (MNHN, Paris) D. Monchaud (Dijon) M. P. Teulade-Fichou (Curie, Orsay), R. Eritja (Barcelona), A. Galeone (Naples) and L. Lacroix (Toulouse) for helpful discussions.

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TEMPLATE ASSEMBLED SYNTHETIC G-QUADRUPLEX (TASQ): A NEW BIOMOLECULAR SYSTEM FOR INVESTIGATING THE INTERACTIONS OF LIGANDS WITH CONSTRAINED QUADRUPLEX TOPOLOGIES

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We reported on the design of a new biomolecular device based on the concept of Template Assembled Synthetic G-Quadruplex (TASQ), where quadruplex DNA structures are assembled on a template allowing the precise control of parallel G-quadruplex conformation. This device is used for investigating by SPR the affinity of ligands for G-quadruplex, providing more precise information about the binding mode of them.

INTRODUCTION

DNA has been long considered as an ideal target for developing novel classes of therapeutic agents. Until recently the focus was on double-stranded DNA structures (duplex-DNA), where two sequences of DNA are held together in an anti-parallel double helical architecture through canonical Watson–Crick A/T and G/C base pairing. More recently, the targeting of alternative DNA architectures has shown an increasing interest; in particular, the more interesting G-quadruplex DNA architecture comprising a four-stranded structure of stacked guanine-tetrads formed by the coplanar arrangement of four guanines, held together by Hoogsteen bonds. Indeed, these structures have been hypothesized to play an important role in many relevant biological processes such as telomere stabilization, oncogene activation, and regulation of the immunoglobulin switch region[1]. A number of small molecules (called G-quadruplex ligands), displaying varying degrees of affinity and more importantly selectivity (*i.e.* the ability to interact only with quadruplex-DNA and not with the duplex-DNA), have been designed to target the G-quadruplex-DNA[2]. This small-molecule approach is not only devoted to the discovery of new anticancer agents but also to the generation of chemical tools for elucidating the biological functions of quadruplexes.

We were interested by the use of surface plasmon resonance (SPR) for monitoring the interaction of ligands with quadruplexes. Indeed this method may provide information on both the thermodynamic and the kinetic parameters of ligand interactions. However, a wide variety of topolo-

gies can be adopted by the G-quadruplex depending on the number of strands involved in the structure, the strand direction, as well as variations in loop size and sequence. Obviously, this can confuse the study of recognition phenomena with potential ligands. In this context, we have designed a novel molecular device based on the concept of Template Assembled Synthetic G-Quadruplex (TASQ) for directing the folding of G-quadruplex into a single conformation. The present communication aims to provide an overview of our research accomplishments and the future challenges in this field.

RESULTS AND DISCUSSIONS

The template used in our approach is a cyclic decapeptide with two independent functionalizable faces due to the orientation of the lysine side chains. On one side, the four oligonucleotides derived from the human telomeric sequence d($^{5'}$TTAGGGT$^{3'}$) were anchored by using the oxime bond formation and a biotin residue was incorporated on the other side for the attachment on surfaces through streptavidin binding. The system **1** (Fig. 1) was thus prepared in satisfactory yield and characterized by mass

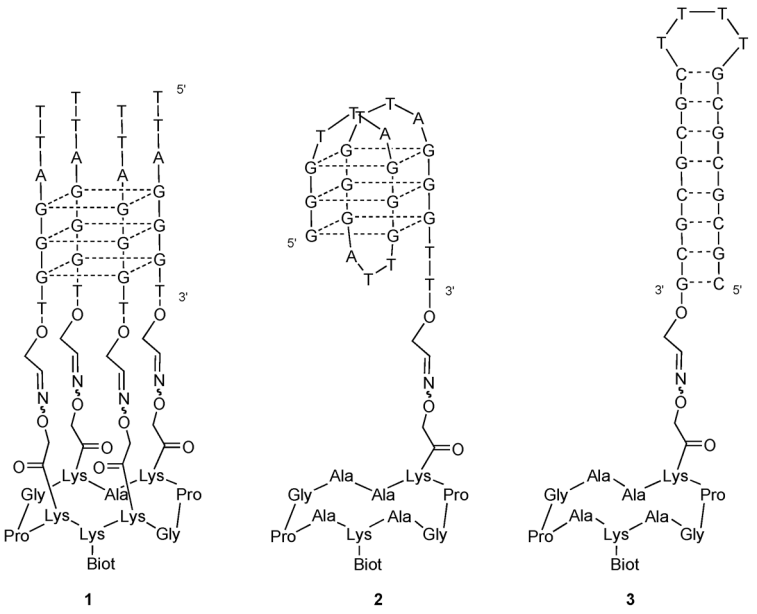

FIG. 1
Parallel G-quadruplex **1**, antiparallel G-quadruplex **2** and duplex **3** used for SPR studies

spectrometry. Circular dichroism (CD) spectroscopy was used to determine the topology adopted by the four ODNs in the quadruplex **1** and confirmed the formation of a parallel G-quadruplex conformation. We also demonstrated that the use of the scaffold allows the formation of the quadruplex motif even without the addition of cations and dramatically increases the stability of the motif[3].

The molecular system can be immobilized on surface for studying by SPR (Surface Plasmon Resonance) the interactions with small organic molecules. The natural human intramolecular quadruplex **2** and duplex **3** have been also anchored on this peptidic scaffold (Fig. 1). In this manner by using SPR, we have investigated the interactions of various ligands with these different nucleic acid conformations (parallel G-quadruplex **1**, antiparallel G-quadruplex **2** and duplex **3**). A representative panel of G-quadruplex ligands, each of them being characterized by a typical binding mode (tetrad, groove or loop recognition or a combination) were studied to establish the proof of concept (Fig. 2).

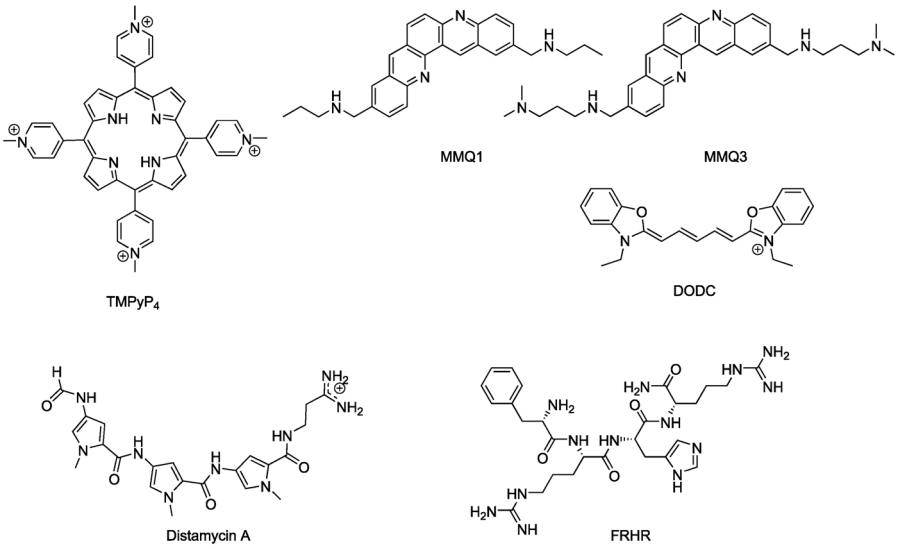

FIG. 2
Structure of the different ligands used in the present study

Interestingly, the various studied ligands have shown differences of behavior that could be correlated with the binding mode of the ligand. As anticipated, ligands displaying a π-stacking binding mode showed a higher binding affinity for intermolecular-like G-quadruplexes **1**, whereas ligands

with other binding modes (groove and/or loop binding) showed no significant difference in their binding affinities for the two quadruplexes **1** or **2**. A numerical parameter, called G-quadruplex Binding Mode Index (G4-BMI), has been introduced to express the difference in the affinity of ligands for intermolecular G-quadruplex **1** against intramolecular G-quadruplex **2**. The G-quadruplex Binding Mode Index (G4-BMI) of a ligand is defined as follows: G4-BMI = KD_{intra}/KD_{inter}, where KD_{intra} is the dissociation constant for intramolecular G-quadruplex **2** and KD_{inter} is the dissociation constant for intermolecular G-quadruplex **1**. This numerical parameter G4-BMI helps to evaluate the binding mode of ligands without any structural studies. A high G4-BMI value correlates with π-stacking as major binding mode, whereas a low G4-BMI value suggests the absence of π-stacking as major binding mode. In addition, the present method also provided information about the selectivity of ligands for G-quadruplex DNA over the duplex-DNA.

CONCLUSION

The molecular devices **1–3** enabled the concomitant study of the affinity for G-quadruplex (system **1** and **2**), the selectivity for quadruplex over duplex-DNA (**1** and **2** *versus* **3**), and the intra-quadruplex-selectivity (**1** *vs* **2**) for a given ligand. This novel SPR-based approach using the peptide scaffold is thus of interest for the screening of G-quadruplex DNA ligands, providing key information on their affinity, selectivity and binding mode. At present, this strategy is used for the formation of other particular conformation of DNA (antiparallel G-quadruplex, *i*-motifs...). In particular, *i*-motifs conformation (which consists in an antiparallel conformation) has been prepared by two successive "click reaction" (*i.e.* oxime bond formation following by Huisgen reaction).

This work was supported by the Région Rhône-Alpes.

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NMR STUDY OF A POTENTIAL ROLE OF ANIONS ON FOLDING OF DIMERIC G-QUADRUPLEX IN AQUEOUS SOLUTION

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Imino regions of ^{1}H 1D NMR spectra demonstrate only a minor influence of anions on folding of dimeric $d(G_4T_4G_4)_2$ quadruplex in the presence of a series of nine sodium salts. In the case of BF_4^- anions formation of quadruplex species is greatly reduced. Few specific differences are attributed to interactions of anions with residues in loop regions.

INTRODUCTION

Guanine-rich DNA sequences can fold into four-stranded G-quadruplex structures composed of G-quartets, planar arrays of four guanines held together by eight Hoogsteen hydrogen bonds (Fig. 1a)[1–3]. G-rich sequences are overrepresented in the promoter regions of a number of genes, including oncogenes, ribosomal DNAs as well as in telomeric DNA regions[1,4]. G-quadruplex DNA structures have been in the past years a subject of great interest since their formation has been suggested to play a role in variety of important biological processes as well as due to their potential therapeutic applications. In addition, G-quadruplexes exhibit a great potential as part of nanomolecular devices due to their well defined structure and self recognition properties.

The first high resolution structure of a G-quadruplex was determined by NMR spectroscopy on oligonucleotide $d(G_4T_4G_4)$[5,6]. The structure exhibits a dimeric and symmetric topology (Fig. 1b). It consists of four G-quartets and two loops consisting of four thymine residues spanning across the diagonals of the outer G-quartets. Each G-rich strand exhibits a neighboring strand in antiparallel and parallel orientations. The presence of cations seems to be prerequisite for G-quartet formation due to their role in reducing repulsions amongst guanine carbonyl oxygen atoms and additionally enhancing base-base stacking interactions. A fairly wide variety of cations is capable of inducing formation of G-quadruplex structures. In general, cations have been localized along the central cavity of G-quadruplex between two G-quartets or in the plane of a G-quartet[7–12]. Recently, it has been shown that the nature of anions has a decisive role in the self-

assembly of guanosine derivatives in aprotic solvents[13]. However, not much is known about the influence of anions on G-quadruplex formation by G-rich oligonucleotides in aqueous solution. The aim of the present work was to assess the influence of anions on the formation of G-quadruplex structure formed by $d(G_4T_4G_4)$.

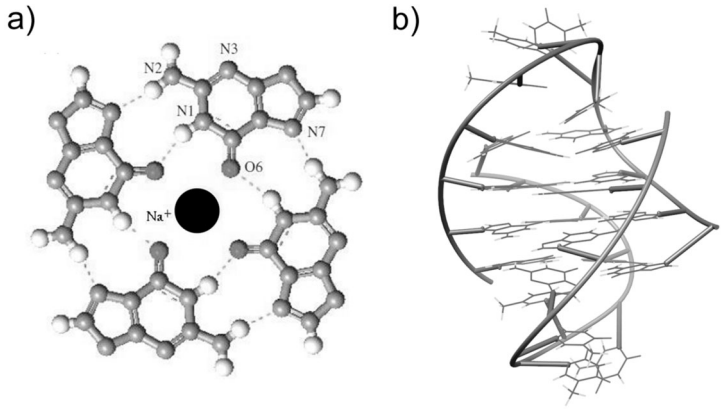

FIG. 1
a) G-quartet with the central sodium ion. b) Structure of $d(G_4T_4G_4)_2$ quadruplex (PDB ID 156D)

RESULTS AND DISCUSSION

Oligonucleotide $d(G_4T_4G_4)$ was dissolved in H_2O and extensively dialyzed against solution of different sodium salts. The DNA was then lyophilized and subsequently redissolved in 90% H_2O/10% ^2H_2O. Sample concentrations were between 2 and 2.6 mM in strand. Concentration of sodium salt in each sample was ca. 30 mM, pH was 5.8. NMR data were collected on a 600 MHz Varian NMR System. ^1H NMR spectrum of $d(G_4T_4G_4)_2$ quadruplex in the presence of NaCl was compared with the spectra in the presence of different anions (Fig. 2). Although ^1H 1D spectra are a rather rough estimate of structural changes, they can be very informative in assessment whether a structure is different or identical to the reference one. Perusal of imino region of ^1H NMR spectra of $d(G_4T_4G_4)_2$ in the presence of different sodium salts shows only a minor influence of anions on its G-quadruplex structure with the exception of BF_4^-. Barely measurable amount of quadruplex is formed in the presence of BF_4^- anions. Anyhow, the pattern of signals in imino region in the presence of BF_4^- retains similar characteristic resonances in addition to a broad signal at δ 10.9 ppm. Characteristic signals are an indication of quadruplex formation albeit in small quantity.

Chemical shifts corresponding to imino resonances are very similar to the values in the presence of other anions, which suggests formation of the same topology.

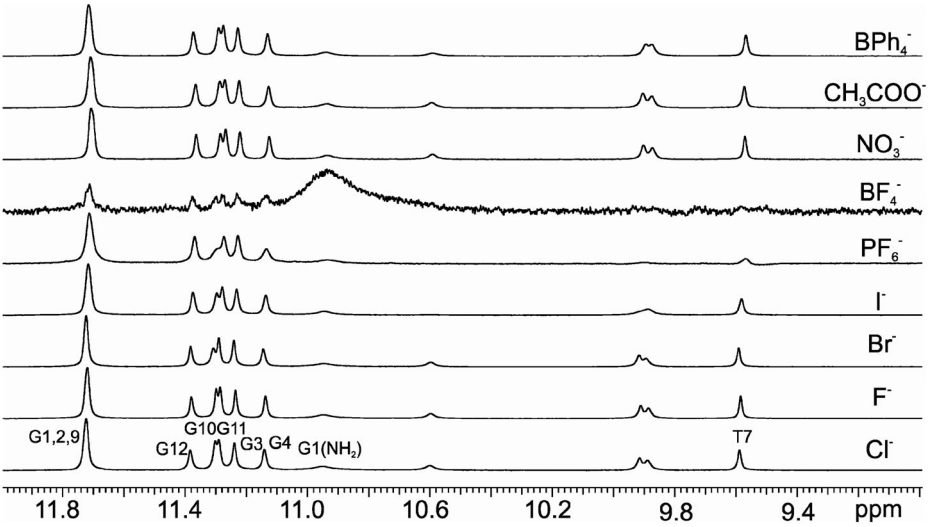

FIG. 2
Imino region of ^{1}H NMR spectra of $d(G_4T_4G_4)_2$ quadruplexes in the presence of different sodium salts. Anions are marked on the right side of individual plots. Assignments of individual guanine and T7 imino protons are marked. Vertical scale of spectrum with BF_4^- anions is increased by 50-times

A minor difference with respect to chloride anions is observed in the case of iodide and PF_6^- anions. In both cases imino resonances of thymine residues display reduced intensity. Chemical shift of T7 at δ 9.50 ppm remains practically unchanged in both spectra. Small deviations are observed for imino resonances of G10 in the presence of iodide, PF_6^- and bromide anions. In the presence of fluoride, NO_3^-, CH_3COO^- and BPh_4^- anions there are no changes in imino region of ^{1}H spectra.

$d(G_4T_4G_4)_2$ quadruplex is formed irrespective of the present halogenide anions. There are some specific changes in thymine loops in the case of iodide. Iodide is the largest of all halogenide anions with ionic radius of 2.2 Å, which affects its interactions with thymine loop and the outer G-quartets. In case of BPh_4^-, CH_3COO^- and NO_3^- anions there are no observable differences in spectra.

In summary, ^{1}H NMR study showed only slight influence of anions on topology of dimeric $d(G_4T_4G_4)_2$ quadruplex. In the case of BF_4^- anions forma-

tion of quadruplex species is greatly reduced. Minor differences were observed in loop regions. Further work utilizing analysis of 2D NOESY spectra is in progress.

The authors acknowledge the financial support of Slovenian Research Agency (ARRS) and the Ministry of Higher Education, Science and Technology of the Republic of Slovenia (Grant Nos. P1-0242 and J1-0986) and COST MP0802.

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LIGAND RECOGNITION OF RIBOSWITCHES

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Synthetic RNAs with site-specific nucleoside modifications are key derivatives to explore the molecular response mechanism of mRNA riboswitches.

RIBOSWITCHES

Non-coding regions of mRNA which bind metabolites with high selectivity and specificity function as so-called riboswitches[1–3]. They represent gene regulation systems that are widespread among bacteria and importantly, they do not rely on the assistance of proteins. Riboswitches consist of a metabolite-sensitive aptamer and an adjoining expression platform. Although impressive progress has been made in revealing three-dimensional structures of metabolite-bound aptamer complexes of various riboswitch classes, less is known about how binding of the metabolite to the aptamer is communicated into a structural change of the expression platform which in turn signals "on" or "off" for gene expression. The simplified picture of bacterial transcription control is that upon metabolite binding either a mRNA terminator structure is formed which causes the RNA polymerase to stop synthesis ("off" regulation) or an existing terminator is disrupted which enables the polymerase to continue synthesis with the mRNA template ("on" regulation). In the case of translational control, accessibility versus sequestration of the Shine-Dalgarno sequence upon metabolite binding is the essence of the response mechanism for "on" versus "off" regulation, corresponding to hindrance or enabeling of bacterial ribosome translation initiation.

In this presentation, we summarize our ongoing studies on the biophysical characterization of riboswitches[4–9] and present two very recent investigations on the metabolite recognition process of SAM-II and $preQ_1$ sensing riboswitches:

Ligand-Induced Folding of a Translationally Controlled SAM-II Riboswitch[10]

While the ligand-bound states of riboswitches have proven amenable to structure determination efforts, structural features of riboswitches in their

ligand-free forms and their ligand-response mechanisms giving rise to regulatory control are lacking. In this presentation, we explore the ligand-induced folding process of the SAM-II riboswitch using chemical and biophysical methods, including NMR and fluorescence spectroscopy, and single-molecule fluorescence resonance energy transfer imaging. The data reveal that the unliganded SAM-II riboswitch is highly dynamic in nature, where its conserved P1/L1 stem-loop element becomes engaged in a pseudoknot fold through base-pairing with nucleosides in the single-stranded 3'-overhang containing the Shine-Dalgarno sequence. The pseudoknot structure is highly transient in the absence of its ligand, S-adenosylmethionine (SAM), and becomes conformationally restrained upon ligand recognition. Binding of magnesium ions preorganizes structural elements of the free riboswitch enabling SAM recognition by a conformational capture mechanism. These insights provide a molecular understanding of riboswitch dynamics that shed new light on the mechanism of riboswitch-mediated translational regulation.

Ligand-Induced Folding of a Transcriptionally Controlled preQ$_1$ Riboswitch[11,12]

7-Aminomethyl-7-deazaguanine (preQ$_1$) sensitive mRNA domains belong to the smallest riboswitches known to date. Although recent efforts have revealed the three-dimensional architecture of the ligand-aptamer complex less is known about the molecular details of the ligand-induced response mechanism that modulates gene expression. We present an *in vitro* investigation on the ligand-induced folding process of the preQ$_1$ responsive RNA element from Fusobacterium nucleatum using biophysical methods, including fluorescence and NMR spectroscopy of site-specifically labeled riboswitch variants. We found evidence that the full-length riboswitch domain adopts two different co-existing stem-loop structures in the expression platform. Upon addition of preQ$_1$, the equilibrium of the competing hairpins is significantly shifted. This system therefore, represents a finely tunable antiterminator/terminator interplay that impacts the in vivo cellular response mechanism. A model is discussed how a riboswitch that provides no obvious overlap between aptamer and terminator stem-loop solves this communication problem by involving bistable sequence determinants.

Financial support from the Austrian Science Fund FWF (P21641, I317) and from the bm:wf (GEN-AU programme "Non-coding RNA", P7260-012-012) is acknowledged.

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SYNTHESIS OF BIARYL-SUBSTITUTED FLUORESCENT NUCLEOSIDES AND NUCLEOSIDE TRIPHOSPHATES AND THEIR INCORPORATION TO DNA

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A series of modified nucleosides bearing the fluorescent substituted biaryl labels attached to nucleobase was prepared and their luminescent properties were evaluated. The modified nucleosides show divers fluorescence from 400 to 560 nm when excited at 340 nm in water depending on structural and substitution pattern. The corresponding biaryl-substituted nucleoside triphosphates (dNTPs) were prepared analogously and used for polymerase incorporation to DNA. Applications in hybridization probes were studied.

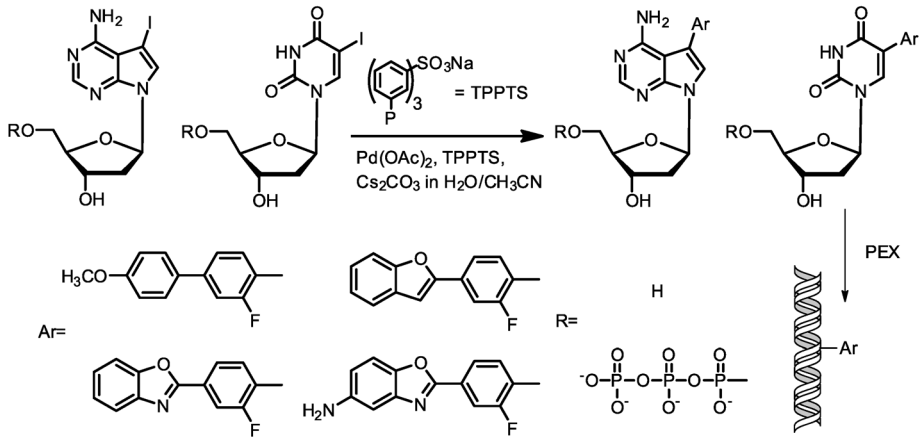
INTRODUCTION

Modified nucleic acids gain growing interest due to their potential applications ranging from therapeutics over catalysis to utilization in biosensors and chips[1,2]. The latter are increasingly utilized in molecular biology and medicine to analyze nucleotide sequences of DNA or RNAs, gene expression and searching for mutations[3]. More fluorescent derivatives are desirable to extend the application of the functionalized DNA. In addition to the classical phosphoramidite method, functionalization of the DNA by PCR or primer extension using modified nucleoside triphosphates becomes considerably significant due to more efficient synthesis of modified nucleic acids[4]. The methodology of the synthesis of modified nucleoside triphosphates via aqueous Suzuki cross-coupling reaction using base-halogenated dNTPs and the boronic acids was developed[5]. Based on these results, this work is focused on the synthesis of functionalized nucleosides bearing fluorescent biaryl labels. Oligonucleotides containing modified fluorescent nucleosides may serve as fluorescent probes for the detection of target sequence, structural studies as well as deletion or mismatch identifiers in analysis of nucleic acid structure.

RESULTS AND DISCUSSION

Different types of biaryl moieties were chosen as fluorescent labels. A series of substituted derivatives of biaryl halides was prepared by Suzuki coupling of appropriate aryl halides and arylboronic acids. The resulting biaryl

halides were borylated according to Miyaura[6] to give pinacolatoboronates, which were then coupled with base-halogenated nucleosides or dNTPs to give modified biaryl nucleosides or dNTPs. The modified nucleosides and nucleoside triphosphates exert fluorescence whose emission maxima rang from 360 to 560 nm when excited by 320–340 nm in methanol or water depending on the structural pattern. The excitation maximum differs from DNA absorption and as well as from emmission maxima enough to achieve convenient measurement. These modified nucleoside triphosphates were subjected to study of incorporation by several DNA polymerases to obtain biaryl functionalized oligonucleotides, which were then studied by fluorescence measurements with respect to structural changes and detection of deletions and mismatches.



SCHEME 1

This work is a part of the research projects Z4 055 0506 supported by the Academy of Sciences of the Czech Republic. It was specifically supported by the Ministry of Education, Youth and Sports of the Czech Republic (LC512), Czech Science Foundation (203/09/0317) and by Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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NUCLEOTIDE STORAGE AND INCORPORATION VIA CHEMICAL PRIMER EXTENSION

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Nucleotides are pivotal compounds. Cells use nucleoside phosphates as building blocks for nucleic acids, energy-rich molecules, second messengers, and recognition elements in co-factors. We have developed oligonucleotide complexes that can store nucleoside mono-, di- and triphosphates, and release them upon gentle heating. Further, we have developed a methodology for the efficient incorporation of nucleotides in growing oligonucleotide chains in enzyme-free, template-directed fashion. These molecular processes help to pave the way for autonomous or self-evolving nucleic acid systems.

INTRODUCTION

Life most probably evolved from simple self-replicating systems. It is likely that these systems relied on nucleic acids as genetic polymer and catalyst[1,2]. It is interesting to ask how the first primitive catalysts[3] arose from short sequences that form by spontaneous oligomerization. Further, it is important to understand how monomers for replication (activated nucleotides) can be bound by short oligonucleotide sequences, and how they can be incorporated into growing sequences, as directed by a template sequence, in the absence of polymerases (Fig. 1). Advances in our understanding of the

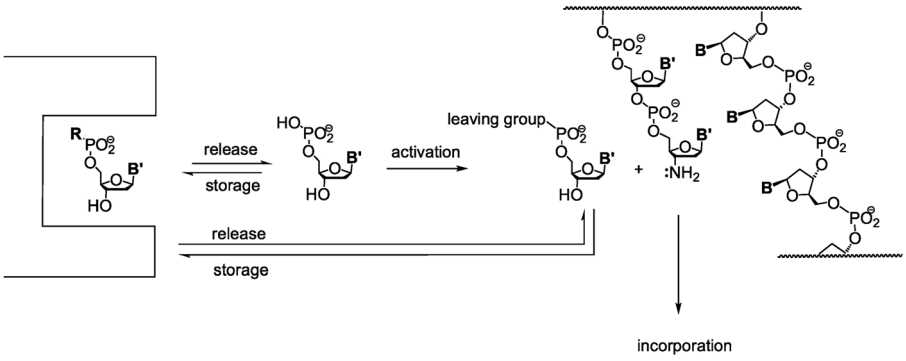

FIG. 1
Storage, activation, and incorporation of nucleotides. The same processes may be induced for RNA

binding and reactivity of nucleotides can also be expected to lead to advances in practical applications, ranging from diagnostics to biotechnology and clean energy processes.

RESULTS AND DISCUSSION

We have recently discovered oligonucleotide complexes that bind one or several nucleoside phosphates with micromolar or sub-micromolar dissociation constant[4]. Two oligonucleotide strands engage the nucleotide in hydrogen bonding, and the termini of other strands can assist the binding through stacking. Depending on the sequence of the oligonuclotides and the buffer conditions, nucleoside phosphates such as ATP, cAMP and cGMP can be bound with high affinity, and released upon warming of the solution or by chemical means.

The template-directed incorporation of nucleotides at the terminus of primers (Fig. 1) occurs spontaneously, in the absence of polymerases, if the nucleotide phosphate group is activated[5]. Figure 2 shows an active amide[6] (T-MeIm) and two active esters of thymidine-5′-monophosphate. Activation may be induced *in situ*[7,8,], starting from free nucleotides. Alternatively, nucleotides may be activated separately[9] and stored or released in activated form, prior to their use in primer extension reactions.

We have previously shown that chemical primer extension occurs efficiently with methylimidazolides[10], such as T-MeIm, but more rapidly with oxyazabenzotriazolides, such as T-OAt [9,11]. A detailed study on the sequence

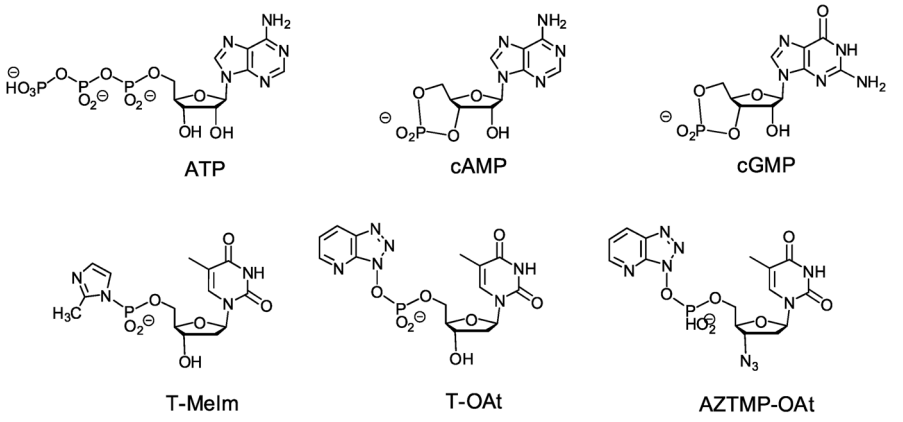

FIG. 2
Structures of biologically relevant nucleoside phosphates (top row) and activated nucleoside monophosphates for enzyme-free primer extension (bottom row)

dependence of the extension reaction with amino-terminal primers showed that any of the 64 possible sequence triplets, where the templating base is at the center position, induces near-quantitative incorporation of activated nucleotides[12]. Rates of incorporation differ by less than two orders of magnitude between the most favorable sequence context and the poorest templates.

Multiple extensions can be induced by employing activated ribonucleotides[13,14] or activated aminonucleotides[15]. In order to gain control over each oligomerization step, and to make the spontaneous incorporation reactions useful for sequencing, reversible termination approaches are required. Protecting groups that can be removed under non-denaturing conditions are one way of achieving this control. This includes composite protecting group that feature a fluorophore, used to read out the bases of the template[16]. Azido groups are latent amines. We have developed a synthesis of the oxyazabenzotriazolide of 3′-azido-3′-deoxythymidine-5′-monophosphate (AZTMP-OAt), starting from AZT. Incorporation in a growing primer is followed by a reduction step that liberates the amino functionality, setting the stage for the subsequent elongation. For AZTMP-OAt, reduction with phosphines is accompanied by a low level elimination reaction that produces a ladder of fragments. This ladder may be used to read out sequences at the end of an assay via MALDI-TOF mass spectrometry[17].

The methodologies described above provide an exciting level of control over nucleotide chemistry in the absence of enzymes.

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SYNTHESIS AND BIOLOGICAL PROPERTIES OF 2′-*O*-MODIFIED OLIGORIBONUCLEOTIDE DERIVATIVES

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In this study, we developed effective methods for the synthesis of 2′-*O*-cyanoethyl- and 2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]-ribonucleoside derivatives using the oxa-Michael reaction using acrylonitrile and acrylates, respectively. Several interesting properties of oligoribonucleotides modified with 2′-*O*-alkylated ribonucleoside derivatives will be reported.

INTRODUCTION

Since the discovery of RNA interference[1] as well as the unveiled new functions of non-cording RNAs [2], much attention has been paid to the chemical synthesis of unmodified and modified RNAs. Particularly, it is of great importance to develop new oligoribonucleotides that have stronger hybridization affinity for DNA or RNA with enzyme resistance[3–5]. A number of studies directed toward creation of nucleic acid drugs having such improved properties have been extensively reported to date[7]. In 2005, we reported the synthesis of 2′-*O*-(2-cyanoethyl)-RNAs where the appropriately protected ribonucleoside 3′-phosphoramidite derivatives were synthesized by a series of reactions involving the Michael reaction of 3′,5′-*O*-TIPS-ribonucleoside derivatives with acrylonitrile in *t*-butanol in the presence of Cs_2CO_3 [8]. However, this original method proved to be somewhat tedious since it required multi-step reactions for the preparation of the 3′-phosphoramidite building blocks. Later, we could improve the method for the synthesis of pyrimidine 3′-phosphoramidite units by using the ring opening reaction of 2,2′-anhydrouridine with trimethylsilyl cyanoethyl ether in the presence of $BF_3{\cdot}OEt_2$ [9]. This straightforward method enabled us to make the U and C units very conveniently. Recently, we also improved the original method for the synthesis of purine ribonucleoside 3′-phosphoramidite derivatives. This improvement involved the highly selective oxa-Michael reaction that allowed the use of *N*-unprotected synthetic intermediates as Michael donor molecules. However, the synthesis of the U-unit required

the protection of the N^3-imido group with a benzoyl group[10]. This new route led to the use of a lesser number of reactions for the construction of the phosphoramidite building blocks.

In this paper, we wish to report our recent studies on the synthesis of 2′-*O*-modified oligoribonucleotides using new synthetic strategies and their biological properties.

RESULTS AND DISCUSSION

Previously, we used the Cs_2CO_3-mediated Michael reaction for the introduction of the cyanoethyl group into the 2′-hydroxyl group of ribonucleoside derivatives, as shown in Fig. 1[8]. In these reactions, we needed the protection of the amino groups of A, G, and C and the imide group of U to

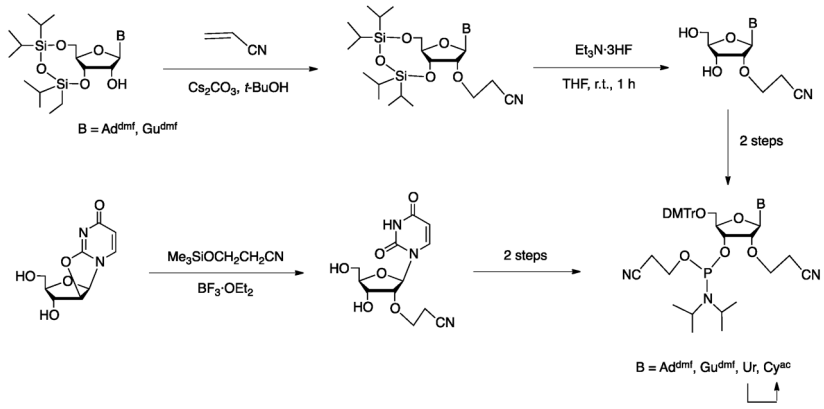

FIG. 1
Previous strategies for the synthesis of 2′-*O*-cyanoethyribonucleoside 3′-phosphoramidite building blocks

avoid the side reactions on the base moieties. However, we found the Cs_2CO_3-mediated Michael reaction did not require any protecting group on the A base. In fact, it turned out that the reaction of 3′,5′-*O*-TIPS-adenosine in *t*-BuOH in the presence of Cs_2CO_3 gave selectively the 2′-*O*-cyanoethylated product in 88% yield.

In the case of guanosine, we succeeded in synthesizing the 2′-*O*-cyanoethylguanosine derivatives via a 2-aminopurine derivative that was selectively deaminated by enzymatic or chemical treatment.

On the other hand, we found the Michael reaction of *N*-free A and C ribonucleoside derivatives with methyl acryl gave selectively the 2′-*O*-alkylated products in high yields. The 2-(methoxycarbonyl)ethyl

substituent introduced into the 2′-hydroxyl group could be converted to an 2-[(*N*-methyl)carbamoyl]ethyl (MCE) group by the reaction with methylamine. In the case of guanosine, 2′-*O*-MCE-guanosine derivatives were synthesized in a manner similar to that described for the synthesis of 2′-*O*-(2-cyanoethyl)guanosine derivatives.

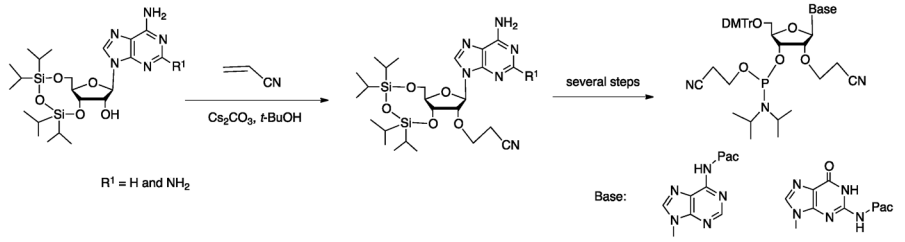

FIG. 2
Selective 2′-cyanoethylation of *N*-unprotected ribonucleoside derivatives

The resulting 2′-*O*-MCE-ribonucleoside derivatives proved to be stable under acidic and basic conditions. Therefore, we used this MCE group as the modifier of the 2′-hydroxyl group to prevent enzymatic degradation in cells when used as antisense molecules. Actually, oligoribonucleotides incorporating 2′-*O*-MCE-ribonucleosides were found to be significantly more resistant to venom phosphodiesterase than the unmodified ones.

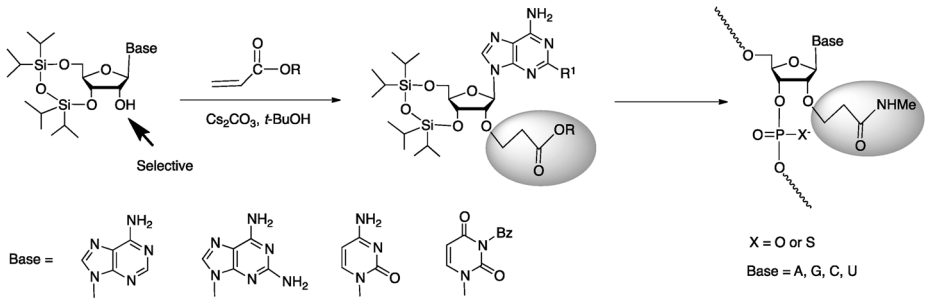

FIG. 3
Selective oxa-Michael reaction of *N*-unprotected ribonucleoside derivatives and its application to the synthesis of 2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]-RNA oligomers

As an application of our 2′-*O*-modified RNAs, we studied the so called exon-skipping of pre-mRNA of the dystrophin gene of mdx52 mice directed toward the gene therapy of muscular dystrophy. It was found that incorpo-

ration of these modified RNA derivatives exhibited significant exon-skipping activities compared with those of the corresponding morpholino nucleic acids[8].

In connection with these synthetic and biological studies, we also developed an effective method for the prediction of 2′-*O*-modified RNAs by computer-based calculations using molecular dynamics simulation. We considered there should be a certain relationship between the experimentally determined melting temperatures of 2′-*O*-modified RNA/RNA duplexes and their deformability estimated from molecular dynamics simulations. To elucidate this relationship, we synthesized several fully modified oligoribonucleotides such as 2′-*O*-cyanoethyl-RNAs and 2′-*O*-methoxyethyl-RNAs and compared the actual melting temperatures of the duplexes with their calculated deformabilities. An increase of the melting temperatures by 2′-*O*-modifications was found to correlate strongly with an increase of the helical elastic constants in U_{14}/A_{14}, $(CU)_7/(AG)_7$, and $(GACU)_3/(AGUC)_3$ sequences. Our results indicated the possibility of predicting the thermal stability of 2′-*O*-modified duplexes at the computer-aided molecular design stage.

In summary, we have successfully developed the oxa-Michael reaction giving rise to the 2′-*O*-cyanoethylated and 2′-*O*-(2-alkoxycarbonyl)ethylated ribonucleoside derivatives that were the key intermediates for the synthesis of 2′-*O*-modified RNA derivatives. These modified oligoribonucleotides proved to have significant exon-skipping activities in the splicing reaction of pre-mRNA of mdx52 mice. In addition, our new method would be useful for the prediction of hybridization ability of synthetic RNA oligomers.

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PROBING FUNCTIONAL NUCLEOTIDES IN DEOXYRIBOZYMES BY COMBINATORIAL MUTATION INTERFERENCE ANALYSIS (CoMA)

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Deoxyribozymes are functional DNA molecules that are capable of catalyzing chemical transformations. Many deoxyribozymes have practical utility for RNA cleavage and RNA ligation reactions. Very little is currently known about the molecular details of the intricate structures and mechanistic principles of DNA catalysis. To provide molecular level insights into the function and mechanism of deoxyribozymes, we report the development of combinatorial mutation interference analysis (CoMA) as a highly efficient method to identify catalytically essential nucleotides in deoxyribozymes. This new strategy allows the simultaneous assessment of all possible mutations in the active site of DNA catalysts.

INTRODUCTION

Synthetic single-stranded DNA molecules that have the ability to catalyze chemical transformations with high selectivity are known as DNA catalysts, deoxyribozymes or DNA enzymes. Deoxyribozymes are identified in the laboratory by in vitro selection from random sequence DNA pools. Since the first report of a DNA enzyme to catalyze the site-specific cleavage of RNA phosphodiester bonds[1], various chemical reactions have been catalyzed by DNA. The majority of reports concern phosphodiester chemistry (e.g. RNA cleavage, RNA ligation, DNA cleavage, DNA ligation, DNA phosphorylation, DNA adenylation), but the catalytic ability of DNA is not limited to oligonucleotide substrates. This is demonstrated by a variety of examples, including nucleopeptide bond formation, C–C bond formation, thymine dimer cleavage, porphyrin metalation, or peroxidation[2]. Several DNA catalysts have found practical applications as analytical tools, computational devices, therapeutic agents, and as reagents for synthesis[2]. For the preparation of long chemically modified RNA, DNA-catalyzed ligation of RNA is an experimentally attractive alternative to protein-catalyzed RNA ligation[3]. A powerful application of DNA catalysts is the synthesis of 2′,5′-branched or lariat RNAs, important RNA splicing intermediates that are difficult to obtain by other chemical methods[4]. The prototype of this class of RNA ligases is the 7S11 deoxyribozyme that catalyzes the nucleophilic attack of a specific internal 2′-hydroxyl group of one RNA substrate (L-RNA) onto the 5′-triphosphate of the second RNA substrate (R-RNA)[5].

The detailed chemical mechanism of DNA-catalyzed RNA ligation is not well known, and the three-dimensional structure of any active deoxyribozyme has not yet been solved. The identification of functional nucleotides required for deoxyribozyme activity is fundamental for understanding DNA catalytic mechanisms. Traditionally, such insights are provided by systematic deletion or substitution of individual nucleotides and careful kinetic analyses of many separate deoxyribozyme mutants[6]. We have recently reported an innovative alternative approach that rapidly provides comprehensive and reliable data sets for all possible single mutants of a deoxyribozyme. Combinatorial mutation interference analysis (CoMA)[7] serves as a general tool for the characterization of functional single-stranded DNA, in particular for the identification of catalytically important nucleotides.

RESULTS AND DISCUSSION

The Concept of Combinatorial Mutation Interference Analysis (CoMA)

CoMA is based on solid-phase synthesis of combinatorial mutation libraries using mixtures of nucleoside phosphoramidites. Mutations are introduced as ribonucleotides, with the 2′-OH group acting as a chemical tag. Each library therefore contains one of the four standard ribonucleotides statistically distributed in the catalytic core of the deoxyribozyme. The CoMA workflow consists of four steps: A) solid-phase synthesis of four 2′-OH-

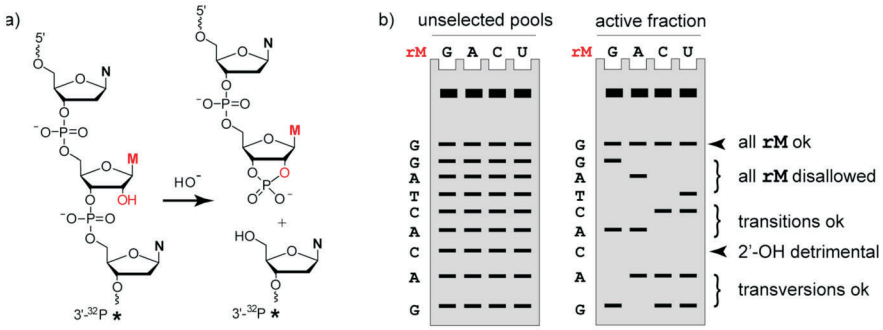

FIG. 1
Concept of CoMA. a) Mutations are incorporated as ribonucleotides into the DNA libraries. The 2′-OH group serves as a chemical tag, because it allows for selective cleavage by alkaline hydrolysis only at the mutated positions. b) Schematic depiction of PAGE analysis of interference effects. Missing bands in the active fraction indicate a detrimental effect of the mutation on DNA catalysis

encoded combinatorial mutation libraries, B) separation of active and inactive library members, C) specific backbone cleavage at mutation sites by alkaline hydrolysis, and D) analysis of interference patterns by denaturing PAGE (Fig. 1). This efficient strategy allows to investigate all mutations at every nucleotide position in the catalytic loop of a deoxyribozyme with a set of only four combinatorial libraries. In a second implementation of this method, the contribution of individual functional groups of essential nucleobases can be probed via modified ribonucleotides.

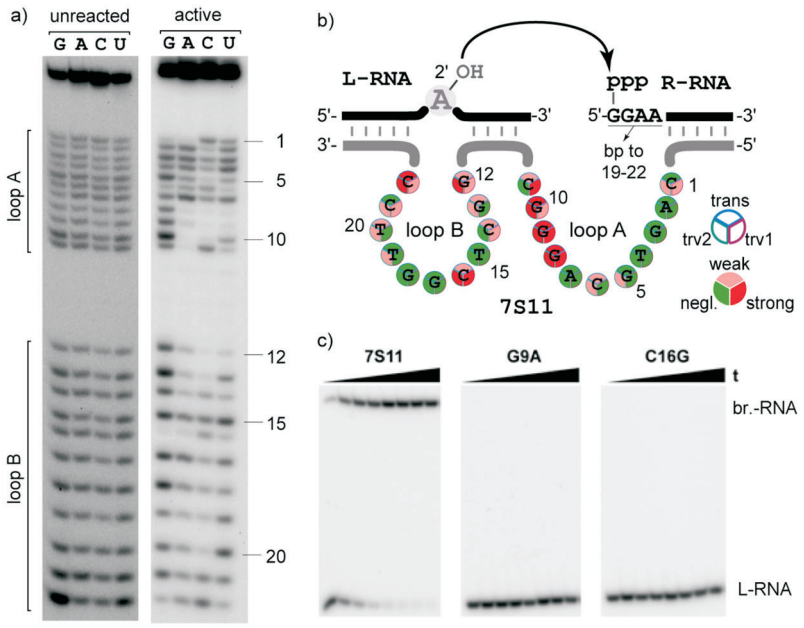

FIG. 2

CoMA of the 7S11 deoxyribozyme that enables the synthesis of 2′,5′-branched RNA by catalyzing the nucleophilic attack of an internal 2′-hydroxyl group of the branch-site adenosine in the L-RNA onto the α-phosphate of the 5′-triphosphate of the R-RNA substrate[5]. a) PAGE analysis of alkaline hydrolysis pattern of unseparated rN-libraries and of active 7S11 mutants with ^{32}P-labels at the 3′-end (therefore 5′ to 3′ nucleotide numbering from top to bottom of the gel). b) Interference effects for transitions (trans, G-A, C-U) and transversions (trv1, G-U, A-C, and trv2, G-C, A-U) depicted as circular color representation summarizing strong (red), weak (pink), and negligible (green) interference effects at all nucleotide positions. c) Analysis of the ligation kinetics of two selected 7S11 point mutants G9A and C16G confirm the CoMA results

CoMA of the 7S11 Deoxyribozyme

Using this combinatorial approach, we studied several examples of RNA-ligating deoxyribozymes, that catalyze the formation of 2′,5′-branched RNA, and the linear ligation of two RNA substrates via a 3′-5′-phosphodiester linkage. Here we show the results for the 2′,5′-branch-forming 7S11 deoxyribozyme (Fig. 2).

The analysis of the 7S11 CoMA data identified three conserved guanosine nucleotides G8, G9 and G10 in loop A that cannot be changed to any other nucleotide without severely affecting catalytic activity. A strong interference effect in loop A was also observed for C11A. In loop B, nucleotides G12, C16, and C22 are most sensitive to alteration and mutations at positions G13 and G14 show medium or small effects. Single nucleotide mutations have also rather small effects in loop B at nucleotides 19-21, which base-pair with the R-RNA substrate, apparently demonstrating that single mismatches at all but the first position of this paired region are acceptable. For selected mutants, the 7S11 CoMA results have been independently confirmed by analyses of the ligation activity. Overall, these experiments demonstrate that CoMA provides comprehensive and reliable mutation data for the nucleotides in the catalytic region of 7S11.

CONCLUSION

Combinatorial mutation interference analysis (CoMA) is a highly efficient method to identify catalytically essential nucleotides in deoxyribozymes. The CoMA results also provide information on non-essential nucleotides and thereby facilitate minimization of the catalytic core regions. We found that the deoxyribose-to-ribose substitution is well tolerated for the large majority of nucleotides. The solid-phase synthesis of deoxyribozyme libraries enables the assessment of all possible mutants in one set of experiments using four distinct libraries. This cannot be achieved by enzymatic methods using template-dependent polymerases, which can only incorporate Watson–Crick-complementary nucleotides. In contrast to conventional mutagenesis, CoMA is unbiased by the need to choose a subset of specific mutants for investigation. Comprehensive results from combinatorial mutagenesis will guide more detailed mechanistic investigations of such intriguing functional DNAs.

We gratefully acknowledge the Max Planck Society for generous support of our work.

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CROSS-LINKING INDUCED BY THE CONJUGATE MALONALDEHYDE-GLYOXAL AND MALONALDEHYDE-METHYLGLYOXAL ADDUCTS OF 2′-DEOXYADENOSINE

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M_1Gx-dA (3-(2′-deoxy-β-D-ribofuranosyl)-8-diformylmethylimidazo[2,1-*i*]purine) and M_1MGx-dA (3-(2′-deoxy-β-D-ribofuranosyl)-8-diformylmethyl-7-methylimidazo[2,1-*i*]purine) represent so called "conjugate adducts", the class of the DNA bases derivatives that contain units derived from the condensation products of electrophliles inducing this type of modifications. M_1Gx-dA and M_1MGx-dA are formed in the reaction of 2′-deoxyadenosine with the endogenous mutagen malonaldehyde, in the presence of compounds causing protein glycation, glyoxal and methylglyoxal, respectively. Both adducts possess the aldehyde groups and present highly reactive electrophiles. The cross-linking potency of these adducts in reactions with N^{α}-acetyllysine has been investigated. The studies resulted in the identification of five new structural modifications of the aminoacid.

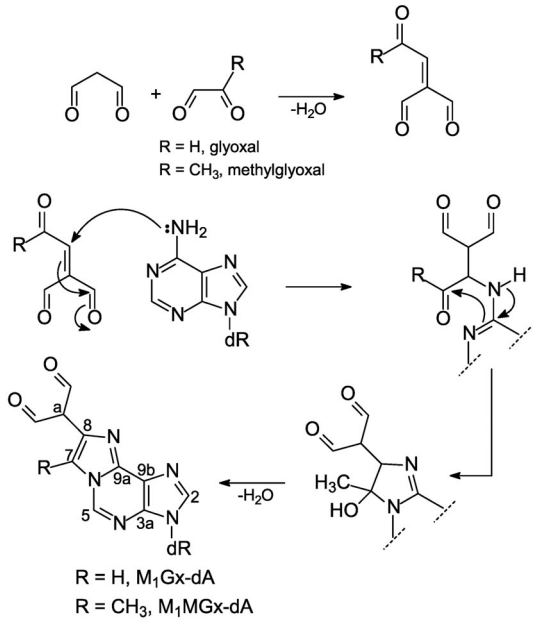
INTRODUCTION

Endogenously formed short-chained α-dicarbonyl compounds such as glyoxal and methylglyoxal are continuously of great interest in biochemical studies. This is due to significant importance of these compounds as mutagens and very potent protein modifiers. Giving rise to DNA adducts these reactive electrophiles are capable to induce damage of the genetic material[1]. Both aldehydes can also react with proteins causing their modification that can lead to the formation of advanced glycation end-products (AGEs) which are involved in the pathology of diabetes, aging, cancer and Alzheimer's diseases[2]. Numerous studies assumed that 2-oxoaldehydes are essential intermediates in the formation of most of the AGEs identified so far[3]. It has been shown that α-dicarbonyl compounds are more reactive in the glycation reactions that reducing sugars and play an important role in protein cross-linking[4]. Protein cross-linking has been suggested to be a key factor in loss of cellular and tissue function[5]. Cross-linked species accumulate in tissues such as the skin, cornea, smooth muscle, cataractous lens and vascular collagen both as a consequence of diabetes and normal aging[5]. Despite the potential importance of these species in diseases, the biochemistry of glycation-mediated protein cross-linking is still not fully understood.

Herein, we investigated the possibility of inducing cross-linking by the conjugate malonaldehyde-glyoxal and malonaldehyde-methylglyoxal adducts of 2′-deoxyadenosine in reactions with N^{α}-acetyllysine. Our studies resulted in the identification of five new structural modifications of the aminoacid.

RESULTS AND DISCUSSION

Recently, in our laboratory the reactivity of malonaldehyde toward 2′-deoxyadenosine simultaneously with glyoxal and methylglyoxal, respectively was examined. The reactions resulted in the formation of one malonaldehyde-glyoxal and one malonaldehyde-methylglyoxal conjugate adduct respectively[6]. These adducts were structurally characterised by mass spectrometry, UV, ^{1}H and ^{13}C NMR spectroscopy. The malonaldehyde-glyoxal adduct was identified as 3-(2′-deoxy-β-D-ribofuranosyl)-8-diformylmethylimidazo[2,1-*i*]purine (M_1Gx-dA), while the malonaldehyde-methylglyoxal one as 3-(2′-deoxy-β-D-ribofuranosyl)-8-diformylmethyl-7-methylimidazo[2,1-*i*]purine (M_1MGx-dA) (Scheme 1).



SCHEME 1
Formation of M_1Gx-dA and M_1MGx-dA

A plausible mechanistic explanation for the formation of M_1Gx-dA and M_1MGx-dA is a Michael addition of the exocyclic amino group of 2′-deoxyadenosine to the initially formed malonaldehyde-glyoxal and malonaldehyde-methylglyoxal conjugates, respectively (Scheme 1). Following tautomerisation to the keto form the endocyclic nitrogen atom of the 2′-deoxyadenosine attacks this carbonyl group of the conjugate that is derived from glyoxal and methylglyoxal respectively to form five-membered ring. Finally loss of a molecule of water yields the product. Both adducts were also observed in calf thymus DNA when incubated with the respective aldehydes under physiological pH and temperature.

M_1Gx-dA as well as M_1MGx-dA possess the aldehydic functionality and seem to be good candidates for explanation of the cross-linking ability of glyoxal and methylglyoxal.

Since the yield of M_1Gx-dA is low the nucleoside analogue of this compound, M_1Gx-A, was synthesised and used in studies concerning cross-linking potency of this kind of adducts. M_1Gx-A and M_1MGx-dA were allowed to react with N^{α}-acetyllysine in 0.1 M phosphate buffer solution at pH 7.4 and 45 °C for 7 days. HPLC analysis of the reaction mixture of M_1Gx-A with N^{α}-acetyllysine revealed the formation of three products marked M_1Gx-A-X1, M_1Gx-A-X2 and M_1Gx-A-X3 (Fig. 1).

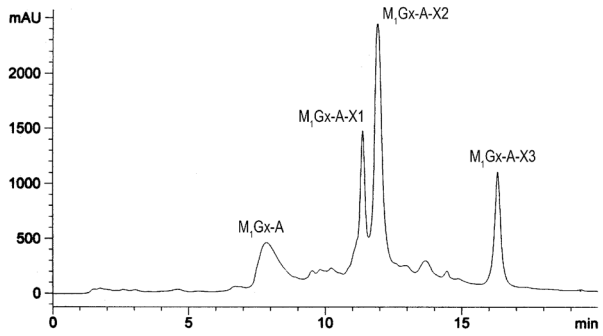


FIG. 1
C18 analytical column HPLC chromatogram of the reaction mixture of M_1Gx-A with N^{α}-acetyllysine

In the positive ion electrospray mass spectra of M_1Gx-A-X1, M_1Gx-A-X2 and M_1Gx-A-X3 protonated molecular ion peaks were observed at *m/z* 702, 532 and 532, respectively indicating that the first product is most likely formed in the condensation reaction of two molecules of N^{α}-acetyllysine with one molecule of M_1Gx-A, while the remaining products are conjugates formed in reaction of one molecule of M_1Gx-A with one of the aminoacid.

The detected cross-links were stable during isolation and purification process. Replacement of M_1Gx-A in the reaction mixture with M_1MGx-dA resulted in the formation of two products marked M_1MGx-dA-X1 and M_1MGx-dA-X2 (Fig. 2).

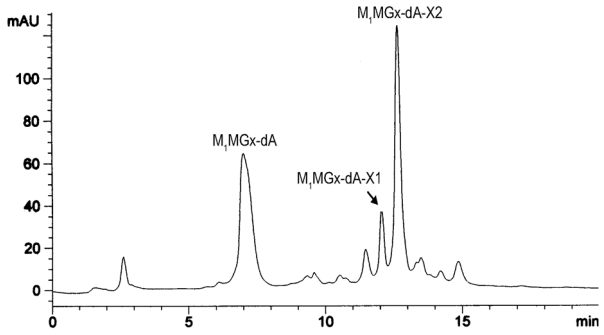


FIG. 2
C18 analytical column HPLC chromatogram of the reaction mixture of M_1MGx-dA with N^{α}-acetyllysine

On the basis of data obtained from mass spectrometry the detected products were identified as the structural analogues of M_1Gx-A-X1 and M_1Gx-A-X2, respectively. However, unlike M_1Gx-A-X2, M_1MGx-dA-X2 was found to be unstable. Prolonged incubation of M_1MGx-dA with N^{α}-acetyllysine led to the increase in the yield of M_1MGx-dA-X1 and a decline in the amount of M_1MGx-dA-X2 indicating that the later compound undergoes conversion into the former one.

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DIALDEHYDE DERIVATIVES OF NUCLEOSIDES AND NUCLEOTIDES AS NOVEL CROSSLINKING REAGENTS AND THEIR COMPARISON WITH GLUTARALDEHYDE

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In medical and pharmaceutical applications, chitosan is used as a component of hydrogels – macromolecular networks swollen in water or biological fluids. Chemical hydrogels are formed by covalent links with primary amino functionalities of chitosan. To date, the most common crosslinkers used with chitosan are dialdehydes such as glutaraldehyde (GA). We have developed new GA-like crosslinkers with additional functional groups, leading to chitosan-based biomaterials with novel properties.

INTRODUCTION

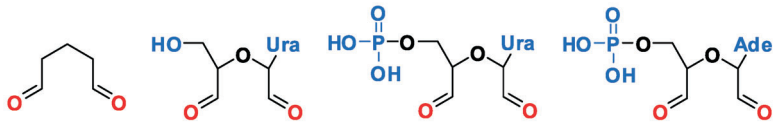
Chitosan is prepared from chitin, the second most abundant natural polymer in the world after cellulose. Chitosan is a copolymer of β-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose and can be obtained by deacetylation of chitin, which is produced from shells of crustaceans, insects, and other sources. The presence of reactive aminogroups in chitosan would be highly beneficial for chemical modifications to construct sophisticated molecular architectures. Due to these properties chitosan is considered as biofunctional polymer having much higher potential than cellulose. Chitosan is a nontoxic, biodegradable, and biocompatible natural polymer and can be used in a wide range of applications such as in the areas of biomedicine, membranes, drug delivery systems, hydrogels, water treatment, food packaging, etc.[1–3].

Crosslinking reagents are widely used for preparation of microspheres, films, gels, absorbents, immobilization of proteins and so on. To date, the most common crosslinkers are dialdehydes and the most effective and reliable is glutaraldehyde (GA)[3]. The main drawback of GA crosslinking is the formation of irregular products due to aldol condensation of GA[4,5]. The crosslinking of chitosan by GA is a complex process: chitosan accelerates the polymerization of GA with the intensive formation of conjugated

bonds –N=CH–CR=CH– and O=CH–CR=C– [5]. This fact should be taken into consideration if the reaction of chitosan with GA is used to create materials for medical or pharmaceutical applications. Under neutral or slightly alkaline conditions and using excess of GA a co-polymer of poly-GA and chitosan may be formed[5].

RESULTS AND DISCUSSION

To improve the characteristics of chitosan-based materials, we have proposed a series of novel and effective reagents. We have noted a striking similarity of GA and oxidized nucleosides and nucleotides[6]. Dialdehyde derivatives of nucleosides and 5′-nucleotides may be readily prepared by reaction of parent natural compounds with an equimolar amount of $NaIO_4$ or HIO_4 in water at room temperature[7].



The kinetics of gelation of chitosan solutions in the presence of crosslinkers and the growth of gel viscosity were investigated. To find optimal crosslinking conditions we have examined several gelation conditions using solutions of 2% chitosan (average molecular weight 190000, degree of deacetylation 90%). The time after which the system lost the ability to flow was used as the gelation point. The duration of gelation decreased with an increase in the GA – chitosan ratio and at pH 5.6 it was much faster than at pH 4.1. The rates of gelation of chitosan with GA, oUMP and oAMP under different concentrations are quite close (Fig. 1). At pH 5.6 the rate of gelation of chitosan in the presence of oUrd was much slower.

It will be of interest to examine physicochemical properties of novel reagents in details and compare with those of GA. NMR spectroscopy was used for the structure elucidation and examination of the conformational equilibrium in aqueous solutions. The analysis of coupling constants led to the conclusion that oAMP exists as equilibrium of four possible diastereomers of the dioxane derivatives without a noticeable amount of the free aldehyde. ^{1}NMR spectra of oUrd is even more complicated because of the presence of 5′-hydroxyl group which may be involved in the formation of hemiacetals. Practically no free aldehydes were present in aqueous solu-

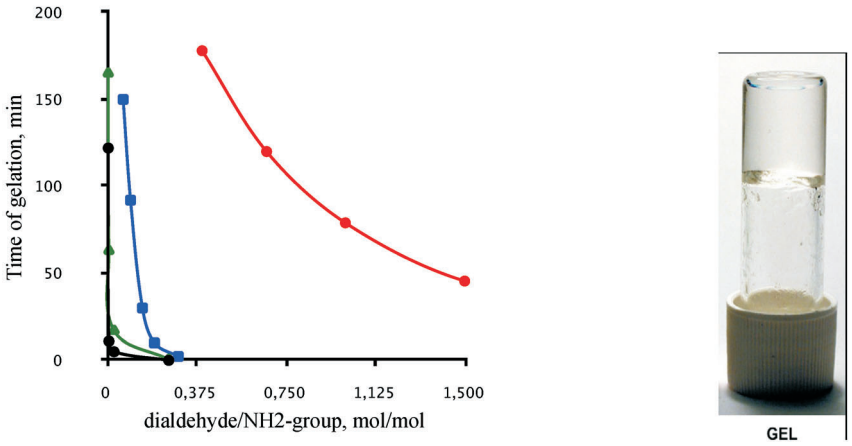


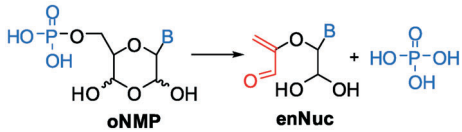
FIG. 1
Influence of the ratio of dialdehyde and aminogroups of chitosan on a time of gelation at 20 °C, pH 5.6 (1–3) and 4.1 (4). 1 – oUrd; 2, 4 – oAMP; 3 – GA

tions of oxidized nucleosides and 5′-nucleotides. In aqueous solutions GA consists of free dialdehyde (20–35%), its hydrates and cyclic hemiacetal, all in equilibria and in different proportions as a function of temperature[5]. The remarkable differences between crosslinking capabilities of oUrd and GA may be due to the presence of substantial amount of free aldehyde in the case of GA. On the other hand, the striking difference in the gelation properties of aldehyde derivatives of nucleosides and nucleotides may be attributed to the presence of the phosphate group which may be involved in additional ionic interactions. Moreover it is known that these nucleotide derivatives are rather unstable due to the β-elimination of the phosphate group[8]. This cleavage may be readily envisaged at 32 °C using NMR spectroscopy and the reaction with oAMP was complete after 15 h at pH 7.0. As a result, conjugated $H_2C{=}CH{-}CH{=}O$ system is formed with free aldehyde proton in ^1H NMR spectrum lying around 9 ppm.

Elimination of the phosphate group in oAMP and oUMP may be readily followed up by UV spectroscopy. A difference spectrum, that of compound en-oNuc minus that of oNMP, showed a new peak with λ_{max} 236 nm (Table I) which could be attributed to the absorption of a double bond conjugated with the aldehyde group. The spectroscopic characteristics of en-oAdo are in good agreement with those prepared from periodate oxidized ATP[8] (Table I). This elimination was investigated in the presence of low molecular weight chitosan (average MW 12200 with degree of deacetylation 98%) at pH 5.6. According to UV spectra at room temperature the re-

action was completed in 20 h (half time of conversion was 4 h) and the increase of absorption at 236 nm was higher (ε 6000) than that those without chitosan. In this case a bigger extinction may be attributed to the absorption of double bond conjugated with the aldimine group. Under the

TABLE I
β-Elimination of the phosphate group in oUMP and oAMP at pH 5.6. Characteristics of UV difference spectra (enNuc-oNMP)



Dialdehyde	*T*, °C	Complete reaction, h	Dialdehyde/NH_2-groups ratio, mol/mol	λ_{max}, nm(ε) enNuc-oNMP
oUMP	37	46	–	236 (4900)
oUMP	20	20	1:10	236 (6200)
oAMP	37	40	–	236 (3800)
oAMP	20	20	1:10	235 (6000)
oATP[8]	30	24	pH 9.1	235 (3700)[8]

same conditions the elimination of phosphate group in oUMP proceeds much slower with estimated half time of conversion being around 40 h. It may be concluded that the presence of chitosan significantly accelerates the elimination of the phosphate group. Using NMR spectroscopy it was shown that elimination is quiet effective even at pH 4.1. As can be judged from ^{31}P NMR spectra without proton decoupling, the singlet at 0.7 ppm is attributed to the formed phosphorous acid and the additional triplet at 1.4 ppm with coupling constant 4.8 Hz to the $(HO)_2P(O)–CH_2–$ fragment of the crosslinking reagent.

In conclusion, the use of dialdehyde derivatives of nucleosides and nucleotides allows the effective crosslinking of macromolecular chitosan chains with simultaneous modification such as introduction of heterocyclic bases and leading to chitosan based biomaterials with novel properties.

This work was supported by the Russian Foundation for Basic Research and Russian Academy of Sciences (Program "Molecular and Cell Biology").

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INVASION OF 2′-*O*-METHYL OLIGORIBONUCLEOTIDES AND THEIR AMINOGLYCOSIDE CONJUGATES TO A ^{19}F LABELLED HIV-1 TAR MODEL

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2′-*O*-methyl oligoribonucleotides and their aminoglycoside conjugates have been targeted to a ^{19}F labeled HIV-1 TAR RNA model and the resulted invasion has been monitored by ^{19}F NMR spectroscopy. In addition to obtained K_d-values, temperature-dependent behavior of the complexes has been studied by ^{19}F shift *versus* temperature profiles. A remarkably enhanced invasion, compared to that resulted by the corresponding unmodified 2′-*O*-methyl oligoribonucleotide (5′-CAGGCUCA-3′), has been obtained by the neomycinconjugate. The increased affinity is resulted by a cooperative effect, in which the complex is stabilized by the concomitant binding of the neomycin moiety and the hybridization, but the invasion is also able to follow the mechanism, in which the first molar equivalent of the conjugate induces hybridization of the second.

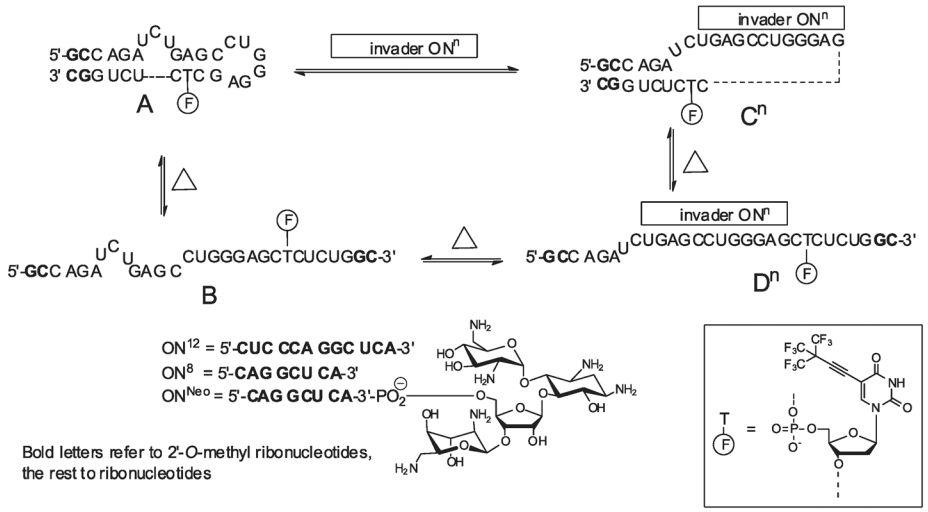
INTRODUCTION

Invasion is a fundamental process of RNA, in which a weak stem region is denatured by an exterior single strand (*i.e.* invader) resulting in a reorganized secondary structure. This process, applied *e.g.* in the antisense approach[1], is well known, but the means with which to follow its progress in detail are rather limited. Recently, the potential of ^{19}F NMR spectroscopy as a tool for analysis of RNA secondary structures has been recognized[2]. ^{19}F is a sensitive nucleus (83% compared to ^{1}H), and its resonance shift is additionally strongly dependent on the spatial vicinity of the ^{19}F label. Thus, even a modest change in the secondary structure of a ^{19}F labelled RNA may result in a detectable resonance signal distinct from that of the initial state. As examples: a 2′-deoxy-2′-fluoronucleoside incorporated in RNA has been used for the identification of site-specific RNA binders[2a], 5-[4,4,4,-trifluoro-3,3-bis(trifluoromethyl)but-1-ynyl]-2′-deoxyuridine for the detection of DNA duplex formation[2b], 5-fluoropyrimidine nucleotides for the characterization of secondary structure of HIV-2 TAR[2c] and 2′-deoxy-2′-fluoro and 2,4-difluorotoluene nucleosides for the quantification of hairpin/hairpin[2d] and duplex/hairpin[2e] equilibrium of self-complementary RNAs. Hairpin/competitive duplex equilibrium, involved in an RNA invasion, is even a more complex reaction system, of which monitoring by ^{19}F NMR spectroscopy has been demonstrated in the present study[3]. Invasion of 2′-*O*-methyl oligoribonucleotides and their aminoglycoside 3′-conjugates to a ^{19}F la-

belled HIV-1 TAR RNA model and the temperature-dependent behaviour of the resulted complexes have been examined.

RESULTS AND DISCUSSION

Applicability of an appropriately positioned ^{19}F probe[2b] to determination of the melting temperature of the TAR model (**A** ↔ **B** in Scheme 1) was first evaluated. At 25 °C, the ^{19}F probe gives one sharp signal at –66.32 ppm



SCHEME 1

(i/Fig. 1a), which upon heating expectedly undergoes a linear temperature-dependent shift to downfield. On approaching the *T*m-value this signal is broadened and a new broad signal appears at 0.4 ppm upfield. The latter signal refers to the denaturated TAR model (**B**). The equilibration between **A** and **B** is hence slow in NMR time scale below the melting temperature. Upon further heating, the signals shift closer to each other, being almost equal sized at 60 °C (~T_m, vii/Fig. 1a). Coalescence to the broad signal at –66.32 ppm is observed at 66 °C (vi/Fig. 1a), which refers to a mixture where **B** predominates. After completion of the denaturation process (>70 °C), the signal is sharpened and a linear temperature-dependent shift to downfield is again observed. A unique negative S-curve (^{19}F shift *versus* temperature, Fig. 2c) is obtained, where the inflection point (60 °C) shows the thermal denaturation (consistently with UV-spectroscopy: T_m = 60.7 °C).

Applicability of ^{19}F NMR spectroscopy for the monitoring of invasion was first tested with **ON^{12}**. Upon titration of **A** with **ON^{12}** at 25 °C, the signal of

A disappears and a novel signal at –66.89 ppm (xvii/Fig. 1c) almost quantitatively (K_d = 0.11 ± 0.06 μmol l^{-1} at 25 °C) appears, referring to formation of an invasion complex. Interestingly, on increasing the temperature, grad-

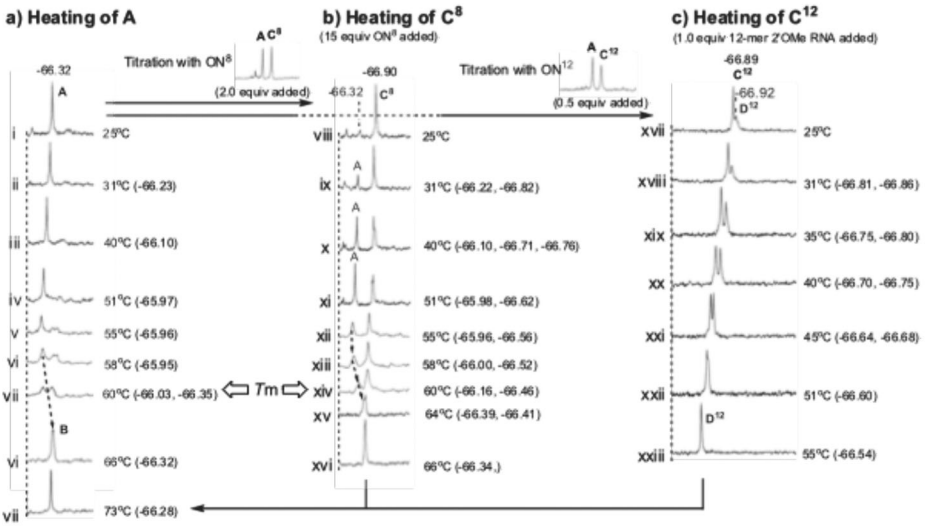


FIG. 1
^{19}F NMR spectra of **A** and its invasion complexes in different temperatures

ual conversion of this signal to another one at 0.05 ppm upfield takes place, the two signals being equal at 45 °C (xxi/Fig. 1c) and at 51 °C (xxii/Fig. 1c) they are merged to a single broad signal. Most likely, the first observed signal refers to a macro-looped complex ($\mathbf{C^{12}}$) and the latter signal to a stable open-chain invasion complex ($\mathbf{D^{12}}$). When the temperature is still increased, the signal of $\mathbf{D^{12}}$ is almost linearly shifted to downfield and eventually it merges with the signal of the single stranded form **B** (Fig. 2c). Thermal denaturation of $\mathbf{D^{12}}$, hence, takes place at a higher temperature than the conversion of the hairpin TAR (**A**) to an open chain form (**B**) (consistently with UV-spectroscopy: T_m 65.7 °C). The signals of $\mathbf{D^{12}}$ and **B** expectedly overlap, since the melting of $\mathbf{D^{12}}$ is not accompanied by any marked change in the immediate vicinity of the ^{19}F probe. Invasion obtained by 8-mer 2′-*O*-methyl oligoribonucleotide $\mathbf{ON^8}$ behaved similarly. As expected, the affinity of $\mathbf{ON^8}$ is weak, but a 1:1 complex is formed (K_d = 18.5 ± 1.1 μmol l^{-1} at 25 °C, Fig. 2b). At 25 °C and in excess (15 equiv) of $\mathbf{ON^8}$ the ^{19}F signal of the macro-looped invasion complex $\mathbf{C^8}$ (–66.90 ppm, viii/Fig. 1b) predominates. As with $\mathbf{ON^{12}}$, the complex exhibits two, albeit

broader and partially overlapped, signals at a higher temperature ($\mathbf{C}^8/\mathbf{D}^8$) (viii-xi/Fig. 1b). The most notable difference between $\mathbf{C}^8$ and $\mathbf{C}^{12}$ was in their stability. In contrast to $\mathbf{A} \leftrightarrow \mathbf{C}^{12}$, $\mathbf{A} \leftrightarrow \mathbf{C}^8$ was a dynamic equilibrium, in which amount of $\mathbf{C}^8$ decreased upon heating (viii-xi/Fig. 1b).

The site-specific binding of neomycin occurs below the bulged nucleotides of native HIV-1 TAR region through the minor groove[4]. Therefore, neomycin, covalently attached to $\mathbf{ON}^8$, may result in a cooperative effect, in which the complex is stabilized by the concomitant binding of the neomycin moiety and the hybridization. This should be involved in the macro-looped complex (**C**), since the open-chain invasion complex (**D**) bears only the canonical double strand with a modest affinity to aminoglycosides. As seen, neomycin conjugate $\mathbf{ON}^{Neo}$ provides a significantly enhanced invasion (K_d = 0.59 ± 0.16 μmol l^{-1}) compared to that obtained by unmodified 2′-*O*-methyl oligoribonucleotide $\mathbf{ON}^8$ (Fig. 2b). An interesting

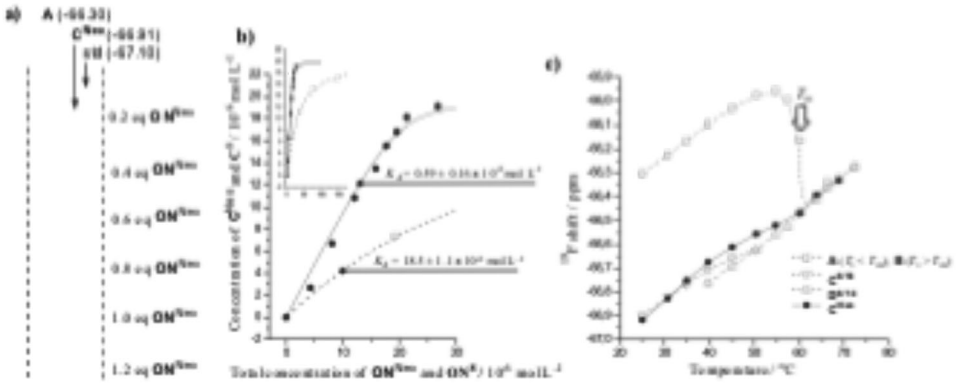


FIG. 2
a) ^{19}F NMR spectra of **A** + increasing concentration of $\mathbf{ON}^{Neo}$, b) titration curves with $\mathbf{ON}^8$ and $\mathbf{ON}^{Neo}$, c) ^{19}F shift *versus* temperature profiles of A ↔ B (□), $C^{8/12} \leftrightarrow D^{8/12} \leftrightarrow$ B (▽/△) and $C^{Neo} \leftrightarrow$ B (●)

observation was additionally resulted, when the mixture of the complex $\mathbf{C}/\mathbf{D}^{Neo}$ was heated. The ^{19}F shift *versus* temperature profile of the complex ($\mathbf{C}/\mathbf{D}^{Neo}$) followed the prolonged downfield curve compared to the complexes with unmodified 2′-*O*-methyl oligoribonucleotides (Fig. 2c). The prolonged downfield curve (●, Fig. 2c) is consistent with the expectation that the enhanced invasion is related to the stabilized macro-looped complex ($\mathbf{C}^{Neo}$). Further studies with other aminoglycoside conjugates (not shown in this abstract) showed that the invasion may also follow the alternative

mechanism, in which the first molar equivalent of the conjugate induces invasion of the second.

This work was financially supported by the Academy of Finland.

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AUTOMATIC OLIGONUCLEOTIDE SYNTHESIZER UTILIZING THE CONCEPT OF PARALLEL PROCESSING

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Described is a system synthesizing DNA in an array of 36 384-well microtiterplates with filters. The plates are placed in holders on a perimeter of a rotating table and positioned under arrays of nozzles delivering individual reagents. Delivery of reagents is verified by cameras as well as the progress of the synthesis is monitored by camera inspection of the plates after delivery of deblocking reagent. As every plate on the table is in a different stage of the synthesis cycle (one cycle of the synthesis equals one turn of the table), the synthesizer is performing all synthetic operations simultaneously and all stations are utilized to the maximum capacity. Since every plate can be taken out independently of the other plates, the synthesizer can be called a "continuous synthesizer".

INTRODUCTION

Genomic technologies are enabled by fast and economical preparation of synthetic oligonucleotides. Solid phase synthesis of these molecules is one of the most efficient and well developed chemistries[1]. Therefore, the challenge in automation of the synthesis of oligonucleotides is in the level of parallelization of the synthetic processes and in the lowering their costs. Illumina needed millions of DNA probes and the cost of available machinery and/or custom synthesized oligos was a major stumbling block in the development of the genotyping technology based on the random distribution and subsequent decoding of micron-sized silica beads[2] immobilized in the genotyping "chips".

RESULTS

We have applied in our first generation of DNA synthesizers the technology of the so-called tilted centrifugation and achieved the throughput of 768 oligos per synthesizer run[3]. The technology of tilted centrifugation[4], in which the solid support is kept in the synthetic vessel (well of the microtiterplate) and the reagent solutions are removed by decantation over the edge of the well, was later improved by melting the CPG (controlled-pore glass) to the bottom of the plate wells. This improvement allowed us to use V-shape wells of 384 well plates and remove the liquids completely by centrifugation without the need to apply any tilt in the centrifugation step.

In this way we improved throughput of our synthesizer to 3024 oligos per run. However, this still did not fulfill the need required by the manufacturing of our chips and increase of the productivity was achieved only by building multiple synthesizers.

We have found the solution in the application of the concept of parallelization of all synthetic processes. In the next generation of our synthesizers we abandoned the concept of centrifugation for liquid removal and we worked with the 384-well filter plates. Thirty-six of these plates were placed on the perimeter of two-meter diameter rotor in special holders allowing their placement under delivery heads and application of vacuum for emptying the wells by filtration. Synthetic protocol was distributed into 36 stations placed above the area through which the plates were carried around by the rotor. The stations were either active – stations in which reagents were added or removed – or passive – stations in which only incubation occurred. In addition, in several stations the plates were inspected by the digital camera to establish whether the reagent was added correctly or whether the reaction (deblocking reaction which creates orange coloring) proceeded to completion. In this arrangement all steps of the synthesis are performed at the same time in the different location of the rotor and no time is wasted by waiting for the completion of the previous step. In two special locations of the rotor the plate holders can be taken out and replaced by the new holder with fresh synthetic substrate (CPG) and the synthesis in the new plate can be started. Therefore, the synthesizer can work on the different oligonucleotides of various length (obviously the oligos of the similar length should be placed in one microtiterplate) and does not have to stop between different batches of oligos. The batch is defined as an individual microtiterplate. Delivery of solutions is achieved by passing the plate wells under an array of 24 nozzles actuated "on the fly" to deliver single digit microliter amount of the reagents in 100 milliseconds per well. Reagents are stored in mini columns located in close proximity to solenoid valves connected to the nozzles. These columns are continually refilled from the large volume storage of reagents and kept under constant pressure. All nozzles are periodically calibrated so that the volume defined by the back pressure and solenoid actuated time is kept constant. Delivery of liquid is verified by the camera and plate is ready for the visit in the next station. One operation, or "tick" of the instrument is about 9 seconds, which gives the instrument the capacity of 3,686,400 couplings per day.

Figure 1 is a schematic diagram of the rotary table and dispensing stations. The plate modules and their associated plate holders can be loaded onto the synthesizer either manually or using an automated robotic arm. A

unique bar code is assigned to each microtiter plate so that each plate is automatically tracked from the time it is placed onto the synthesizer until it is removed. A computer control system monitors the progress of each microtiter plate as it moves onto and off the rotary plate. A bar code scanner is associated with the automated handling device such that each plate which is loaded or unloaded from the synthesizer is scanned and tracked within a LIMS. Each well of the microtiter plate is assigned a different oligonucleotide to be synthesized. Based on this assignment, a LIMS instructs each dispenser to output the proper reaction solutions into the proper well.

Once a plate holder is loaded onto a plate module on the rotary table, the plate module enters below the wash station dispenser and the valves within the dispenser output a wash solution into the microtiter wells. After a wash solution is dispensed into each well, and it has been allowed to incubate for a desired time, the solution is removed by vacuum. The vacuum is in continuous communication with the plate modules as the rotary table rotates. A valve is placed between the vacuum source and the plate for attenuated communication between the vacuum source and the plate. The rotary union allows a vacuum line to communicate with each of the microtiter plates and to travel with each of the microtiter plates while the rotor is moving and while the vacuum source is maintained at a static location. The vacuum supply to each multi-well plate is controlled by separate valves. An electrical slip ring of the rotary union contains a series of electrical circuits allowing operating solenoids to travel with each of the multi-well plates. By connecting a solenoid valve to each of the moving plates, the vacuum to each plate is turned on or off by the system at any time during the synthesis cycle.

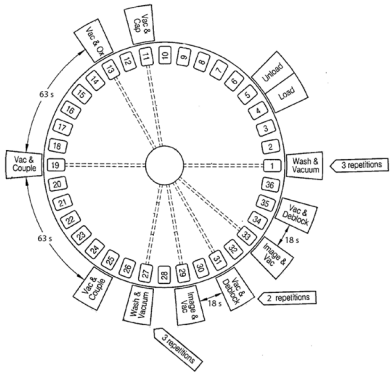

FIG. 1
Schematic diagram of the array of stations within the continuous oligonucleotide synthesizer

Prior to evacuation of the ACN wash, an image of the multi-well plate is taken by one of the digital cameras, and sent for processing to the image processing system. The image processing system analyzes the image to determine if each well of the microtiter plate was properly treated with a wash. If a well is not found to have a wash solution, then that well can be marked as bad, or can be marked to be treated with an additional wash at a later cycle. The image processing system is used in a similar fashion to determine if solution has been sufficiently removed from a multi-well plate based on an image acquired after an evacuation step.

Following the wash step, the plate modules move to a position under the deblock dispenser. This dispenser dispenses a deblocking solution containing, trichloroacetic acid that removes a dimethoxytrityl (DMT) group from the last nucleotide. The release of DMT is readily identifiable by a bright orange color that is imaged by another camera. The optical density of the orange color of each well in the microtiterplate is determined from the image and used to evaluate coupling efficiency. The plate module is then evacuated to remove the deblocking solution. The delivery of deblocking solution and imaging of the plate is repeated twice more before the plate is washed by acetonitrile three times.

After being washed, the plate module travels under the coupling dispenser where the appropriate nucleotide is added to each of the wells. The dispenser is composed of four banks of 24 nozzles delivering A, C, G, and T nucleotides into each of the wells of the microtiter plate. The next dispenser is composed of three banks which can be used to deliver modified nucleotides, and one bank delivering an activator (dicyanoimidazole).

Following delivery from the two coupling dispensers and incubation, the plate module moves to an oxidation dispenser. This solution is imaged and evacuated, and the plate module moves to a capping mixture dispenser for the final stage in the synthesis cycle.

Illumina is using two of these world's highest throughput DNA synthesizers and tested their performance by the synthesis of more than 40 million oligonucleotides up to 110 bases long.

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MODELING THE GENERAL ACID/BASE CATALYZED RNA CLEAVAGE OF SMALL RIBOZYMES

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For elucidating the mechanism of the general acid/base catalyzed cleavage of RNA phosphodiester bonds, a number of cleaving agents having two cyclen moieties tethered to a 1,3,5-triazine core have been prepared and their ability to bind and cleave UpU studied over a wide pH range. Around neutral pH, the cleaving agents form highly stable ternary complexes with UpU and Zn^{2+} through coordination of the uracil N3 and the cyclen nitrogen atoms to the Zn^{2+} ions. Under conditions where the deprotonated neutral form of the triazine ring predominates, hydrolysis of UpU, but not of ApA, is accelerated by approximately two orders of magnitude in the presence of the cleaving agents, consistent with general base catalysis.

INTRODUCTION

An increasing number of cases are reported, where ribozymes employ their nucleobases as general acid/base catalysts for the cleavage of phosphodiester bonds[1]. General acid/base catalysis of RNA cleavage has been extensively studied with simple buffer acids and bases, such as imidazole, morpholine or acetic acid. In such systems, the observed catalysis is so weak that high buffer concentrations have to be employed, making elimination of medium effects difficult. The results are, hence, somewhat controversial and still open to various interpretations[2]. One approach to increase the local concentration of the general acid/base catalyst while avoiding undesired solvent effects is to anchor the catalyst close to the scissile phosphodiester linkage. In this paper we apply a new type of structure to achieve this. The catalyst is anchored to uridylyl-3′,5′-uridine (UpU) exploiting the strong binding of Zn^{2+} chelates of small azacrowns to the deprotonated N3 atom of a uracil base, first reported by Kimura (Fig. 1)[3]. The Zn^{2+}:cyclen anchors are attached to the catalytic core through spacers that are sufficiently flexible to allow the departing 5′-linked nucleoside to adopt an apical position within the phosphorane intermediate. The soundness of this approach has been previously demonstrated with related bi- and trinuclear azacrown derivatives (**1a**, **1b**) forming a highly stable complex with UpU. The binuclear bis(azacrown) compound (**1b**) is catalytically inactive owing to both of its Zn^{2+}-chelates being engaged in binding to the

uracil bases but still allows the spontaneous cleavage of UpU to proceed at an unaltered rate[4].

In the new scaffolds (**2h**, **2i**, **2n** and **2o**) employed in the present work, the two UpU anchoring Zn^{2+}:cyclen chelates are linked to a common 1,3,5-triazine ring instead of the benzene ring applied of **1a** and **1b**. The former may act as a "shuttle" mediating proton transfer from the attacking 2′-OH to a non-bridging oxygen of the phosphorane intermediate and, finally, to the leaving group. A similar mechanism has been proposed for catalysis by the hammerhead and hairpin ribozymes, with a guanine base transferring a proton from the attacking 2′-oxygen to the *pro*-R_P non-bridging phosphoryl oxygen[5]. Because the Zn^{2+} chelate of cyclen is a poorer catalyst for phosphodiester cleavage than the Zn^{2+} chelate of 1,5,9-triazacyclododecane used in the catalytically inactive compound **1b**, the Zn^{2+}:cyclen moieties are expected to be involved only in binding with the uracil bases and not in the actual catalysis (Fig. 1)[6].

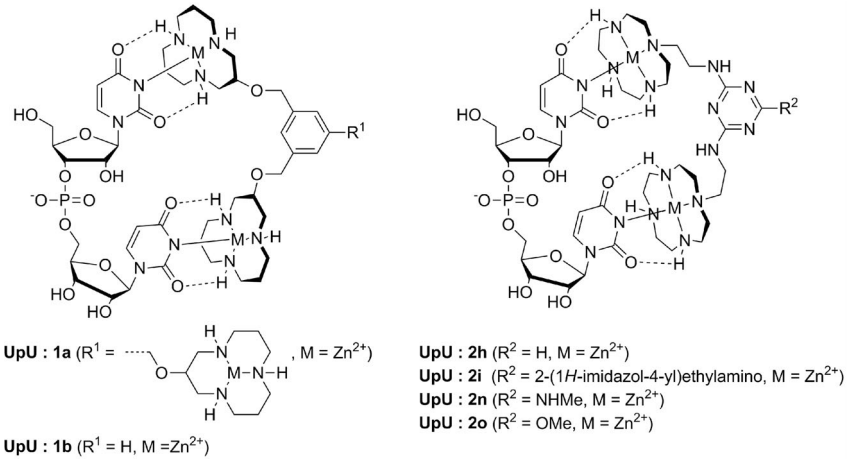

FIG. 1
Complexes of UpU with bi- and trinuclear Zn^{2+} chelates of 1,5,9-triazacyclododecane (**1a** and **1b**) and cyclen (**2h**, **2i**, **2n** and **2o**)

RESULTS AND DISCUSSION

To have thermodynamic data on the interaction between Zn^{2+} and the 1,3,5-triazine-based ligands, as well as between UpU and the dinuclear Zn^{2+} complexes, protonation of 2′-*O*-methyluridylyl-3′,5′-uridine (mUpU) and **2n** (L) and the complex formation in the Zn^{2+}-L and Zn^{2+}-mUpU binary and in the Zn^{2+}-mUpU-L ternary systems were studied by potentiometric and spectrophotometric titrations. The species distribution curves for the

Zn^{2+}-L-mUpU (2:1:1) system are depicted in Fig. 2. Between pH 7 and 10 the complex having both of the cyclen moieties bound to the uracil bases is overwhelmingly the most stable species.

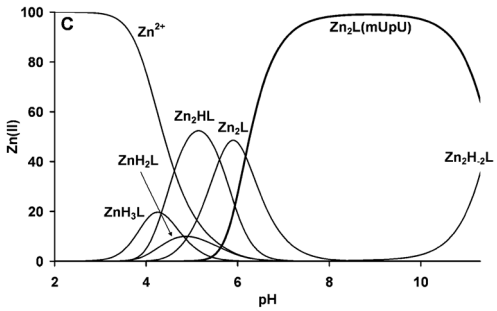

FIG. 2
Species distribution curves for the Zn^{2+} complexes in the Zn^{2+}-L-mUpU (2:1:1) system (T = 25 °C, I (NaCl) = 0.1 mol l^{-1}, [L] = 1 mmol l^{-1})

To verify the catalytic activity of the binuclear Zn^{2+}:cyclen compounds synthesized, cleavage of UpU was followed at various concentrations (0–80 μmol l^{-1}) of the methylamino derivative **2n** (T = 90 °C; pH* 6.57). Cleavage of ApA was followed under the same conditions for comparison. The dependence of the observed pseudo first-order rate constant for the cleavage of UpU and ApA as a function of [**2n**] is presented in Fig. 3A.

Cleavage of UpU follows simple first-order kinetics and attains a maximum acceleration of approximately two orders of magnitude at sufficiently high concentrations of **2n**. In contrast, cleavage of ApA is hardly accelerated by **2n**, indicating that binding of the nucleobases to the Zn^{2+}:cyclen moieties of the cleaving agent is essential for catalytic activity. In principle, one might argue that only one of the azacrown chelates is engaged in UpU binding, the other one serving as an intracomplex catalyst. However, as discussed earlier, the related Zn^{2+}:azacrown complex **1b**:2Zn^{2+} does not catalyze the cleavage of UpU, even though its Zn^{2+}:1,5,9-triazacyclododecane moieties are known to be better catalysts for phosphodiester cleavage than the Zn^{2+}:cyclen moieties of **2n**:2Zn^{2+}.

The pH-rate profiles for the disappearance of UpU in the presence (60 μmol l^{-1}) or absence[7] of the cleaving agents are presented in Fig. 3B. In the presence of the cleaving agents, the reaction becomes roughly second-order in [OH^-] at pH* 4, pH-independent at pH* 6 and finally first-order in [OH^-] at pH* 8. No data for the hydrolysis promoted by the cleaving agents **2h**, **2i**, **2n** and **2o** could be obtained at pH* < 4 owing to the instability of the ternary complexes under these conditions. Over the pH range studied, mu-

tual isomerization between 2′,5′- and 3′,5′-UpU is neither facilitated nor retarded by the cleaving agents. The results are consistent with a mechanism where the triazine core mediates proton transfer from the attacking 2′-OH to the phosphorane intermediate and, finally, to the leaving group.

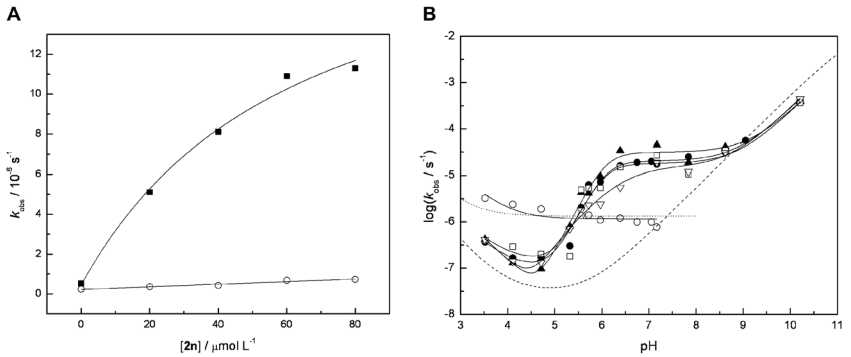

FIG. 3

(A) Pseudo first-order rate constants for the cleavage of UpU (■) and ApA (○) as a function of the concentration of the cleaving agent **2n** at 90 °C, pH* 6.57, $I(NaClO_4)$ = 0.10 mol l^{-1}, [UpU] = [ApA] = 5.0 μmol l^{-1}; (B) pH-rate profiles for the cleavage of UpU in the absence (dashed line) and in the presence of the cleaving agents **2h** (□), **2i** (▽), **2n** (●) and **2o** (▲) and the isomerization of UpU in the absence (dotted line) and in the presence of **2n** (○) at 90 °C, $I(NaClO_4)$ = 0.10 mol l^{-1}, [2] = 0/60 μmol l^{-1}

Financial support from the Academy of Finland and from the Hungarian Scientific Research Found (OTKA K63606) is greatfully acknowledged.

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SUBSTRATE RECOGNITION BY ALKYLTRANSFERASE-LIKE (ATL) PROTEINS FROM *S. pombe* AND *T. thermophilus*

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Alkyltransferase-like (ATL) proteins are highly conserved and show substantial homology to O^6-alkylguanine-DNA alkyltransferases (AGTs). AGTs repair DNA containing the highly toxic and mutagenic lesion O^6-alkylguanine by transfer of the alkyl group to an active site Cys. In ATL proteins this Cys is replaced with Trp or Ala. ATL proteins bind tightly to O^6-alkylguanine-containing DNA but are incapable of direct repair. In *S. pombe* and *T. thermophilus* that both lack an AGT, ATL proteins protect against the mutagenic and toxic effects of alkylating agents, indicating a role in DNA repair. It has been proposed that Atl1 from *S. pombe* forms a relatively stable and bulky complex with alkylated DNA, which is subsequently recognised and repaired by the NER machinery. We have shown that two ATL proteins, Atl1 from *S. pombe* and TTHA1564 from *T. thermophilus*, have the ability to bind with high affinity single- and double-stranded oligodeoxyribonucleotides containing a broad range of O^6-alkylguanine adducts, including many that are poor substrates for human AGT.

INTRODUCTION

O^6-Alkylguanine-DNA alkyltransferases (AGTs) are the major form of defence against the mutagenic, cytotoxic and recombinogenic effects of O^6-alkylguanine lesions in DNA[1–4]. Recently a related family of proteins has emerged that, while capable of recognising adducts of this type, have no ability to repair them[5,6]. Due to the structural homology of these proteins, along with conservation of key functional motifs such as those in the highly conserved AGT active site sequence PCHR (PW/AHR in ATLs), they have been termed alkyltransferase-like proteins (ATLs)[7]. ATL proteins have been characterized in a number of organisms, the best studied being derived from *E.coli* [5,8,9] (eATL or YbaZ), *S. pombe* [6,10] (Atl1) and *T. thermophilus* [11,12] (TTHA1564). The latter two organisms are of particular interest since neither contains an AGT protein. The ATL protein from *S. pombe*, Atl1, has been shown to inhibit the action of MGMT [6] and recent crystal structures have shown marked similarities in the way these proteins bind alkylated DNA; by interaction with the minor groove and base flip-

ping of the damaged guanine into the active site pocket of the protein[10]. In the case of Atl1 the binding pocket for the damaged base is substantially larger than that found in other AGT proteins[10,13,14]. This appears to permit binding of DNA containing alkylguanines with much bulkier alkyl groups such as O^6-(4-oxo-4-(3-pyridyl)butylguanine (PobG)[10,15]. In addition, the NMR structure of the ATL protein from *Vibrio parahaemolyticus* (vpAtl) suggests that the binding pocket exhibits conformational flexibility which may facilitate recognition of a broad range of O^6-alkylguanine adducts[16]. Atl1 bends DNA by an angle of approximately 45° upon binding[10], and it is thought that this helically distorted, bulky complex is suitable for recognition by nucleotide excision repair (NER) proteins that are known to repair large lesions in DNA such as those caused by UV radiation. If the role of ATL proteins is to recognise O^6-alkylguanine lesions in DNA and flag them for repair by the NER apparatus, the ability to bind, with high affinity, DNA containing a broad range of O^6-alkylguanine adducts (including those which are not effectively repaired by AGT proteins) would be considered desirable. In order to explore the substrate specificity of ATL proteins, a series of short (13-mer) fluorescently-labeled (SIMA-HEX) oligodeoxyribonucleotides (ODNs) were synthesised and subsequently modified to contain a variety of different O^6-alkylguanine lesions (Fig. 1). These ODNs were then used in fluorescence-based binding assays to quantify the recognition of ATL proteins Atl1 from *S. pombe* and TTHA1564 from *T. thermophilus*.

RESULTS AND DISCUSSION

In order to create a wide range of fluorescently-labelled O^6-alkylguanine-ODNs for studying substrate recognition by the two ATL proteins, we were interested in incorporating alkyl groups that varied in size, charge, polarity and hydrophobicity (Fig. 1). To achieve this, we used our previous strategy[17] which relies on reacting the appropriate alcohol under basic conditions with ODNs containing the reactive base 2-amino-6-methylsulfonylpurine. 5′-SIMA(HEX)-labelled ODNs were used as this label has sufficient stability under basic conditions (unlike HEX [18]) to be used in displacement reactions of this type whilst also displaying similar fluorescent properties to HEX which allow dissociation constants in the low nanomolar range to be measured[19].

The addition of either ATL protein to a solution of fluorescently labelled ODN resulted in a concentration-dependant decrease in total fluorescence intensity. Thus, titrations were performed to determine dissociation con-

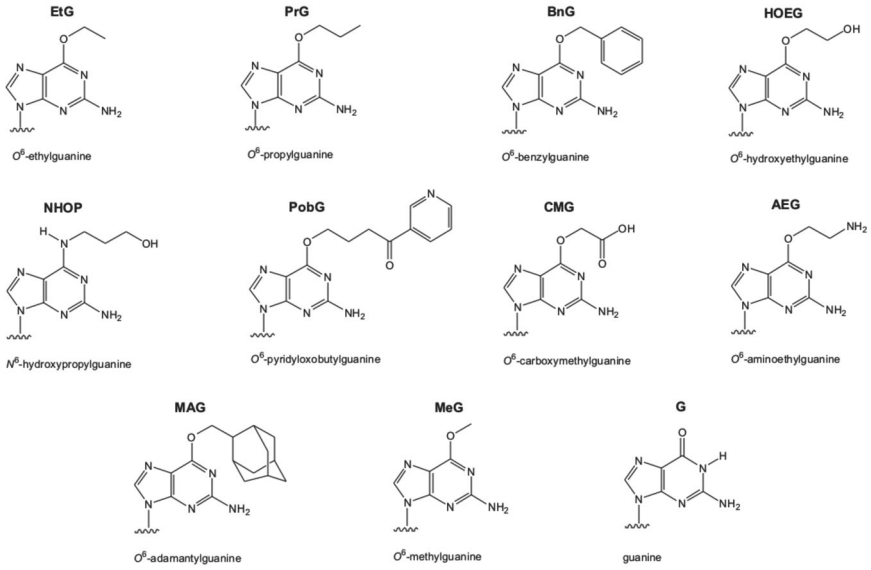

FIG. 1
O^6-Alkylguanine lesions (X) incorporated into 13-mer ODNs for the purposes of this study

TABLE I
K_D values for ATL proteins binding to single-stranded oligodeoxyribonucleotides (ODNs) d(GCCATGXCTAGTA) containing O^6-alkylguanine determined by measurement of changes in fluorescence intensity. Values given are the average of those determined by three separate titrations

O^6-Modification	Atl1 K_D, nM	MBP-TTHA1564 K_D, nM
Propyl (PrG)	0.389 ± 0.02	1.8 ± 0.14
Methyladamantyl (MAG)	0.554 ± 0.03	1.86 ± 0.18
Benzyl (BnG)	0.632 ± 0.01	1.63 ± 0.15
Hydroxyethyl (HOEG)	0.727 ± 0.04	1.94 ± 0.09
Aminoethyl (AEG)	1.01 ± 0.02	3.61 ± 0.13
Ethyl (EtG)	1.09 ± 0.12	1.72 ± 0.17
Pyridyloxobutyl (PobG)	1.1 ± 0.02	1.82 ± 0.1
N^6-PropylOH (NHOP)	1.13 ± 0.14	3 ± 0.22
Carboxymethyl (CMG)	2.03 ± 0.06	3.55 ± 0.03
Methyl (MeG)	2.2 ± 0.12	1.23 ± 0.13
None (G)	721 ± 105	102.6 ± 2

stants for Atl1 and TTHA1564 with single-stranded (ss) ODNs containing all of the lesions shown in Fig. 1 (Table I). The results clearly show that all the O^6-alkylguanine-containing ODNs were recognised with high affinity (compared to the control sequence containing guanine), with only small differences in K_D value between most of the bound substrates. Interestingly, both ATL proteins recognise O^6-aminoethylguanine (AEG), pobG, CMG and HOEG, all of which are reported to be poor or non-substrates for MGMT [17,20,21]. This result, along with the fact that the AGT proteins Ogt and Ada from *E. coli* are known to be less capable than the human protein of repair of bulky O^6-alkylguanine adducts[22–25] would seem to vindicate the conclusion that ATLs have a much broader substrate specificity than AGTs. The evidence would indicate that, although devoid of O^6-alkyltransferase activity, ATL proteins seem likely to have the role of efficient damage recognition sensors which can bind lesions that are poor substrates or refractory to direct repair by AGTs.

This project was funded by the BBSRC and JSPS.

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DEVELOPMENT OF INSULATOR BASE PAIRS FOR THE DRASTIC ENHANCEMENT OF QUANTUM YIELD

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Perylenediimide (PDI) is highly quenched by nucleobases, which greatly restricts its application as a fluorescent probe. Here, we propose "insulator base pairs" tethering cyclohexane ring through D-threoninol. When "insulator base pairs" were inserted between PDI and nucleobases, the quantum yield of PDI drastically increased by several thousand-fold. The "insulator base pairs" reported here also have the potential to increase the quantum yields of other fluorophores.

INTRODUCTION

Fluorescent-labeled oligonucleotides are powerful tools for biochemical and biological research. However, some fluorophores, even those with high potential, cannot be utilized for the labeling of DNA or RNA due to their strong quenching by natural nucleobases. In order to protect these fluorophores from severe quenching, a new methodology that can shield these fluorophores from natural nucleobases is required. Kool et al. first reported an "insulator molecule" (5,6-dihydro-2′-deoxythymidine) which enhances the emission of pyrene in a single strand[1].

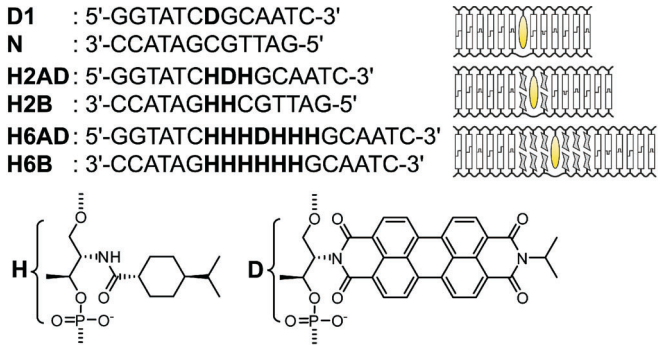


FIG. 1
Sequences of the modified DNAs synthesized in this study

Here, we propose "insulator base pairs" that can shield a fluorophore from nucleobases in order to achieve a high quantum yield in a duplex. We incorporated *trans*-isopropylcyclohexane moiety (**H** in Fig. 1), which has

one rigid cyclohexane ring with no π electrons[2], as an insulator. The absence of π electrons should efficiently suppress electron transfer. In our design, **H** moieties were introduced into both strands of a DNA duplex to form tentative "base pairs". Because **H** moieties have high hydrophobicity, they should face to the inside of the duplex and form "base pairs". The "insulator base pair" would therefore be expected to shield a fluorophore from quenching by nucleobases.

We selected the fluorophore perylenediimide (PDI: **D**) to test the shielding effect of the "insulator base pair". PDI has been widely applied to material use because of its high electron affinity, high brightness, strong π-π stacking interaction and photostability. Furthermore, PDI has also been used for the functionalization of DNA[3–10]. However, even though the PDI monomer shows a high quantum yield and photostability, the quenching of PDI by nucleobases, due to electron transfer from nucleobases, has severely limited its application as a fluorophore[8–10]. Thus, we introduced "insulator base pairs" between PDI and nucleobases in order to enhance the fluorescence of PDI. Sequences of modified DNA are shown in Fig. 1. Two or six "insulator base pairs" were introduced between PDI and nucleobases on D-threoninol as a scaffold[11,12]. DNA containing one PDI but no insulator was also synthesized as a control (**D1**).

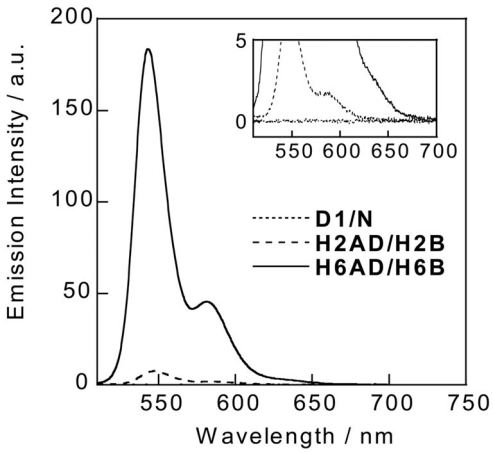


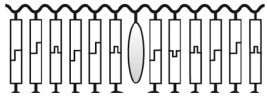
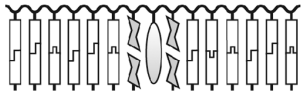
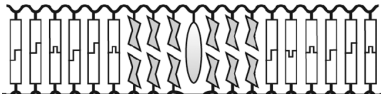
FIG. 2
Fluorescence emission spectra at 20 °C of **D1/N**, **H2AD/H2B** and **H6AD/H6B**. Solution conditions were as follows: [DNA] = 1.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer). Excitation wavelength: 500 nm

RESULTS AND DISCUSSION

For the hybridization of **D1** with a complementary strand (**N**), almost no fluorescence emission was detected from PDI (see the dotted line in the inset of Fig. 2). This extremely low emission is attributable to the almost complete quenching of PDI by electron transfer from nucleobases to PDI[9]. However, when two **H–H** pairs were inserted between PDI and its neighboring nucleobases (**H2AD/H2B**), distinct emission at 550 nm was observed from PDI. As summarized in Table I, the quantum yield (Φ) of **H2AD/H2B** was 0.020 whereas that of **D1/N** was less than 0.001. These results clearly demonstrated that the "insulator base pairs" successfully suppressed electron transfer from the nucleobases to PDI.

Since the value of Φ remained still lower than 0.1 following the introduction of two insulator pairs, we determined if insertion of additional insulator pairs might enhance PDI emission. Indeed, the emission intensity of **H6AD/H6B** was dramatically higher than that of **H2AD/H2B** (Fig. 2B). The Φ value of **H6AD/H6B** was as high as 0.59, which is about 30 times higher than that of **H2AD/H2B** (see Table I). Consequently, quantum yield of PDI increased approximately several thousand-fold compared to that of **D1/N** without insulators. The Φ value of **H6AD/H6B** is comparable to those of common fluorophores, indicating that this duplex can be utilized for labeling. This dramatic increase in quantum yield can also be detected even by the naked eye (data not shown). A solution exhibits bright orange color by incorporating six "insulator base pairs".

TABLE I
Effect of duplex sequence on the quantum yield (Φ) and melting temperature

Sequence		Φ^a	T_m, °C[b]
D1/N		<0.001	41.0
H2AD/H2B		0.020	44.2
H6AD/H6B		0.59	62.0

[a] Quantum yield was determined using the quantum yield of Rhodamine 6G in ethanol (0.94) as a reference. [b] [DNA] = 5.0 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer).

The melting temperatures (T_ms) of the duplexes are also summarized in Table I. Incorporation of insulator pairs into DNA unexpectedly stabilized the duplex even though insulators have non-planar structures. For example, the T_m of **H2AD/H2B** was 44.2 °C, which was 3.2 °C higher than that of **D1/N**. Furthermore, the introduction of six **H–H** pairs greatly enhanced the thermodynamic stability of the duplex: the T_m of **H6AD/H6B** was as high as 62.0 °C. It can be concluded that **H–H** pairs strongly stabilize the duplex probably due to hydrophobic interactions. This high stability of **H–H** pairs supports the idea that insulator moieties form "base pairs" in a DNA duplex and disturb π-π stacking between PDI and the nucleobases. Shielding of PDI from natural nucleobases was also substantiated by the UV-Vis spectra; absorption maximum of **H2AD/H2B** was 535 nm whereas that of **D1/N** was 546 nm (data not shown). Concurrently, absorbance of **H2AD/H2B** was larger than that of **D1/N**. These blue-shift and hyperchromic effect show that interactions between PDI and nucleobases were disturbed by insulator base pairs.

In conclusion, the quantum yield of PDI dramatically increased following the introducing of "insulator base pairs" with cyclohexane moieties[13]. The quantum yield of PDI was increased from <0.001 to as high as 0.59 when six **H–H** pairs were introduced between PDI and nucleobases. Thus, this duplex has the potential to be used for labeling of DNA or RNA. Because even PDI, whose fluorescence is completely quenched by natural nucleobases, showed high quantum yield, the "insulator base pairs" could be utilized for the enhancement of quantum yield of other fluorophores. We recently reported the assembly of fluorophores in DNA duplexes, in which the fluorophores and the natural base pairs were alternately introduced[14]. The "insulator base pairs" can also be utilized to assemble fluorophores without decreasing the quantum yield.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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NEW INDUCIBLE NUCLEIC ACID CROSS-LINKING METHODOLOGY BASED ON OXIDATION OF INCORPORATED FURAN MOIETIES: SCOPE AND LIMITATIONS

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The high specificity by which oligonucleotides recognize nucleic acids has caused considerable interest in the design of modified and reactive oligonucleotides. These customized nucleic acid derivatives can be used for a range of therapeutic, diagnostic and analytical purposes. Recently, a furan-oxidation based cross-linking strategy was developed. A series of furan modified nucleoside building blocks were synthesized and incorporated into oligodeoxyribonucleotides. Selective oxidation of the furan moiety triggers conversion into a reactive keto-enal moiety which can subsequently be used in DNA interstrand cross-linking reactions. Detailed investigations of the influence of the specific furan modified building block upon duplex stability and cross-link selectivity have been carried out. The structure of the cross-linked species was unambiguously characterized by coinjection of the enzymatic digestion reaction mixture with the synthesized dinucloside. Cross-linking is fast and efficient and allows to isolate stable adducts in good yield. In view of the efficacy of the methodology applications in chemical biology and material science can be now envisaged as a toolbox of building blocks is now available that allow fine-tuning of yield and selectivity.

INTRODUCTION

Oligonucleotides that form interstrand crosslinks (ICLs) have found widespread application in chemical biology research areas. Therefore, chemical and enzymatic methods have been developed to incorporate crosslinks into helical regions of DNA and RNA[1]. Examples include the synthesis of a duplex incorporating a preformed crosslinked dinucleotide[2,3] or post-synthetic modification of duplexes by bifunctional crosslinking reagents[4,5]. However, in some cases, the site-specific introduction of a crosslink is problematic due to formation of a mixture of monoadducts, intrastrand and interstrand crosslinks. Introduction of reactive moieties at a specific position within the duplex can circumvent the selectivity issue as is shown for photocrosslinking with 4-thiouridine[6], 5-bromouridine[7], 5-methyleneaminouridine[8] or 8-azidoadenosine[9]. Furthermore, a thio-modified oligo-nucleotide (ON) can form disulfide bonds post-synthetically[10,11]. However such ICLs are highly dependent on the proper positioning of thiol groups and, more

importantly, require modification of both strands. More recent studies on site-specific ICL formation involve modified phenylselenyl derivatives of thymidine or 5-methyl-2′-deoxycytidine[12], 1,4-dioxobutane abasic lesion[13], alkyl-connected 2-amino-6-vinylpurine[14] and 4-amino-6-oxo-2-vinyl-pyrimidine[15]. We have earlier developed (Fig. 1) a complementary methodology incorporating furan-modified 2′-amido-uridine **1** and an acyclic building block **2** to give a site-specific ICL upon oxidation with *N*-bromosuccinimide (NBS)[16,17]. We have shown that cross-linking occurs in a very fast and efficient way and that substantial amounts of stable, site-selectively cross-linked species can be isolated.

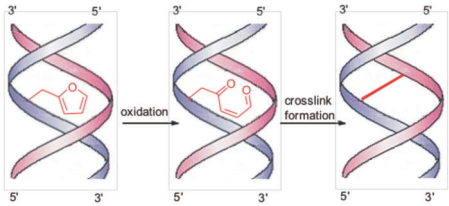


FIG. 1
Furan-oxidation triggered nucleic acid cross-linking

RESULTS AND DISCUSSION

In a further extension of the furan-oxidation cross-link study acyclic and cyclic systems with a phenyl furan moiety (cfr. building blocks **3** and **4**) replacing the natural base were studied[18]. With respect to the previous generation of duplexes incorporating building block **2**, stabilisation can be observed when incorporating an extra phenyl unit as in building block **3**. However, the extra stabilisation expected for a restored entirely cyclic backbone was absent. Duplexes incorporating **4** were in most cases even somewhat lower in stability. Whereas building block **3** benefits from enhanced stacking interactions resulting from the increased aromatic surface (when comparing to **2**), restriction of conformational flexibility and restoration

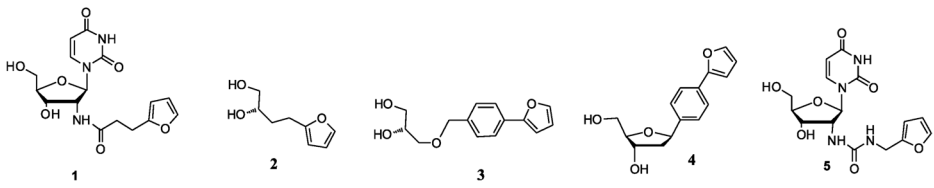
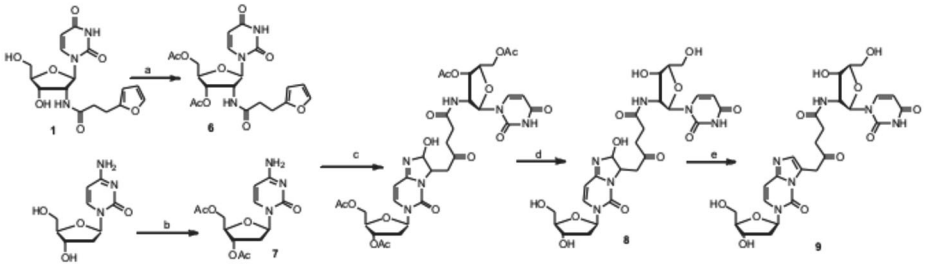


FIG. 2
Different furan-modified building blocks for nucleic acid cross-linking

of the backbone in building block **4** does not result in an increased duplex stability but a more pronounced C-selectivity is observed.

In continuation of our earlier preliminary studies with **1** we decided to reconsider this type of building blocks introducing the furan moiety by incorporation of 2′-modified uridine residues presenting the furan moiety through an ureide- (**5**) linker. Whereas building blocks **1** through **4** consistently show cross-linking to complementary A or C bases, building block **5** revealed an unprecendented C-selectivity[19]. A detailed investigation of sequence selectivity and linker influence has been carried out and thorough structural characterization has now shed light on the exact crosslinking mechanism.



a) Ac_2O, DMAP, NEt_3, ACN, 2 h, rt, 67%; b) Ac_2O, DMAP, NEt_3, ACN, overnight, rt, 68%; c) NBS, pyridine, THF/acetone/H_2O 5/4/2, overnight, rt; d) NH_3/MeOH, overnight, rt 80% (over 2 steps); e) 0.1 M HCl, 9 days, rt, quantitative

FIG. 3
Chemical synthesis of crosslinked dinucleoside derived from 2′-amido-modified **1** and dC

Following protection, crosslinking was performed by oxidation of the furan-moiety in protected nucleoside **6** with 1.1 eq of NBS, after which 3′-5′-bis-acetyl protected cytidine **7** was added[59] After straightforward deprotection with methanolic ammonia, the desired crosslinked dinucleoside **8** was obtained in good yield. Dinucleoside **8** is however present as an unseparable diasteromeric mixture, rendering NMR characterization complicated. To simplify identification, it was attempted to convert dinucleoside **8** to the aromatized nucleoside **9**. Heating of dinucleoside **8** in water or acetonitrile led to the formation of unwanted side products. Upon evaluating several conditions, aromatization in 0.1 M HCl_{aq} solution for 9 days gave the cleanest conversion to compound **9** (Fig. 4). The structure of this compound could be unambiguously confirmed by NMR.

Enzymatic digestion of the cross-linked duplex was performed with snake venom phosphodiesterase (SVPD). After removal of the phosphate groups with alkaline phosphatase, the reaction mixture containing the desired

dinucleotide can be analyzed by coinjection with the synthesized crosslinked dinucleoside (Fig. 4), confirming the identity of the crosslink structure.

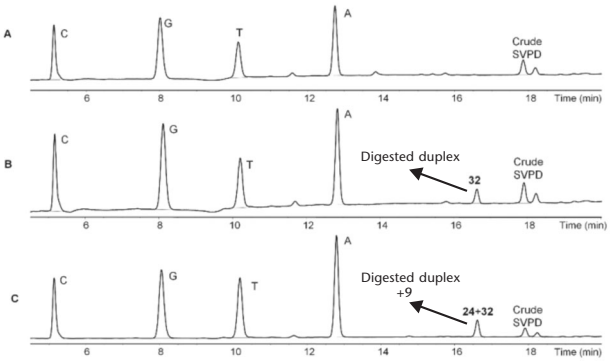


FIG. 4
Coinjection experiments; A: Reference sample: injection of A, C, T and G in the presence of crude SVPD, B: Reaction mixture after overnight reaction with SVPD and 1 h reaction with alkaline phosphatase; C: Coinjection of the digestion mixture and synthesized dinucleoside **9**

CONCLUSIONS

Through synthesis of a series of furan modified nucleosides, a toolbox of building blocks has now become available for the fine-tuned cross-linking of nucleic acids. Yields are high (up to 76% of isolated cross-linked species) and covalent bond formation occurs in a site-selective fashion. Further applications in triplex cross-linking and secondary structure immobilization are currently under study.

MOdB is indebted to the Agency for Innovation by Science and Technology in Flanders (IWT) and the Special Research Fund of Ghent University (BOF-GOA2007, BOF-BAS 01B04405). We further thank the FWO Vlaanderen for financial support (FWO-KAN 1.5.137.09N, FWO-KAN 1.5.186-03). Computational resources and services used in this work were provided by Ghent University.

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THE CARBA-LNA OLIGOS AS RNA TARGETED THERAPEUTICS

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Synthesis of carba-LNA- and carba-ENA nucleotides[2] have been achieved by free-radical cyclization reaction strategy developed in the Uppsala lab[1,2]. Carba-LNA modified oligos (both antisense and siRNA) have been proven[3–5] to be very effective candidate for RNA-directed therapeutics in both antisense and siRNA approach, reasons being their unique blood serum stability and thermodynamic stability, the former being 100-fold more, and later being comparable, than those of LNA-counterparts.

Many novel 2′,4′ conformationally-constrained nucleosides, namely carba-LNA (cLNA), carba-ENA (cENA) and α-L-carba-LNA, and their oligos have been synthesized during the past 4 to 5 years from the Uppsala lab[2]. Carba-LNA/ENA modified oligonucleotides have been found to be significantly more nuclease resistant than the LNA and ENA modified counterparts without compromising other therapeutically important features, which make these types of carba-nucleos(t)ide modifications very promising candidates for therapeutic and diagnostic applications. We briefly describe here their synthetic strategy (Fig. 1) as well as the target RNA affinity, nuclease resistance, and RNase H elicitation of oligonucleotides with these carba modifications. Several recent reports on down-regulation of target gene in cultured cell using cLNA modified antisense oligonucleotides or siRNA are also briefly discussed to highlight the promising potential of cLNA and cENA modified oligos as RNA targeted therapeutics.

In 2007, we reported the synthesis of 7′*R*/*S*-Me-carba-LNA-T (**2**) and 8′*S*-Me-carba-ENA-T (**3**) through radical cyclization reaction, which has its origin in the free-radical cyclization reaction introduced by us in the nucleoside chemistry in 1991–1992 [1] to give the 2′,3 or 3′,5′-fused nucleotides. After that, different kinds of carba-LNA, carba-ENA and α-L-carba-LNA nucleosides have been synthesized[2] by the free-radical ring closure strategy (Fig. 1). All of these nucleosides have been incorporated to antisense oligonucleotides and a comparison of their (A) thermal stability: [ΔT_m (ΔT_m = T_m of modified AON:RNA hybrid – T_m of native AON:RNA hybrid)], (B) RNA selectivity: [$\Delta\Delta T_m$ ($\Delta\Delta T_m$ = ΔT_m of AON:RNA hybrid – ΔT_m of AON:DNA duplex)], and (C) nuclease stability have been reported[2]. The most striking feature of Carba-LNA/ENA modified oligonucleotides is all of

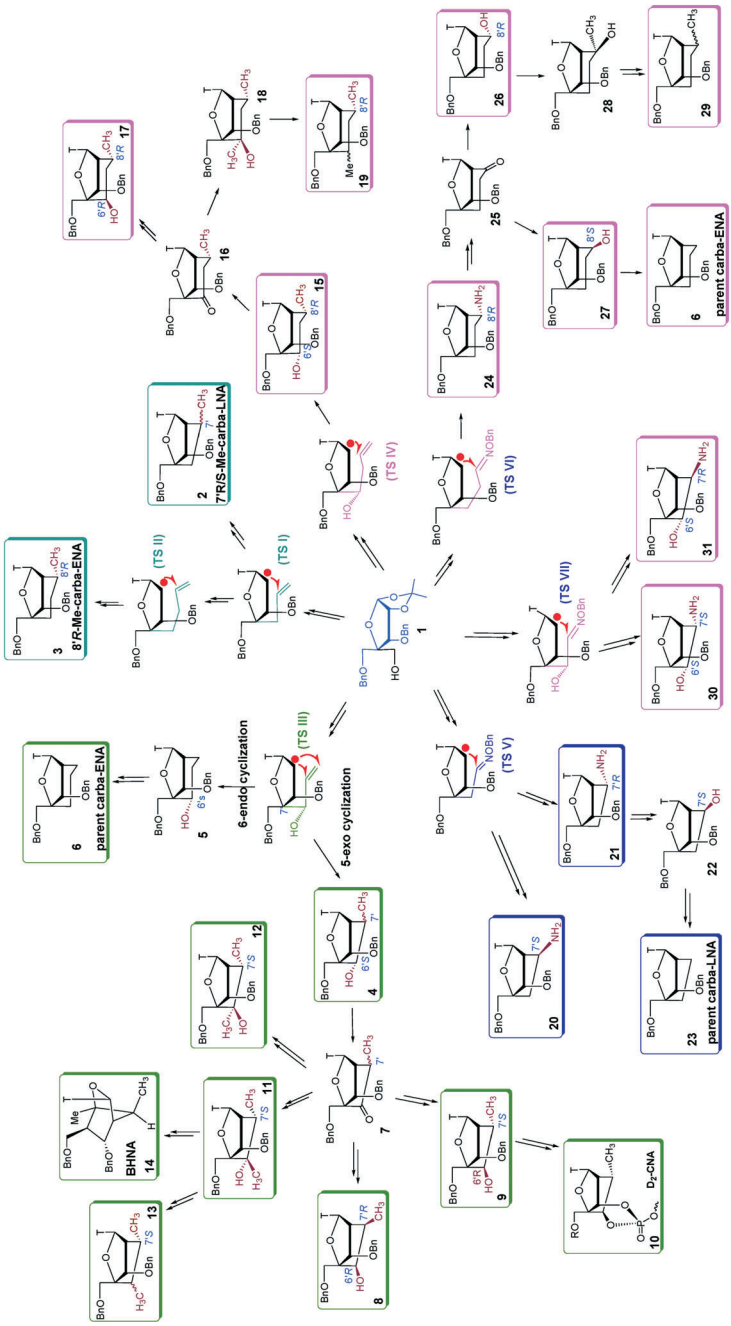

FIG. 1
Synthesis of carba-LNA and carba-ENA nucleosides by radical ring closure reaction

them are much more nuclease resistant than the LNA and ENA modified counterparts. Most of them have been found to be more RNA selective than LNA and ENA modified counterparts. Moreover, just as LNA modified antisense oligonucleotides, all the carba-LNA/ENA modified AONs are very good targets for RNase H and can be digested with high efficiency.

7′-Me-carba-LNA (**2**) and 8′*R*-Me-carba-ENA (**3**) modified siRNAs have been synthesized to target eGFP gene in Hela cells It was found[3] these modifications can be well tolerance in the seed region. siRNAs containing one 7′-Me-carba-LNA (JC-F1) or 8′*R*-Me-carba-ENA (JC-S1) modification at the position 3 (from 5′ end) of guide strand showed the best gene silencing efficiency, which is much higher than that of siRNAs containing UNA, aza-ENA or LNA modification in the same region. They are compatible with the siRNAs containing a HNA modification in the seed region as well as with unmodified siRNA (Fig. 2). Interestingly, some siRNAs such as JC-F1/W131 and JC-S1/DO003 (Fig. 6B) that formed by annealing JC-F1 or JC-S1 with modified passenger strands even showed better silencing potency than the native counterpart. More interestingly, a single 7′-Me-carba-LNA (**2**) or 8′*R*-carba-ENA (**3**) modification at the position 3 (from 5′ end) of guide strand obviously reduce off-target effects. Moreover, 7′-Me-carba-LNA (**2**) or 8′*R*-carba-ENA (**3**) modified siRNAs have been found less toxic than the native siRNA. Hence, incorporating a single carba-LNA or carba-ENA modification at position 3 of the guide strand can produce siRNAs with high knock down activity but reduced off-target effects and cellular toxicity.

The ability of down-regulation of target gene in cultured cell by carba-LNA/ENA modified antisense oligonucleotides or siRNA has been demonstrated and compared with other type of modifications recently[4]. The results show that 7′-Me-carba-LNA[2a] modified antisense oligonucleotides (G C<u>T</u>G C<u>T</u>G C<u>T</u>G C<u>T</u>G C<u>T</u>G C<u>T</u>G C<u>T</u>G G, <u>T</u> denote the modification) have been shown to selectively silence Huntingtin gene expression by targeting the CAG repeat region in the mRNA of mutant HTT gene[5]. The IC_{50} for 7′-Me-carba-LNA modified AON is only 15 nM and allele-selectivity for mutant gene over normal gene is more than 6 fold, which is much better than that for both LNA- modified oligo counterparts (IC_{50} = 40 nm, 2.5 fold of allele selectivity). Taking into account of very low concentration required for silencing and high allele selectivity, the 7′-Me-carba-LNA (**2**) modified AONs targeted to the CAG repeat of HTT are providing proof-of-concept for development of oligonucleotide-based therapeutics that selectively inhibits expression of mutant HTT gene in patients with Huntingtin disease.

We have also recently[5] investigated the relative inhibition efficiency of the HIV TAR RNA by cLNA modified siRNAs compared to those of the LNA counterparts, in that the former were found to exhibit improved silencing efficiency and displayed enhanced stability in human serum with negligible cytoxicity compared to those of the latter. A single cLNA substitution as the 3′-overhang in the modified siRNAs displayed near native-like IC_{50} value [of 4.01 ± 0.87 nM compared to the nearly two-fold higher IC_{50} value of 7.15 ± 1.57 nM for LNA modified counterparts, and of the native siRNA of 1.84 ± 0.16 nM] and significantly higher $t_{1/2}$ value for the stability in serum (11.9 h for cLNA, 6.8 h for LNA and 3.0 h for native), thereby showing that the effi-

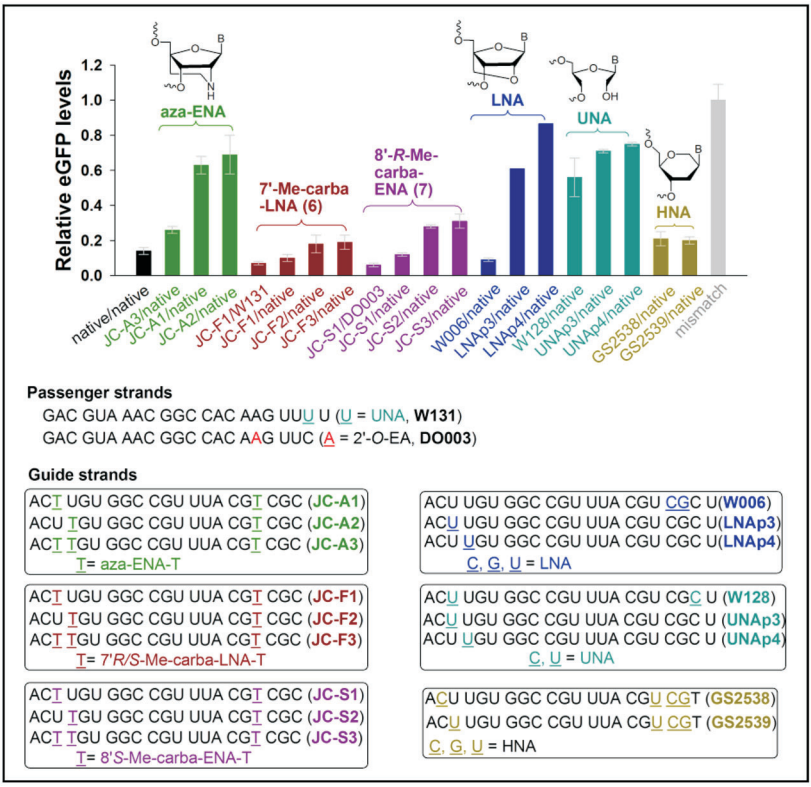

FIG. 2

Comparision of silencing activity of siRNAs in which the guide strands contain different chemical modification types. Hela cells stably expressing eGFP were tranfected with 10 nM siRNAs complexed with *INTERFERin*™. The eGFP levels showed here are the values after 72 h post-transfection (taken from ref.[3])

ciency of jcLNA-modified-siRNAs directly correlates well with the stability without compromising the native-like efficiency and target RNA recognition and subsequent down-regulation. Amongst all the modified siRNAs so far used to down-regulate the target RNA of the HIV-1 TAR region, the best IC_{50} value was obtained for the doubly-modified siRNA in which cLNA substitution was introduced both at position 1 and 20 (T^1 + T^{20}, *i.e.* cLNA11) which showed IC_{50} value of 0.54 ± 0.14 nM). The IC_{50} of this doubly-modified siRNA was more than three-fold lower than that of the native and two-fold lower than that of LNA modified counterpart, *i.e.* LNA12: IC_{50}: 1.13 ± 0.27 nM.

Hence strategy to chemically modify the native siRNAs by substitution with the cLNA can be considered as a significant development, leading to both the enhanced siRNA efficiency and serum stability over that of the native[5].

All coworkers (www.boc.uu.se) are gratefully acknowledged, whose names appear in the reference list, for their excellent contribution and Swedish Research Council (VR), Uppsala University and EU-FP7 for generous financial supports

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ENHANCEMENT OF EXON SKIPPING AND DYSTROPHIN PRODUCTION BY 3′-PEPTIDE CONJUGATES OF MORPHOLINO (PMO) OLIGONUCLEOTIDES IN A *MDX* MOUSE MODEL OF DUCHENNE MUSCULAR DYSTROPHY

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Conjugation of the cell penetrating peptide Pip5e to the 3′-end of a 25-mer PMO oligonucleotide targeting exon 23 of dystrophin pre-mRNA in a *mdx* mouse model of Duchenne muscular dystrophy (DMD) was achieved using peptide-like coupling conditions. 3′-conjugated Pip5e-PMO was equally active in *mdx* mouse cells in exon skipping compared to a 5′-conjugated Pip5e-PMO, and also showed equally good exon skipping and dystrophin production when injected intravenously into *mdx* mice. Pip5e-PMO is an important lead in the development of potential treatments for DMD.

INTRODUCTION

Steric blocking oligonucleotides (ONs) with uncharged backbones, such as peptide nucleic acids (PNA)[1] and phosphorodiamidate morpholino oligonucleotides (PMO)[2], have advantages for RNA targeting applications because of their lack of cell and *in vivo* toxicity and high metabolic stability. In cell culture, attachment of a cell-penetrating peptide (CPP), such as Penetratin, Tat (48-60), Transportan or (R-Ahx-R)$_4$Ahx-βAla (Ahx = aminohexyl), to PNA or PMO enhances cell delivery substantially in various model systems[3–6].

We used a splicing correction assay involving up-regulation of luciferase in HeLa pLuc705 cells[7] to show that whereas Tat or (Lys)$_8$ conjugates of an 18-mer PNA705 required co-incubation with 100 μM of endosomolytic agent chloroquine in order to see significant activity[8], EC_{50} values in the μM range could be obtained in the absence of chloroquine for (R-Ahx-R)$_4$-PNA and (R-Ahx-R)$_4$-PMO constructs[4,9]. We later reported that a new CPP R$_6$-Penetratin (R6Pen)[10] when disulfide-conjugated to PNA705 was able to correct aberrant splicing at sub-μM levels in the HeLa pLuc705 cell model[11].

Duchenne muscular dystrophy (DMD) is a degenerative muscle disorder of young boys resulting from mutations in the dystrophin gene that lead to non-functional dystrophin protein. Antisense ONs targeted close to the mutation site restore the open reading frame of dystrophin pre-mRNA transcripts by exon skipping and give rise to functional dystrophin, the principle involved in current human clinical trials with two ON types, 2′-*O*-methylphosphorothioates and PMO[12,13]. Enhanced systemic delivery of PMO ONs in a *mdx* mouse model of DMD (having a point mutation in exon 23 of the dystrophin gene) has been achieved for PMO conjugated to the Arg-rich CPP (R-Ahx-R)$_4$Ahx-βAla or its derivative (R-Ahx-R-R-βAla-R)$_2$-Ahx-βAla, known as B peptide[14,15].

We generated more serum-stable derivatives of R6Pen, known as Pip (PNA/PMO Internalising Peptides). Pip2a and Pip2b conjugates of 18-mer PNA705 showed sub micromolar EC_{50} values in the HeLa pLuc705 cell assay[16]. Pip2a and 2b conjugates to a 20-mer PNA targeting exon 23 showed stronger exon skipping in the mouse *mdx* cell model than a B-PNA control, and substantially enhanced dystrophin production when injected into the *tibialis anterior* muscle of *mdx* mice[16]. The shortened and simplified Pip5e peptide (R-Ahx-RR-βAla-RR-Ahx-RILFQYR-Ahx-R-βAla-R-Ahx-R-βAla) conjugated at the 5′-end of a 25-mer PMO targeting exon 23 showed uniformly high dystrophin production in all muscle types after intravenous delivery into *mdx* mice with a single 25 mg/kg dose and particularly in heart, where B-PMO had shown much less activity (Yin et al., manuscript submitted). We now show that high *in vivo* dystrophin production is also achieved when Pip5e is directly conjugated to the PMO at the 3′-end.

RESULTS AND DISCUSSION

5′-Conjugation of Pipe5e to PMO was achieved previously using a 5′-amino functionalized PMO and conjugation to Pip5e containing an additional C-terminal cysteine residue *via* a bifunctional GMBS cross-linking agent (Fig. 1A) (Yin et al., manuscript submitted). A 3′-conjugate of B-PMO was reported recently but without accompanying synthesis methods[14]. We carried out direct conjugation of the morpholino residue at the 3′-end of 25-mer PMO *via* an amide linkage to the C-terminal carboxylic acid of Pip5e or B peptides using TBTU/HOAt/DIEA in *N*-methylpyrrolidinone (NMP)/DMSO (Fig. 1B) to yield peptide-PMO conjugates.

We then compared the exon skipping activity of Pip5e-PMO conjugated either at the 5′- or 3′-ends by incubation with differentiated *mdx* mouse myotubes in the absence of any transfection agent. The RT-PCR results

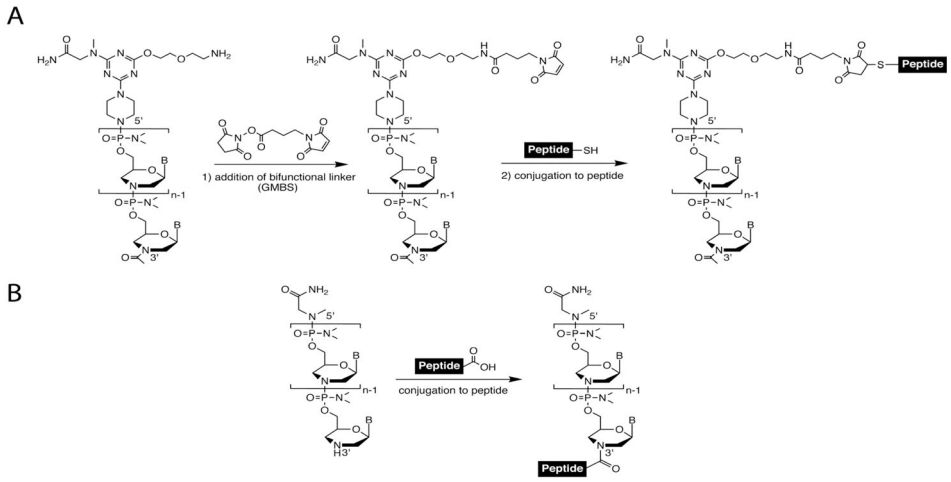

FIG. 1

Peptide-PMO conjugate synthesis schemes. **A**) 5′-thioether linkage: a 2-fold excess of GMBS linker was 5′-coupled in sodium phosphate buffer (pH 7.2), 20% acetonitrile to PMO (100 nmoles) for 1 h at room temperature. After acetone precipitation, C-terminal thiol peptide (2-fold excess) was coupled in sodium phosphate buffer (pH 6.5), 20% acetonitrile for 2 h. The conjugate was purified by RP HPLC using an acetonitrile/0.1% heptafluorobutyric acid (HFBA) system. **B**) 3′-amide linkage: PMO (100 nmole) in DMSO was coupled with 2-fold excess of peptide using TBTU:HOAt:DIEA (2.5:1.8:1.7 molar excess over peptide) in NMP at 37 °C for 2 h. The conjugate was purified by cation exchange HPLC (Source 15S, GE Healthcare) and desalted on a HLB column (Waters)

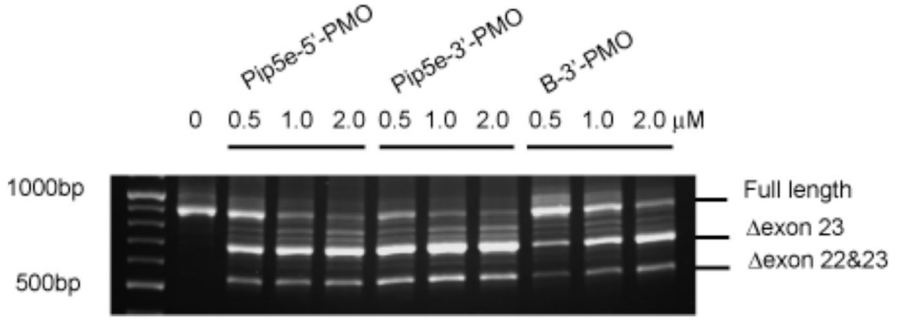

FIG. 2

Representative agarose gel of nested RT-PCR assay using 1 μg of total RNA as a template, following incubation of peptide-PMO conjugates with differentiated H2K *mdx* myotubes as described previously[16]

showed that the exon-skipping capacity of the dystrophin transcript (Δexon23) was very similar for both Pip5e-5′-PMO and Pip5e-3′-PMO and in both cases more efficient than control B-PMO (Fig. 2).

Similarly, a single intravenous injection of 3′-conjugated Pip5e-PMO into *mdx* mice showed similarly high dystrophin production in a number of muscle types (Fig. 3). Exon skipping in heart muscle was much higher for Pip5e-3′-PMO (25%) than for B-PMO (3%) as judged by qRT-PCR (data not shown). The results show that Pip5e-3′-PMO is an important lead for development of a peptide-PMO conjugate for potential treatment of DMD.

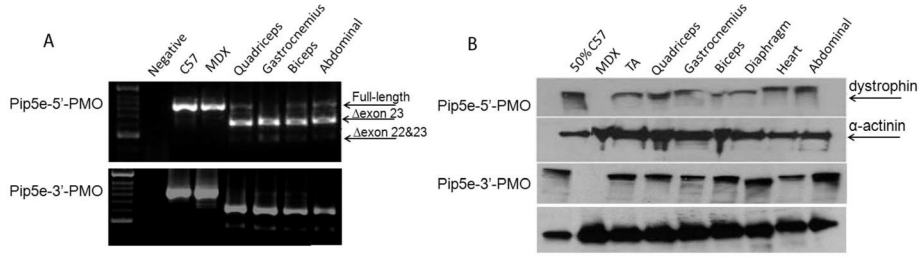

FIG. 3

Comparison of 3′- and 5′-conjugated Pip5e-PMO for exon skipping and dystrophin production in adult *mdx* mice treated with a single intravenous injection of 25 mg/kg. Body wide tissues were harvested 2 weeks later as described[17]. **A**) RT-PCR to detect exon-skipped products in 5′ and 3′-conjugated Pip5e-PMO treated *mdx* muscle groups; unskipped or deleted for exon 23 or exon 22 and 23 are indicated. **B**) Western blot to detect dystrophin protein in indicated muscles from treated *mdx* mice compared with *C57BL6* and untreated *mdx* controls. 50% total protein loaded for the *C57BL6* control. α-Actinin expression was used as a loading control

We thank David Owen and Donna Williams for provision of synthetic peptides and PNA. This work was supported by research grants from Action Duchenne to M.J.A.W. and M.J.G. (Unit programme U105178803).

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SYNTHESIS OF NUCLEOSIDES AND NUCLEOSIDE TRIPHOSPHATES BEARING ANTHRAQUINONE SUBSTITUENTS AS REDOX PROBES AND THEIR ENZYMATIC INCORPORATION TO DNA

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Modified 2′-deoxynucleosides and nucleoside triphosphates (dNTPs) bearing anthraquinone (AQ) via acetylene or propargylcarbamoyl linkers were prepared by single-step Sonogashira cross-coupling reactions halogenated nucleosides (7-iodo-7-deaza-2′-deoxyadenosine and 5-iodo-2′-deoxycytidine) or dNTPs with *N*-(2-propynyl)-anthraquinone carboxamide and 2-ethynylantraquinone. Polymerase incorporation of the AQ-labelled dNTPs into DNA has also been studied. Square-wave voltammetry of the AQ-labelled nucleosides and nucleotides showed one reversible peak at –0.5 V.

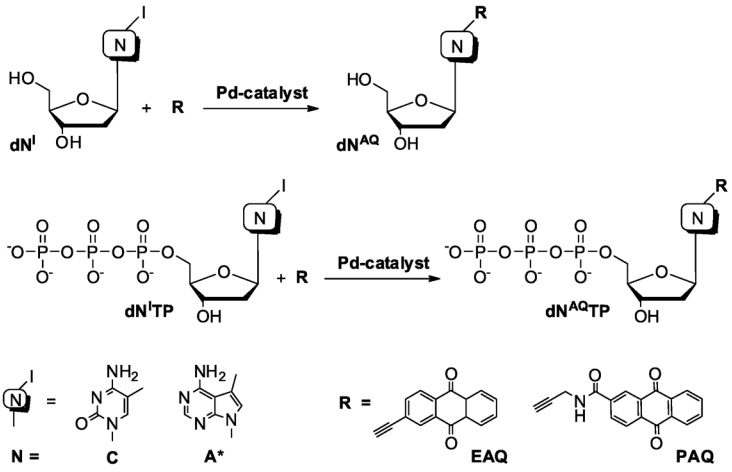
INTRODUCTION

Electrochemical detection[1] is a less expensive but comparatively sensitive alternative to common optical methods. Particularly efficient is the single-step synthesis of modified dNTPs by cross-coupling reactions followed by polymerase incorporation. We have recently used this novel approach for the synthesis of DNA bearing amino acids[2], ferrocenes[3], amino- and nitrophenyl groups[4] and [Ru/Os-(bpy)$_3$][5] complexes. In combination with previously reported dNTPs bearing ferrocene, aminophenyl, and nitrophenyl tags, the Os-labelled dATP has been successfully used for "multicolor" redox labelling of DNA and for DNA minisequencing[5]. Anthraquinones are attractive redox labels suitable for labelling of biomolecules[6]. Our goal was to prepare modified dNTPs bearing anthraquinones and their polymerase incorporation to DNA.

RESULTS AND DISCUSSION

Modified 2′-deoxynucleosides and dNTPs bearing anthraquinone attached through an acetylene or propargylcarbamoyl linker to the 5-position of pyrimidine (C) or to the 7-position of 7-deazaadenine were prepared by Sonogashira cross-coupling (Pd-catalyzed, Cu-mediated cross-coupling) of halogenated dNTPs with 2-ethynylanthraquinone (EAQ)[7] or N-(2-propynyl)-anthraquinone carboxamide (PAQ)[8] (Scheme 1). Polymerase incorporations

of the AQ-labelled dNTPs into DNA by primer extension with KOD polymerase have been successfully developed. Electrochemical properties of the AQ-labelled nucleosides, nucleotides and DNA were studied using cyclic and square-wave voltammetry to show a distinct reversible couple of peaks around –0.5 V.



SCHEME 1

This work was supported by the Academy of Sciences of the Czech Republic (Z4 055 0506), the Ministry of Education (LC512,1M0508), Grant Agency of the Academy of Sciences of the Czech Republic (IAA400040901), Czech Science Foundation (203/09/0317) and by Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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MODULAR SYNTHESIS OF 5-SUBSTITUTED THIOPHENE AND FURAN *C*-NUCLEOSIDES AND THEIR ANALOGUES

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A new modular and efficient methodology for the preparation of 5-substituted thiophen-2-yl and 5-substituted furan-2-yl *C*-nucleosides was developed. A Friedel–Crafts-type of *C*-glycosidation of 2-bromothiophene or 2-bromofuran with bis-toluoyl protected methyl-2′-deoxyribofuranoside in presence of Lewis acid gave the desired bis-toluoyl protected 5-bromothiophne and 5-bromofuran *C*-nucleosides in good yields. They were used as key intermediates for Stille or Suzuki coupling whith (hetero)arylstannanes or boronic acids to afford a series of 5-(hetero)aryl thiophene and 5-(heteroaryl)furan *C*-nucleosides.

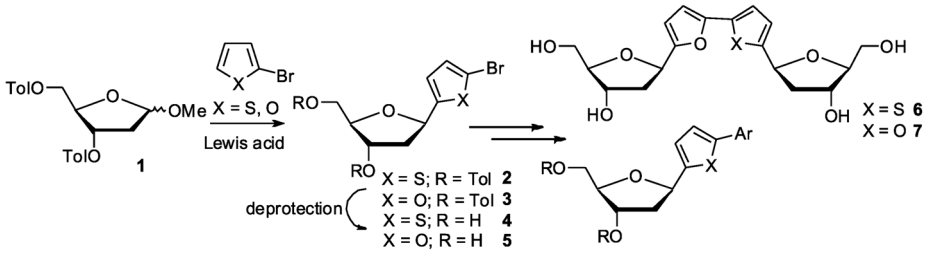
INTRODUCTION

C-Nucleosides bearing hydrophobic aryl groups as nucleobase surrogates attracted great attention due to their use in the extension of the genetic alphabet[1]. In oligonucleotide duplexes, they selectively pair with the same of other hydrophobic nucleobase due to increased stacking and favourable desolvation energy as compared to canonical hydrophilic nucleobases. Triphosphates of some of the *C*-nucleosides are efficiently incorporated to DNA by DNA polymerase[2].

RESULTS AND DISCUSSION

Our modular methodology for the synthesis of 5-thiophen-2-yl and 5-furan-2-yl *C*-nucleosides is based on a Friedel–Crafts type *C*-glycosidation[3]. We have screened a wide range of Lewis acids and selected most efficient conditions. Reaction of 2-bromothiophene or 2-bromofuran with easily available methyl-2′-deoxyribofuranoside **1** in presence of $SnCl_4$ or $BF_3{\cdot}Et_2O$ in DCM gave the desired toluoyl-protected β-*C*-nucleosides **2** in 60% (key thiophene *C*-nucleosides) and **3** 45% (key furane *C*-nucleosides). Deprotection of toluoyl-protected *C*-nucleosides **2** and **3** gave free 5-bromo-(hetero)aryl *C*-nucleosides **4** and **5** almost quantitatively. Protected 5-bromo *C*-nucleosides **2** and **3** were used directly for Stille cross-coupling with (hetaro)arylstannanes in organic media, whereas the unprotected *C*-nucleosides **4** and **5** were efficiently used for the Suzuki cross-couplings reactions with (hetero)arylboronic acids in aqueous system to afford a series of

5-(hetero)arylthiophene and 5-(hetero)arylfurane *C*-nucleosides. The resulting 5-(heteroaryl) *C*-nucleosides possess interesting luminescent properties with emission maxima varying from 320 to 410 nm. In order, to prepare novel biaryl covalent analogues of nucleoside base pairs **6** and **7**, we have prepared corresponding boronates either by borylation of 5-bromothiophene **2**, 5-bromofuran **3** or by Ir-catalyzed C–H borylation of non-brominated *C*-nucleosides.



SCHEME 1
Synthesis and cross-coupling on 5-bromo key intermediates **2** and **3**

This work is a part of the research project from the Academy of Sciences of the Czech Republic Z4 055 0506. It was supported by the ministry of Education, Youth and Sports of the Czech Republic (LC512), by Grant Agency of the Academy of Sciences of the Czech Republic (IAA400550902) and by Gilead Sciences, Inc.

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3-FLUORO-2-(PHOSPHONOMETHOXY)PROPYL HYPOXANTHINE AND GUANINE DERIVATIVES AS INHIBITORS OF PLASMODIAL HYPOXANTHINE-GUANINE-XANTHINE PHOSPHORIBOSYLTRANSFERASES

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A new methodology for the synthesis of ANPs containing 9-[2-(phosphonoethoxy)ethyl] (PEE) moiety has been developed. Several 9-[3-fluoro-2-(phosphonoethoxy)propyl] (FPEP) derivatives with hypoxanthine or guanine moiety attached were prepared using this method and evaluated as inhibitors of the *Plasmodial* hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT). The FPEP compound containing the guanine moiety exhibited inhibition activity against the enzyme in micromolar range without any toxic effect on human cells.

INTRODUCTION

Acyclic nucleoside phosphonates (ANPs)[1,2] containing a 3-fluoro-2-(phosphonomethoxy)propyl (FPMP)[3] moiety are known for their antiretroviral activities[4]. Derivatives of these compounds with a 6-oxopurine base attached to the phosphonate moiety have recently been identified as inhibitors of *Plasmodium falciparum* and *Plasmodium vivax* hypoxanthine-guanine-(xanthine) phosphoribosyltransferase[5] (HG(X)PRT). They show selectivity for the *Plasmodium* enzymes compared with the corresponding human enzyme (*h*HGPRT). The potency and selectivity of the inhibition depends on the length and chemical substitutions in the phosphonate linker[5,6]. We deduce that the fluorine atom in acyclic chain is responsible for the increased selectivity for *Pf*HGXPRT. Our aim in the present study was to synthesise elongated 3-fluoro-2-(phosphonoethoxy)propyl (FPEP) derivatives with hypoxanthine or guanine as the base, and then to determine their inhibition constants and biological activity.

RESULTS AND DISCUSSION

The synthesis of both FPEPs (Fig. 1), with elongation of the acyclic chain by vinyl phosphonate as the key step, started from commercially available

O-tritylglycidols and 6-chloropurines. 9-[3-Fluoro-2-(phosphonomethoxy)-propyl]guanine[3,5] (FPMPG) has affinity to *Pf*HGXPRT and human HGPRT in the micromolar range (K_i = 3.6 μM and 22.7 μM, respectively). A comparison of this parent compound with our FPEPs shows that attachment of elongated phosphonate linker leads to better binding affinity to *Pf*HGXPRT (K_i = 0.2 μM) when guanine is the nucleobase (FPEPG). The hypoxanthine derivative (FPEPHx) was inactive. On the other hand, the improved binding constant also correlates with better affinity of FPEPG for human HGXPRT (K_i = 1.1 μM). From this study, we conclude that the higher affinity of the FPEPG to *Pf*HGXPRT compared with parent FPMPG is accompanied by a decrease of selectivity between *Plasmodial* and human enzyme. Compounds were tested for antiviral and immunomodulatory properties and they did not show any significant activity.

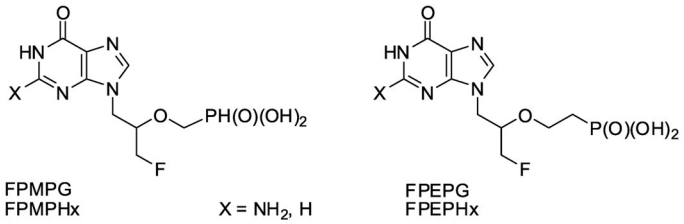

FIG. 1
FPMP-ANPs and FPEP derivatives with extended phosphonate linker

This work is a part of the research project AVOZ40550506 of the Institute of Organic Chemistry and Biochemistry and was supported by the Grant Agency of the Czech Republic (grant No. P207/11/0108) and Centre for New Antivirals and Antineoplastics (1M0508). This study was also supported by funds from the National Health and Medical Research Council, Australia (grant No. 569703) and by Gilead Sciences (Foster City, CA, U.S.A.).

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SYNTHESIS AND REACTIONS OF 2,6-BIS-(4-SUBSTITUTED-1,2,3-TRIAZOL-1-YL)-9-(β-D-ARABINOFURANOSYL)PURINES

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Facile synthesis of protected 2,6-diazidopurine arabinonucleoside starting from tetra-*O*-acetyl-D-arabinose and 2,6-dichloropurine via 1-bromo-tri-*O*-acetylarabinose has been developed. A series of 2,6-bis-(1,2,3-triazolyl)-substituted purine arabinonucleosides has been synthesized in Cu(I) catalysed Huisgen 1,3-dipolar azide-alkyne cycloaddition reactions in moderate to good yields. The reaction intermediate 2-triazolyl-6-azidopurine nucleoside and side product 2-triazolyl-6-aminopurine nucleoside were isolated. Reactions of bis-triazolyl purine nucleosides with *N*-nucleophiles proceed smoothly resulting in 6-*N*-substituted-2-triazolyl-arabinoadenosine derivatives. For example, in reaction with dimethylamine in water, 2-(4-hydroxypropyl-1,2,3-triazol-1-yl)-6-*N*,*N*-dimethylaminopurine arabinonucleoside was obtained in good yield.

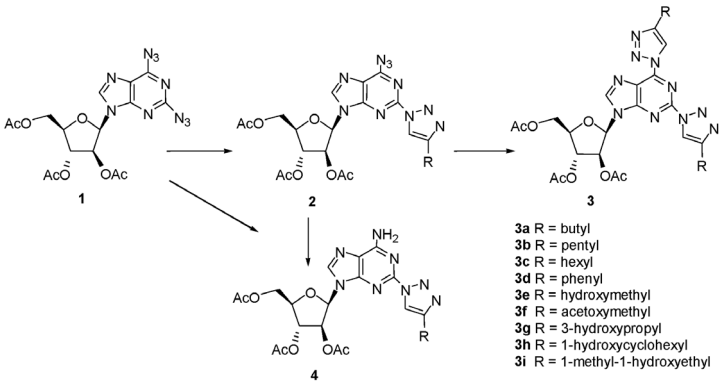
INTRODUCTION

Azide-alkyne 1,3-dipolar cycloaddition reaction (*click* reaction) have found a variety of applications in nucleoside, nucleotide and oligonucleotide chemistry[1]. A number of 1,2,3-triazolyl-functionalized nucleosides were synthesized and investigated in recent years. Substances with antiviral and anticancer activity, enzyme inhibitors, adenosine receptors agonists and antagonists were discovered. Recently, the synthesis and biological activity of ribo- and deoxyribonucleoside analogs containing 2- or 6-(1,2,3-triazolyl)purines have been reported[2–4]. Azide-tetrazole equilibrium of C-2 and C-6 azidopurine nucleosides was also discussed in these papers. The aim of this study was to develop method for synthesis of novel 2,6-bis-(1,2,3-triazolyl)-substituted purine arabinonucleosides and to investigate their reactions with *N*-nucleophiles.

RESULTS AND DISCUSSION

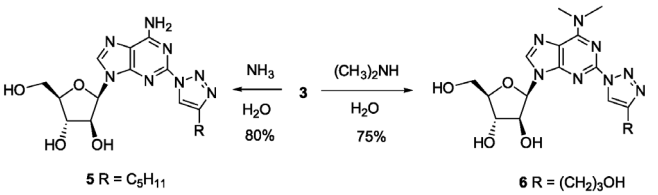
The key intermediate, 9-(tri-*O*-acetyl-β-D-arabinofuranosyl)-2,6-diazidopurine (**1**) was synthesized in 3 steps. Reaction of tetra-*O*-acetyl-D-arabinose with HBr/AcOH afforded α-1-bromo-tri-*O*-acetylarabinose. Coupling with 2,6-dichloropurine gave 9-(tri-*O*-acetyl-β-D-arabinofuranosyl)-2,6-dichloropurine, which was reacted with sodium azide in boiling 80% ethanol. After

column chromatography we obtained diazide **1** with 28% yield in 3 step sequence from tetra-*O*-acetylarabinose.



SCHEME 1
Reaction of diazides **1** with alkynes. Reagents and conditions: $CuSO_4 \cdot 5H_2O$, sodium accorbate, *t*BuOH/H_2O, 30–40 °C, 12–72 h

Diazide **1** was reacted with various terminal alkynes in copper catalysed 1,3-dipolar cycloaddition reaction (*click* reaction) using $CuSO_4 \cdot 5H_2O$ and sodium ascorbate as a catalyst generating system and tert-butanol-water as a solvent. Typically, 4-fold excess of alkyne, 20 mole % of $CuSO_4 \cdot 5H_2O$ and 10 mole % of sodium ascorbate were used. Reaction time at 30–40 °C varied from 12–72 h, depending on nature of alkyne. After work-up bis-triazolyl-nucleosides **3** were isolated by silica gel column chromatography. The isolated yields of **3a–3i** ranged from 40–65%. We observed formation of intermediate monoazide **2** in the first hours of reaction. Some of azides **2** and amino derivatives **4** (R = C_5H_{11}; C_6H_{13}; $(CH_2)_3OH$) were isolated and their structure determined. We found no evidence for presence of tetrazole tautomeric form of azides **1** and **2**.



SCHEME 2
Reaction of bis-(1,2,3-triazolyl)nucleoside with ammonia and dimethylamine

Observation that 6-(1,2,3-triazolyl) moiety easy undergoes nucleophylic displacement promted us to investigate reactions of **3** with *N*-nucleophiles. As examples are reactions of **3b** with NH_3 and **3g** with dimethylamine in water solution.

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ENZYMATIC SYNTHESIS OF ESTER PRODRUGS OF DHPA AND RELATED COMPOUNDS BY LIPASES

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Acylation of hydroxyl groups is an alternative method to synthesize prodrugs. These newly formed molecules are more lipophilic and more soluble in biological membranes. In this work, we present a fast and easy method to synthesize esters by lipases using vinyl esters. The desired products were formed in reasonable yields and could be easy and fast isolated in high purity.

INTRODUCTION

Lipophilisation of drug molecules can lead to a higher transport through biological membranes. The parent drug is than released in the cell by an enzymatic process, in most cases by enzymatic hydrolytic reactions. Also acylation of appropriate functional groups is a good way to enhance a lipophility of molecules. The enzymatic approach for acylation of hydroxyl groups exhibits additional advantages – low costs, regiospecificity, no need of protecting groups and mild conditions.

Penetration through the membranes is extraordinary important for biologically active of compounds applied to the skin in a form of creams, e.g. the antiherpetic drug DHPA (Dihydroxypropyladenine, Duviragel). DHPA is transported to the cell by diffusion. Due to a high hydrophility of DHPA, diffusion into the cell is very slow. The enhancing of lipophility can thus enhance the bioavailability in the cell and so bodyresponse to the therapy.

An enzymatic method was used for esterification of DHPA and related compounds with vinyl esters and catalyzed by different lipases in non-aqueous media (DMF). Esters of DHPA and other compounds could be synthesized and purified in satisfactory yields.

METHODS

A set of lipases (lipase from *Geotrichum candidum* 4013, pig pancreas, *Mucor miehei*, *Candida rugosa*, *Candida antarctica*, *Aspergillus niger* and *Pseudomonas cepacia*) was tested for the ability to acylate DHPA and related compounds

(non-acyclic and acyclic nucleoside analogs). Palmitate, butyrate and acetate were chosen as acyl donors.

A stirred mixture of 0.111 M acyl-acceptor (DHPA or other nucleoside analog) and 0.555 M vinyl ester in DMF was incubated at 30–35 °C for 15 min. The enzyme was added to an overall activity of 29 U/ml (activity according to producer data). After three days a new enzyme (29 U/ml) was added and the reaction proceeded for further three days. The progress of the reaction was monitored by TLC (mobile phase $CHCl_3$:CH_3OH = 80:20).

The solid was filtered off and washed with DMF. The solvents were evaporated and the residue disolved in a mixture of chloroform and methanol. The mixture was adsorbed on a silica gel by evaporation. The product was purified by a column chromatography on a silica gel (mobile phase $CHCl_3$:CH_3OH = 80:20). Alternatively, the reaction mixture was separated by a preparative HPLC on C-18-column, or by a preparative TLC (mobile phase $CHCl_3$:CH_3OH = 80:20).

RESULTS AND DISCUSSION

We have found that all so far tested lipases are able to catalyze the desired reaction. The reaction yields depended on a chosen lipase, vinyl ester and on a nucleoside analog structure. The achieved conversion was observed between 15 and 55% by TLC. Isolated yields depended on a chosen method.

The reaction rate is determined by a substrate specificity of the enzyme to different acyl lengths. For example, lipase from candida antharctica can catalyze the transesterification reaction with butyrate much faster than with palmitate. The vinyl moiety of the ester is a good leaving group and vinyl esters are thus good acyl donors for this reaction, because the reaction rate is high and the cleavage of the ester bond is non-reversible (additionally, the resulting vinylalcohol rearranges to acetaldehyde). The newly formed enzyme-acyl complex is then attacked by hydroxyl group of DHPA. For the nucleophilic attack the primary hydroxyl group can be used; that means that the reaction is regiospecific. An absence of water is important to avoid hydrolysis of the substrate and the product.

The steric hindrance of the reacting hydroxyl group has some effect on the reaction rate and yield. Dihydroxypropyl analogs of nucleosides are good substrates for this method; 2-methyldihydroxypropyl derivatives give lower yields and cannot react so fast. Ribosides or 2′-deoxyribosides do not undergo such reactions et all.

This work is a part of the research project of the IOCB AV0Z40550506 and Z40550506. It was supported by the Centre of New Antivirals and Antineoplastics 1M0508 by the Ministry of Education, Youth and Sports of the Czech Republic and by Gilead Sciences, Inc. (Foster City, U.S.A.).

THE EFFICIENT SYNTHESIS OF 2-ARYL SUBSTITUTED PYRIMIDINE ACYCLIC NUCLEOSIDE PHOSPHONATES USING LIEBESKIND–SROGL CROSS-COUPLING

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A series of novel acyclic nucleoside phosphonates with a built-in 2-arylsubstituted pyrimidine moiety has been prepared using the Liebeskind–Srogl cross-coupling protocol. The reactions of highly functionalised 2-methylsulfanylpyrimidines with various arylboronic acids were studied and optimised.

INTRODUCTION

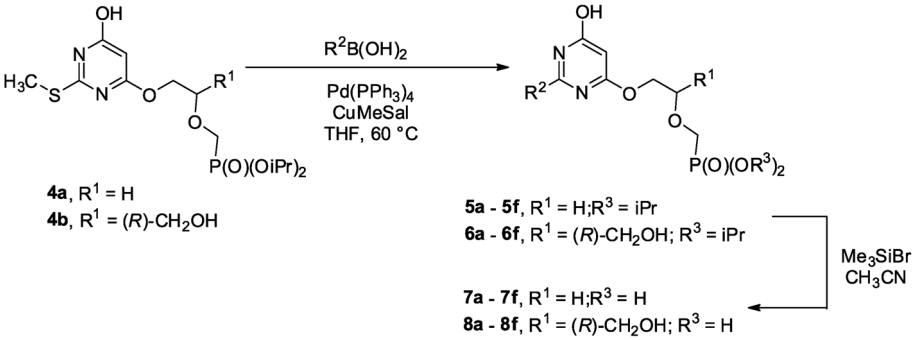
Acyclic nucleoside phosphonates (ANPs) are nucleotide analogues containing a stable phosphonomethyl moiety. ANPs possess a broad spectrum of biological activities, e.g. antiviral, cytostatic, and antiprotozoal. ANPs are currently used as successful drugs against HIV, HBV, and herpes viral infections. The choice of the heterocyclic base of antivirally active ANPs is limited, with the exception of a cytosine derivative, to the derivatives of purine[1]. A second generation of ANPs is derived from 2,4-diamino-6-hydroxy- and 2-amino-4,6-dihydroxypyrimidine, with the phosphonomethoxyalkyl chain attached to the oxygen atom at position C-6 of the pyrimidine ring[2]. These compounds mimic purine acyclic nucleoside phosphonates and are recognised by HIV-1 reverse transcriptase as purine nucleotides[3].

Cross-coupling reactions of heteroaromatic compounds with various organometallic reagents are widely used in drug design for the synthesis of modified purine[4] and pyrimidine[5] bases. The Liebeskind–Srogl[6] cross-coupling of thioorganics with arylboronic acids extends the versatility of the Suzuki–Miyaura and Stille cross-coupling reactions. The Liebeskind–Srogl cross-coupling methodology used in our synthetic approach comprises Pd-catalysed and copper(I)-mediated reactions of thioethers with arylboronic acids under the base-free conditions.

RESULTS AND DISCUSSION

2-Methylsulfanylpyrimidine derivatives bearing the 2-(phosphonomethoxy)ethoxy (PMEO) and (*R*)-3-hydroxy-2-(phosphonomethoxy)-

propoxy (HPMPO) side chain were readily prepared by an alkylation of commercially available 4,6-dihydroxy-2-methylsulfanylpyrimidine with the phosphonate-bearing building blocks according to the previously described procedure[7]. Phosphonates **4** and phenylboronic acid were chosen as model compounds for the optimisation study of the Liebeskind–Srogl cross-coupling reaction (Scheme 1). The influence of various Pd catalysts ($Pd(PPh_3)_4$, $Pd(PPh_3)_2Cl_2$, and $Pd_2(dba)_3/Ph_3P$) and various reaction conditions was studied. All compounds were evaluated on the antiviral activity.



SCHEME 1

This work is a part of the research project AVOZ40550506 of the Institute of Organic Chemistry and Biochemistry and was supported by Centre for New Antivirals and Antineoplastics (1M0508). This study was also supported by Gilead Sciences (Foster City, CA, U.S.A.).

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THE OPTIMIZED MICROWAVE-ASSISTED DECOMPOSITION OF FORMAMIDES AND ITS SYNTHETIC UTILITY IN THE AMINATION OF PURINES AND PYRIMIDINES

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The microwave-assisted decomposition of DMF was thoroughly studied and the reaction conditions (temperature, solvent effect, and effect of additives such as acids, bases, and salts) were optimized for its use in the amination reactions. The applicability of this expedient methodology in purine and pyrimidine chemistry and with various formamides is demonstrated. The procedure proved to be the method of choice for the introduction of substituted amino functions into organic molecules and for the facile and convenient modification of compounds of biological interest.

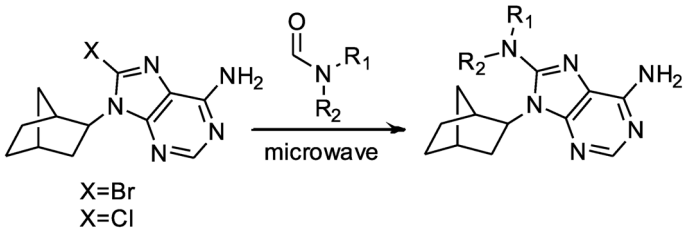
INTRODUCTION

N,*N*-Dimethylformamide (DMF) is one of the most common protic polar solvents in organic synthetic chemistry and is the solvent of choice for a wide range of organic reactions. Nonetheless, DMF is much more than a solvent since it is quite a reactive molecule. DMF can react either as a nucleophilic or electrophilic agent and also has interesting coordinating properties. Depending on the reaction conditions, DMF can be the source of various key intermediates in organic synthesis, such as carbon monoxide, dimethylamine, formyl, and formate[1].

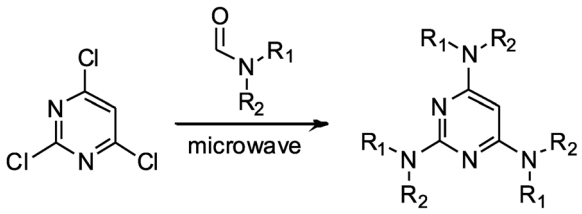
RESULTS AND DISCUSSION

We have recently published the study on microwave-assisted decomposition of formamides and its synthetic utility in the amination of the purine moiety in the positions C-2 and C-6 [2]. In the present work, the general formation of the alkylamino and dialkylamino compounds using formamides as a source of amines is described in detail, with special emphasis on the applicability of the procedure in both purine and pyrimidine chemistry. This novel synthetic methodology was used for the introduction of substituted amino groups into the position C-8 of the purine ring (Scheme 1), for the preparation of 2,4,6-tris(dialkylamino or monoalkylamino)pyrimidines

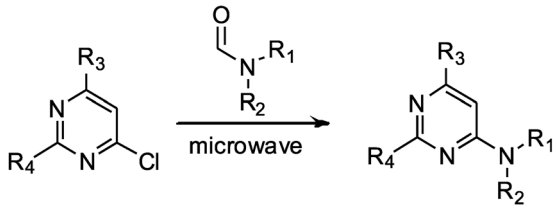
starting from 2,4,6-trichloropyrimidine (Scheme 2), as well as for the amination of various monochlorinated pyrimidines (Scheme 3).



SCHEME 1
Microwave-assisted synthesis of 8-substituted purine derivatives



SCHEME 2
Microwave-assisted amination of 2,4,6-trichloropyrimidine



SCHEME 3
Microwave-assisted amination of monochlorinated pyrimidine derivatives

This work is a part of the research project AVOZ40550506 of the Institute of Organic Chemistry and Biochemistry and was supported by Centre for New Antivirals and Antineoplastics (1M0508). This study was also supported by Gilead Sciences (Foster City, CA, USA).

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DICHOTOMY IN REGIOSELECTIVITY OF Pd-CATALYZED DIRECT C–H ARYLATION OF PROTECTED URACILS

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Regioselective Pd-catalyzed and/or Cu-mediated direct C-H arylations of 1,3-dibenzyluracil with diverse electron-rich aryl halides were studied. The follow-up deprotection gave a series of 5- and 6-arylated uracils.

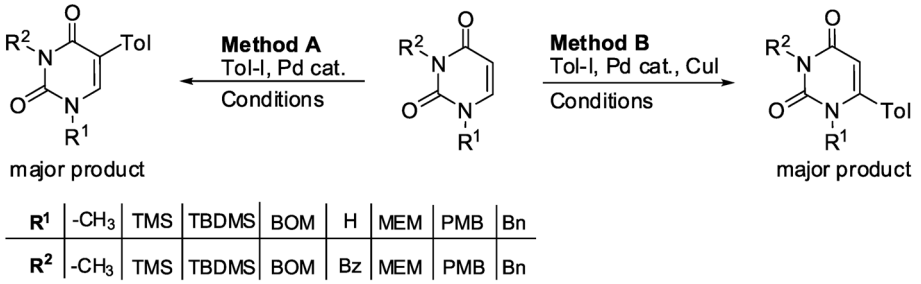
INTRODUCTION

Uracil bases and nucleosides bearing aryl groups in positions 5 or 6 are an important class of compounds and display wide range of biological activities[1]. In addition, arylation in position 5 is often used for labeling of nucleotides, oligonucleotides and DNA for applications in bioanalysis or chemical biology[2]. The 5- or 6-aryluracils can be prepared by heterocyclization or by cross-coupling reactions of halouracils with arylboronic acids or stannanes or metallated uracils with aryl halides. Direct C–H arylation of uracil is an alternative to classical cross-couplings where the preparation of reactive organometallic reagent is avoided. Recently, we have developed regioselective Pd-catalyzed and/or Cu-mediated direct C–H arylations of 1,3-dimethyluracil as a model compound for pyrimidine bases to position C-5 or C-6 [3].

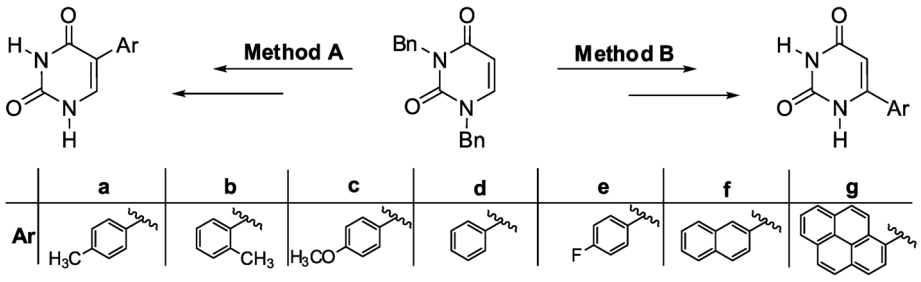
RESULTS AND DISCUSSION

In this work we focused on application of this methodology in regioselective synthesis of libraries of 5- and 6-arylated uracil bases. We started the screening to find a suitable protecting group for uracil and successfully use two different conditions for regioselective direct C–H arylation with *p*-tolyl iodide under specific conditions (A) $Pd(OAc)_2$ in absence of CuI and (B) the same conditions in presence of 3 equiv of CuI (Scheme 1). Finally, we selected 1,3-dibenzyluracil for further direct arylation with diverse aryl halides (**a**–**g**), because together with deprotection provided best results (Scheme 2). The reactions with *p*-Tol-I (**a**), *o*-Tol-I (**b**), 4-MeOPh-I (**c**), Ph-I (**d**) under conditions A gave 1,3-dibenzyl-5-aryluracils as major products in 44–70% yields, while under conditions B the regioselectivity was reversed to afford 6-aryl derivatives as major products in 28–66% yields. In all

cases minor amounts of the other regioisomer were isolated (with the exception of reaction with *o*-Tol-I (**b**), which gave 6-isomer as the only one product). Aryl bromides *p*-F-Ph-Br (**e**), phenyl-Br (**f**) and pyrenyl-Br (**g**) were also successfully used for the C–H arylation under the same conditions A and B to show similar conversion and selectivity. Electron-poor aryl halides (3-iodopyridine, 2-bromothiofene, 6-iodo-benzyl-purine, 2-bromofurane) were also examined in these reactions under conditions A and B but in all cases no reactions (or very low conversions <10%) were observed.



SCHEME 1
C–H arylation of various protected uracil



SCHEME 2
C–H arylation of 1,3-dibenzyluracil

This work is a part of the research project Z4 055 0506. It was supported by the "Centre for Chemical Genetics" (LC06077) and by Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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THERMOLABILE PROTECTING GROUPS IN OLIGONUCLEOTIDE SYNTHESIS

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A new class of universal thermolabile protective group (TPG) is presented. Their main advantage is a quick release the aqueous environment only by the increase of temperature. However, the instability at lower temperatures was observed reducing their wider application. In present paper a new method for attaining higher stability of thermolabile protecting groups (TPG) is demonstrated at phosphate center by intramolecular cyclisation through a "click-clack" approach and at hydroxyl function as protectant using a "chemical switch".

INTRODUCTION

The development of present-day methods of chemical synthesis of complex natural product or bioactive nanostructures and nanodevices triggers the use of new systems of protecting groups in order to save reactive functional places. A need to develop new protecting systems that would least damage the structure of a protected molecule and at the same time would result in a high efficiency of blocking and unblocking reaction initiates a search for new protecting groups. Thermolabile Protecting Groups (TPGs) have emerged as a new strategy for manipulation of protecting groups in organic synthesis. Their basic feature is their removal in the aqueous environment only as a result of an increase of temperature. Owing to their practical properties, TPGs can be widely used in contemporary bioorganic chemistry. Their practical application concerns nanotechnology and arranged syntheses on microarrays, i.e. oligonucleotides. In order to avoid the occurring problems, a new approach was developed in the synthesis of DNA oligonucleotides on microarrays with the use of thermolabile protecting groups[1].

RESULTS AND DISCUSSION

Some TPG are based on N-(2-pyridyl)aminoethanol as a precursor and are referred to as 2-pirydyl TPG. The thermolabile groups are successfully used as protectant of phosphorate centre during chemical synthesis of DNA[2]. A mechanism for the removal of 2-pyridyl TPG from a phosphate center is based on an intramolecular cyclisation initiated by a nucleophilic attack of the pyridine moiety. The reaction considerably advanced in higher temper-

ature consists in a leaving phosphate anion as a result of intramolecular, stereospecific cyclisation of alkyl in water or aqueous organic environment. Temperature highly influences kinetic of this reaction but the elimination proceeds at moderate rate also at room temperature. To eliminate this drawback a method to increase thermostability of a 2-pyridyl TPG via temporary engagement of a phosphate centre in a cyclic structure have been investigated. The idea of temporary protection of thermolabile properties of TPGs through a process was called "click-clack"[3].

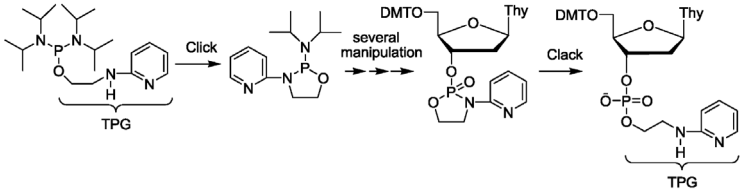

FIG. 1
The idea of a "click-clack" approach using 3-pyridyl-[1,3,2]-oxazaphospholidine as temporary protection

An intramolecular cyclization was demonstrated to be helpful in the protection of thermolabile properties of TPGs bearing an N-(2-pyridyl)aminoethyl moiety. The 3-pyridyl-[1,3,2]-oxazaphospholidine ring was found to be very susceptible to acid hydrolysis and thus enabling the recovery of a thermolabile form of TPG. This "click-clack" approach (cyclisation followed by hydrolysis) offers a simple and efficient way to control thermolabile properties of protecting groups.

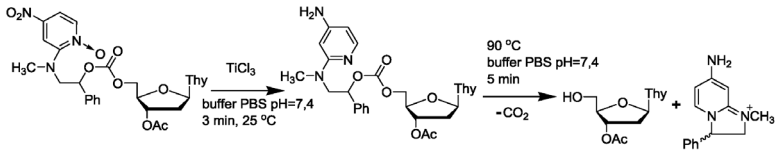

FIG. 2
Conversion nitro substituents to amino and deprotection of TPG

Similar to phosphate TPGs, instability of thermolabile hydroxyl groups is observed at room temperature. This defect, effectively limits the wide application of these groups. The increase of stability TPGs and keep effective level of thermodeprotecting was done by "chemical switch" approach.

It has been demonstrated a method changes characters of TPGs using transformation of nitro group into amine group by reduction witch $TiCl_3$.

This shift from electron withdrawing group to electron donating group gives an aceleration of thermodeprotection reaction[4].

This work was supported by National Cohesion Strategy POIGUDA-01.03.01-30-045/09.

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PHOTOSWITCHABLE MOLECULAR GLUE FOR HYBRIDIZATION OF NUCLEIC ACIDS

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Sequence-dependent hybridization of DNA and RNA is essential for their biological functions and render them promising functional material. In order to regulate the diverse functions triggered by the nucleic acids hybridization, we have developed a series of photoswitchable ligands that enable the reversible control of hybridization of two natural unmodified DNAs and RNAs by external light stimuli.

INTRODUCTION

Spontaneous hybridization between two complementary DNAs occurs with high specificity, and provides a unique basis for the DNA-based technology. In order to regulate the diverse functions triggered by DNA hybridization, studies toward controlling or modulating the DNA hybridization in response to external stimuli have drawn considerable interest. To this end, we have developed photoswitchable molecular glue that controls DNA and RNA hybridization reversibly by light stimuli.

RESULTS AND DISCUSSION

We have reported that DNA mismatch binding ligands (MBL) functioned as a molecular glue for DNA[1,2]. **NCD** is a MBL selective for GG-mismatch, which consists of two guanine recognizing naphthyridine carbamate moieties connected with an dipropylamino linker (Fig. 1a)[1]. **NCDs** bind to DNAs containing GG mismatch sites, and the resulting **NCD**-DNA complex increases the stability of the double-stranded DNAs (dsDNA). While **NCD** can induce the conversion of single-stranded DNAs (ssDNA) into dsDNA by selecting an appropriate sequence, the function was limited to the unidirectional and irreversible control of DNA hybridization. To achieve completely reversible and bidirectional control of DNA hybridization, we have investigated the incorporation of a photo-responsive unit into MBL to drive hybridization/dehybridization cycle by external light stimuli. **NCD** was integrated into photoswitchable molecular glue (**NCDA**) by incorporating a photochromic azobenzene between two base-recognizing naphthyridine heterocycles (Fig. 1a)[3]. A reversible *Z*/*E* photoisomerization of azobenzene

changes the relative orientations and positions of the naphthyridine moieties, resulting into the switching of the **NCDA** ability to adhere two ssDNAs containing the GG-mismatch (Fig. 1b).

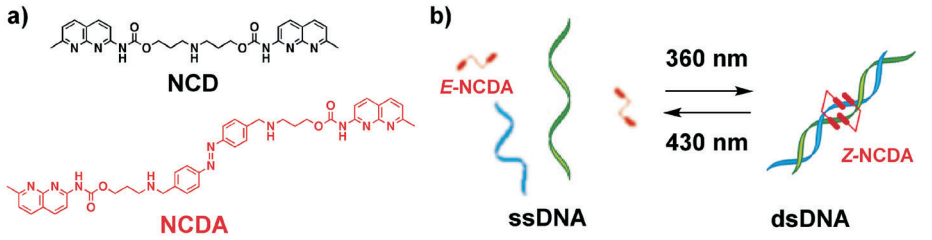

FIG. 1
(a) Structures of **NCD** and **NCDA**. (b) Schematic illustration of reversible photoswitching of DNA hybridization by **NCDA**

The photoswitchable ligand **NCDA** can be reversibly isomerized from *E* to *Z* or *vice versa* by using 360 nm and 430 nm photoirradiation, respectively. Since a *Z*-azobenzene linkage in *Z*-**NCDA** allows two naphthyridine moieties to be placed in the appropriate positions for the binding, only *Z*-**NCDA** can bring two ssDNAs containing GG mismatch site (CGG/CGG sequence) together. The photoisomerization of **NCDA** in fact controlled the hybridization and dehybridization of the DNA duplex containing the mismatch site in response to the *Z*/*E* ratio of **NCDA** adjustable by external light stimuli (Fig. 1b)[3]. The combination of **NCDA** and a fluorescent pyrene-modified DNA enabled us to produce a light-driven, DNA-based switching device, that reversibly changes the fluorescence of a pyrene derivative from monomer (blue, 430 nm) to excimer (green, 520 nm) emission or *vice versa* by photoirradiation[4]. Light-controlled DNA hybridization with **NCDA** indeed provided the switching of pyrene blue/green fluorescence under isothermal conditions.

NCDA, which consists of MBL connected with a photo responsive azobenzene linker, permits reversible control of DNA hybridization by external light stimulus. **NCDA** is a new class of molecule, which functions as a photoswitchable molecular glue for DNA, and will be a useful tool for wide-ranging DNA-based technology. The concept of molecular glue is not limited to DNA but can be applicable to RNA. Molecular glue for RNA can control RNA secondary structures that are deeply involved in diverse RNA functions.

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THE MECHANISM OF ISOTOPIC EXCHANGE REACTION OF HYDROGEN H-5 OF URACIL DERIVATIVES IN WATER AND IN NON-PROTIC SOLVENTS

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The mechanism of isotopic exchange reaction of hydrogen H-5 of uracil and its methyl-derivatives in water and in organic solvents was studied. The key intermediate of the reaction is a C-5 tautomer of uracil. We have used ^{1}H NMR spectroscopy to follow the kinetics of the hydrogen-to-deuterium exchange reaction. In aqueous media, a general base catalysis was observed and in organic solvents we propose a reaction mechanism with participation of the solvent molecules. The transition rates determined by NMR could be rationalized by density functional computations. We have shown that the hydrogen-to-deuterium exchange reaction is much faster in suitable nucleophilic solvents than in water. These findings could be effectively used for the tritium labeling of pyrimidine nucleic acid bases.

INTRODUCTION

Uracil is one of the most common nucleic acid bases, its tautomeric forms have been thoroughly investigated in recent years. Six tautomers (one diketo, four hydroxy-keto and one dihydroxy) have traditionally been considered. Spectroscopic experiments indicate that the 2,4-dioxo form of uracil is the most stable tautomer. Only recently, a new type of uracil tautomers, with a proton transferred to the C-5 carbon atom, has been proposed[1,2]. Here, we present results of our mechanistic studies of the isotopic exchange reaction of the hydrogen H-5. We monitor the exchange by NMR spectroscopy in various buffers and solvents containing an amount of D_2O. Our hypothesis is that the C-5 tautomers of uracil are the key intermediates of the exchange reaction.

RESULTS AND DISCUSSION

The H-5 exchange reaction was apparently a first-order reaction. We measured the exchange rates in D_2O at different pD, in acetate buffer and in phosphate buffers of different concentrations. The reaction rate was not dependent on pH in the neutral region but it depended on the buffer concentration. These features are typical characteristics of a general base-catalyzed reaction. The hydrogen-to-deuterium exchange reaction of the H-5 of uracil

was also studied in a series of protic and non-protic solvents. The reaction rates depended strongly on the solvent used. The highest reaction rate was observed in 4-methoxypyridine, in which the reaction was three orders of magnitude faster than in acetone and two orders of magnitude faster than in water. We suggest the following mechanism of isotopic exchange, depicted in Fig. 1. The exchange reaction involves a C-5 uracil tautomer. The organic solvents could work as catalysts in the rare tautomer formation. In the transition state, a solvent molecule could form a complex with the uracil molecule, and the free electron pair on the solvent heteroatoms could lower the barrier of the proton shift from oxygen to carbon C5. The influence of the electronic structure of the solvent molecules is best demonstrated on the series of pyridine derivatives. The electron-donating substituents ($–CH_3$, $–OCH_3$) in position 4 of pyridine increased (with respect to pyridine) the reaction rates. To obtain further insight into the mechanism of the isotopic exchange, we measured the reaction rates of the methylated uracil derivatives.

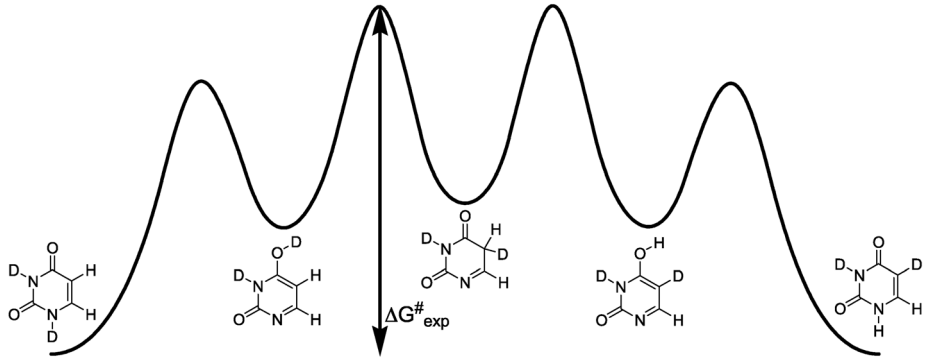

FIG. 1
The mechanism of the isotopic exchange of hydrogen in position 5 of uracil

Financial support from the Grant Agency of the Academy of Sciences of the Czech Republic through Project KJB400550903 is acknowledged.

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NOVEL TRIPHENYLAMINE-BASED DNA MINOR GROOVE BINDERS FOR USE IN TWO-PHOTON EXCITED MICROSCOPY

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Herein we report the synthesis and characterization of a series of fluorescent triphenylamine-DNA ligands displaying large two-photon absorption cross-sections. Interaction with various DNA secondary structures has been investigated by UV-vis and fluorescence spectroscopies. Circular dichroism measurements indicated a binding mode *via* minor groove insertion. Finally fixed cells observed by confocal microscopy showed a specific staining of nuclear DNA with low background fluorescence.

INTRODUCTION

Triphenylamine derivatives have long been shown to be promising materials for two-photon absorption (2PA). However, their large size and strong aromatic characteristics are incompatible with biological conditions[1].

In our ongoing research aimed at nucleic acids/small molecules interactions, we have developed new triarylamine derivatives which combine a high 2PA cross-section and biocompatible properties (small size and water solubility).

We recently described a first generation of triphenylamines (TP-Py) which display enhanced two-photon excited fluorescence upon binding to double-stranded DNA (Fig. 1)[2,3].

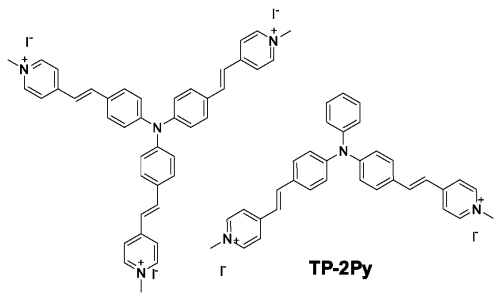

FIG. 1
Structure of the vinylpyridinium triphenylamines (TP-Py)

RESULTS AND DISCUSSION

Based on the same triphenylamine pattern, several heterocyclic electron-withdrawing groups were introduced to optimize the optical properties. The new dyes display the same light up property in DNA and were fully characterized for their linear and nonlinear optical properties (Fig. 2A).

Dissociation constants of the dye/DNA complexes were calculated by nonlinear fitting of titration curves and were all found in the low micromolar range. Measurement in various forms of nucleic acids (duplex, single strand, RNA and G-quadruplex) showed a selective binding of the two-branched compounds to B-DNA, whilst the three-branched molecules display a poor structural preference.

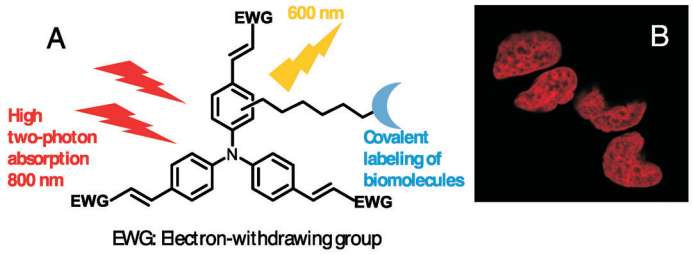

FIG. 2
A) General structure of the triphenylamine dyes. B) Stained fixed MRC-5 cells under two-photon excited confocal microscopy

Confocal microscopy of fixed cells incubated with the dyes showed a very bright and specific staining of the nuclear DNA with a very low cytoplasmic fluorescence background (Fig. 2B).

The optimized markers were also derivatized to allow bioconjugation to oligonucleotides, in view of fluorescent detection of hybridization, and specific targeting of tumor cells.

In conclusion, we developed a series of highly specific DNA stainers with optimized optical properties, suitable for one- and two-photon cellular imaging.

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ON SUPPORT FLUORESCENT ASSAYS BASED ON FUNCTIONALIZED OLIGONUCLEOTIDES TO MONITOR SPECIFIC DNA REPAIR ACTIVITIES

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New devices based on supported oligonucleotide probes were developed to detect base excision repair activities. These bioanalytical tools allow a rapid, specific and quantitative analysis of DNA N-glycosylases and AP-endonucleases functionalities as well as a high throughput screening of specific inhibitors.

INTRODUCTION

The removal of DNA damages by dedicated repair pathways plays a key role in the maintenance of the integrity of genomes. The study of the processing of each lesion and the expression of the corresponding enzymatic activities is essential to better understand molecular mechanisms of several diseases and resistance phenomena in cancer chemo- and radiotherapy[1]. To achieve this goal there is a need to develop new devices aim at monitoring DNA repair activities in a quick, easy and specific manner. Moreover, such tools can be useful to develop HTS assays to search for specific inhibitors. The base excision repair (BER) pathway[2] takes in charge bit bulky DNA adducts, mainly resulting from alkylation, oxidation or desamination processes of nucleobases. Briefly, a DNA N-glycosylase recognizes the damaged base and cleaves the N-glycosidic bond forming an abasic site (AP site). Then, a second enzyme, namely an AP-endonuclease, incises the phosphodiester bond close to the AP-site, generating a nick in the strand. Some DNA N-glycosylases are bifunctional and possess an additional AP-lyase activity. AP sites and single strand breaks are equally repair by the BER pathway. A majority of study uses radioactive or fluorescent labeled oligonucleotides coupled with an electrophoretic separation to analyze DNA N-glycosylases and AP-endonucleases activities[3]. In the current work we have designed and then prepared original on support DNA biosensors able to detect specific BER activities.

RESULTS AND DISCUSSION

Our devices are based on a set of immobilized fluorescent DNA probes, each of them being a substrate for a specific BER enzymatic activity by incorporating a defined lesion in the double strand. In the present work, several oligonucleotides containing specific lesions have been synthesized, namely uracile, inosine, 8-oxo-guanine and AP site analog (THF), to target Uracile N-glycosylase (UNG), Alkyl-Adenine N-glycosylase (Aag), 8-oxo-guanine N-glycosylase (Ogg1) and AP-endonucleases respectively. These DNA probes are also modified so that they can be immobilized on solid supports such as glass slides or microbeads, allowing a rapid quantification of the different targeted activities in a parallel manner by a fluorescent detection. To validate our functional DNA biosensors we have first investigated the initiation of the BER pathway (i.e. strand scission induced by a bifunctional ADN N-glycosylase activity or by a joint activity of a monofunctional DNA N-glycosylase and an AP-endonuclease) by purified enzymes. Another important point addressed in this study was the evaluation of the benefit provided by the immobilized platform in comparison with the classical in solution format towards the non-specific degradation by nucleases present in biological samples. To investigate that point, we have analyzed the processing of our immobilized fluorescent DNA probes by nuclear cell extracts (Fig. 1). In this experiment fluorescent intensity directly reflects the enzymatic cleavage of the probes. A comparative analysis by direct fluorescence measurements and polyacrylamide gel electrophoresis (PAGE) revealed that the substrate DNA probes are significantly less degraded when attached on microbeads. Altogether, these results validated our bioanalytical devices

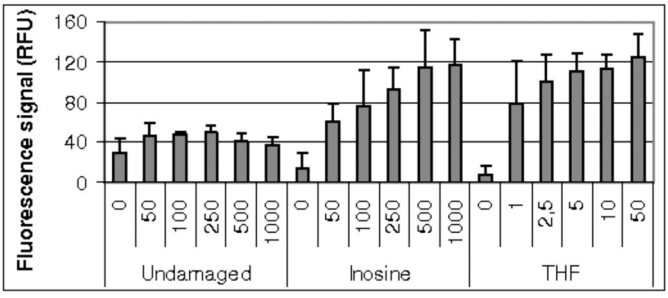

FIG. 1
On support cleavage of lesion-containing DNA probes (1 pmol) immobilized on microbeads by HeLa nuclear extracts (in μg/ml)

and the corresponding functional assays to analyze DNA repair activities in a parallelized and miniaturized fluorescent on support format.

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SYNTHESIS OF 2,2′-BIPYRIDINE METAL COMPLEXES AS POTENTIAL G-QUADRUPLEX DNA LIGANDS

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A series of 2,2′-bipyridine derivatives has been synthesized, including several Cu(II) and Pt(II) metal complexes. All ligands incorporate in their structure DNA recognition motifs, i.e., either DNA intercalating subunits or minor group binders. Because they have an extended surface available for π stacking interactions, the 2:1 (ligand to metal ratio) metal complexes may selectively interact with G-quadruplex DNA structures, and their preparation may provide a basis for the development of novel anti-cancer agents.

INTRODUCTION

Derivatives of the 2,2′-bipyridine and 1,10-phenanthroline systems are widely known to interact with DNA. As polycyclic planar aromatic ligands, these compounds are specially well-suited to favor π stacking interactions with the DNA base pairs. Moreover, the two nitrogens confer these derivatives the ability to bind metal ions, thus acting as versatile chelating agents[1]. The goal of this work is to prepare a series of new small molecules that can preferentially recognize G-quadruplex DNA structures, *versus* duplex DNA. These secondary structures have been observed in different regions of the genome, such as several gene promoters and human telomeres[2]. Therefore, the preparation of metallo-organic molecules that can selectively bind and stabilize G-quadruplex DNA might represent a promising approach in the identification of novel anti-cancer therapeutics[3]. In this study we have obtained several 2,2′-bipyridine copper(II) and platinum(II) complexes designed to bind G-quadruplex DNA.

RESULTS AND DISCUSSION

The synthesis of the 2,2′-bipyridine ligands connected to naphthalene, anthracene and acridine as DNA intercalating subunits (Fig. 1) was carried out as previously reported[4–6]. 2:1 (ligand to metal ratio) copper(II) complexes were then prepared by direct reaction of the ligands with $CuNO_3$ in refluxing ethanol. In the case of platinum(II) complexes, the preparation of complexes resulted more challenging. The reaction of the previously ob-

tained 1:1 complexes with excess ligand proved to be difficult due the low solubility of the starting materials, and we are currently exploring alternative synthetic routes.

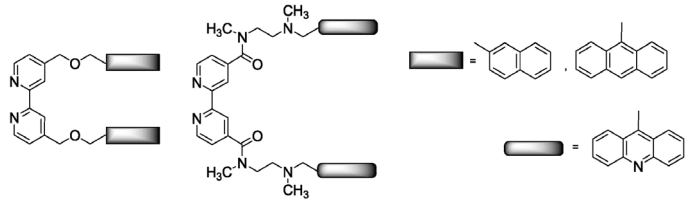
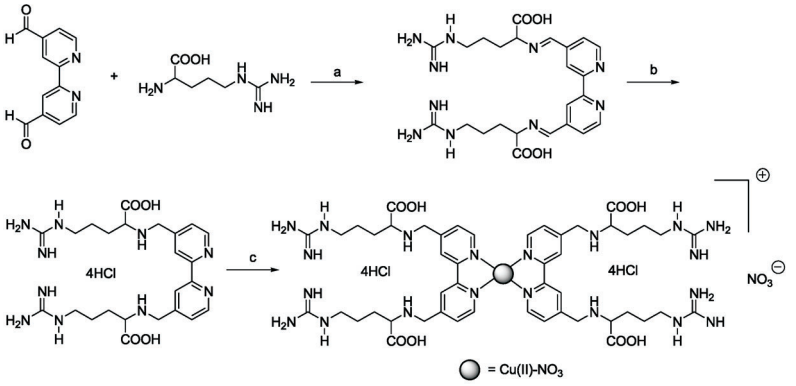


FIG. 1

In addition, two 2,2′-bipyridine derivatives containing aliphatic chains linked to guanidinium groups capable of interacting with the DNA grooves were synthesized (an example using the amino acid arginine is shown in Scheme 1). The 2:1 copper(II) metal complexes were prepared following a similar procedure as the one used for the 2,2′-bipyridine derivatives linked to DNA intercalating subunits.



SCHEME 1

a) EtOH, reflux; b) i: $NaBH_4$, EtOH and ii: $HCl{\cdot}Et_2O$, EtOH; c) $CuNO_3{\cdot}3H_2O$, H_2O, reflux

The interactions between the synthesized compounds and G-quadruplex DNA will be evaluated by different biochemical techniques. We expect to find in the near future selective G-quadruplex ligands that can be tested for their therapeutic applications as telomerase inhibitors.

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ISOXAZOLE LINKED OLIGONUCLEOTIDE CONJUGATES BY ON RESIN AND PREVIOUSLY CLICKED NITRILE OXIDE ALKYNE CYCLOADDITIONS

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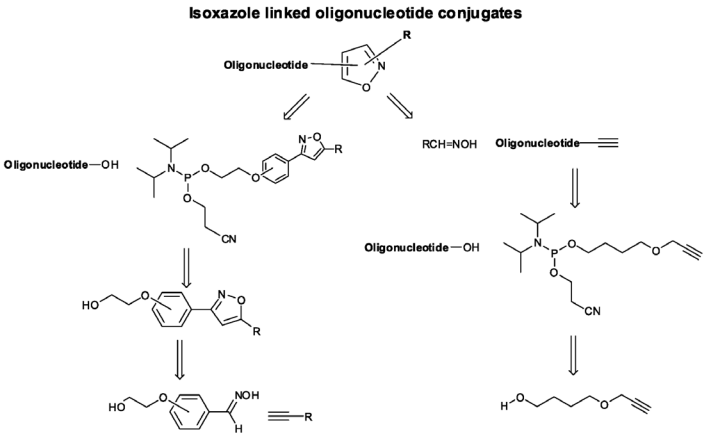
Bioconjugation protocols in environments free from residual copper or other catalytic components are important for therapeutic and biomedical applications as well as in living systems. In this communication we discuss the versatility of the catalyst free, isoxazole generating nitrile oxide alkyne Huisgen cycloaddition for provision of chemically modified oligonucleotide conjugates. Two distinct approaches will be demonstrated. In the first we discuss on resin cylcoaddition between *in situ* generated nitrile oxides and oligonucleotides bearing alkyne functionalities at the either, or both, the 3′- or the 5′-termini[1–3]. In the second we discuss the construction of oligonucleotide conjugates by phosphoramidite chemistry of previously-clicked isoxazole derivatives. Examples of conjugates prepared each approach and the merits of the previously clicked and on resin click approaches will be discussed.



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SYNTHESIS AND REARRANGEMENTS OF DEWAR BENZENE DEOXYRIBOSIDES

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A diastereomeric mixture of *C*-(Dewar benzene)-deoxyribosides was synthesized from the corresponding propynoates. These compounds undergo a thermal or UV light initiated rearrangement and give rise to new highly substituted *C*-aryldeoxyribosides.

INTRODUCTION

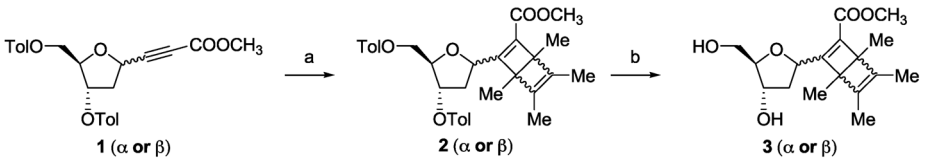
Dewar benzene is an interesting compound which, although conceived in 19th century, was first synthesized in 1960s. It is in fact bicyclo[2.2.0]-hexadiene with very strained bonds and the species without any substituents gradually rearranges to benzene, the compound is much more stable when an electron withdrawing group is present as a substituent. The uses of Dewar benzenes in organic synthesis are quite rare, but they are no less interesting. They have been utilized in the syntheses of e.g. pentamethylcyclopentadienyl-organometallic compounds[1,2], in the synthesis of permethylated ladderanes[3]. There are also reports of their conjugates with ferrocene[4] and their application in the synthesis of ter- and quaterphenyls[5]. An interesting use of Dewar benzene is as a protecting group[6].

We decided to synthesize new *C*-(Dewar benzene)-deoxyribosides as a way to obtain a new synthetic route to highly substituted *C*-aryldeoxyribosides. These aromatic compounds arise once the corresponding Dewar benzenes are subjected to high temperatures or UV light.

RESULTS AND DISCUSSION

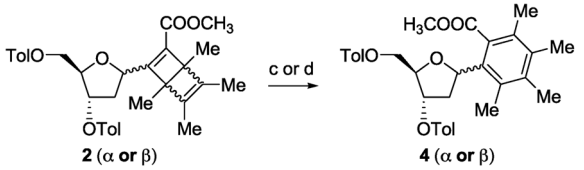
We started the synthesis from a toluoyl-protected halogenose and its reaction with ethynylmagnesium bromide gave rise to an anomeric mixture of the corresponding ethynylriboside. Lithiation of this mixture with LDA and subsequent reaction with TBDMS-Cl furnished products with a bulky protecting group allowing the separation of the anomers by simple column chromatography. TBDMS group was removed by treatment with TBAF. Next, the ethynylribosides were lithiated with LDA and the reaction with

methyl chloroformate gave rise to propynoates **1**, these were suitable substrates for the Dewar benzene synthesis thanks to the electron withdrawing functionality (ester group). The reaction of but-2-yn and aluminium chloride formed the tetramethylcyclobutadiene-aluminium trichloride complex that reacted with the activated propynoate **1** to form an inseparable mixture of diastereomers of Dewar benzenes **2**. These compounds were then treated by MeONa/MeOH and furnished the deprotected deoxyribosides **3**.



SCHEME 1
a) i: $AlCl_3$, but-2-yne and ii: DMSO; b) MeONa in MeOH

The synthesized Dewar benzenes were very stable; when we attempted to rearrange them thermally, the reaction did not proceed with a satisfactory reaction rate until the temperature reached 150 °C. The flaw to this method was the degradation of the sugar moiety that occurred at these temperatures. Therefore the rearrangement was carried out in a quartz tube under UV irradiation at laboratory temperature. Under these conditions it proceeded fast to form a benzene derivative as a single stereoisomer **4**.



SCHEME 2
c) 150 °C, DMSO; d) 20 °C, UV, $CHCl_3$

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SYNTHESIS OF SPIN-LABELED RNA AND PROBING OF RNA SECONDARY STRUCTURES BY PULSED EPR SPECTROSCOPY

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Spin-labeled RNA was readily prepared by postsynthetic modification of prefunctionalized oligonucleotides. This approach provides access to paramagnetic RNA with site-specific TEMPO groups at cytidine, adenosine, and guanosine nucleobases. The application of TEMPO-modified RNA was demonstrated for the investigation of alternative RNA secondary structures by distance measurements using pulsed EPR spectroscopy.

INTRODUCTION

Electron paramagnetic resonance (EPR) spectroscopy provides useful information on local and global dynamic properties of nucleic acid structures and has been recognized as powerful tool to investigate RNA folding events[1]. Since natural RNA is diamagnetic, the application of EPR spectroscopy depends on the ability to incorporate paramagnetic centers into RNA at specific positions. Pulsed electron double resonance (PELDOR) yields long-range structural restraints by measuring the distance between two spin labels. PELDOR can therefore be used to detect alternative RNA conformations and to monitor RNA refolding. Several strategies have been reported for the attachment of spin labels to RNA[1], but convenient access to high-quality spin-labeled RNA remains the bottleneck for the widespread application of EPR in RNA research. The goal of our study therefore was to develop a protocol for the facile and efficient preparation of paramagnetic RNA for PELDOR experiments.

RESULTS AND DISCUSSION

The nitroxyl radical-based nucleobase spin labels used in this study[2] are designed to preserve the Watson–Crick base-pairing capability of labeled nucleotides and not to interfere with alternative base-pairing patterns in different RNA conformations. The paramagnetic TEMPO group was directly attached to the exocyclic nucleobase amino groups of adenosine, cytidine and guanosine by the convertible nucleoside approach (Fig. 1). In this strat-

egy, appropriately functionalized precursor nucleosides[3] were incorporated into RNA by solid-phase synthesis. Installation of the TEMPO substituent occurred at the same time as cleavage from the polystyrene support and deprotection of phosphate and nucleobase protecting groups.

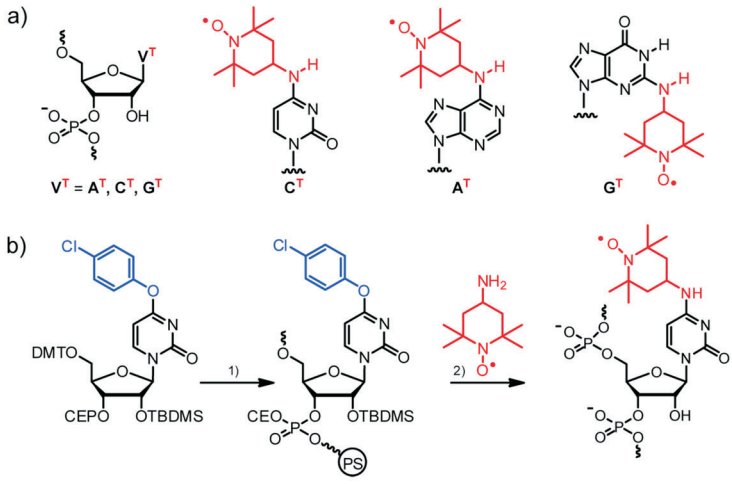

FIG. 1
a) Spin-labeled RNA with nitroxide-containing nucleosides. b) Synthesis of cytidine-labeled RNA by substitution of 4-chlorophenyluridine. 1) Solid-phase synthesis under standard conditions. 2) i) 2 M TEMPO-NH_2 in methanol, 42 °C, 24 h; ii) 1 M TBAF in THF, 25 °C, 12 h. DMT 4,4′-dimethoxytrityl, TBDMS *tert*-butyldimethylsilyl, CEP = β-cyanoethyl diisopropylphosphoramidite

This strategy allowed us to place the TEMPO spin labels in the major groove (for A and C) or in the minor groove (for G) of Watson–Crick base paired RNA duplexes. The influence of the spin label on RNA conformation and secondary structure was investigated by UV melting experiments and CD spectroscopy. PELDOR measurements on TEMPO-labeled RNA successfully detected different RNA conformations on the secondary structure level[2]. Examples involved hairpin, duplex, and quadruplex conformations, which are important for studies of regulatory RNA elements. The narrow distance distributions obtained from the high-quality PELDOR data on our spin-labeled RNAs are promising for future studies of conformational rearrangements in larger RNAs and RNA-protein complexes.

We gratefully acknowledge the Max Planck Society for generous support of our work.

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A NOVEL, HIGHLY STEREOSELECTIVE SYNTHETIC APPROACH FOR THE PREPARATION OF SUBSTITUTED 2,5-DIHYDRO-2,5-DIHYDROXYFURANS

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A novel highly stereoselective reduction of 5-substituted furan-2(5*H*)-ones has been developed as a key step in the synthesis of 2,5-dihydro-5-hydroxyfuran-2-yl nucleosides. This synthetic procedure was used for the preparation of compounds of biological interest and new compounds with antiproliferative activity were identified.

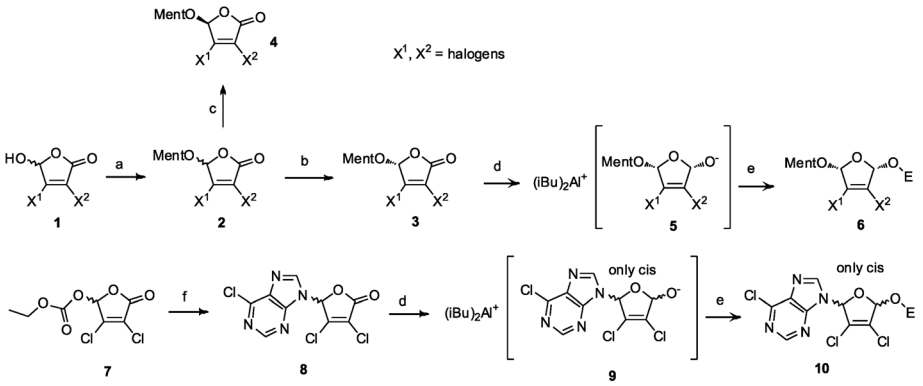
INTRODUCTION

(2*R*,5*R*)-9-[2,5-Dihydro-5-(phosphonomethoxy)furan-2-yl]adenine was described as a compound with promising anti-HIV activity[1]. Recently, new 3-fluro derivative of this compound was studied as a potential anti-HIV drug candidate[2]. Nevertheless, the SAR study of this group of compounds is complicated since the synthesis of the 2,5-dihydro-5-hydroxyfuran-2-yl nucleosides and nucleoside phosphonates is quite challenging.

RESULTS AND DISCUSSION

The aim of this study was to develop an alternative stereoselective synthesis of *cis*-2,5-dihydro-5-hydroxyfuran-2-yl nucleosides that would allow for a scale-up. Our novel procedure (Scheme 1) stars from very cheap mucuhalogenic acids **1** which are manufactured from furfural. It was already published that these compounds can be transformed into the menthol bearing acetals **2** which could be isolated as a single (*R*) enantiomer **3** by crystallization and the mixture of enantiomers could be recycled under acid catalysis[3]. At this point, we have found the conditions for isolation of pure (*S*) enantiomer **4**. That was a challenging task since the (*S*) enantiomer usually co-crystallizes off with the (*R*) enantiomer. Next step of the synthesis was the diastereoselective reduction of the carbonyl group in the position 2 of the furan ring with DIBAL to produce the pure *cis* diastereoisomer. This is caused by the fact that a bulky substituent in the position 5 (e.g. menthol) rotates over the 2,5-dihydrofuran ring and totally blocks the possible attack of the DIBAL reagent from that side. This was also confirmed by theoretical calculations. Hydrogen atom is then introduced only into the *trans* configu-

ration to the menthol group and thus the newly formed single bond between the furan ring and the oxygen has the *cis* configuration. The intermediate **5** was then subjected to one-pot reaction with various electrophiles (e.g. Ac^+ by the use of Ac_2O) to produce pure (*R*,*R*) diastereoisomers **6**. The optical purity of the prepared diastereoisomer is very high (>99%) and we were not able to detect any presence of the second diastereoisomer (NMR, GC/MS, HPLC/MS). Absolute stereochemistry (*R*,*R*) was confirmed by solving the X-ray structure.



SCHEME 1

a) L-menthol, H^+/toluene, reflux; b) crystallization from light petroleum; c) light petroleum evaporation in an open system; d) DIBAL/THF, r.t.; e) e.g. Ac_2O; f) 6-chloropurine, $AlMe_3$, $Pd[P(Ph)_3]_4$, dppf/acetonitrile, 0 °C

The model racemic compound **7** was successfully modified with 6-chloropurine under conditions for palladium-catalyzed allylic substitution to form nucleoside analogue **8**. This type of reaction proceeds with the retention of configuration[4]. Subsequent reduction with DIBAL and reaction with electrophile (one-pot) afforded the racemic mixture of both *cis* diastereoisomers (again *cis* only).

Compounds **3** and **4** are endowed with antiproliferative activities that are stereospecific.

This work is a part of the research project AVOZ40550506 of the Institute of Organic Chemistry and Biochemistry and was supported by Centre for New Antivirals and Antineoplastics (1M0508). This study was also supported by Gilead Sciences (Foster City, CA, U.S.A.).

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NEW α-THYMIDINE 5′-PHOSPHONATE DERIVATIVES

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A set of α-thymidine 5′-phosphonate derivatives was obtained and studied their stability in different conditions, cytotoxicity and penetration into K562 cell cultures.

INTRODUCTION

At present tuberculosis (TB) together with AIDS are the most dangerous infection diseases. It kills about two million people every year[1]. Therefore, the search of new antituberculosis drugs is an essential aim. Among modified nucleosides displaying a noticeable anti-mycobacterial effect on experimental models, the inhibitors of *M. tuberculosis* thymidine monophosphate kinase (TMPKmt) were effective against *M. tuberculosis* growth[2,3].

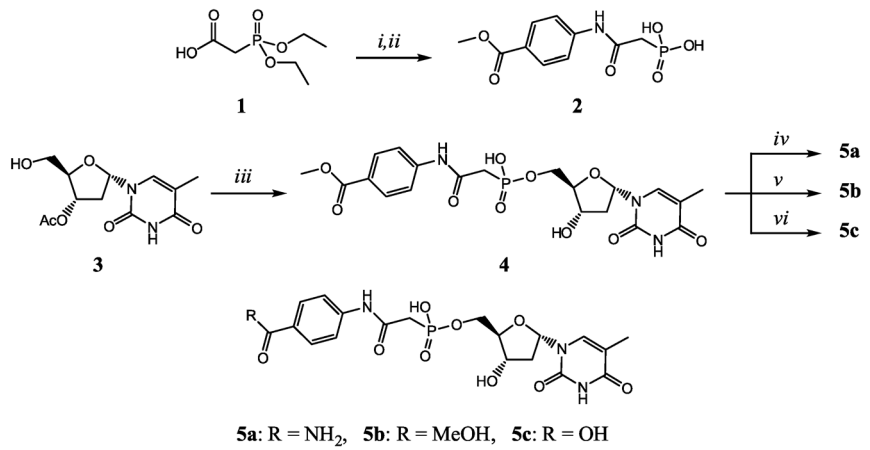
Comparison of 5′-deoxy-5′-arylthiourea derivatives of α-thymidine, which exhibited significant *in vitro* anti-tubercular activity[2] and its 5′-arylaminocarbonylmethyl phosphonate derivatives using the Accelrys Discovery Studio program, demonstrated spatial similarity of these compounds. In this work we describe the synthesis of a set of α-thymidines 5′-phosphonates as potential inhibitors of *M. tuberculosis* growth, evaluation of their stability in different conditions, cytotoxicity in Vero and K562 cells and penetration into K562 cell cultures.

RESULTS AND DISCUSSION

The starting α-thymidine was obtained as described in[4]. The reaction of *p*-anilinemethoxycarboxylic acid with CDI-activated diethyl phosphonoacetate **1** followed by deblocking of ethyl groups using Me_3SiBr in DMF yielded compound **2**. Its coupling with α-thymidine **3** in the presence of DCC in pyridine[5] gave N-[4-(methoxycarbonyl)phenyl]aminocarbonylmethyl phosphonate of 2′-acetyl-α-thymidine **4**. Aminocarbonyl-, methoxycarbonyl- and carboxy- derivatives **5a**, **5b** and **5c** were obtained by treatment of phosphonate **4** with aq. NH_3, MeONa/MeOH or aq. NaOH, respectively.

Compounds **5a–5c** were stable in buffer solutions at pH 2, 7.4 and 9 and in fetal calf serum for more than 24 h. Phosphonates **5a–5c** were not

cytotoxic at concentrations up to 500 μM in *Vero* and up to 250 μM in K562 cells.



SCHEME 1
i: 4-MeO_2C-C_6H_4-NH_2, CDI, DMF; *ii*: Me_3SiBr, DMF; *iii*: **2**, DCC, DMF; *iv*: aq. NH_3, *v*: MeONa, MeOH, *vi*: aq. NaOH

We also studied the capacity of **5a**, **5b** to penetrate into cells After incubation of phosphonates **5a** and **5b** in K562 cell cultures these compounds were detected in the cell lysates at concentrations of 1 nmol and 0.1 nmol, respectively, per 1 million cells.

The anti-TB-activity will be reported.

This study was supported by the Fundamental Research Program of the Presidium of the Russian Academy of Sciences "Molecular and Cell Biology" and the Russian Foundation for Basic Research (grant No. 11-04-00603).

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BIOLOGICAL PROPERTIES OF THE C-8 SUBSTITUTED ANALOGUES OF 9-[2-(PHOSPHONOMETHOXY)ETHYL]ADENINE (PMEA)

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A series of 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) derivatives substituted at the C-8 position of the purine moiety was evaluated for their biological properties. Although none of the compounds exhibited any pronounced antiviral or antimalarial activity, both 8-hydroxy and 8-sulfanyl derivatives of PMEA possess significant immunostimulatory activities, increasing levels of inflammatory cytokines (TNF-α, IL-6) and chemokine (RANTES), thus up-regulating production of NO.

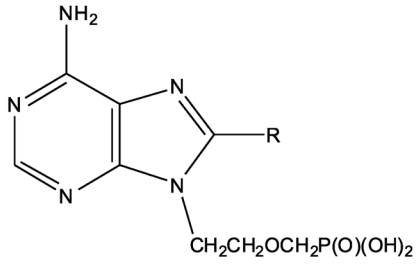
INTRODUCTION

Acyclic nucleoside phosphonates[1] (ANPs) are nucleotide analogues with a wide range of biological activities, especially antiviral[2], antiparasitic[3], and cytostatic[4]. Several mono- or dialkyl, cycloalkyl, alkenyl, and alkynyl N^6-substituted ANPs have been found to possess remarkable immunostimulatory activities[5], that may contribute to their overall antiviral effectiveness. In the present study, biological properties of the C-8 substituted 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA) derivatives have been evaluated.

RESULTS AND DISCUSSION

The 8-substituted PMEA derivatives involved in this study were prepared according to the reported procedure[6], i.e. by the reaction of 8-bromo-9-{2-[(diisopropoxy)methoxy]ethyl}adenine with the corresponding nucleophiles (S_NAr), followed by standard deprotection of the phosphonate moiety. In contrast to the parent 8-unsubstituted derivative (PMEA), none of the prepared compounds (**Z-118**, **Z-174**, **Z-177**, **Z-178**, **Z-179**, **Z-196**, Fig. 1) was active at subtoxic concentrations against the viruses tested (HIV-1, HIV-2, MSV, HSV-1, HSV-2, CMV, VZV) and none of them revealed any inhibitory effects on hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRTase), a key enzyme of the purine metabolism of *Plasmodium falciparum*, a protozoan parasite causing malaria.

Next, immunomodulatory properties of the 8-substituted PMEA derivatives were studied under in vitro conditions using mouse resident peritoneal cells. Stock solutions (10 mM) of compounds **Z-118**, **Z-174**, **Z-177**, **Z-178**, **Z-179**, and **Z-196** were prepared in apyrogenic water/0.05 M NH_3 solution. The samples were passed through the centrifugal filters with a mol. wt. 3000 cut-off (Amicon Ultra, Millipore). The procedure eliminates bacterial lipopolysaccharide and lipoteichoic acid that might be a potential source of a bias in evaluating the intrinsic immunomodulatory activities of the compounds. The 8-hydroxy and 8-sulfanyl derivatives of PMEA, compounds **Z-174** and **Z-179**, respectively, have been found to possess immunostimulatory properties. Substantially augmented levels of inflammatory cytokines (TNF-α and IL-6) and chemokine (RANTES) were observed at as low concentrations as 10 μM. Enhanced cytokine secretion is a plausible explanation for the up-regulatory effects of these compounds on biotransformation of L-arginine to NO, primarily triggered by IFN-γ.



Z-118 R = NH_2
Z-174 R = OH
Z-177 R = NHMe
Z-178 R = $N(Me)_2$
Z-179 R = SH
Z-196 R = SMe

FIG. 1
8-Substituted PMEA derivatives

This work is a part of the research project AVOZ40550506 of the Institute of Organic Chemistry and Biochemistry and was supported by the Grant Agency of the Czech Republic (grant No. P207/11/0108) and Centre for New Antivirals and Antineoplastics (1M0508). This study was also supported by funds from the National Health and Medical Research Council, Australia (grant No. 569703) and by Gilead Sciences (Foster City, CA, U.S.A.).

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THE UNIQUE IMPACT OF MICROWAVE IRRADIATON ON THE CHEMISTRY OF ACYCLIC NUCLEOSIDE PHOSPHONATES

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Latest improvements of the syntheses of ANPs using microwave irradiation are presented and discussed, namely a novel, efficient, and environmentally friendly synthesis of dialkyl haloalkylphosphonates *via* the microwave-assisted Michaelis–Arbuzov reaction and microwave-assisted hydrolysis of phosphonate diesters.

INTRODUCTION

Synthetic phosphonates are now widely used as herbicides[1], pesticides[2], detergents[3], chelating agents for di- and trivalent metals ions[4], agents that inhibit crystal growth and scale formation[5], reagents for Wittig–Horner reactions[6], hybrid organic-inorganic supports and catalysts[7], antiviral agents[8], agents with antitumor activity[9] or as chemical weapons of mass destruction (the V-series of nerve agents)[10]. Thus, the improved syntheses of ANPs are of a great scientific, as well as economic value. Examples of the microwave-assisted (MW-assisted) procedures for the preparation of ANPs are presented here.

RESULTS AND DISCUSSION

Microwave-assisted (MW-assisted) organic synthesis has become a very rapidly developing area of chemistry as this progressive methodology provides a number of advantages over the standard heating techniques.

The selective formation of the desired dialkyl haloalkylphosphonates by the microwave-assisted Michaelis–Arbuzov (M–A) reactions of dihaloalkanes can be reached very effectively by the accurate control of the reaction temperature[11]. The selective substitution of only one halogen atom performed well at 180–190 °C for dichloroalkanes, at 130–170 °C for dibromoalkanes, and at 90–100 °C for diiodoalkanes. Surprisingly, the selectivity for the monosubstitution by M–A reaction is also preserved in the case of dihaloalkanes with five carbon atoms between the two halogen atoms, where the steric effects cannot play a significant role. We can speculate that this phenomenon is caused by much higher absorption of micro-

wave irradiation by dihaloalkanes compared to dialkyl haloalkylphosphonates and/or by the different absorption by the corresponding transition states formed during the M–A reaction. Thus, under the MW-assisted conditions, the reactivity of the halogen atom would be higher in dihaloalkanes compared to dialkyl haloalkylphosphonates.

Another example of MW-assisted methodology is the efficient hydrolysis of phosphonate diesters to the corresponding free phosphonic acids. This procedure also enables the hydrolytic transformation of the heterocyclic bases substituted by halogen atom. Thus, profitable one-pot synthesis of ANPs bearing guanine, hypoxanthine, and xanthine bases was developed. The new method is convenient for the preparation of various acyclic nucleoside phosphonates of biological interest.

This work is a part of the research project AVOZ40550506 of the Institute of Organic Chemistry and Biochemistry and was supported by Centre for New Antivirals and Antineoplastics (1M0508). This study was also supported by Gilead Sciences (Foster City, CA, USA).

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CHEMICAL SYNTHESIS OF PRODRUGS DERIVED FROM 5,6-DIHYDRO-5-AZACYTOSINE AND ITS NUCLEOSIDES USING VINYL ESTERS

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5,6-Dihydro-5-azacytosine nucleosides exhibit some biological activity. These features could be enhanced by prodrug synthesis. We described an easy, fast and selective method for synthesis of prodrug forms using vinyl esters. The aminogroup of the 5,6-dihydro-5-azacytosine is acylated under mild conditions and without protecting of any other reacting groups in the molecule.

INTRODUCTION

5-Azacytosine nucleosides exhibit strong biological activity explained by their epigenetic mechanism of action[1,2]. 2′-Deoxy-5-azacytidine[3–5] and 5-azacytidine[6] were approved for the treatment of myelodysplastic syndromes. Other nucleoside analogs are being investigated in clinical trials[7–9]. 2′-Deoxy-5,6-dihydro-5-azacytidine exhibits additionally anti-HIV potency, especially if applied as prodrug form (Fig. 1)[10–12].

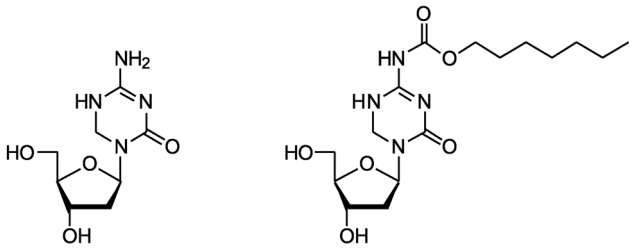

Fig. 1
2′-Deoxy-5,6-dihydro-5-azacytidine and its prodrug form

We have found that such acylation of amino group of 2′-deoxy-5,6-dihydro-5-azacytidine can be achieved by reaction with vinyl esters under mild conditions (DMF, 50 °C). This approach offers a selective way to synthesize lipophilic prodrugs of 5,6-dihydro-5-azacytosine nucleosides without protecting and deprotecting steps.

RESULTS AND DISCUSSION

5,6-Dihydro-5-azacytosine and its nucleosides were incubated at different temperatures in a presence of vinyl acetate, butyrate or palmitate. Desired compounds (Fig. 2) were isolated and characterized.

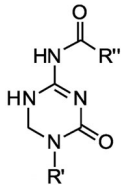

FIG. 2
General structure of prepared compounds (R′ = H, ribose, 2′-deoxyribose; R″ = CH_3, $CH_2CH_2CH_3$, $(CH_2)_{14}CH_3$)

It was found, that such structure can be formed at room temperature in good yields in 7 days. The temperature 50 °C was found as optimal – the reaction could be stopped after 2–3 days and only traces of site-products were observed. The reaction performed at 75 °C proceeded very fast (9–12 h), but the formation of undefined site-products led to more complex mixtures.

This work is a part of the research project of the IOCB AV0Z40550506. It was supported by the Centre of New Antivirals and Antineoplastics 1M0508, by the Ministry of Education, Youth and Sports of the Czech Republic and by Gilead Sciences, Inc. (Foster City, U.S.A.).

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SYNTHESIS AND PROPERTIES OF DINUCLEOTIDE CAP ANALOG FOR mRNA 5′ END LABELING

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Synthesis of dinucleotide cap analog functionalized with amino group at the 2′-position of 7-methylguanosine moiety has been accomplished. Subsequently, biotin activated *in situ* as NHS-ester has been efficiently attached to the 2′-NH_2 group. The biotin tag at the 2′-postion of 7-methylguanosine does not disturb cap's interaction with eukaryotic initiation factor 4E, moreover, such labeled analog is incorporated into mRNA during transcription *in vitro*. The modified transcripts are efficiently translated in rabbit reticulocyte lysate system. PAGE analysis has shown characteristic gel migration shift for biotin labeled transcript–streptavidin complex.

INTRODUCTION

All eukaryotic mRNAs possess at 5′ends 7-methylguanosine linked *via* 5′,5′ triphosphate bridge to the first transcribed nucleotide, which is called cap. This structure, unique for nucleic acids, fulfills important role in several steps of gene expression[1]. This makes synthetic cap analogs invaluable tools for studies of initiation of translation, mRNA turnover, intracellular transport and maturation[2]. In order to further facilitate studies on gene expression, in this article we present the first example of efficient labeling of mRNA 5′ end with biotin tag, by a combination of chemical and enzymatic methods.

RESULT AND DISCUSSION

A dinucleotide cap analog has been modified at the 2′-position of 7-methylguanosine with amino group as a position of biotin attachment. Previously, it has been shown that modification at the 2′-position of cap dinucleotide assures exclusive incorporation into mRNA chain during *in vitro* transcription and, additionally, it does not influence the interaction with eukaryotic initiation translation factor 4E (eIF4E)[3]. The synthesis has been performed as shown in Fig. 1. The starting material, 2′-amino-2′-deoxyguanosine (**1**), was phosphorylated with $POCl_3$ and, subsequently, the product (**2**) was methylated with methyl iodide. Interestingly, methylation occurred at the *N*7 position, but not at the 2′-NH_2 group. The product, $m^7$2′-NH_2GMP (**3**),

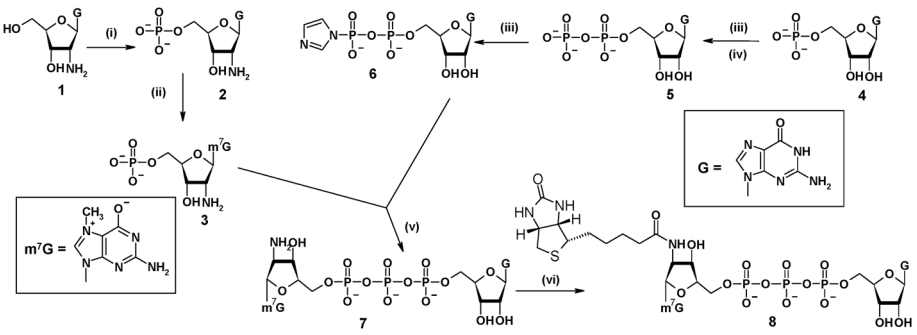

FIG. 1

Synthesis of biotin labeled cap analog: (i) 1. $POCl_3$, $PO(OCH_3)_3$ and 2. H_2O; (ii) CH_3I, DMSO; (iii) imidazole, 2,2′-dithiodipyridine, PPh_3, DMF; (iv) PO_4^{3-}/TEA salt, DMF, $ZnCl_2$; (v) DMF, $ZnCl_2$; (vi) biotin, TSTU, DMSO

was then coupled with P-imidazolide of GDP (**6**) in DMF with $ZnCl_2$ as a catalyst. The biotinylation of m^{7}2′-NH_2GpppG (**7**) was performed in 0.1 M borate buffer (pH 8.5) using NHS strategy. Biotin was converted *in situ* into active NHS-ester using TSTU, and then quantitatively coupled with aliphatic NH_2 group of **7**. The biotin labeled cap analog (**8**) was efficiently incorporated into mRNA by SP6 RNA polymerase. Although fluorescence studies have shown slight decrease in affinity of **8** for eIF4E, the transcripts were translated i*n vitro* with satisfactory efficiency (Table I). PAGE analysis confirmed interaction of the 5′-biotinylated transcript with streptavidin and thus proved usefulness of biotinylated cap analogs in biological studies (Fig. 2).

TABLE I

Biochemical properties of novel cap analogs

Cap analog	K_{as}, μM^{-1} [a]	Relative translation efficiency[b]
m^{7}GpppG	9.4 ± 0.4	1
m$^{7,2'OMe}$GpppG	10.2 ± 0.4	1.4 ± 0.20
m^{7}2′NH_2GpppG	5.58 ± 0.60	0.91 ± 0.10
m^{7}2′NHBiotGpppG	3.80 ± 0.10	1.01 ± 0.07

[a] Association constants of cap–eIF4E complexes determined by fluorescence quenching titration. [b] Efficiency of translation *in vitro* for capped transcript in rabbit reticulocyte lysate system.

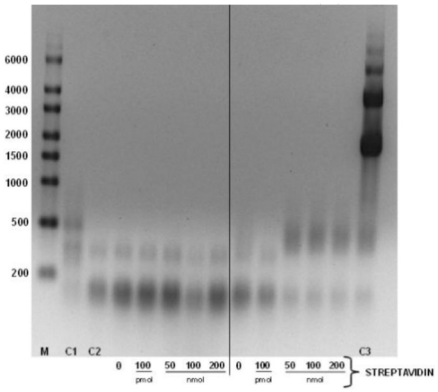

FIG. 2
PAGE analysis of 150-nt transcripts capped with m^7GpppG (left) or biotinylated cap (right) incubated with streptavidin

Financial support from Polish Ministry of Science and Higher Education (NN204 089438) is gratefully acknowledged.

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SYNTHESIS OF NUCLEOTIDE SUGARS AND NUCLEOSIDE 5′-PHOSPHOSULFATES BY $MgCl_2$ MEDIATED COUPLING

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Divalent metal cation mediated coupling of imidazolide derivatives with various phosphate nucleophiles have proved to be useful method allowing for versatile naturally occurring nucleotides and their analogues. Here, we present straightforward method for preparation of two classes of biologically important nucleotides: nucleotide shugars and nucleoside 5′-phosphosulfates. The procedure employs a rapid and efficient coupling of an appropriate nucleotide P-imidazolide with trialkylammonium salt of either sugar phosphate or sulfate (VI) in DMF and in the presence of $MgCl_2$ excess and subsequent purification by DEAE Sephadex. The preparative yields ranged from 80 to 100%.

INTRODUCTION

Nucleotide sugars fulfill important biological role as donors of activated monosaccharides in glycosylation reactions. They are useful to study specificity and mechanism of various glycosylases, hence, many methods have been developed for their preparation[1,2]. Adenosine 5′-phosphosulfate (APS), on the other hand, is a donor of activated sulfate moiety. It plays a central role in sulphur assimilation in some bacteria and plants, but has also been exploited for DNA pyrosequencing method[3]. Moreover, its 3′-phosphorylated derivative (PAPS) is involved in sulfate metabolism in mammals.

In this work we describe a rapid and very efficient method for the synthesis of nucleotide sugars and nucleoside 5′-phosphosulfates without any sugar or nucleobase protections. Employing this method we obtained adenosine, guanosine, uridine 5′-diphosphoglucose and 5′-diphoshogalactose (**1–4**) derivatives and various nucleoside 5′-phosphosulfates with isolated yields exceeding 85%.

RESULTS AND DISCUSSION

The synthesis of nucleotide sugars (**1–4**) and nucleoside 5′-phosphosulfates (**5–8**) is depicted in Fig. 1. An appropriate nucleoside 5′-monophosphate P-imidazolide was coupled in DMF either with glucose phosphate triethylammonium salt to produce nucleotide sugar or with bis(tributylammonium) sulfate (VI) to produce nucleoside 5′-phosphosulfate. The reactions were

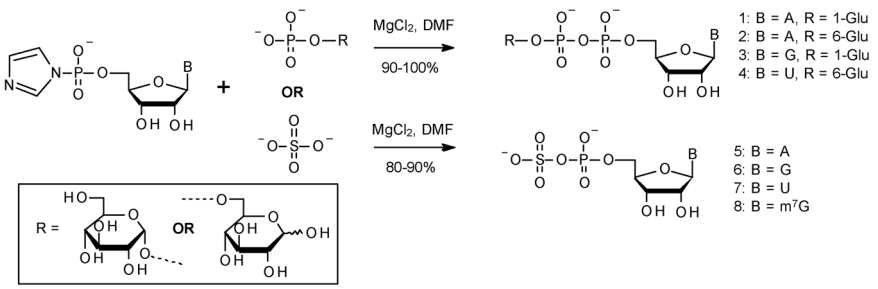

FIG. 1
Synthesis of compounds **1–8** by $MgCl_2$ mediated coupling in DMF

mediated by 10–16 fold excess of metal (II) chloride (Zn or Mg). In case of either mediator the reactions were very clean and fast (15–30 min for completion) as observed by RP HPLC (Fig. 2), however, in the reaction mixtures with $ZnCl_2$ a relatively fast, gradual hydrolysis of products to nucleoside monophosphates was observed, whereas in the presence of $MgCl_2$ products were fairly stable. The isolation by DEAE Sephadex and precipitation of sodium salts by $NaClO_4$ solution in acetone yielded desired compounds in excellent yields (>80%).

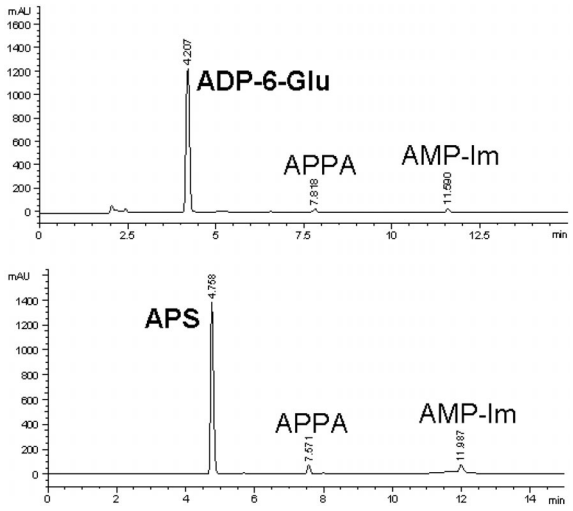

FIG. 2
RP HPLC profiles of $MgCl_2$-mediated synthesis of a) ADP-6-glucose and b) APS after 15 min from reaction start

Financial support from Polish Ministry of Science and Higher Education (IP2010020170 and NN204 089438) is gratefully acknowledged.

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SYNTHESIS OF NUCLEOTIDES BEARING OLIGOPYRIDINE LIGANDS AND THEIR INCORPORATION INTO DNA

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The synthesis of 7-deaza-dATPs and dCTPs bearing bipyridine or terpyridine ligands via acetylene tethers was accomplished through Pd-catalyzed cross-coupling reactions. Different DNA polymerases were used for their incorporation into DNA.

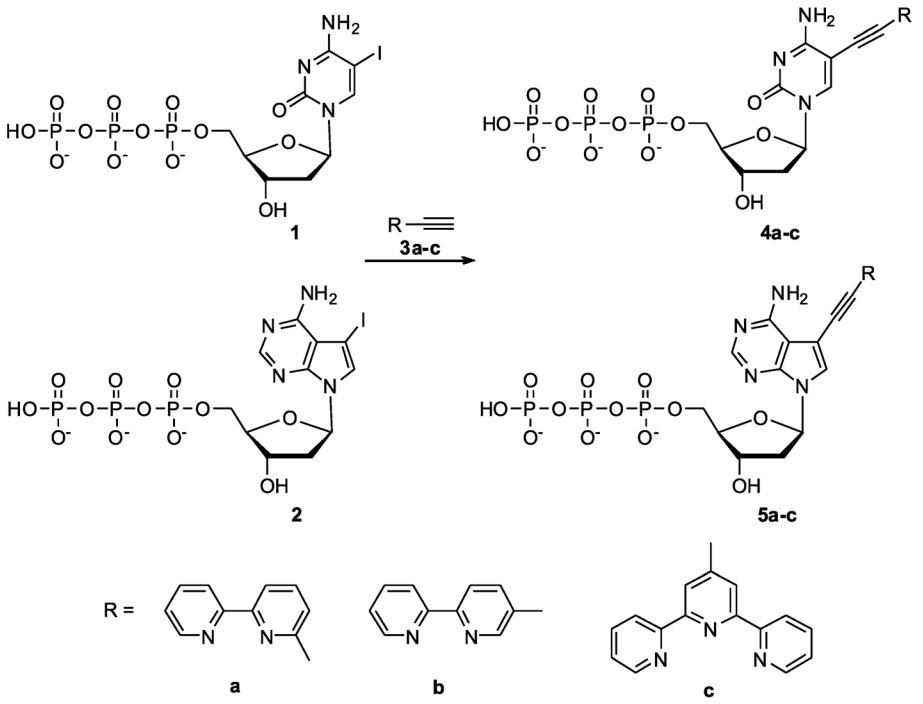
INTRODUCTION

Functionalization of DNA with diverse transition metals exert redox activity, photoactivity, magnetic and catalytic properties as well as enhanced stability[1,4]. Functionalized oligonucleotides can be prepared via enzymatic incorporation of modified nucleoside triphosphates bearing modifications in position 5 of pyrimidine or 7 of 7-deazapurine base, because they are substrates for DNA polymerases[2,3]. Recently we have reported on the synthesis of base-modified DNA labeled with $[Ru(bpy)_3]^{2+}$ and $[Os(bpy)_3]^{2+}$ [4]. Here we report on the synthesis of dNTPs bearing oligopyridine ligands and their enzymatic incorporation into DNA.

RESULTS AND DISCUSSION

Modified nucleoside triphosphates can be easily prepared in two steps. First step is phosphorylation of halogenated nucleosides[3] and the second step is the Sonogashira cross-coupling of like this prepared halogenated nucleoside triphosphates **1** and **2** with terminal acetylene **3a–3c** (Scheme 1). Reaction was performed in the presence of 5 mole % of $Pd(OAc)_2$, TPPTS, CuI and Hünig's base in the mixture water/acetonitrile (2:1). Reaction mixture was heated at 80 °C for 1 h. Desired products **4a–4c** and **5a–5c** were isolated in the yields from 40 to 67%.

These triphosphates were then incorporated into DNA by primer extension experiment, where different templates (designed for incorporation of either one or four modified triphosphates) and different polymerases (Vent (*exo*-), Pwo, DyNAzyme II, Phusion, KOD XL, Deep Vent and Deep Vent (*exo*-)) were tested.



Scheme 1
Shonogashira cross-couplingd of halogenated nucleosides triphosphates

This work is a part of the research projects Z4 055 0506 supported by the Academy of Sciences of the Czech Republic. It was specifically supported by the Ministry of Education, Youth and Sports of the Czech Republic (LC512), Czech Science Foundation (203/09/0317) and by Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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POLYMERASE SYNTHESIS OF MODIFIED DNA CONTAINING CYTOSINE IN MAJOR GROOVE

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(Cytosine-5-yl)ethynyl derivatives of pyrimidine and 7-deazaadenine 2-deoxyribonucleosides and nucleoside triphosphates (dNTPs) were prepared by aqueous Sonogashira coupling. All modified dNTPs were excellent substrates for DNA polymerases to give DNA bearing acetylene-linked cytosine(s) in the major groove.

INTRODUCTION

Editing enzymes such as DNA methyltransferases[1] or DNA glycosylases[2] use nucleotide-flipping mechanism in which a nucleobase is flipped out of the DNA duplex. We envisaged that the modification of DNA duplexes by acetylene-linked cytosine in major groove might mimic the flipped-out state. Here we report the synthesis of modified DNA by a two-step methodology based on cross-coupling of halogenated dNTPs followed by enzymatic synthesis of modified DNA by polymerase incorporation of modified nucleoside triphosphates (dNTPs)[3,4].

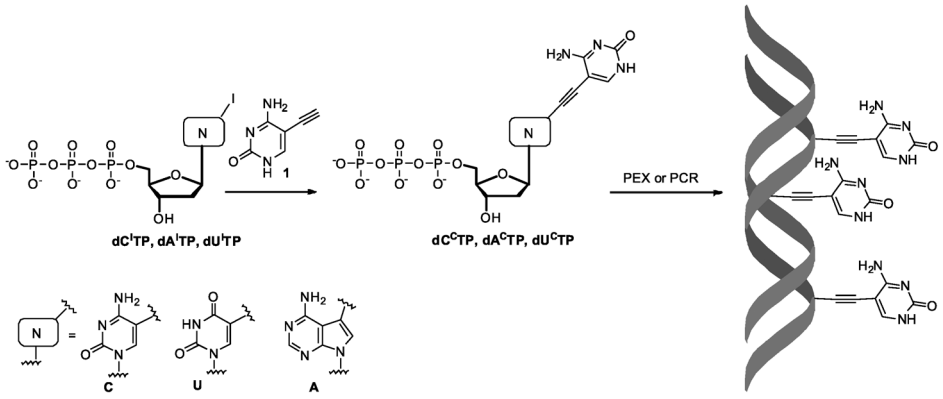

FIG. 1
Synthesis and incorporation of double-nucleobase nucleotide triphosphates

RESULTS AND DISCUSSION

The starting 5-ethynylcytosine[5] (**1**) was prepared by Sonogashira coupling of iodocytosine[6] with TMS-acetylene followed by the deprotection. The aqueous Sonogashira cross-coupling reactions of halogenated nucleosides and nucleoside triphosphates **dC^{I}**, **dA^{I}** and **dU^{I}** with **1** gave the desired modified nucleosides and **dC^{C}TP**, **dA^{C}TP** and **dU^{C}TP**.

All novel functionalized dNTPs – **dC^{C}TP**, **dU^{C}TP** and **dA^{C}TP** were successfully incorporated with *KOD XL* DNA polymerase in primer extension experiments (PEX) and polymerase chain reactions (PCR) using various templates with different number of modification in resulting DNA.

This work is a part of the research projects Z4 055 0506 supported by the Academy of Sciences of the Czech Republic. It was specifically supported by the Ministry of Education, Youth and Sports of the Czech Republic (LC512), Czech Science Foundation (203/09/0317) and by Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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CLICK CONJUGATION OF 4′-*C*-MODIFIED OLIGONUCLEOTIDES

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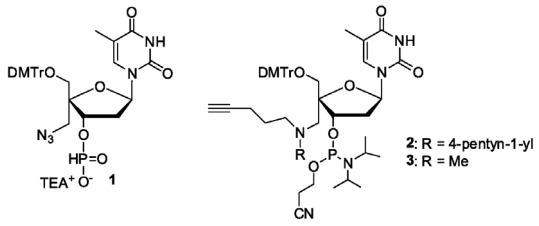
Azido and alkyne bearing 4′-modified thymidine building blocks, *i.e.* 4′-*C*-azidomethylthymidine 3′-(H-phophonate) (**1**) and 4′-*C*-[*N,N*-di(4-pentyn-1-yl)aminomethyl]thymidine and 4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine 3′-phosphoramidites (**2**, **3**) were synthesized and incorporated into 15mer oligodeoxyribonucleotide. These monomers enable the click conjugation in both ways, *i.e.* to have azido function present either on the oligonucleotide or ligand side. The conjugations on a solid support or in a solution were accomplished with derivatives of mannose, aminoglycosides and *p*-aminomethylbenzene. Melting temperature studies revealed that the 4′-position accommodates even rather large molecule clusters without appreciable destabilization of the duplex with DNA and 2′-*O*-Me RNA targets.

INTRODUCTION

4′-Modified oligonucleotides, in which the 4′-branch is facing the minor groove upon hybridization, are shown to form rather stable duplexes with unmodified complementary sequences and, additionally, they are less prone to enzymatic degradation than their unmodified counterparts[1]. To develop a conjugation method for 4′-glycoconjugates, potentially useful for delivery and targeting of therapeutic oligonucleotides, 4′-*C*-azidomethylthymidine 3′-(*H*-phophonate) (**1**) and 4′-*C*-[*N,N*-di(4-pentyn-1-yl)aminomethyl]thymidine and 4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]-thymidine 3′-phosphoramidite (**2**, **3**) monomers were prepared[2,3]. Using

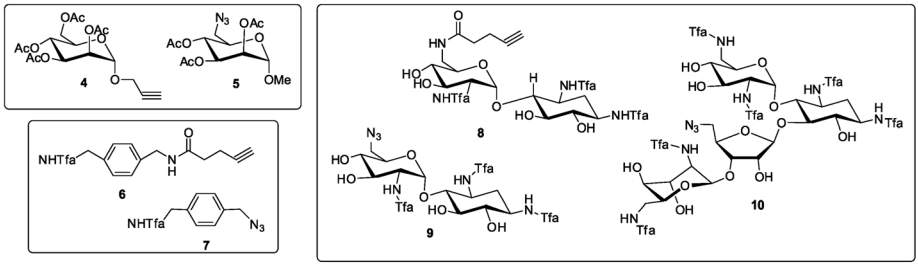


click chemistry, *i.e.* 1,3-dipolar cycloaddition of alkynes and azides[4], the oligonucleotides incorporating these building blocks can be functionalized with alkynyl or azido derivatized ligands. Nucleoside **2** bearing two 4-pentyn-1-yl groups is designed for high density functionalization of oligonucleotides whereas nucleoside **1** and **3** allows one-armed conjugations.

Hybridization properties of the mannose, aminoglycoside and *p*-aminomethylbenzene conjugates were assessed by UV-melting studies.

RESULTS AND DISCUSSION

4′-*C*-Branch was introduced to thymidine as previously reported[5]: 5′-OH was oxidized to aldehyde and 4′-*C*-hydroxymethyl group was achieved by aldol condensation with formaldehyde followed by Cannizzaro reduction. Using triflate as a leaving group, the 4′-position was azidated with NaN_3 to afford 4′-*C*-azidomethyl thymidine[2]. For alkyne derivatives, the 4′-*C*-azido function was converted to amino group by Staudinger reaction and the amino group was reductively alkylated[3]. In the presence of $NaBH_3CN$, 4′-*C*-[*N*,*N*-bis(pentyn-4-yl)aminomethyl]thymidine was received using 2 eq of 4-pentynal and 4′-*C*-[*N*-methyl-*N*-(pentyn-4-yl)aminomethyl]thymidine was prepared by consecutive alkylation steps with 4-pentynal and formaldehyde. After tritylation of the 5′-hydroxyls, the nucleosides were converted to *H*-phosphonate (**1**) and phosphoramidite (**2**, **3**) monomers to be used on an automated DNA synthesizer.



Monomer **1** was coupled to the oligonucleotide using *H*-phosphonate chemistry since intramolecular Staudinger reaction between the azido and phosphoramidite group makes the phosphoramidite building block highly unstable[2]. One to three modified units were incorporated into central part of the oligodeoxyribonucleotide 5′-CAT CTG GTT CTA CGA-3′ and click conjugated with mannose (**4**, **5**), *p*-aminomethylbenzene (**6**, **7**) and aminoglycoside (**8–10**) derivatives on a solid support or in a solution.

The influence of the 4′-conjugations on duplex stability with DNA and 2′-*O*-methyl RNA targets was studied. The melting temperatures observed for the oligonucleotides containing 4′-*C*-(alkynylaminomethyl)thymidine units (**2**, **3**) and their conjugates are higher than those observed for their 4′-*C*-azidomethylthymidine (**1**) counterparts. This implies that the alkyl

chain allows a more flexible alignment of the triazole ring and conjugate groups upon hybridization. Also the tertiary amino group, which probably is protonated under physiological conditions, further stabilizes the duplex formation. Generally, the results were encouraging for glycotargeting consept.

This work is supported by the Academy of Finland

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SYNTHESIS AND EVALUATION OF 5-SUBSTITUTED-2′-DEOXYURIDINE MONOPHOSPHATE ANALOGUES AS INHIBITORS OF FLAVIN-DEPENDENT THYMIDYLATE SYNTHASE IN *Mycobacterium tuberculosis*

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A series of 5-substituted-2′-deoxyuridine monophosphate analogues has been synthesized and evaluated for its inhibitory activity and selectivity on thyX, a flavin-dependent thymidylate synthase in *Mycobacterium tuberculosis*. Of the synthesized compounds, derivatives **2** and **3** were the most active. In addition, derivative **2** bearing an *N*-(prop-2-ynyl)-octanamide side chain shows low activity on the classical thymidylate synthase, thyA.

INTRODUCTION

Tuberculosis (TB) is the leading cause of death among adults worldwide. According to the WHO[1], around 1.7 million people died from TB in 2010 with 98% of deaths have been reported in developing countries where HIV-coinfection plays an important role. Currently, patients with active TB are treated in a six-month regimen consisting of either isoniazid, rifampin, pyrazinamide and either streptomycin or ethambutol. The emergence of multi-drug resistant (MDR) and extremely multi-drug resistant (XDR) strains is considered as a major threat to global health and new treatment strategies are required[2].

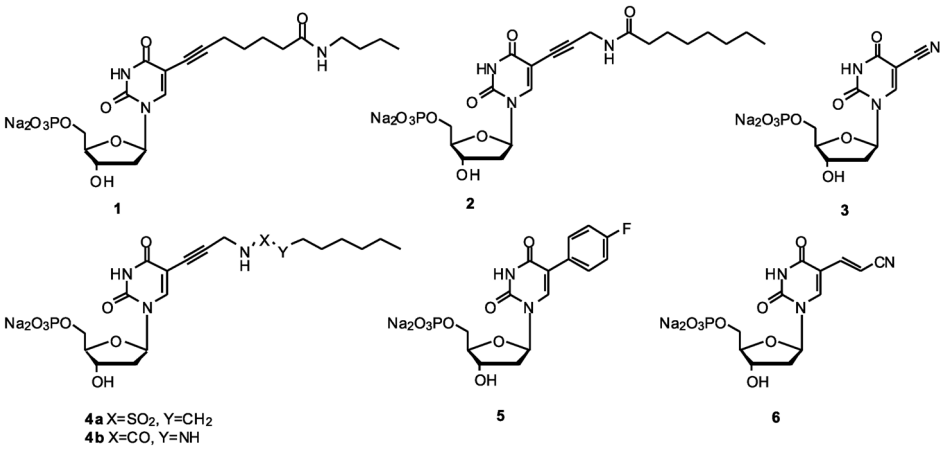
A number of pathogenic microorganism solely depend on thyX[3], a novel flavin-dependent thymidylate synthase (FDTS) which has been discovered a few years ago and is absent in the human genome. Interestingly, the genome of *Mycobacterium tuberculosis* contains the genes encoding for both, thyX and the classical thymidylate synthase thyA, but it is yet unclear whether either of the two genes is essential.

Based on the available biochemical reaction mechanism and the crystal structure[4] of Mtb thyX the aim of this study was to design, synthesize and to evaluate selective thyX-inhibitors which show no activity on thyA.

RESULTS AND DISCUSSION

It is well known that 5-alkynyl deoxy and arabinonucleosides show good antimycobacterial activity in a cellular assay[5]. These results prompted us to investigate whether 5-substituted deoxyuridine monophosphates are capable of inhibiting thyX.

Title compounds **1** to **6** have been prepared *via* classical Heck, Sonogashira or Suzuki–Miyaura coupling reactions, respectively, starting from protected or unprotected 5-iodo-2′-deoxyuridine. Deprotection (if required) followed by Yoshikawa phosphorylation furnished our desired derivatives (Scheme 1).



SCHEME 1
Designed and synthesized inhibitors of FDTS

The synthesized compounds show good levels of inhibition of Mtb-thyX with derivatives **2** and **3** having the lowest IC_{50}-values of 0.9 and 0.13 μM, respectively. It has to be pointed out, however, that 5-cyano dUMP **3** is a known inhibitor of the classical TS in *L. casei* [6]. On the other hand, derivative **2** bearing a *N*-(prop-2-ynyl)octanamide side chain exhibits only very low levels of Mtb-thyA-inhibition.

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DEVELOPMENT OF TETRAMERIC NAPHTHYRIDINE DERIVATIVES FOR DNA AND RNA CONTAINING A G-G MISMATCH

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Naphthyridine carbamate dimmer (**NCD**) is a mismatch binding ligand that displays high binding affinity and selectivity for DNA G-G mismatch. We herein report the design and synthesis of a series of tetrameric naphthyridine derivatives (**NCTX**s), which consist of two **NCD**s connected with various linkers. We revealed the influence of the various linkers on the **NCTX**-DNA complex formation. Since *cis*-stilbene linker of (***Z***)-**NCTS** makes the base-recognition units pre-organized in the complex formation, (***Z***)-**NCTS** showed the highest binding affinity with both of DNA and RNA mismatches by the entropically favorable complex formation.

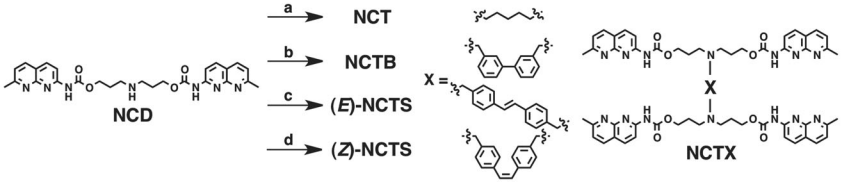
INTRODUCTION

We have developed a series of synthetic ligands which selectivity bind to characteristic local structures of DNA, such as DNA bulges, mismatched base pairs, and trinucleotide repeats[1]. Mismatch binding ligands (**MBL**s) selective for G-G mismatch have *N*-acyl-2-amino-1,8-naphthyridine moieties as base-recognition units, which can recognize a guanine base with hydrogen bondings. Naphthyridine carbamate dimer (**NCD**) containing two naphthyridine moieties is one of the highest-affinity ligands for G-G mismatch. We previously reported that the complex formation between two **NCD**s and DNA containing a single G-G mismatched site, 5′-CGG-3′/5′-CGG-3′, resulted in significant stabilization of the DNA duplex[2]. The binding stoichiometry for the **NCD**-DNA complex is 2:1, since four naphthyridine moieties are needed for the recognition of four guanine bases in the CGG/CGG. Binding with a 1:1 stoichiometry is expected to be more favorable for the stable complex formation in terms of the entropic factor. We herein report the design and synthesis of new tetrameric **MBL**s which selectivity bind to the G-G mismatched site with a 1:1 stoichiometry (Fig. 1a).

RESULTS AND DISCUSSION

We synthesized a series of tetrameric naphthyridine derivatives (**NCTX**s), which consist of two **NCD** molecules connected with various linkers such

as five methylenes (**NCT**), biphenyl (**NCTB**), *cis*-stilbene ((***Z***)-**NCTS**), and *trans*-stilbene ((***E***)-**NCTS**) (Scheme 1). These ligands have different conformational flexibility depending on the linkers, which is an important factor for thermodynamics of the **NCTX**-DNA complex formation.



SCHEME 1

Synthesis of **NCTX**s. (a) glutaraldehyde, $NaBH_3CN$, MeOH, $CHCl_3$, AcOH, 23%; (b) 3,3′-bis-(bromomethyl)biphenyl, KI, K_2CO_3, dry DMF, 34%; (c) (*E*)-4,4′-bis(bromomethyl)stilbene, dry DMF, 18%; (d) (*Z*)-4,4′-diformylstilbene, $NaBH_3CN$, MeOH, $CHCl_3$, AcOH, 18%

Binding of **NCTX**s with a DNA duplex containing a G-G mismatch was evaluated by melting temperature (T_m). T_m values of an 11-mer duplex (**ODN1**/**ODN2**), 5′-(CTAA CGG AATG)-3′/5′-(CATT CGG TTAG)-3′, were measured in the absence and presence of tetrameric ligands (Fig. 1b). All **NCTX**s except for (***E***)-**NCTS** selectively stabilized the G-G mismatch DNA duplex, and, especially, (***Z***)-**NCTS**-DNA complex provided the highest T_m value among the tested ligands. Cold-spray ionization time-of-flight mass spectrometry (CSI-MS) measurements revealed that this efficient stabilization was attributed to the complex formation with a 1:1 stoichiometry. We also tested bindings of **NCTX**s with RNA mismatches, and revealed that (***Z***)-**NCTS** showed the highest binding affinity also with the RNAs. Since

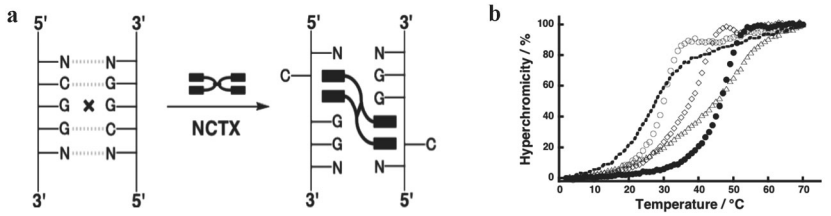


FIG. 1

(a) Schematic illustration of the binding of **NCTX**s to a mismatched site with a 1:1 stoichiometry. (b) Thermal melting profiles of DNA duplex **ODN1**/**ODN2** (4.5 μM) in the absence and presence of **NCTX** (9.1 μM). The absorbance at 260 nm was measured in 10 mM Na cacodylate buffer (pH 7.0) containing 100 mM NaCl and 0.1% (v/v) Tween 20. Plots in the absence of **NCTX**, dashed line; **NCT**, open triangle; (***E***)-**NCTS**, open triangle; (***Z***)-**NCTS**, closed circle

cis-stilbene linker of (***Z***)-**NCTS** makes the base-recognition units pre-organized in the complex formation, the strongest binding of (***Z***)-**NCTS** is most likely explained by the entropically favorable complex formation.

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SYNTHESIS AND BIOLOGICAL PROPERTIES OF THE 2′-TRIFLUORO-METHYL ANALOGUES OF TENOFOVIR

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A series of acyclic nucleoside phosphonates substituted at the aliphatic part of the molecule by the trifluoromethyl group was prepared and evaluated for their biological properties. Surprisingly, none of the prepared phosphonic acids exhibits any anti-HIV or anti-HCV activities.

INTRODUCTION

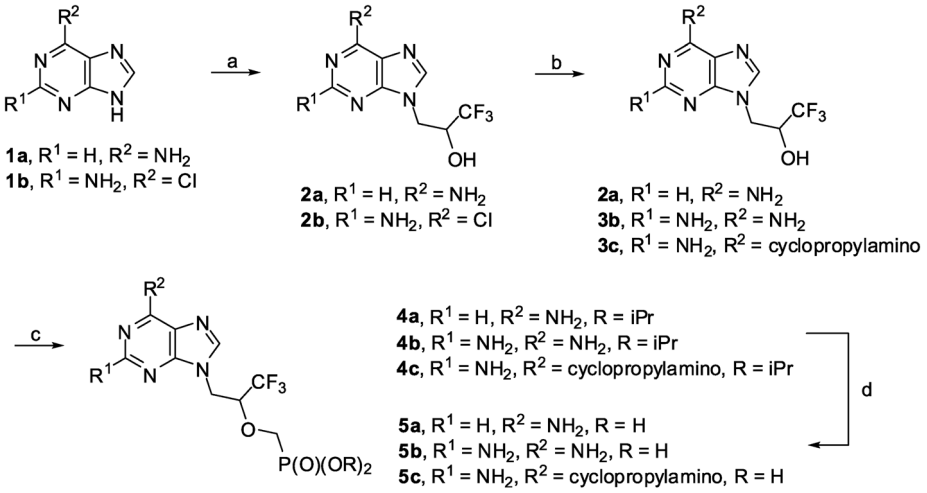
Acyclic nucleoside phosphonates[1] (ANPs) are nucleotide analogues with a wide range of biological activities, especially antiviral[2], antiparasitic[3], and cytostatic[4]. Tenofovir disoproxil fumarate (Viread), as a prodrug of tenofovir (PMPA), was approved by FDA for the treatment of HIV infection. The aim of this SAR study was to replace the 2′-methyl group in the structure of tenofovir with the more polar trifluoromethyl group.

RESULTS AND DISCUSSION

As the former synthesis of the trifluoromethylated analogues of PMPA *via* alkylation of purines with 3-bromo-1,1,1-trifluoropropan-2-ol was shown to be inefficient[5], new methodology based on alkylation of purine derivates with trifluoromethyloxirane was used in the present work. This methodology was previously used for the preparation of pyrimidine containing ANPs[6].

Alkylation of adenine (**1a**) or 2-amino-6-chloropurine (**1b**) with trifluoromethyloxirane afforded the corresponding 1,1,1-trifluoropropan-2-ol intermediates **2** (Scheme 1). The treatment of the intermediates **2** with ammonia or cyclopropylamine afforded compounds **3**. In order to obtain the phosphonate derivatives **5**, the compounds **3** were reacted with diisopropyl bromomethylphosphonate[7] and subsequently hydrolyzed under the standard conditions using Me_3SiBr/MeCN.

All prepared compounds were screened for their antiviral activities. Surprisingly, none of the phosphonic acids **5** exhibits any anti-HIV or anti-HCV activities.



SCHEME 1

a) trifluoromethyloxirane, Cs_2CO_3/DMF; b) NH_3/EtOH or cyclopropylamine/MeCN; c) $BrCH_2P(O)(OiPr)_2$, NaH/DMF; d) Me_3SiBr/MeCN

This work is a part of the research project AVOZ40550506 of the Institute of Organic Chemistry and Biochemistry and was supported by Centre for New Antivirals and Antineoplastics (1M0508). This study was also supported by Gilead Sciences (Foster City, CA, USA).

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PIPERIDINE NUCLEOSIDE PHOSPHONIC ACID DERIVATIVES

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A series of novel phosphonic acid derivatives of piperidine nucleosides was prepared and synthesis of both enantiomeric and diastereomeric derivatives is discussed. Preparation of diphosphate analogs of phosphonic acids derivatives is also described. The prepared nucleotide analogs were tested for their potential biological properties.

INTRODUCTION

Among modified nucleosides and nucleotides there are many structures exhibiting interesting biological properties. They could act either as inhibitors of nucleotide processing enzymes or in the form of triphosphates as terminators of DNA or RNA polymerisation. In the later case, to become active, nucleosides must be phosphorylated by cellular kinases to give the corresponding 5′-mono-, -di-, and -triphosphates. Phosphonate analogues which mimic nucleoside monophosphates do not require the first intracellular phosphorylation (often the rate limiting step) necessary for activation of nucleosides. Several types of phosphonic acid derivatives were prepared in our group including prolinol[1], and pyrrolidine[2,3] based derivatives. Four azanucleotide compounds **1–4** were found active against SD rat T-cell lymphoma thymidine phosphorylase (Fig. 1).

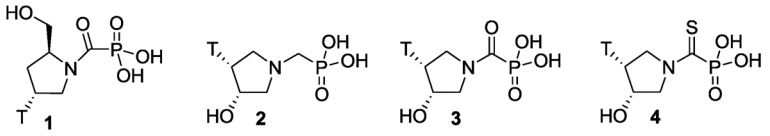


FIG. 1

RESULTS AND DISCUSSION

In the course of our previous work we synthesized piperidine nucleosides[4] (Fig. 2), which served as starting compounds in the preparation of desired piperidine nucleotides.

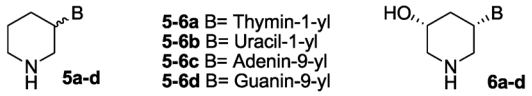
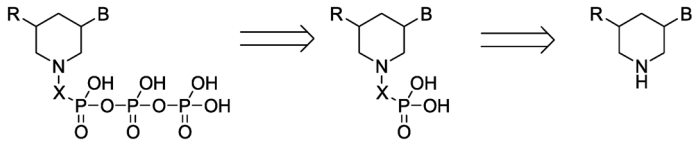


FIG. 2

The reaction of the piperidine ring nitrogen atom with several phosphonylation reagents led to novel *N*-phosphonocarbonyl, *N*-phosphonoacetyl, *N*-phosphonothiocarbonyl, *N*-phosphonomethyl and *N*-phosphonoethyl nucleotide analogs. Synthetic strategy is indicated in the Scheme 1.



SCHEME 1
R = OH, H; B = nucleobase, X = various

For the introduction of various phosphonate moieties, phosphonylation reagents were synthesized according to published procedures. The phenyl diisopropylphosphono-formate was prepared *via* the Arbuzov reaction, from phenyl chloroformate and triisopropylphosphite[5]. The methyldiisopropylphosphono-dithioformate was prepared from diisopropyl phosphite and carbon disulfide, followed by treatment with methyl iodide[6]. Diisopropyl 2-phosphonoacetic acid was prepared *via* the Arbuzov reaction, from triisopropyl phosphite and ethyl 2-chloroacetate[7]. Phosphonomethyl function was introduced by Mannich reaction of piperidine nucleosides with aqueous formaldehyde and diisopropyl phosphite. Phosphonoethyl piperidine derivatives were prepared by Michael addition of piperidine nucleosides to diethyl vinylphosphonate.

Chosen analogs of nucleotides were then transformed into the diphosphate derivatives of phosphonic acids representing nucleoside triphosphate analogs. These NTP analogues has been tested as potential inhibitors of RNA polymerase from *E. coli*.

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CYANOETHYL DERIVATIVES OF PHOSPHATE AND THIOPHOSPHATE – NEW REAGENTS FOR EFFICIENT SYNTHESIS OF PHOSPHATE MODIFIED NUCLEOTIDES

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We present two simple, phosphate-derived reagents **1a** and **1b**, which allow for oligophosphate chain elongation of nucleotides bearing terminal thio-, seleno-, boranophosphate or (methylenebis)phosphonate moieties. We employed these new reagents for the synthesis of nucleoside 5′-(1-boranodiphosphates), (2-thiotriphosphates), (2-selenotriphosphates), (1,2-dithiodiphosphates) and other synthetically challenging nucleotides.

INTRODUCTION

Nucleotide analogs modified within (oligo)phosphate moieties serve as tools for enzymes' structural and functional studies and as more stable counterparts of natural compounds, which often have potential applications in biotechnology and medicine. Here, we present a new method for introducing phosphate or thiophoshate group into terminal position of variously P-modified 5′-nucleotides. The method employs coupling of imidazole-activated and 2-cyanoethyl-protected (thio)phosphate (reagent **1a** or **1b**) with an appropriate modified nucleotide in the presence of Mg, Zn or Mn(II) chloride in DMF and a subsequent removal of the cyanoethyl group in basic conditions. The main advantage of using reagents **1a** and **1b** is that they enable avoiding problematic P-activation of phosphate-modified nucleotides. To demonstrate usefulness of this approach we synthesized several nucleoside di- and triphosphates modified with a single or double phosphorothioate, phosphoroselenoate or boranophosphate groups and nucleoside tri- and tetraphosphates modified with (methylenebis)phosphonate moiety.

RESULTS AND DISCUSSION

Reagent **1a** was synthesized from commercially available barium (2-cyanoethyl)phosphate by a conversion into triethylammonium salt and activation with imidazole employing triphenylphosphine/dithiodipyridine activation system (Fig. 1). The product was easily isolated from reaction

mixture by precipitation with lithium perchlorate solution in acetone. Reagent **1b** was synthesized analogously from (S-cyanoethyl)phosphorothiolate sodium salt[1].

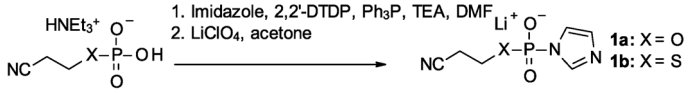

FIG. 1
Synthesis of reagents **1a** and **1b**

These reagents were then employed for the synthesis of various modified nucleotides (Fig. 2). Coupling **1a** with adenosine or guanosine 5′-(2-thiodiphosphates)[2] in DMF in the presence of 16-fold anhydrous $ZnCl_2$ or $MgCl_2$, yielded corresponding (2-cyanoethyl)-protected nucleoside 5′-(2-thiotriphosphates) with excellent HPLC yields (>90%). Subsequent deprotection with DBU at 50 °C for 3–6 h and purification by DEAE Sephadex afforded nucleoside 5′-(triphosphates) with 60–-70% isolated yields. Similar

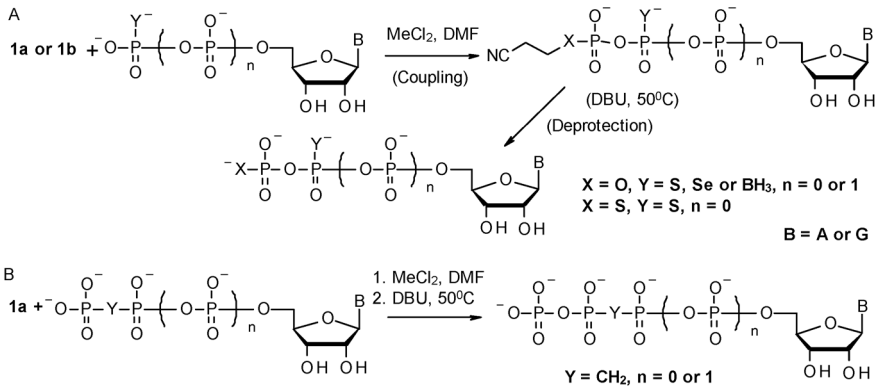

FIG. 2
Syntheses of modified nucleoside di-, tri- and tetraphosphates employing reagents **1a** and **1b**

approach was used to obtain nucleoside 5′-(1-thiodiphosphates), 5′-(2-selenotriphosphates), 5′-(1-boranodiphosphates) and 5′-(2-boranotriphosphates) with good isolated yields (50–70%). Reagent **1b** was so far employed for the synthesis of nucleoside 5′-(1,2-dithiodiphosphates) from corresponding 5′-phosphorothioates. We also show that reagent **1a** can be beneficial for the synthesis of some (methylenebis)phosphonate derivates. The synthesis of nucleoside 5′-(1,2-methylenetriphosphates) and 5′-(2,3-

methylenetetraphosphates) is much more efficient by using reagent **1a** and an appropriate modified nucleotide than by the previously reported route[3].

Financial support from Polish Ministry of Science and Higher Education (IP2010020170) is gratefully acknowledged.

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SYNTHESIS AND PROPERTIES OF NEW THIO-SUBSTITUTED mRNA CAP ANALOGS

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We report synthesis and preliminary properties of two new anti-reverse cap analogs modified by oxygen to sulphur substitution at various positions of dinucleotide cap structure which appear to be beneficial for applications based on efficient mRNA expression.

INTRODUCTION

Analogs of eukaryotic mRNA 5′ end (cap) are useful tools for studying mechanism of protein translation and mRNA degradation and have application in biotechnology and, potentially, medicine. One of the most significant applications is their use as reagents for preparation of 5′-capped mRNAs by transcription *in vitro* catalyzed by bacteriophage (T7, T3, SP6) RNA polymerase. This method gives also unique possibility to prepare mRNAs chemically modified within their 5′ ends.

Previously, we have developed a series of mRNA cap analogs modified with phosphorothioate moieties at different positions of 5′,5′-triphosphate bridge[1,2]. Some of these analogs were additionally modified with methyl group at the 2′-*O* position of 7-methylguanosine to ensure exclusively correct incorporation into mRNA during transcription *in vitro* (so called anti-reverse cap analogs, ARCA). The ARCA analogs modified at the β position of 5′,5′-triphosphate bridge notably increased mRNA stability and translation efficiency both in cultured cells and *in vivo*, which makes them especially beneficial for applications such as mRNA-based gene therapy[2,3].

Here, we describe two new anti-reverse cap analogs (**1** and **2**) designed to further explore potential advantages of oxygen to sulfur substitution (Fig. 1): analog **1** bearing phosphorothioate group in the 5′,5′-triphosphate bridge and 2′,3′-isopropylidene group[4] instead of usual 2′- or 3′-methoxyl group and analog **2** bearing 6-thioguanine (^{6S}G) as a second nucleobase and 2′-*O*-methyl ARCA modification.

RESULTS AND DISCUSSION

The synthesis of new cap analogs depicted in Fig. 1 was accomplished by previously developed approach[1,5]. The final $ZnCl_2$ mediated coupling leading to analog **2** was monitored by HPLC at 260 nm and 340 nm (λ_{max} for ^{6S}Guo). Both analogs were isolated with ~60% yield. The analog **1** existed in form of two P-diastereomers (**1a** and **1b**), which were resolved by preparative RP HPLC and named D1 and D2 (due to the elution order).

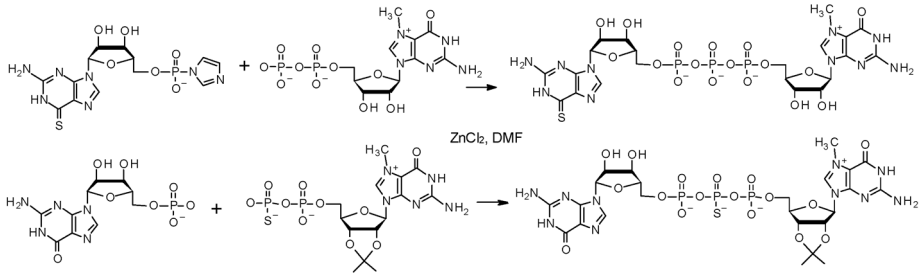

FIG. 1
Syntheses of sulfur containing ARCA dinucleotides

The results of fluorescence quenching titration of new analogs compared with their parent compounds are collected in Table I. Binding affinities of **1a**, **1b** and **2** for translation initiation factor 4E were higher than for unmodified counterparts. Although the bulky 2′,3′-isopropylidene group generally decreased the affinity of cap analogs for eIF4E, the translation

TABLE I
Association constants of cap-eIF4E complexes determined by fluorescence quenching titration

Cap analog	K_{as}, μM^{-1} [a]
m^7GpppG	9.4 ± 0.4
$m^{7,2'OMe}$GpppG	10.2 ± 0.4
m^7*i*PrGpppG	2.9 ± 0.4
m^7*i*PrGpp$_s$pG (D1)	5.5 ± 0.1
m^7*i*PrGpp$_s$pG (D2)	9.2 ± 0.2
$m^{7,2'OMe}$Gppp^{68}G	16.7 ± 0.8

efficiency of mRNAs capped with **1a** and **1b** was not lower than for $m^{7,2'\text{-OMe}}$GpppG-mRNA. 6-Thioguanosine present as a first base of transcript also increased translation efficiency of mRNA. These finding confirm that analogs **1** and **2** may be beneficial for applications based on efficient mRNA expression. Finally, analogue **2** may be employed in photo-cross-linking experiments (λ = 340 nm).

Financial support from Polish Ministry of Science and Higher Education (NN301 096339 and NN204 089438) is gratefully acknowledged.

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9-[2-HYDROXY-3-(PHOSPHONOMETHOXY)PROPYL] ("iso-HPMP") DERIVATIVES OF PURINE BASES AND THEIR SIDE-CHAIN MODIFIED ANALOGUES: SYNTHESIS AND ANTIMALARIAL ACTIVITY

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Hypoxanthine and guanine 9-[2-hydroxy-3-(phosphonomethoxy)propyl] derivatives substituted in 2′-position with short polar groups containing carbon chains (CH_2COOEt, CH_2COOH, CH_2CONH_2) were prepared and studied as potential inhibitors of a key enzyme of the malarial parasite *Plasmodium falciparum Pf*HGXPRT.

INTRODUCTION

Purine acyclic nucleoside phosphonates (ANPs) are potent inhibitors of hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT), a key enzyme of purine salvage pathway of the malarial parasite *Plasmodium falciparum*[1]. Based on these data, a new series of ANPs, 2-hydroxy-3-(phosphonomethoxy)propyl ("iso-HPMP") derivatives[2], were synthesized.

RESULTS AND DISCUSSION

Two "iso-HPMP" derivatives were synthesized: 9-[2-hydroxy-3-(phosphonomethoxy)propyl]hypoxanthine (**1**) and 9-[2-hydroxy-3-(phosphonomethoxy)propyl]guanine (**2**) (Fig. 1). These compounds were tested as possible inhibitors of human HGPRT and *Plasmodium falciparum* HGXPRT.

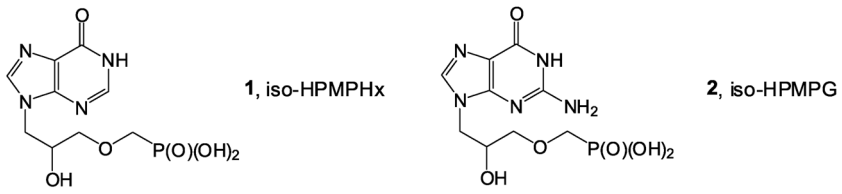
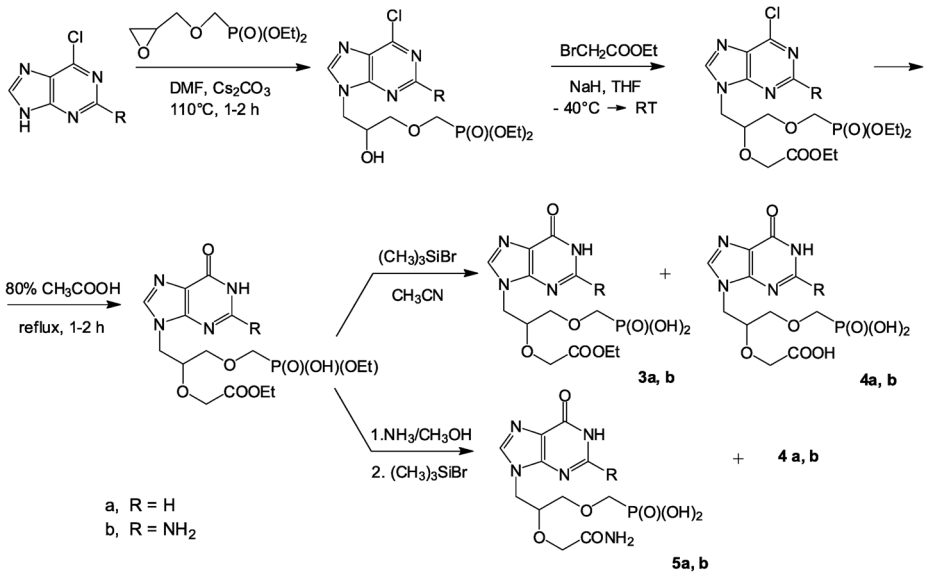


FIG. 1
Structures of iso-HPMPHx (**1**) and iso-HPMPG (**2**)

The hypoxanthine derivative **1** inhibited *Pf*HGXPRT with K_i = 1.9 μM; no inhibition of human HGPRT was observed. The addition of pyrophosphate resulted in a decrease of the K_i value for *Pf*HGXPRT to 0.3 μM[3]. This suggests that the addition of polar groups and/or elongation in 2′-position could decrease K_i values. On the basis of this hypothesis, derivatives **3a**, **3b–5a**, **5b** were prepared (Scheme 1). Antimalarial screening of these new structures is under way.



SCHEME 1

This work was performed as a part of the Research program of IOCB AV0Z40550506. It was supported by the Center for New Antivirals and Antineoplastics 1M0508 by the Ministry of Education, Youth and Sports of the Czech Republic, the Grant Agency of the Czech Republic (grant No. P207/11/0108), funds from the National Health and Medical Research Council, Australia (grant No. 569703) and by Gilead Sciences & IOCB Research Centre.

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NUCLEOSIDES CONTAINING 8-AZA-7,9-DIDEAZAXANTHINE

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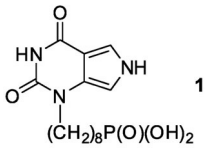
8-Aza-9-carboxy-7,9-dideaza-3-(β-D-ribofuranosyl)- and -3-(β-D-galactopyranosyl)xanthine were prepared from 5-nitrouracil by a Vorbrüggen glycosylation with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose and 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose, respectively, followed by a Barton–Zard reaction with benzyl isocyanoacetate, and deprotection.

INTRODUCTION

Pyrrolo[3,4-*d*]pyrimidines (8-aza-7,9-dideazapurines) were not so far exploited for preparation of analogs of nucleosides and nucleotides except for 3-(8-phosphonooctyl)-8-aza-7,9-dideazaxanthine (**1**, Chart 1) and some its congeners, that we found to be potent inhibitors of bacterial as well as human thymidine phosphorylase[1,2]. Our aim was to prepare some 8-aza-7,9-dideazaxanthine nucleosides and investigate their potential biological activity.



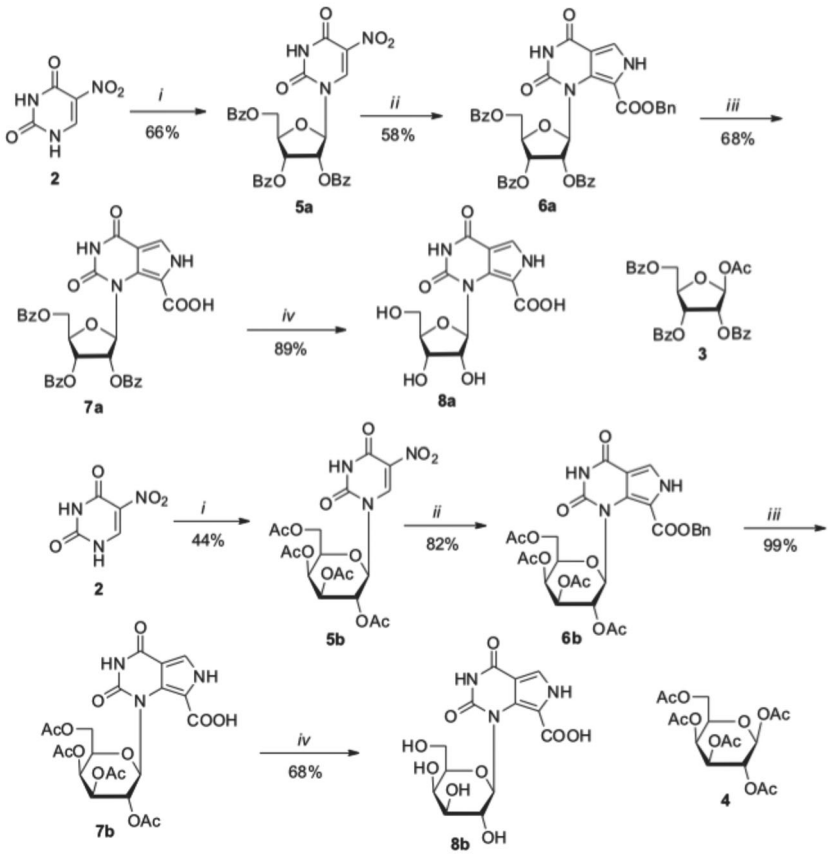
1

CHART 1

RESULTS AND DISCUSSION

The synthesis started from 5-nitrouracil (**2**) which was glycosylated according to Vorbrüggen with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (**3**) and 1,2,3,4,6-penta-*O*-β-D-galactopyranose (**4**), respectively, affording the protected nucleoside analogues **5a**, **5b**. Compounds **5a**, **5b** were treated with benzyl isocyanoacetate according to the literature[3] under the conditions of a Barton–Zard reaction[4] to form the pyrrolo[3,4-*d*]pyrimidines **6a**, **6b**. Catalytic hydrogenation of compounds **6a**, **6b** afforded free carboxylic acids **7a**, **7b**, which were subsequently deprotected by the treatment with sodium methoxide to nucleoside analogs **8a**, **8b**.

The final compounds **8a**, **8b** could be considered as analogs of orotidine, however, no antitumor or antiviral activity was found.



SCHEME 1

(i) 1. BSA, DCM, 10 min; 2. **3** or **4**, $SnCl_4$, 0 °C, 1 h; 3. $NaHCO_3$, MeOH, ice. (ii) Benzyl isocyanoacetate, DBU, dioxane, overnight. (iii) H_2/Pd, MeOH–AcOH (9:1). (iv) MeONa/MeOH

This work is a part of the research project of the Institute No. Z40550506. It was supported by the Centre for New Antivirals and Antineoplastics 1M0508 by the Ministry of Education, Youth and Sports of the Czech Republic and by Gilead Sciences, Inc. (Foster City, USA). The authors' thanks are due to Dr. P. Fiedler for the IR spectra, the staff of the mass spectroscopy (Dr. J. Cvačka, Head) and analytical departments (Dr. S. Matějková, Head) of the Institute.

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COFACTOR-LINKED RNAs

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Recently, covalent conjugates of RNA and two well-known enzyme cofactors (NAD – nicotinamide adenine dinucleotide and CoA – coenzyme A) were discovered in the bacteria *Escherichia coli* and *Streptomyces venezuelae*. The methodology used in these studies did not allow the exact determination of length, sequence and function of these conjugates. Our new approach should lead to the synthesis of model cofactor-RNA linked conjugates and the development of the capturing techniques involving the tools of organic chemistry and biochemistry for the further characterization of these molecules.

INTRODUCTION

Very recently, an unexpected and surprising modification of RNA was discovered: RNA conjugates with covalently bound derivatives of CoA (Coenzyme A)[1] and NAD (Nicotinamide adenine dinucleotide)[2] (Fig. 1) and related small molecules indicate new roles of RNA conjugates in biological systems. Both these conjugates were detected in *Escherichia coli* and *Streptomyces venezuelae*.

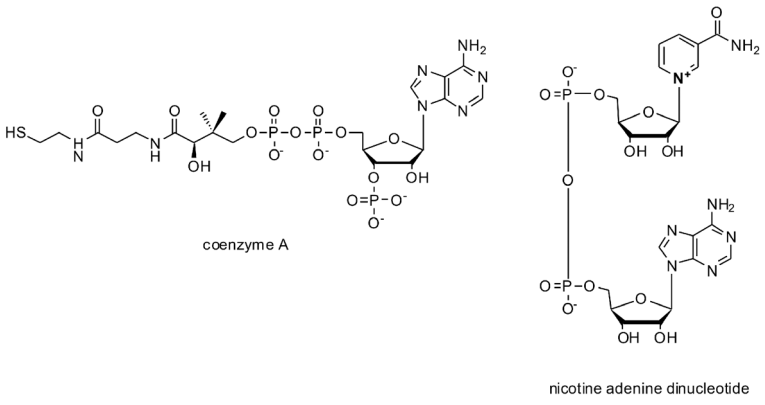

FIG. 1
Structure of coenzyme A and nicotine adenine dinucleotide

Liu *et al.* developed a methodology for the detection of chemically labile cellular small molecule-RNA conjugates employing size-exclusion chromatography followed by LC/MS analysis. Investigation of the size distribution

of coenzyme linked-RNAs in cell extracts demonstrated the presence of CoA-RNA and NAD-RNA predominantly in the fraction below ~200 nucleotides in length. This finding excludes the possibility of nonspecific transcriptional initiation being the originator of the cofactor-RNA conjugates as it would be expected to generate a broader size distribution of the conjugates. Moreover, these molecules are quite abundant; more than 3000 copies per cell were detected in both organisms. In addition, the presence of other cofactors e.g. FAD (flavin adenine dinucleotide)-linked RNA molecules is intriguing. Since the cofactors are common parts of classical protein enzymes in cell, this discovery indicates completely new catalytic function of non-protein coding RNA in cell machinery and may lead to elucidation of an ancient transition between RNA-templated chemistry as an early form of metabolism to protein enzymes systems as known nowadays.

RESULTS AND DISCUSSION

The first task of our project was the synthesis of model cofactor-linked RNAs. We employed the methodology developed by Huang[3] relying on the direct incorporation of CoA (or more precisely: 3′-dephospho-CoA), NAD and FAD as initiator nucleotides into RNA by *in vitro* transcription with T7 RNA polymerase. The T7 class II promoter enables the preparation of RNAs with adenosine at their 5′-termini. Nevertheless, this protocol has not been used for quantitative preparation of the pure cofactor-linked RNAs. In parallel, we are developing strategies for capturing these model RNA molecules by using various chemical and enzymatic reactions. The target reactions should meet two requirements: to be mild enough to not destroy very sensitive RNA molecules, and to be specific to modify only the cofactor moiety.

This work is supported by the Alexander von Humboldt foundation.

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SEQUENCING OF COMBINATORIAL LIBRARIES WITH MASS SPECTROMETRY

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Synthetic combinatorial oligonucleotide libraries are amongst useful approaches used to find oligomers with required biological and physical properties e.g. looking for aptamers. However, the approaches used till now can be applied to libraries made of natural, DNA or RNA, units. In the present communication we report a method of sequencing of elements of synthetic combinatorial oligonucleotide libraries carrying modified nucleotide units by the use of MALDI-TOF mass spectrometry. This development largely increases the scope of applications of combinatorial analysis of oligonucleotides.

INTRODUCTION

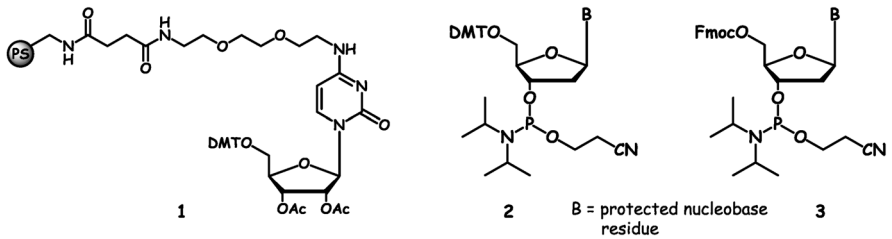
Combinatorial approach[1] to synthesis and analysis of properties of chemical compounds has found interesting applications in nucleic acids field. This is well illustrated by the use of SELEX approach relies on DNA sequencing of cloned oligonucleotide sequences that went through the numerous amplification and selection procedures. However, due to the nature of amplification and sequencing procedures this approach cannot be applied to oligonucleotide sequences carrying modifications in any party of nucleic acid chain. If the approach of direct analysis of synthetic combinatorial oligonucleotide libraries carrying modified units would be developed it would largely increase the scope of application of this approach as general methods useful in combinatorial analysis such as recursive deconvolution[2] or tagging methods are rather hardly applicable as material and labour demanding approaches[1–3].

RESULTS AND DISCUSSION

Although many methods of oligonucleotide sequencing were elaborated, all of them require relatively high amounts of sequencing material. In our studies the synthetic oligonucleotide library prepared on a resin with beads of 50–100 μm diameter can carry upmost picomole oligonucleotide quantities when final loading of oligonucleotide of several μmole/g of support is achieved[4]. Thus, we had to develop own approach that would be suitable for our purposes. We choose mass spectrometry as direct and adequate tool

for oligonucleotide libraries sequencing that would allow in principle read sequences with any modification within oligomer unit.

The selection of the oligonucleotides with appropriate functionality is performed after removal of protecting groups. Therefore, oligonucleotides must be linked to the solid support by a linker that is stable not only during the chemical synthesis of oligonucleotide combinatorial library but through selection procedure as well. We have turned our attention to linkers that would be stable during library synthesis and analysis (selection step) and will be quantitatively cleaved prior to MS analysis without degradation of oligonucleotides carrying modifications of interest. Our earlier experience with oxidative cleavage of ribonucleoside 2′,3′-diol led us to the linker (**1**) shown below[5].



Then, the linker **1** treatment with aqueous periodate followed by methionine allowed effective cleavage of oligonucleotide that could be performed using a single polystyrene bead of ca. 50 μm diameter.

The sequencing was based on the analysis of masses of oligonucleotide fragments that were generated during the library synthesis. To control the quantities of shorter oligonucleotides the synthesis by phosphoramidite approach was performed with the mixtures of appropriate building block amidites that consisted mostly of 5′-*O*-dimethoxytrityl monomers **2** and partly of 5′-*O*-fluorenylmethoxycarbonyl (*Fmoc*) ones **3** acting as terminators in oligonucleotide synthesis. The sequencing experiments proved unequivocally that one can read the oligonucleotide sequence attached to a single bead of polystyrene resin during MALDI-TOF MS analysis[6]. The results of analysis of a model oligonucleotide combinatorial library with fluorescently labelled probes[7] fully corroborated effectiveness of our sequencing approach[6].

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CHEMICALLY MODIFIED PHOSPHOROTHIOATE DNA AND 2′-OMe RNA AS ANTISENSE AGENTS

Paul D. MARZENELL, Helen HAGEN, Larisa KOVBASYUK and Andriy MOKHIR

Phosphorothioate desoxyribonucleic acids (PT DNAs) and 2′-OMe RNAs find applications as antisense agents for regulation of gene expression and RNA interference. One applies typically >20-mers to achieve the sufficient affinity towards target ribonucleic acids.

However, such long probes exhibit low mismatch discrimination and may therefore, induce unspecific effects. Shorter probes are more specific, but their affinity towards nucleic acids is not sufficiently high.

We explored different terminal chemical modifications to improve the RNA affinity of such shorter probes without affecting their sequence discriminating ability. The results of this exploratory study will be presented in the poster.

NEW INSIGHTS IN TO PHOTOREACTIVATION OF (6-4) PHOTOLESIONS

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A series of oligonucleotides containing either the T(6-4)T or the T(6-4)C photoproduct has been synthesized in efficient amounts. These photoproducts were used in repair studies with (6-4) photolyase from *D. melanogaster*.

INTRODUCTION

Ultraviolet light irradiation of DNA causes the formation of three different types of DNA lesions, termed *cis-syn* cyclobutane pyrimidine dimers (CPD photoproducts) **1**, pyrimidine(6-4)pyrimidone ((6-4) photoproducts) photolesions **2** and **3** and Dewar valence isomers **4** and **5**, which are formed from the corresponding (6-4) lesions **2** and **3**, respectively, by irradiation with UV-A/B light[1–3].

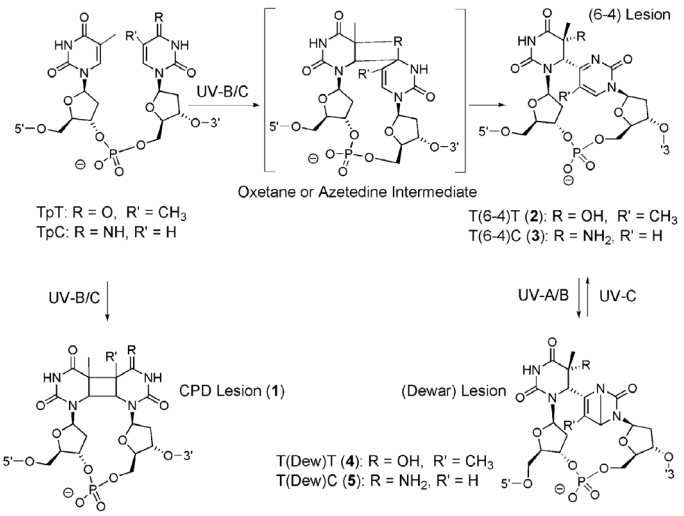

FIG. 1
Formation of CPD photolesion **1**, (6-4) photoproducts **2** and **3** and its Dewar valence isomers **4** and **5** under UV light

RESULTS AND DISCUSSION

The repair mechanism of (6-4) photolesions and also the mechanism behind repair of Dewar lesion are yet not fully understood. After the discovery of the (6-4) photolyase it was assumed that (6-4) photolyases bind the (6-4) lesions and that they rearrange the lesion in a dark reaction, back into the oxetane/azetidine intermediates[4].

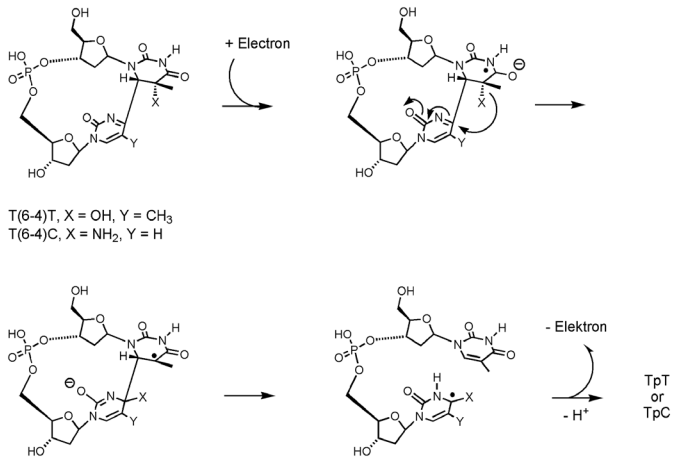

FIG. 2
Proposed repair mechanism for (6-4) lesion

We determined the first X-ray crystal structure of a (6-4) photolyase in complex with a DNA double strand containing the T(6-4)C lesion and showed that both lesions T(6-4)T and T(6-4)C are bound almost identically in the active site. The structure shows a cytosine containing photolesion which supports the idea that the critical electron transfer from the protein to the lesion occurs directly into the lesion and not into a strained four-membered ring intermediate[5].

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AN ENZYMATIC SYNTHESIS OF 2′-DEOXYRIBOSIDES OF SOME 8-AZA- AND 8-AZA-7-DEAZAPURINES

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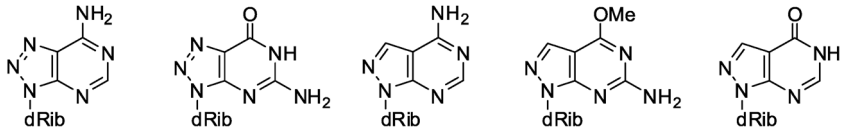
An enzymatic synthesis of 2′-deoxyribosides of some 8-aza- and 8-aza-7-deazapurines has been studied. Two methods have been used, *viz.*, (*i*) transglycosylation reaction employing 2′-deoxyguanosine as a donor of the pentofuranose residue and recombinant *E. coli* purine nucleoside phosphorylase (PNP) as a biocatalyst and (*ii*) one pot synthesis from 2-deoxy-D-ribose and heterocyclic bases employing recombinant *E. coli* ribokinase (RK), phosphopentomutase (PPM) and PNP as biocatalysts. A good substrate activity was observed for all the studied bases.

It is well known that the use of nucleoside phosphorylases (NP), in particular *E. coli* NP, as biocatalysts for the synthesis of natural as well as base and sugar modified nucleosides is a very efficient methodology (for recent reviews, see[1]). In the comprehensive study of Doskočil and Holý[2] on *E. coli* purine nucleoside phosphorylase (PNP) was shown that 8-azaguanine is a good ribosyl acceptor in the synthetic reaction (see also[1]). Later on, Votruba et al. have studied alginate gel-entrapped cells of auxotrophic thymine-dependent strain of *E. coli* as a biocatalyst for the transfer of 2-deoxy-D-ribofuranosyl moiety of 2′-deoxyuridine to purine and pyrimidine bases as well as their aza and deaza analogues[3]. The reaction proceeded regio- and stereospecific affording, *inter alia*, 8-aza-2′-deoxyadenosine (**1**) and -guanosine (**2**). It was also noted that the presence of the N^7-atom of purines and their isosteric analogues is a prerequisite for the reaction[8]. From this conclusion are, however, several exceptions. Thus, 5-aza-7-deazaguanine[4] and *N*-(1,3,4-thiadiazol-2-yl)cyanamide[5], which generally is not derived from heterocyclic structures of nucleic acids, are substrates of bacterial PNP.

The work presented here reports on the enzymatic synthesis of N^9-2′-deoxy-β-D-ribonucleosides of 8-azaadenine (**1**) and 8-azaguanine (**2**) as well as 8-aza-7-deazaadenine (**3**), 2-amino-8-aza-7-deaza-6-methoxypurine (**4**)

and 8-aza-7-deazahypoxanthine (**5**) employing 2′-deoxyguanosine as a donor of the sugar residue and recombinant *E. coli* PNP[6] as a biocatalyst (**A** – transglycosylation reaction) and the cascade transformation of 2-deoxy-D-ribose into the nucleosides in the presence of recombinant *E. coli* ribokinase (RK)[7], phosphopentomutase (PPM)[8] and PNP (**B** – one-pot synthesis[8]).



1 **2** **3** **4** **5**

SCHEME 1

Standard reaction conditions: A - *K,Na*-phosphate buffer (10 mM, pH 7.0); a donor/acceptor ratio was 1.5:1.0 (mol); the 1000 units of *E. coli* PNP [6] was used per 1 mmol of base; 50 °C, 48–50 h; silica gel column chromatography; isolated yields for **1–5**: 66, 60, 77, 76 and 68%, respectively. B - total volume 2 ml; 2 mM ATP, 50 mM KCl, 3 mM $MnCl_2$, 20 mM Tris·HCl (pH 7.5); 1.3 mM 2-deoxy-D-ribose, 1 mM heterocyclic base; units of enzymes: RK [7] (9), PPM [8] (4) and PNP [6] (14); 40 °C; yields by HPLC of nucleoside (in %) and time of the reaction (in h): **1** (10; 15), **2** (25; 25), **3** (55; 25), **4** (50; 20) and **5** (60; 10)

Reaction conditions in both modes of reactions have not been optimized. Despite this, all the tested heterocyclic bases showed satisfactory substrate properties and the desired nucleosides have been prepared in good yields. In all the experiments, we have not observed the formation of the regioisomeric nucleosides. The structure of all isolated nucleosides was proved by integrity of the spectral (^{1}H and ^{13}C NMR, UV) methods.

Financial support by the International Science and Technology Centre (project #B-1640) is gratefully acknowledged.

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AN ENZYMATIC SYNTHESIS OF NUCLEOSIDES OF N^2-ACETYL-O^6-[2-(4-NITROPHENYL)ETHYL]GUANINE

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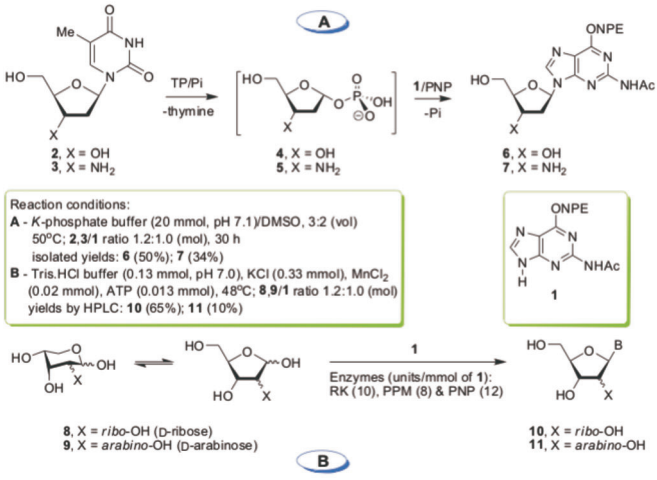
An enzymatic synthesis of nucleosides of N^2-acetyl-O^6-[2-(4-nitrophenyl)ethyl]guanine (**1**) is reported. The base **1** was found to be a satisfactory substrate of recombinant *E. coli* purine nucleoside phosphorylase (PNP). It was used as an acceptor in the synthesis of (*i*) N^9-2′-deoxy-β-D-*erythro*-furanosyl (**6**) and N^9-3′-amino-2′,3′-dideoxy-β-D-*erythro*-furanosyl (**7**) nucleosides using thymidine (**2**) and its 3′-aminodeoxy derivative (**3**) as donors of the sugar residues and recombinant *E. coli* thymidine phosphorylase (TP) and PNP as biocatalysts, and (*ii*) the *ribo*- and *arabino*-nucleosides **10** and **11** in the cascade transformation of the respective pentoses **8** and **9** in the nucleosides in the presence of recombinant *E. coli* ribokinase (RK), phosphopentomutase (PPM) and PNP.

We have earlier described an enzymatic synthesis of nucleosides of N^6-benzoyladenine and N^2-acetylguanine using the respective acylated bases as acceptors of the pentofuranose residues and recombinant *E. coli* PNP as a biocatalyst[1]. In continuation of these studies, we report here on the use of N^2-acetyl-O^6-[2-(4-nitrophenyl)ethyl]guanine (**1**)[2] as an acceptor in (*i*) the transglycosylation reaction (for recent reviews, see[3]) employing thymidine (**2**) and its 3′-aminodeoxy derivative (**3**) as donors of the sugar residues and recombinant *E. coli* TP and PNP[4] as biocatalysts (Scheme 1, **A**), and (*ii*) the synthesis of the *ribo*- and *arabino*-nucleosides **10** and **11** in the cascade transformation[5] of the corresponding D-pentoses **8** and **9** into the nucleosides in the presence of recombinant *E. coli* RK[6], PPM[5] and PNP (Scheme 1, **B**).

The base **1** is poorly soluble in the phosphate buffer and a 3:2 (vol) mixture of *K*-phosphate buffer (20 mM; pH 7.1) and DMSO was, therefore, used in the studied reactions. It was found that the base **1** displays satisfactory substrate activity for *E. coli* PNP and the nucleosides **6** and **7** form in the reaction mixture in 64 and 42% (HPLC data), respectively, after 30 h at 50 °C (Scheme 1). Both individual nucleosides have been isolated by silica gel column chromatography in 50 and 34% yields, respectively. The use of the acceptor/donor ratio of 1.0:1.5 (mol) gave rise to the formation of the

nucleosides **6** and **7** in 74 and 55% yields (HPLC), respectively, after 48 h at 50 °C.

A good substrate activity of the base **1** for *E. coli* PNP prompted us to test it in the one-pot synthesis of nucleosides from D-pentoses in the cascade transformation in the presence of recombinant *E. coli* RK, PPM and PNP (Scheme 1, **B**). It was found that the riboside **10** forms in the reaction mixture in 65% yield after incubation for 48 h at 48 °C. Under similar reaction conditions, the formation of the arabinoside **11** proceeds more slowly affording 10% of the product after 48 h.



SCHEME 1

Remarkably, a good substrate activity of **1** with voluminous NPE group points to a large hydrophobic pocket in the *E. coli* PNP active site that can accommodate this group

Financial support by the International Science and Technology Centre (project #B-1640) is gratefully acknowledged.

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CHEMO-ENZYMATIC SYNTHESES AND BIOLOGICAL EVALUATION OF 5,6-DISUBSTITUTED BENZIMIDAZOLE RIBO- AND 2′-DEOXYRIBO-NUCLEOSIDES

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A number of 5,6-disubstituted benzimidazoles have been prepared and their substrate properties for recombinant *E. coli* purine nucleoside phosphorylase (PNP) in the transglycosylation reaction investigated. The heterocyclic bases showed good substrate activity for PNP and the ribo- and 2′-deoxyribo-nucleosides have been synthesized. The efficient formation of the nucleosides with voluminous substituents in the studied bases points to a large hydrophobic pocket in the *E. coli* PNP active site that can accommodate these groups.

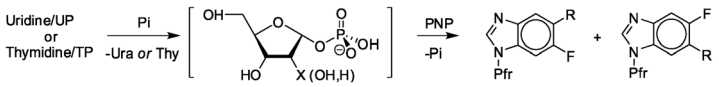
It was earlier shown that benzimidazole (BI) and its derivatives with substituents in the benzene ring are good substrates of *E. coli* PNP in the transglycosylation reaction[1–4]. In the present study we synthesized 5,6-difluorobenzimidazole and its derivatives, one fluorine atom of which is replaced with methoxy, ethoxy, isopropoxy, 4-morpholino and *N*-methylpiperazino groups, and investigated their ribosylation and 2-deoxyribosylation using relevant nucleosides as donors of the pentofuranose residues and recombinant *E. coli* nucleoside phosphorylases[5] as biocatalysts (Table I).

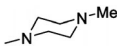
The reaction conditions have been optimized in regard to the donor/base ratio, quantity of the recombinant *E. coli* enzymes and temperature of the reaction mixture. It was found the 3:1 to 10:1 molar donor/base ratio and the use of 40 units of uridine phosphorylase (UP) per 1 mmol of uridine (60–160 UP units for 2′-deoxyuridine) and 155–400 PNP units afforded the ribosides **1–12** in good isolated yields (Table I). Reactions were conducted at 52 °C monitoring the formation of the products by HPLC, the conversion of base into nucleoside(s) was ≥98.5%. Compounds **1** and **2** were crystal-

lized out directly from the reaction mixtures, the other nucleosides were isolated by C18 RP column chromatography. It is noteworthy that the synthesis of 2′-deoxyribosides was completed in 1–3 h, whereas the transribosylation proceeded much slowly and 22–28 h required achieving high yields of products.

The *regio*-isomeric structure of all isolated nucleosides was proved by scrupulous analysis of the ^{1}H and ^{13}C NMR data (incl. [^{1}H,^{1}H] and [^{1}H,^{13}C] 2D: COSY, HSQC, HMBC and NOESY spectra).

TABLE I



Compd	Pfr[a]	R	Isolated yield, %	Ratio of isomers	
1	Rib	–F	77		–
2	dRib		68		–
3	Rib	–OMe	54	95	5
4	dRib		51	41	59
5	Rib	–OEt	98	89	11
6	dRib		85	70	30
7	Rib	–O*i*Pr	75	100	–
8	dRib		65	96	4
9	Rib		75	100	–
10	dRib		80	100	–
11	Rib		79	100	–
12	dRib		68	100	–

[a] Pfr = β-D-Pentofuranosyl (Rib = D-ribofuranose; dRib = 2-deoxy-D-ribofuranose).

A good substrate activity of BI derivatives with voluminous substituents points to a large hydrophobic pocket in *E. coli* PNP active site that can accommodate these groups.

None of the compounds were cytostatic in cell culture, neither showed significant inhibitory activity against replication of a broad variety of DNA and RNA viruses.

Financial support by the International Science and Technology Centre (project #B-1640) and the Ministry of Education and Science of Russian Federation (State Contract 02.740.11.0260) are gratefully acknowledged.

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SELECTIVE CLEAVAGE OF ACYL PROTECTING GROUPS IN 3′,5′-*O*-(TETRAISOPROPYLDISILOXANE-1,3-DIYL)RIBONUCLEOSIDES

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Stability of TIPDS protection in nucleosides in ammonia/amine solutions in MeOH and EtOH was studied. It was found that $MeNH_2$-EtOH solution is a reagent of choice for selective deacylation of *N*- and *O*-acyl protected nucleosides without notable cleavage of 3′,5′-TIPDS group.

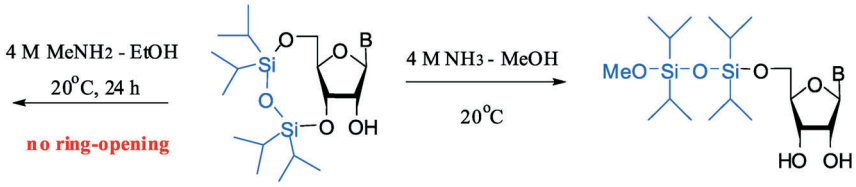
INTRODUCTION

Tetraisopropyldisiloxane-1,3-diyl (TIPDS) group developed by Markiewicz[1] is widely used for simultaneous protection of 3′,5′-hydroxyl groups in ribonucleosides and subsequent manipulation with 2′-OH group of ribonucleosides: deoxygenation, oxidation, alkylation, glycosylation, protection, preparation of 2′-amino-2′-deoxynuclesides, and many others[2–9]. It is known that TIPDS protection has limited stability both in acidic and basic media. Under alkaline conditions (0.2 M NaOH in dioxan–water 4:1, 20 °C, 1 h) 3′,5′-TIPDS group is cleaved selectively forming 5′-*O*-isomer[1].

RESULTS AND DISCUSSION

We have widely used 3′,5′-*O*-TIPDS-ribonucleosides for the preparation of 2′-*O*-pentafuranosylnucleosides and 2′-*O*-hydroxyalkoxymethylnucleosides[6–9]. Hydroxyl and amino groups of additional residue were usually protected with acyl blocking groups. TIPDS group is not enough stable when using common deacylation procedures such as NH_3 in methanol and MeONa in methanol. As a result the yields of the desired partially protected derivatives were moderate. First we studied the stability of protected uridine derivative (B = Ura) in basic conditions[10]. It undergoes partial cleavage forming 5′-*O*-isomer with 40% yield (NH_3–MeOH) or 75% yield (DBU–MeOH). Cleavage of 2′-deoxy derivative 3′,5′-*O*-TIPDS-thymidine under the same conditions results in formation of two regioisomers 5′- and 3′-derivatives in 2:1 ratio. In order to improve yields and optimize the orthogonal protection strategy we examined the stability of TIPDS-ribonucleosides, containing acyl groups, under ammonolysis/aminolysis conditions in details[10].

Prolonged reaction with NH_3 in methanol gave significant amounts of open-ring products and replacement of MeOH with EtOH strongly reduced this side reaction. It appeared that $MeNH_2$–EtOH was even more active in deacylation than NH_3–MeOH: deacylation was completed for 5 and 30 min for N^4-acetyl-TIPDS-cytidine and N^4-benzoyl-TIPDS-cytidine, respectively (for comparison: 10 min and 3.5 h in the case of NH_3–MeOH). N^2-*iso*-butyroyl-TIPDS-guanosine reacted with $MeNH_2$–EtOH more slowly, the time of complete deacylation being 2.5 h. The formation of open-ring products in all cases was less than 2%. The usage of $EtNH_2$ or $PrNH_2$ in EtOH resulted in decrease of deacylation rate.



In conclusion, the simple substitution of NH_3–MeOH for commercially available alkylamine-EtOH results in substantial increase of stability of TIPDS protection group with retention of deacylation capacities.

This work was supported by the Russian Foundation for Basic Research and Russian Academy of Sciences (Program "Molecular and Cell Biology").

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SYNTHESIS OF N^6-SUBSTITUTED ADENOSINES

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An efficient and reliable method for the preparation of N^6-substituted adenosines by alkylation of N^6-acetyl-2′,3′,5′-tri-*O*-acetyladenosine has been developed.

INTRODUCTION

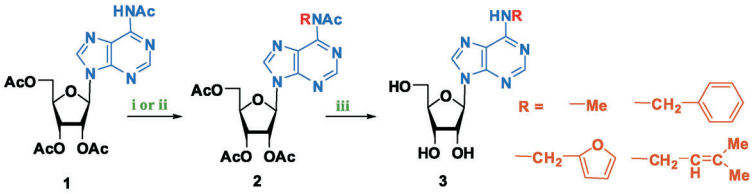
Adenine and adenosine derivatives play an important role in numerous biochemical processes. Among *N*^{6}-alkylated adenines several compounds were found to possess high cytokinine activity[1]. Cytokinines regulate many processes in plants such as seed germination, leaf propagation, proliferation and others. They occur endogenously as free bases, nucleosides or nucleotides. Several N^6-substituted adenosines have pronounced anticancer activity[2]. Moreover N^6-methyladenosine, N^6-isopentenyladenosine and some others were isolated from tRNA[3]. Several approaches for preparation of *N*^{6}-alkylated adenosines have been developed: 1-*N*-alkylation of adenosine with subsequent Dimroth rearrangement; aminations of inosine[5]; nucleophilic substitution of halogen in 6-position with amine[6]; N^6-alkylation under phase-transfer catalysis conditions[7].

RESULTS AND DISCUSSION

In spite of a number of available methods for the preparation N^6-substituted adenosines there is still a room for the development of simple and reliable methods for the synthesis of this group of important natural compounds. Here, we report on successful application of *N*^{6}-acetyl-2′,3′,5′-tri-*O*-acetyladenosine (**1**)[8] for selective *N*^{6}-alkylations under base-catalyzed and Mitsunobu conditions. Tetraacetate **1** can be alkylated at ambient temperature under traditional base assisted or Mitsunobu conditions. As a base we used 1,8-diazabicyclo[5.4.0]undec-7ene (DBU). It is a sterically hindered strong base which is soluble in most organic solvents that allows to conduct alkylation reactions homogeneously.

Alkylation of **1** in all cases gives solely the product of N^6-alkylation **2**. No other products were detected by TLC in traceable amounts. Ammonolysis of **2** give free nucleosides **3** in high overall yields. The compound **1**

can be alkylated with activated halides (MeI, BnBr, $Me_2C{=}CHCH_2Br$ and others) affording **2** with good yields. To broaden the scope of utilization of **1** for the synthesis of adenosine analogues we applied Mitsunobu conditions for its alkylation. We found that regioselectivity of reaction with alcohols (BnOH, furfuryl alchol and others) under these conditions is the same as in the base assisted alkylation with alkyl halides. The disadvantage of Mitsunobu protocol is often connected with tedious chromatographic purification of the desired compound from the excess of reagents and their products. Nevertheless, when alkyl halides are not available this protocol utilizing parent alcohols is the only possible way.



SCHEME 1
i) DBU, MeCN, RX, 3 h, 20 °C; ii) ROH, DEAD, Ph_3P, THF, 3 h, 20 °C; iii) 5 NH_3 in MeOH, 24 h, 20 °C

The position of alkylation was unambiguously assigned by comparison of ^{1}H NMR spectra of methylation product **3** (R = Me) with authentic samples of *N*1- and N^6-methyladenosines[4]. For the preparation of N^6-substituted adenines we have used recombinant purine nucleoside phosphorylase from *E. coli* which catalyze reversible phosphorolysis of nucleosides. To insure the completeness of transformation of nucleoside to adenine alkaline phosphatase was added.

In conclusion, we have developed the reliable preparation of N^6-alkyl adenosine derivatives both under base assisted alkylation with alkyl halides and Mitsunobu conditions. The choice of conditions depends upon availability of either halide or parent alcohol.

This work was supported by the Russian Foundation for Basic Research and Russian Academy of Sciences (Program "Molecular and Cell Biology").

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PENTAFERROCENYL PHOSPHORAMIDATE α-OLIGONUCLEOTIDES FOR ELECTROCHEMICAL DETECTION OF NUCLEIC ACIDS

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α-Oligonucleotides exhibiting five phosphoramidate linkages a bearing ferrocenyl (Fc) moiety were synthesized. Their electrochemical properties were studied in solution by cyclic voltammetry. We observed a strong difference in the Fc environment when the probe is alone or hybridized with a complementary strand allowing an electrochemical detection of the duplex formation.

INTRODUCTION

Nucleic acids (DNA and RNA) play a key role in living organisms and are currently the most targeted biomolecules in the field of diagnostics[1]. Among the different strategies to develop a fast and selective diagnostic system, microarrays with electrochemical detection have received considerable attention due to their intrinsic properties: easy integration, sensitivity, rapidity and low cost[2].

RESULTS AND DISCUSSION

Our approach is based on the use of ferrocene (Fc)[3] as redox compound to obtain the highest difference of environment between a single-stranded and a double stranded Nucleic acid structure. For this purpose, Fc was introduced on the internucleosidic linkage as a phosphoramidate function. To this purpose, α-oligonucleotides were used since we have shown that P-modifications have a slight effect on the duplex stability[4–9] in contrast to β-oligonucleotides where P-modifications have a strong negative effect.

Herein, we report the synthesis of α-oligonucleotides exhibiting five phosphoramidate linkages bearing Fc moieties (Fig. 1) and their electrochemical behavior studied by cyclic voltammetry (CV).

Thermodynamic experiments (Tm) showed that Fc moieties introduced into α-DNAs have little influence on the stability of the duplexes formed with the complementary β-DNA target (–0.3 °C < ΔTm/Fc < +0.4 °C).

The electrochemical characterization of Fc_5-α-DNA (**3a**, **3b**, **6**) was achieved by cyclic voltammetry (CV) on a gold electrode microarray[10]. We observed a strong decrease of the current intensity at the oxidation peak (*Ipa*) together with an enlargement of the peak-to-peak potentials (Δ*Ep*) between the single strand and the duplex allowing to determine the formation of the duplex.

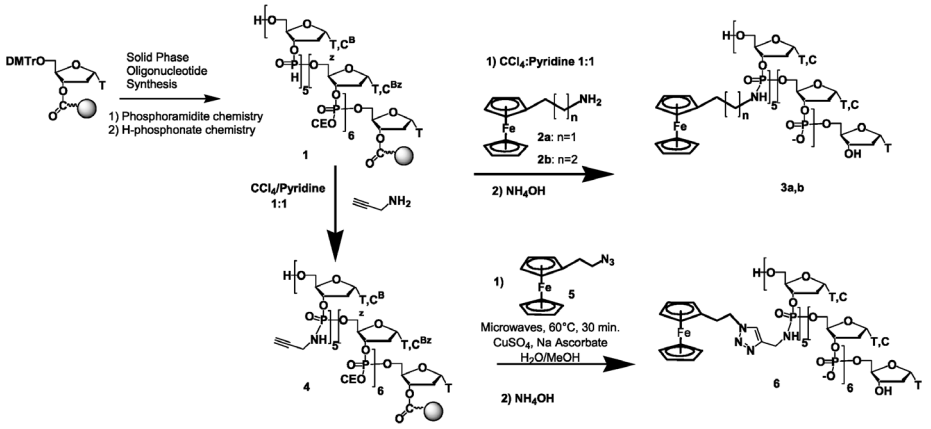

FIG. 1
Synthesis of the Fc_5-α-DNAs

Work is in progress to immobilize these probes on the electrode with the aim of strongly improving the system sensitivity and further developing DNA biochips.

This research is supported by the ANR (French national research agency) through the project VirProbe (PIRIBio program), the Lyonbiopole and Eurobiomed clusters.

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EFFECT OF A URACIL SPECIFIC BINDING FOR THE NUCLEASE ACTIVITY OF BIS(AZACROWN)CONJUGATED 2′-*O*-METHYL OLIGORIBONUCLEOTIDES

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2′-*O*-Methyl oligoribonucleotides bearing dinuclear Zn^{2+} complex of 1,3-bis[(1,5,9-triazacyclododecan-3-yl)oxymethyl]benzene at the 3′-end were synthesized. The goal is that these two azacrowns in synergy with the sequence recognition by hybridization were attached in the vicinity to act co-operatively, the one recognizing the uracil base of the target and the other one cleaving the phosphodiester bond of the target.

INTRODUCTION

Artificial ribonucleases, i.e., oligonucleotide conjugates that sequence-selectively cleave complementary RNA targets have attracted considerable attention during the past decade. Such man-made catalysts are believed to find applications as artificial restriction enzymes with which large RNA molecules could be tailored in a pre-design manner *in vitro* and hopefully also as chemotherapeutic agents capable of silencing an over-expressed gene by recognizing and destroying the respective mRNA. We have previously shown that 2′-*O*-methyl oligoribonucleotides bearing two 3-(3-hydroxypropyl)-1,5,9-triazacyclododecane ligands cleave oligoribonucleotide targets at a single phosphodiester bond and show turnover[1]. The most efficient one of such conjugates, viz. the 3′-terminal conjugate containing 2-hydroxyethyl 3′-*O*-(2-hydroxyethyl)-β-D-ribofuranoside as the branching unit, exhibits 1000-fold cleaving activity compared to the free monomeric Zn^{2+} chelate of 1,5,9-triazacyclododecane. Our group has also previously studied base moiety selectivity in cleavage of short oligoribonucleotides by the dinuclear Zn^{2+} complex of 1,3-bis[(1,5,9-triazacyclododecan-3-yl)oxymethyl]benzene and the trinuclear Zn^{2+} complex of 1,3,5-tris[(1,5,9-triazacyclododecan-3-yl)oxymethyl]benzene[2]. These ligands are interesting because they, besides their marked cleaving activity, recognize uracil and thymine bases. The results show that anchoring of one of the Zn^{2+} azacrown moieties to the uracil base keeps the other moiety in the vicinity of the neighboring phosphodiester bonds, resulting in site-selective cleavage. The present study is aimed at exploiting this anchoring ability in synergy

with the sequence recognition by a 2′-*O*-methyl oligoribonucleotide probe. To achieve this goal, two azacrowns were attached in the vicinity to act co-operatively, the one recognizing the uracil base of the target and the other one cleaving the phosphodiester bond of the target.

RESULTS AND DISCUSSION

Figure 1 shows the two cleaving agents synthesized. They both have dinuclear Zn^{2+} complex of 1,3-bis[(1,5,9-triazacyclododecan-3-yl)oxymethyl]benzene attached at the 3′-end of the 2′-*O*-methyl oligoribonucleotide, and the difference between the cleaving agents is in the length of the spacer attaching azacrown moiety to the oligonucleotide probe. The cleaving ability of these conjugates is tested using chimeric 2′-*O*-methylribo/ribooligonucleotide targets shown in Fig. 1. The targets differ from each other in the sense that they either have or do not have uridine near the cleavage site.

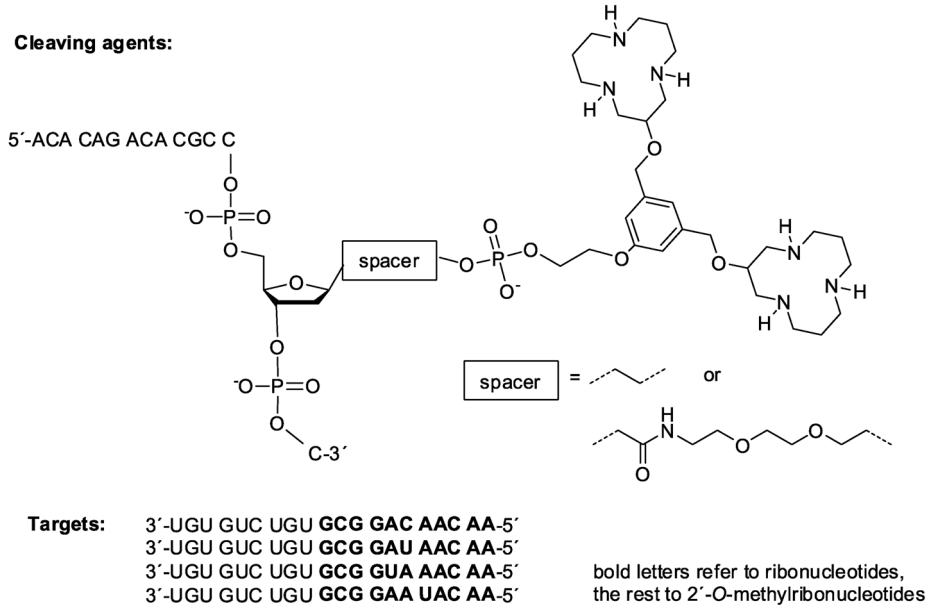

FIG. 1

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THE METHOD FOR SYNTHESIS OF NEW BIFUNCTIONAL CONJUGATES OF OLIGONUCLEOTIDES

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We have proposed a method for the synthesis of 5′- or 3′-bifunctional conjugates of oligonucleotides that contained two different ligands on the terminal phosphate group. The conjugates of oligo(2′-*O*-methylribonucleotides) containing two pyrene residues introduced by different linkers or pyrene and perylene residues attached to same phosphate were synthesized and their fluorescent properties were investigated.

INTRODUCTION

There is a large variety of synthetic methods for the preparation of oligonucleotide conjugates. We picked out the post-synthetic strategy of conjugate synthesis using the modified Mukaiyama reaction[1,2].

RESULTS AND DISCUSSION

The 5′- or 3′-phosphates of oligo(2′-*O*-methylribonucleotides) were synthesized by automatic phosphoramidite method. Conjugates (I) and (II) containing 1-pyrenylmethylamine and 1-pyrenebutyric acid (2-aminoethyl)-amide attached to the same terminal phosphate group and conjugate (III) containing 1-pyrenylmethylamine and (3-perylenylmethylamine)propylamine were prepared (Fig. 1, Table I).

Synthesis of these conjugates was carried out in two stages. First, activated terminal phosphate group reacted with ethylenediamine or 1-pyrenylmethylamine (Fig. 1). In the case of ethylenediamine linker the presence of aliphatic amino group permits to attach various activated esters, such as 1-pyrenebutyric acid *N*-hydroxysuccinimide ester. At the second stage the terminal phosphamide group of mono-conjugate was activated for the subsequent reaction with nucleophylic NH_2-group of the second ligand[3,4]. Conjugates (IV)–(VI) containing two 1-pyrenylmethylamine residues on the 5′- or 3′-terminal phosphate of oligonucleotide were synthesized as controls. The structure of the conjugates was confirmed by MALDI-TOF MS and UV and fluorescent spectra. Fluorescent properties of synthesized conjugates and their duplexes with RNA and DNA were studied.

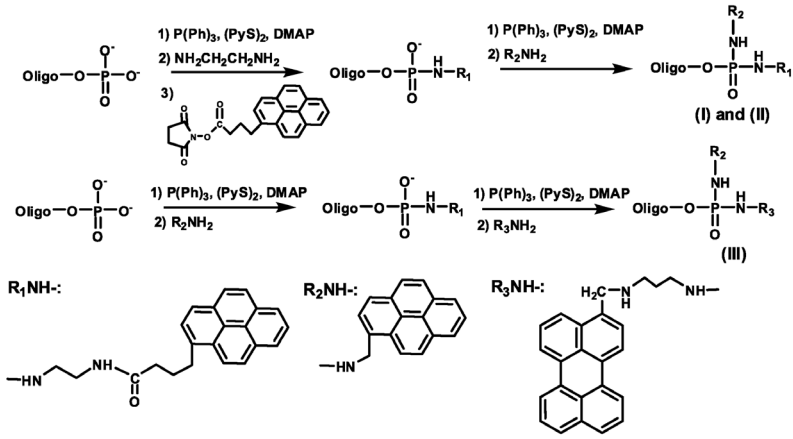

FIG. 1
The synthesis of bifunctional conjugates

TABLE I
Synthesized oligo(2′-*O*-methylribonucleotide) conjugates

N	Conjugate	Molecular mass[a]	
		Found, mz	Calcd
I	5′-**(Pyr1)**(Pyr2)pG^{m}A^{m}G^{m}C^{m}C^{m}G^{m}A^{m}U^{m}U^{m}U^{m}C^{m}A^{m}U^{m}C^{m}A^{m}$_{inv}$***T***	5856.1	5857.6
II	5′-G^{m}G^{m}A^{m}G^{m}A^{m}A^{m}G^{m}G^{m}U^{m}G^{m}U^{m}C^{m}U^{m}G^{m}C^{m}G^{m}G^{m}G^{m}A^{m}G^{m}p**(Pyr1)(Pyr2)**	7467.2	7468.9
III	5′-**(Pyr1)(Per)**pG^{m}C^{m}A^{m}U^{m}C^{m}A^{m}A^{m}G^{m}C^{m}U^{m}C^{m}C^{m}A^{m}G^{m}G^{m}C^{m}	–	5917.2
IV	5′-**(Pyr1)**$_2$pG^{m}A^{m}G^{m}C^{m}C^{m}G^{m}A^{m}U^{m}U^{m}U^{m}C^{m}A^{m}U^{m}C^{m}A^{m}$_{inv}$***T***	5756.2	5757.0
V	5′-G^{m}G^{m}A^{m}G^{m}A^{m}A^{m}G^{m}G^{m}U^{m}G^{m}U^{m}C^{m}U^{m}G^{m}C^{m}G^{m}G^{m}G^{m}A^{m}G^{m}p**(Pyr1)**$_2$	7369.4	7368.0
VI	5′-**(Pyr1)**$_2$pG^{m}C^{m}A^{m}U^{m}C^{m}A^{m}A^{m}G^{m}C^{m}U^{m}C^{m}C^{m}A^{m}G^{m}G^{m}C^{m}	5810.1	5809.7

Pyr1, 1-pyrenylmethylamine; **Pyr2**, 1-pyrenebutyric acid (2-aminoethyl)amide; **Per**, (3-perylenylmethylamine)propylamine; $_{inv}$***T***, thymidine attached by 3′-3′-phophodiester linkage. [a] Molecular mass found by MALDI-TOF MS analysis.

The proposed method can be used for the synthesis of bifunctional oligonucleotide conjugates with various chemical groups.

This study was supported by the Russian Foundation of Basic Research, project no. 08-04-01634-a and Federal Target Program "Scientific and science educational personnel of innovative Russia" (State contract N P1334).

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OPTIMIZATION OF CELL SELEX PROTOCOL FOR 2′-F-MODIFIED RNA APTAMERS

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Specific cell targeting is one of the most acute tasks facing the modern fundamental medicine. Using of escort RNA aptamers recognizing cells of certain type represents a promising approach to address this issue. We proposed and tested a new method for recovery of modified escort RNA aptamers from total cellular RNA during cell SELEX. The method is based on using of small artificial ribonucleases instead of RNase A. The possibility of employing the newly developed method in cell-based SELEX was demonstrated by means of selection of modified RNA aptamers against a cell surface receptor.

INTRODUCTION

Aptamers are single-stranded nucleic acids obtained by SELEX (Systematic Evolution of Ligands by EXponential enrichment) that bind tightly to their targets due to stable and well-defined three-dimensional structure. Aptamers recognizing specifically the cells of certain type (so-called escort aptamers) could be used for addressed delivery of different agents to the cells for diagnostic or therapeutic purposes. To improve biological stability of aptamers, various chemical modifications are employed, particularly the replacement of all pyrimidine nucleotides by their 2′-F analogs. The goal of the present study was to optimize the selection protocol for escort RNA aptamers as well as to evolve new cell internalizing RNA aptamers against human insulin-like growth factor I receptor (IGF-IR). Insulin-like growth factor I (IGF-I) and its receptor (IGF-IR) are involved in cell differentiation, proliferation and apoptosis[1]. Since IGF-I/IGF-IR system takes part also in cell malignant transformation, IGF-IR is considered now as a promising cancer target[2].

RESULTS AND DISCUSSION

Escort RNA aptamers are generally obtained by cell-based SELEX using whole living cells as a target[3]. One of the routine steps of cell SELEX proto-

col is cell lysis followed by isolation of bound and/or internalized sequences. Standard method of isolation of modified RNA aptamers mixed with total cellular RNA includes the treatment of the whole mixture with ribonuclease A. We proposed here an alternative method based on using of artificial ribonucleases, small RNA-cleaving compounds mimicking RNase A[4,5]. The method allows to avoid an accidental RNase A contamination as well as to exclude an additional step for enzyme inactivation on every selection round. First, we have shown in a model system that the treatment of total cellular RNA by artificial ribonucleases results in the formation of short RNA fragments while 2′-F-modified RNA aptamer remain intact and can be subjected then to specific RT-PCR. A total of seven artificial ribonucleases were compared, and ribonuclease Dp12 built of two 1, 4-diazabicyclo[2.2.2]octane residues bearing alkyl substituents and connected through benzene ring was found to be the most efficient (the lowest concentration required for sufficient RNA cleavage), so we chose it for the subsequent experiments. The next task was to establish the feasibility of the newly developed method in cell-based SELEX protocol. Two mice fibroblast lines were used for selection: R+ and R– cells, expressing and non-expressing human IGF-IR, correspondingly. Each SELEX round included two sequential steps: 1) negative selection using R– cells; 2) positive selection with the R+ cells. This scheme provides a way to discard the aptamers nonspecifically recognizing any other cell surface proteins. During the SELEX process we observed an enrichment of RNA library with the sequences possessing an increased affinity to the R+ cells (Fig. 1).

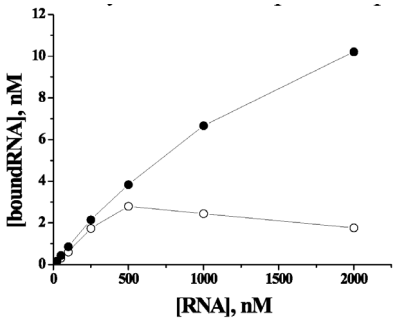

FIG. 1
Binding curves of enriched and initial RNA pools. RNA pools were radioactively labeled and incubated at different concentrations on cell monolayers. Open circles correspond to initial RNA pools, closed circles correspond to enriched RNA pool (after 5 rounds)

To summarize, we developed a new effective approach for the separation of modified RNA aptamers from total cellular RNA during cell SELEX using artificial ribonucleases. The method was successfully applied for the selection of RNA aptamers that specifically bind human IGF-I receptor on the cell surface. These escort aptamers can be considered as promising delivery tools in tumor therapy.

This work was supported by grant from the Government of Novosibirsk region for young scientists and RFBR grant N 11-04-01014. The authors are grateful to Dr. V. N. Silnikov, (ICBFM SB RAS, Novosibirsk, Russia) for kindly provided artificial ribonucleases. We acknowledge Dr. R. Baserga for providing R+ and R– cells.

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RNA APTAMERS AGAINST AUTOREACTIVE IMMUNOGLOBULINS ASSOSIATED WITH MULTIPLE SCLEROSIS

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Multiple sclerosis is an autoimmune pathology characterized by presence of autoantibodies hydrolyzing myelin basic protein. RNA aptamers with 2'-fluoro pyrimidine nucleotides that bind polyclonal antibodies against myelin basic protein were obtained using SELEX techniques.

INTRODUCTION

Multiple sclerosis (MS) is a chronic progressive demyelinating disease of the central nervous system. It is now generally considered that MS is an autoimmune pathology characterized by presence of autoantibodies towards myelin basic protein (MBP). These antibodies were shown to possess a proteolytic activity against MBP [1,2] thus contributing to the pathological destruction of myelin sheath. A development of new approaches to the selective detection of anti-MBP antibodies and inhibition of their proteolytic activity could be of importance for diagnostic and therapeutic purposes.

Aptamers are short single stranded DNA or RNA molecules, binding with high affinity, attributed to their specific three-dimensional shapes, to a large variety of targets, ranging from small molecules to complex mixtures. It was shown that RNA aptamers can bind even to non-RNA binding proteins like antibodies[3] and also can inhibit the physiological functions known to be associated with the target proteins in animal models[4].

The goal of the present work was the selection of RNA aptamers for the specific targeting of anti-MBP autoantibodies.

RESULTS AND DISCUSSION

Polyclonal IgGs were isolated from sera of patients with different types of multiple sclerosis, and the fraction with affinity to human MBP was used as a target in SELEX procedure. To produce 2'-fluoro modified RNA aptamers the selection procedure[5] including immunoglobulins' adsorption to the PCR tubes was employed (Fig. 1). After 10 rounds of selection, an enriched

library was obtained which bound with high affinity to MS-associated IgGs and also inhibited degradation of human MBP by polyclonal IgGs. The library was then cloned and sequenced. The clones were divided into groups according to their similarities. Individual 2′-F-pyrimidine RNA aptamers from each group were synthesized and characterized in terms of their secondary structure, binding affinity and ability to inhibit the proteolytic activity of autoantibodies.

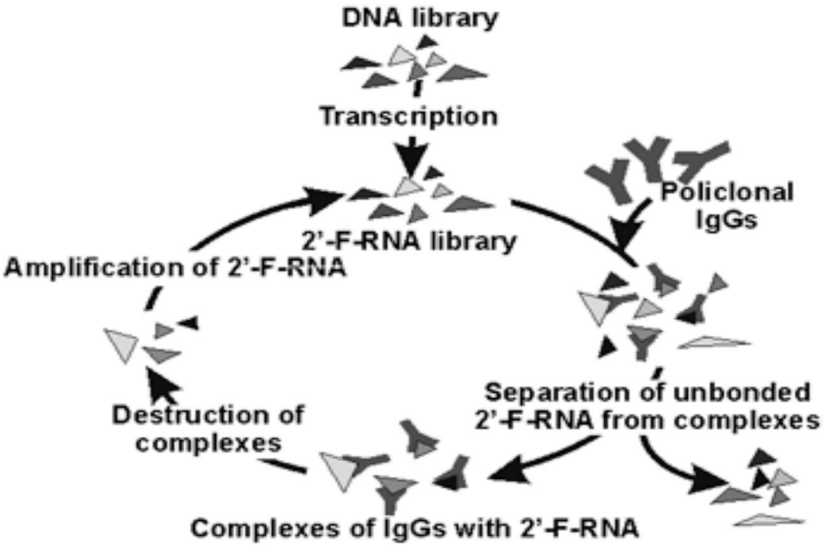

FIG. 1
Selection of 2′-F-RNA aptamers to polyclonal IgGs associated with MS

The obtained aptamers to MS-associated autoantibodies can be considered as promising basis for developing of new precise tools for diagnostic and therapy of multiple sclerosis.

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NON-COVALENT FLUORESCENT HYBRIDS OF CARBON NANOTUBES WITH OLIGONUCLEOTIDES

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The method of preparation of fluorescent SWNT hybrids with nucleic acid fragments containing pyrene residues at 5′-ends was developed. Physicochemical properties of hybrids obtained were examined. Such hybrids can be visualized during nucleic acid delivery experiments.

INTRODUCTION

Delivery of nucleic acids (NA) and NA-based constructions into cells is the actual problem of nanobiotechnology and biomedicine. The usage of non-viral nanoparticles for NA transfection is one of the promising approaches. Carbon nanotubes possessing chemical passivity and compatibility with biomacromolecules and cells are prospective intracellular NA-transporters. Such nanoparticles should contain reporter groups to permit the monitoring of transfection and clearance processes.

Here we propose the approach to the preparation of multifunctional noncovalent hybrids of pyrene-modified oligonucleotides with fluorescently labelled single-walled carbon nanotubes (SWNT) (Fig. 1).

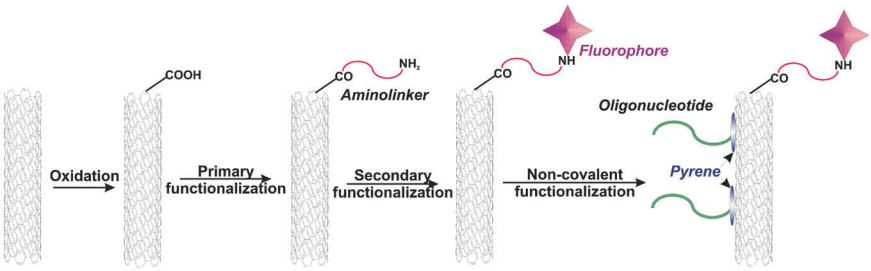

FIG. 1
Strategy of SWNT multi-functionalization

RESULTS AND DISCUSSION

Four types of fluorescently modified SWNT containing residues of fluorescein or cyanine dye Cy5 attached to nanotube surface via aliphatic (hexamethylenediamine) or dendrimer (polyamidoamine dendrimer G3.0)

linkers were prepared. Functionalized nanotubes were characterized by infrared spectroscopy, Raman spectroscopy, thermogravimetric analysis, elemental analysis, transmission and scanning electron microscopy.

Conjugates of oligodeoxyribonucleotides with pyrene residues attached to the 5′-phosphate group directly or via hexa(ethyleneglycol) phosphate linker (Fig. 2) were synthesized as described previously[1] and used for preparation of hybrids with SWNT. Non-covalent functionalization of SWNT and four types of fluorophore-modified ones by 5′-pyrene-containing oligonucleotides was performed. Pyrene residues immobilization on SWNT surface takes place due to stacking interactions between their aromatic systems. Pyrene residues fluorescence quenching observed has been interpreted as an evidence of pyrene residues sorption on the nanotube surface.

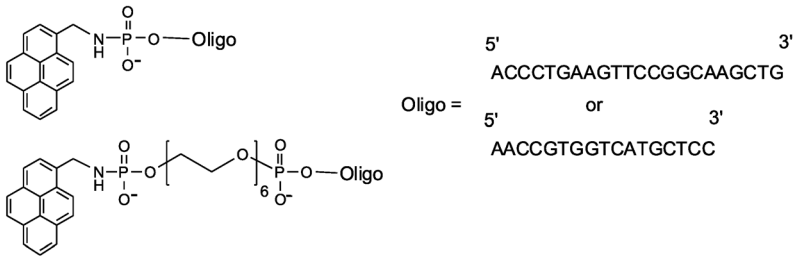

FIG. 2
5′-Pyrene conjugates of oligonucleotides used for non-covalent functionalization of SWNT

Complete sorption of oligonucleotide (90–95%) onto the nanotube surface was achieved with concentration of SWNTs about 50 μg/ml irrespective of the nanotube functionalization type and oligonucleotide length. Capacity of carbon nanotubes as potential intracellular oligonucleotide transporters was estimated from pyrene conjugates of oligonucleotides adsorption data and amounted to about 100 μmol/g. Methylene blue treatment caused the desorption of pyrene-modified oligonucleotides from SWNT surface and thus demonstrated the non-covalent character of interaction.

The results obtained demonstrate the availability of proposed approach for performing rational design of oligonucleotide biohybrids with carbon nanotubes. Such non-covalent hybrids may be used as bionanotransporters of functional NA.

This work was supported by the RFBR grant 11-04-01014-a, Federal Target Programme "Scientific and scientific-educational personnel of the innovative Russia" (grant P1334) and the RAS Presidium program of basic research No. 27 (project 62).

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2′-BISPYRENE OLIGO(2′-*O*-METHYLRIBONUCLEOTIDES) AS NOVEL FLUORESCENT PROBES FOR RNA DETECTION

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A series of novel 2′-bispyrene-labelled oligo(2′-*O*-methylribonucleotides) as fluorescent RNA specific probes was developed. Synthesis and biophysical properties studies of probes obtained were described. High thermal stability their duplexes with complementary oligonucleotides and possibility to detect in solution extended RNA fragments were demonstrated.

INTRODUCTION

Fluorescently labeled oligonucleotides that display an enhanced signal upon hybridization with their RNA or DNA targets have been the subject of intense research over the last several decades[1]. Among them pyrene nucleic acids probes are very promising ones for nucleic acids recognition and detection.

Here we present novel multipyrene-modified oligo(2′-*O*-methylribonucleotides) as high sensitive fluorescent probes for the homogeneous detection of extended RNA. Oligo(2′-*O*-methylribonucleotides) containing 3′-terminal "inverted" thymidine were used as basis for design of these probes due to its high affinity to nucleic acids and nuclease resistance[2–4].

RESULTS AND DISCUSSION

A series of 2′-bispyrene conjugates of oligo(2′-*O*-methylribonucleotides) that containing from one to three 2′-bispyrenylmethylphosphorodiamidate uridines ($\boldsymbol{U}^{bpyr}$) or cytidines ($\boldsymbol{C}^{bpyr}$) was synthesized by analogy with[5] (Fig. 1). The structures of conjugates obtained were confirmed by mass spectrometry, UV and fluorescent spectroscopy. The synthetic fragment of MDR 1 mRNA (5′-UGGCCGUUCCAAGGAGCGCGAGGUC, 113-137 nt) (r25) and its deoxyriboanalogue (d25) were used as model targets. It was shown by thermal denaturation method, that the incorporation of 2′-modified nucleotides led to the slight decrease of the thermal stability of probes duplexes with model targets. It should be noted that the thermal stability of these duplexes depends on quantity and location of 2′-bispyrene nucleo-

tides. The alteration of 2′-bispyrene-modified nucleotides quantity from 1 to 3 provoked the increase of eximer fluorescence intensity, at that fluorescence intensity of probes containing U^{bpyr} was greater than fluorescence intensity of analogous probes containing C^{bpyr}. The eximer fluorescent intensity of probes was increased (up to 10 times) upon hybridization with complementary model RNA (r25).

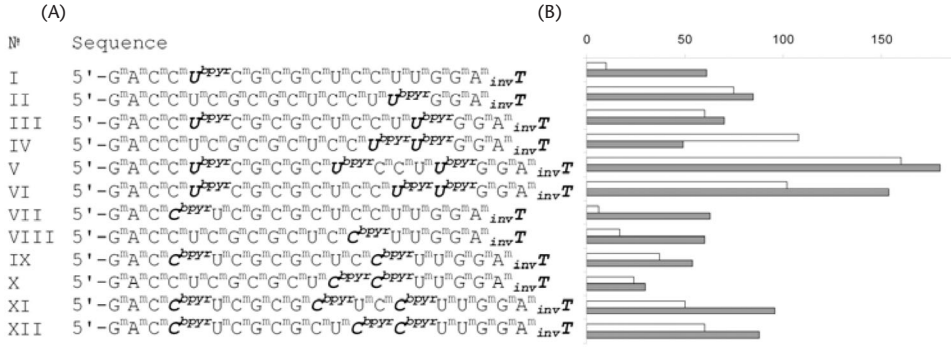

FIG. 1

(A) Sequences of 2′-bispyrene probes ($_{inv}T$ - thymidine introduced by 3′-3′-phosphodiester linkage; U^{bpyr},C^{bpyr} – 2′-bispyrene-modified uridine and cytidine). (B) Intensities of excimer fluorescence (480 nm) of 2′-bispyrene probes (I–XII) (white column) and their duplexes with model RNA (r25) (gray column). Spectra were recorded at 25 °C and λ_{ex} = 345 nm in buffer containing 0.1 M NaCl, 10 mM sodium cacodylate, pH 7.4, and 1 mM Na_2EDTA; concentration of oligonucleotides was 100 nM

The hybridization of the most sensitive probes with the 5′-terminal fragment of mRNA of *mdr1* gene (r678) was studied. The linear dependence of the eximer fluorescence of probes on RNA target concentration was obtained.

The results obtained confirm that 2′-bispyrene-labled oligo(2′-*O*-methylribonucleotides) are prospective fluorescent probes for homogenous detection of extended RNA.

This work is supported by Federal Target Program "Scientific and science educational personnel of innovative Russia" (State contract N P1334).

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RECOGNITION OF 7-DEAZAPURINE-SUBSTITUTED BINDING SITES BY TUMOUR SUPPRESSOR p53 PROTEIN

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The 7-deazapurines, 7-deazaguanine (G*), or 7-deazaadenine (A*), analogues of natural purine nucleobases, were used in primer extension assay (PEX) for generation site-specific modified DNAs of p53 DNA consensus binding site (p53DBS), using series of ^{32}P-labelled primers with distinct length covering p53DBS region. Subsequently, interactions of generated PEX products with p53 tumour suppressor protein were monitored using electromobility shift assay (EMSA). Incorporation of G* instead of guanine (G) residues within both p53DBS half sites completely abolished sequence-specific p53 binding. On the other hand, in cases of modification within only one half site (or less) the p53 binding capability was preserved. Incorporation of any adenine residue within overall p53DBS did not affect p53 protein DNA binding activity at allIncorporation of G* instead of guanine (G) residue within both p53DBS half sites completely abolished p53 binding, however in case of modification within only one half site the p53 binding capability was preserved. Incorporation of any adenine residue within overall p53DBS did not affect p53 protein DNA binding activity at all.

INTRODUCTION

The 7-deazapurines, 7-deazaguanine (G*), or 7-deazaadenine (A*) are analogues of natural purine nucleobases, retaining ability to form Watson–Crick base pairs, maintaining natural nucleobases pairing specificity, but unable to form Hoogsteen pairs due to absence of the N7 atom (which is substituted by CH group, Fig. 1). Thus, the 7-deazapurines cannot be involved in formation of triplex and tetraplex DNA structures. The 7-deazapurines are acceptable substrates for DNA incorporation by DNA polymerases in primer extension assay (PEX) or polymerase chain reaction (PCR)[1].

Although the rate of incorporation of G* or A* by DNA polymerases has been reported to be lower, compared to the "parent" purines[2], both of these nucleobase analogues allow efficient for sequence-specific DNA amplification by PCR and it has been possible to prepare PCR products with a high density of the corresponding modifications. Site specific modification of purine nucleosides for G* or A* could be very sophisticated tool for monitoring DNA-protein interaction and possible tolerability of nucleobase modification or damage. A good model for such studies could be interac-

tion of tumour suppressor protein p53 with p53 DNA binding sequence (p53DBS).

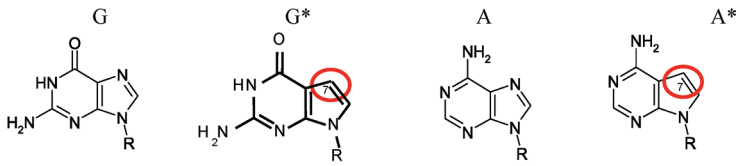

FIG. 1
Formulas of purine nucleobases (A, G) and their 7-deaza analogues (A*, G*)

RESULTS AND DISCUSSION

The function of p53 protein is closely related to recognition of p53DBS which typically consist of two tandem copies of the motif RRRCWWGYYY (p53DBS half site; where R = A or G, Y = C or T, and W = A or T), which may be separated by one or more base pairs[3]. In our work, we used 50-mer oligonucleotide containing p53DBS with two slightly different p53DBS half-sites (Fig. 2) and a set of ^{32}P-radicatively labelled primers of different lengths partly or fully covering the p53DBS region for selective incorporation of G* or A* by PEX using KOD polymerase. Generated sets of PEX products were subjected to binding with p53 protein and finally protein–DNA complexes were analysed by electrophoretic mobility shift assay (EMSA) in native polyacrylamide gel. Gels were dried and autoradiographed.

FIG. 2
Scheme of DNA substrate used in this work (p53 DNA binding sequence (p53DBS) – underlined, key p53 binding residues within p53 binding half site – bold)

Incorporation of G* instead of guanine (G) residues within both p53DBS half sites completely abolished sequence-specific p53 binding. On the other hand, in cases of modification within only one half site (or less) the p53 binding capability was preserved. Incorporation of any adenine residue within overall p53DBS did not affect p53 protein DNA binding activity at all.

This work was supported by the Academy of Sciences of the Czech Republic (Z5 004 0507 and Z5 004 0702), the Ministry of Education, Youth and Sports of the Czech Republic (LC06035), Grant Agency of the Academy of Sciences of the Czech Republic (IAA400040901) and Czech Science Foundation (P301/11/20766).

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P-53

SYNTHESIS AND STRUCTURAL ASSIGNMENT OF NOVEL 5′-EPIMERIC 3′-DEOXY-3′,4′-DIDEHYDRONUCLEOSIDE-5′-*C*-PHOSPHONATES

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Epimeric 5′-(*RS*) dialkyl 3′-deoxy-3′,4′-didehydro-5′-*C*-phosphonates were prepared by nucleophilic addition of various dialkyl phosphites to 3′-deoxy-3′,4′-didehydronucleoside-5′-aldehydes. Successful separation, crystallisation and X-ray crystallographic analysis of a pair of epimeric 5′-*C*-phosphonates, followed by correlation with a series of NMR parameters, led to efficacious configuration assignment of individual epimers in the mixtures.

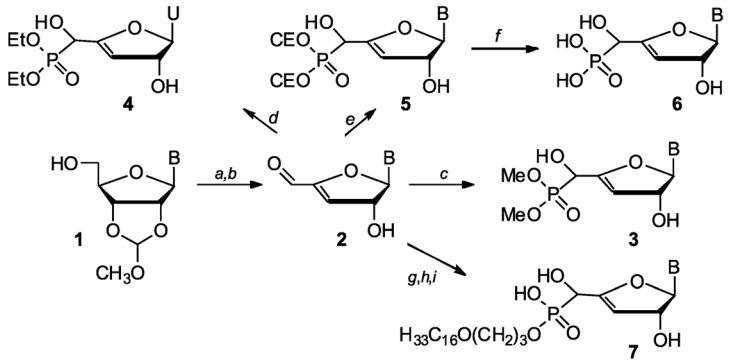
INTRODUCTION

Exploiting the recently reported synthesis of 3′-deoxy-3′,4′-didehydronucleoside-5′-aldehyde synthons[1], we prepared epimeric 3′-deoxy-3′,4′-didehydronucleoside-5′-*C*-phosphonates as isopolar, non-isosteric analogues of 5′-nucleotides. The novel compounds combine in their scaffold both the enzymatically stable *P–C* bond instead of the ester *P–O* linkage[2], and the 3′,4′-unsaturated nucleoside motif[3,4], which renders the potential of biological activity of these derivatives.

RESULTS AND DISCUSSION

3′,4′-Unsaturated 5′-aldehydes[1] **2** were subjected to Abramov Et_3N-mediated nucleophilic addition of dialkyl phosphites[5,6], and epimeric 5′-(*RS*) dialkyl 3′-deoxy-3′,4′-didehydro-5′-*C*-phosphonates were obtained. In the first series, the starting 5′-aldehydes **2** were prepared in situ from 2′,3′-*O*-methoxymethylidene ribonucleosides **1** (by modified Moffat oxidation using DMSO/DCC, TFA/Py, followed by Et_3N-mediated elimination of the orthoester moiety) and subjected to the addition of dimethyl phosphite. Since a substantial cleavage of the nucleosidic bond was observed during TMSB deprotection of dimethyl phosphonates **3**, in the second series, we switched to bis(2-cyanoethyl)phosphite[7] as the phosphonylating agent and conducted the reaction with 5′-aldehydes **2** in DMF in the presence of Et_3N. The obtained diesters **5** were smoothly deprotected via β-elimination in 8 M $MeNH_2$ in EtOH. It was also found that water can be used as the solvent of choice; thus, the crystalline 5′*R*/*S* diethyl phosphonate **4** was obtained.

Thirdly, using 3-hexadecyloxypropyl methyl phosphite[7] followed by the treatment with 60% aq. Py at 50 °C (removal of the methyl ester group) and with methanolic NH_3 or 8 M $MeNH_2$ in EtOH (removal of *N*-acyl groups), lipophilic 3-hexadecyloxypropyl phosphonates **7** were prepared.



SCHEME 1
a) DMSO, DCC, pyridine, TFA; *b*) Et_3N; *c*) $HP(O)(OCH_3)_2$; *d*) $HP(O)(OEt)_2$-Et_3N, H_2O; *e*) $HP(O)(OCE)_2$-Et_3N, DMF; *f*) $MeNH_2$, EtOH, 50 °C; *g*) $H(O)P(OCH_3)(OC_3H_6OC_{16}H_{33})$-$Et_3N$, DMF; *h*) 60% aq. Py, 50 °C; *i*) NH_3 in MeOH or $MeNH_2$ in EtOH (B = protected/deprotected purine/pyrimidine nucleobase)

Due to the absence of the H4′ atom, the previously reported direct NMR assignment method[5], employing the characteristic values of the *J*(P,H4′) and *J*(H5′,H4′) coupling constants, was inapplicable. Nevertheless, based on the data obtained from X-ray analysis of a pair of HPLC resolved epimers **4**, after correlation with selected NMR parameters, the assignment of the C5′-*R*/*S* configuration of individual epimers in a mixture was feasible.

Obtained free phosphonic acids **6** and 3-hexadecyloxypropyl esters **7** were tested against HCV in a replicon system. No activity was found.

Financial support provided by the grants 203/09/1919 and 203/09/0820 (Czech Science Foundation) and by Research Centers KAN200520801 (Academy of Sciences of the Czech Republic) and LC06077 (Ministry of Education, Youth and Sports of the Czech Republic), under the Institute research project Z40550506, is gratefully acknowledged.

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G-QUADRUPLEX DNA STABILIZING AGENTS BASED ON 1,8-NAPHTHYRIDINE

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1,8-Napththyridine-bis(quinolinium) derivatives were synthesized and evaluated as quadruplex-DNA stabilizing agents. Results indicate that the lignads bind with quadruplex DNA with high affinity and also have shown considerable selectivity over the duplex DNA.

BACKGROUND

Quadruplexes are four-stranded higher order nucleic acids structures made up of multiple G-quartets (G-tetrad) vertically stacked over each other. Each planar G-quartet consists of four guanine bases held together by hydrogen bonding[1]. G-quadruplex DNA forming sequences are of high biological significance[2]. They are present at the chromosomal ends called telomeres, promoter regions of DNAs and at the 5′ and 3′ untranslated regions (UTRs) of mRNAs [2]. Formation of G-quadruplex by the stabilizing agents (ligands) is therapeutically appealing. For example, quadruplex formation at telomeric level can inhibit the function of telomerase which is over-expressed in around 85% of cancer cells[3]. Therefore, inhibiting the function of telomerase is an attractive target for cancer treatment since it is highly specific to the cancer cells. Moreover formation and stabilization of quadruplex in the promoter regions controls the gene expression at the transcriptional level[4] and UTR quadruplex regulates genes at the translation level[5]. The main challenge involves in designing ligand is that it should be highly selective toward targeted quadruplex over other quadruplexes and duplex DNA[2]. Though numerous ligands have been studied with various quadruplex forming sequence, many of them bind to all kinds of quadruplexes or duplexes. In this context, naphthyridine based ligands were explored as quadruplex stabilizers.

RESULTS AND DISCUSSION

Naphthyridine-bis(quinolinium) based ligands have been synthesized, and the binding affinity with quadruplex forming sequences were tested by fluorescence intercalator displacement assay (FID)[6]. With telomeric quadru-

plex DNA, the ligands have the binding affinity in the lower micro molar range (0.2 μM). Selectivity of ligands was also tested with duplex forming sequences. It was found that ligands were 15–33-fold more selective towards telomeric quadruplex over duplex DNA. Moreover, quadruplex stabilization by the ligands tested by concentration-dependent polymerase stop assay shows lower IC_{50} (~0.8 μM). CD studies have shown that telomeric sequence was induced to form antiparallel quadruplex structure upon addition of ligands. To get insight into the binding mode and interaction of ligands to G-quadruplex DNA, docking and molecular dynamics studies were performed with anti-parallel (PDB ID: 143D) structure. From MM-PBSA and Nmode analysis based on MD trajectories, it was observed that the end-stacking binding mode was favored with lower binding energy (ΔG = –81 to –89.7 kcal/mol). For all the studies, well-studied quadruplex DNA binding ligand pyridine-bis(quinolinium), **360A**, was chosen as a reference compound[7].

This work was financially supported by IRCC-IIT Bombay and Council of Scientific and Industrial Research (CSIR, 01-2233/08/EMR-II)-Government of India.

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TAIL LABELLED OLIGONUCLEOTIDE PROBES FOR THE DETECTION OF DNA-PROTEIN INTERACTIONS

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DNA-protein interactions can be monitored via different ways. We introduce novel, fast and simple approaches in DNA-protein interaction detection based on electrochemical measurements of DNA alone (structure-sensitive DNA sensing), or DNA modified with osmium tetroxide bearing nitrogenous ligands, or measurements of redox-active moieties enzymatically attached to the end of a DNA substrate thus forming a labeled tail (by terminal transferase).

INTRODUCTION

Electrochemical analysis proved a promising detection platform for the development of novel microanalytic techniques applicable in DNA-protein binding assays. A number of these techniques utilize magnetic beads with immobilized either nucleic acid[1], or protein[2] for pull-down of the other interacting partner from a sample solution. To detect a DNA-protein recognition event, intrinsic electrochemical or electrocatalytic activity of the protein[1] or DNA[2] can be used. For further improvement of sensitivity and selectivity of the assay, specific DNA targets can be redox labeled to obtain a distinct electrochemical signal differing from those produced by non-specific DNA molecules.

RESULTS AND DISCUSSION

Labeling of specific DNA targets with redox markers was attained through attaching a single stranded (ss) tails to 50-mer oligonucleotide (ON) duplexes encompassing the p53 binding site[3]. We used two techniques to introduce electroactive moieties into ss ON tail. The first was based on using terminal deoxynucleotidyl transferase (TdT), an enzyme attaching nucleotides at the 3′-OH terminus of DNA using dNTPs as substrates. A single-stranded primer can thus easily by extended by a labeled homonucleotide stretch (when a single labeled dNTP is used). We found 3-nitrophenyl-7-deazaG as the most useful label because it was efficiently incorporated by

the TdT to form long tail-labels at any oligonucleotide used, and the nitrogroup produced a well developed electrochemical signal. The specific part of the extended ON was hybridized with a complementary strand to create the duplex target. We demonstrated that tumour suppressor p53 protein was able to recognize a specific binding site within the tail-labelled DNA.

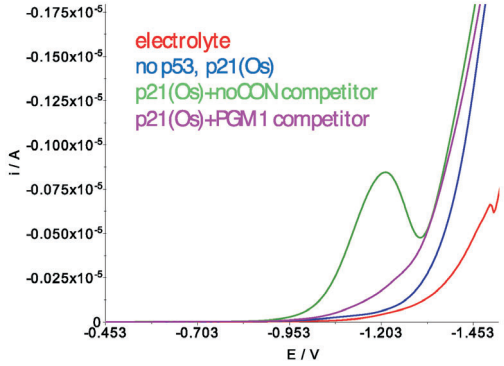

FIG. 1
Electrochemical responses of with Os,bpy-labelled ON p21 alone (blue), p21 in the presence of competitor DNA lacking the p53CON (green; here p53 binds the Os,bipy labeled ON and the signal is observed) and p21 in the presence of competitor DNA containing the p53CON (violet; in this case p53 binds preferentially to the specific competitor and the signal is absent) at HMDE. DNA was recovered from magnetic beads after p53-specific immunoprecipitation of the protein-DNA complexes

The other approach was based on the ON probe tail-labelling with osmium tetroxide in the presence of 2,2′-bipyridine (Os,bpy). In this case, a duplex ON with $(dT)_{20}$ ss overhang was designed in which the oligo(dT) tail was chemically modified with the Os,bpy [4]. Since Os,bpy reacts only with unpaired thymines, those in the specifc duplex segment are protected and p53 binding to the tail-labelled target is retained. We used immunoprecipitation on magnetic beads for studying structure- or sequence-specific DNA binding by tumour suppressor p53 protein to competitor plasmid DNA substrates using the Os,bipy-labelled ON as indicator substrate (Fig. 1).

This work was supported by the ASCR (Z4 055 0506, Z5 004 0507 and Z5 004 0702), the Ministry of Education, Youth and Sports of the Czech Republic (LC512, LC06035), GA ASCR (IAA400040901), GA CR (P301/11/2076) and by Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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USE OF 1,3-DIOXOLANES IN THE SYNTHESES OF α-BRANCHED ALKYL AND ARYL N-9-[2-(PHOSPHONOMETHOXY)ETHYL]PURINES

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Syntheses of various alkyl and aryl substituted N-9-[2-(phoshonomethoxy)ethyl]purines from a number of 2-alkyl(aryl)-1,3-dioxolanes were developed in preparative yields. The cleavage of a dioxolane ring with Lewis acids was chosen for preparation of suitable phosphonate building blocks as a key reaction step followed by their Mitsunobu reaction with purine bases. Obtained phosphonate derivatives were tested as potential HG(X)PRTase inhibitors. In contrast to previously published N-9-[2-(phosphonoethoxy)ethyl]purines, no inhibitory activity towards this enzyme was observed.

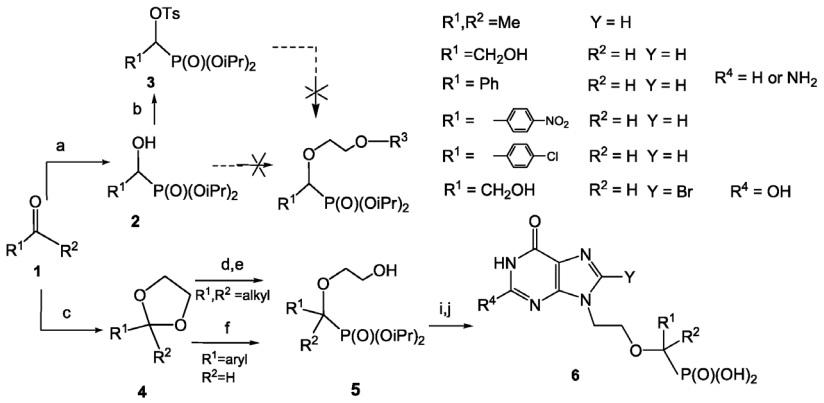
INTRODUCTION

In recent years, acyclic nucleoside phosphonates (ANPs), known as significant antiviral agents[1], have been intensively investigated for their ability to inhibit *Plasmodium falciparum* hypoxanthine-guanine-xanthine phosphoribosyltransferase (*Pf*HGXPRT)[2]. This enzyme plays a significant catalytic role in the formation of 6-oxopurine nucleoside monophosphate as an important component for many cellular processes and its activity is essential for the replication and survival of the *Plasmodium* parasites causing one of the most widespread infectious diseases in the world-malaria.

To date, it is known that some purine ANPs, e.g. N-9-[2-(phoshonomethyl)ethyl]guanine (PMEG), N-9-[2-(phoshonoethyl)ethyl]guanine (PEEG) can mimic the leading structure of nucleoside monophosphate product Hregarding their promissing inhibitory activity towards *Pf*HGXPRT [3]. These findings have encouraged their further modifications (i.e. hypoxanthine PEE derivatives (PEEHx)[3] and various side-chain substituted PEE phosphonates[4]). In this study, we described the synthesis of new side-chain modified phosphonomethoxyethyl (PME) derivatives (Scheme 1). These compounds were designed to investigate an effect of branching of a (phosphonomethoxy)ethyl chain on their ability to inhibit *Pf*HGXPRT. Because the methods of introduction of alkyl and aryl substituents to the PMEG phoshonomethoxy group are not completely documented in literature[5,6], their development still remains a synthetic challenge.

RESULTS AND DISCUSSION

Currently, the methods of introduction of any substituents to the phosphonomethoxy group seem to be limited (e.g. due to a worse reactivity of secondary alkohols **2** or tosylates **3** as key precursors, see Scheme 1).



SCHEME 1

(a) $HP(O)(OiPr)_2$, MeONa, MeOH, Et_2O; (b) TsCl, Et_3N, DMAP, CH_2Cl_2, rt; (c) $HOCH_2$-CH_2OH, *p*-TsOH, reflux; (d) TMSI, $HP(O)(OiPr)_2$, NaH, cyclohexene, –78 °C → rt; (e) 10% aq. KF, EtOH, rt; (f) $SnCl_4$, $P(OMe)_3$, THF, –78 °C; (i) 6-chloropurines, Ph_3P, DIAD, THF, 0 °C; (j) Me_3SiBr, MeCN, rt, followed by HCl, H_2O, 100 °C

Despite these difficulties, syntheses using the 1,3-dioxolane cleavage with Lewis acids afford the appropriate building block **5** in good preparative yields. Subsequent condensation reactions of **5** with guanine or hypoxanthine were performed under Mitsunobu reaction conditions. The obtained phosphonates **6** showed a marginal inhibitory activity in comparison with the corresponding α- or β-branched PEE ANPs [4].

This work was performed as a part of the Research program of IOCB 0Z40550506. It was financially supported by the Center for New Antivirals and Antineoplastics 1M0508 by the Ministry of Education, Youth and Sports of the Czech Republic and Gilead Sciences & IOCB Research Centre.

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DIRECT ENZYMATIC SYNTHESIS OF ALDEHYDE-FUNCTIONALIZED DNA AND ITS CONJUGATION WITH HYDRAZINES AND AMINES

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A new simple methodology for DNA conjugation or staining was developed. 2′-Deoxyribonucleoside triphosphates (dNTPs) bearing reactive aldehyde group were prepared by one-step Suzuki cross-coupling reaction of halogenated dNTPs with boronic acid. These modified dNTPs were enzymatically incorporated into DNA by primer extension (PEX) or amplified by polymerase chain reaction (PCR) using different DNA polymerases. The follow-up reaction between aldehyde-modified PCR products and hydrazine derivatives gave coloured DNA conjugated hydrazones. This methodology was also used for further conjugations of aldehyde-modified 2′-deoxyribonucleoside monophosphates (dNMPs) with amines by reductive amination.

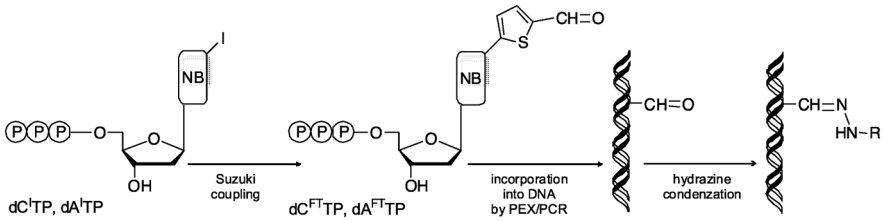
INTRODUCTION

Functionalized nucleic acids are frequently used in different areas such as bioanalysis, nanotechnology and chemical biology. They could be used for DNA-conjugates formation via the cross-link reaction between modified DNA and biomolecules (peptides, proteins) or various markers. Functionalized nucleic acids can be prepared either chemically or enzymatically. Here we present a novel approach to functionalized DNA based on the incorporation of reactive aldehyde functions and their transformation to hydrazones and amines.

RESULTS AND DISCUSSION

Modified cytidine (dC^{FT}TP) and adenosine (dA^{FT}TP) nucleoside triphosphates bearing reactive aldehyde group were prepared by aqueous Suzuki cross-coupling reaction of halogenated nucleoside triphosphates (dN^{I}TPs) with 5-formylthiophene-2-boronic acid (Scheme 1). These modified dN^{FT}TPs were then used as substrates for three different DNA polymerases for the incorporation into DNA[1]. All tested polymerases were efficient in their incorporation by PEX. The best results were obtained with Vent(exo-) DNA polymerase. For the quest of efficiency and fidelity of incorporation, we also proved that aldehyde modified dN^{FT}TPs were excellent substrates for the amplification of DNA. Three different templates and two DNA polymer-

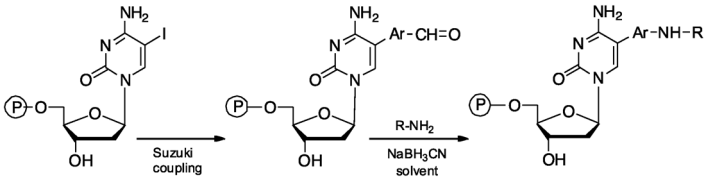
ases (*Pwo* and KOD XL DNA Polymerase) showed full-lenght products. Furthermore, $dA^{FT}TP$ gave more efficient amplification compared to $dC^{FT}TP$.



SCHEME 1
Preparation of aldehyde modified $dN^{FT}TPs$, their incorporation into DNA and hydrazon formation

The products of amplification (aldehyde-modified DNA) were used for condensation of with two different arylhydrazines to give the corresponding orange or violet hydrazones[2]. To facilitate the condensation, we used aqueous ammonium acetate and aniline according to the Dawson protocol for aqueous conjugation of peptides[3].

The synthetic pathway for the formation of amino-derivatives is depicted in Scheme 2. The treatment of various aldehyde derivatives with amino acids and/or peptides by using $NaBH_3CN$ as the reducing agent in either organic or aqueous[4] solvents, gave products of reductive amination as models for bioconjugation of functionalized DNA and peptides or proteins. Reaction conditions will be discused in more details.



SCHEME 2
Synthetic pathway for amino-derivatives formation

This work was supported by the Academy of Sciences of the Czech Republic (Z4 055 0506), the Ministry of Education, Youth and Sports of the Czech Republic (LC512), the Czech Science Foundation (203/09/0317), and Gilead Sciences, Inc. (Foster City, CA, U.S.A.).

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LOCKED NUCLEIC ACID ANTISENSE OLIGONUCLEOTIDES TARGETING APOLIPOPROTEIN B: THE EFFECT OF SHORT SEQUENCES AND α-L-LNA INSERTION

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The reduction in length of a 16 nucleotide LNA gapmer targeting apoB resulted in a length dependent increase in activity on both mRNA expression in liver and serum cholesterol levels. EC_{50} was reduced from 25 to 2.5 mg/kg when gapmer length was reduced from a 16-mer to a 12-mer. Higher levels of serum ALT after long term dosing of some oligonucleotides could be revoked by the insertion of α-L-LNA units in the sequence without losing potency.

INTRODUCTION

Oligonucleotides can be used to target specific mRNAs and thereby inhibit the protein translation of the target. LNA is a nucleic acid modification that increases affinity and improves stability of oligonucleotides[1]. These characteristics are of major importance in the development of highly potent therapeutic oligonucleotides shorter than 20 nucleotides. A stereoisomer of LNA called α-L-LNA has been shown to be able to recruit RNase H and can therefore potentially be placed in the DNA stretch of an LNA-DNA gapmer without loss of activity[2]. Metabolic disorders like hypercholesterolemia is typically associated with increased plasma levels of VLDL and LDL. ApoB is important for assembly of VLDL in liver and for the clearance of VLDL and LDL particles from plasma. Reduction of apoB mRNA expression is known to lower the secretion of VLDL from the liver and to reduce plasma cholesterol, which decreases the risk of developing atherosclerosis[3]. We have previously shown that by using a 16-mer LNA oligonucleotide, the expression of apoB can be downregulated in liver, leading to reduction in plasma cholesterol levels[4].

RESULTS AND DISCUSSION

A series of LNA gapmer oligonucleotides with lengths of 12 to 16 nucleotides were synthesized and screened *in vivo* in mice. Target ApoB mRNA levels in liver, total cholesterol levels in serum and serum ALT levels were

monitored to evaluate the efficiency of the compounds. It was shown that shortening the oligonucleotides from 16- and 15-mers down to 14-, 13- and 12-mers resulted in a marked increase in activity both on target mRNA expression and serum cholesterol levels. This increase in activity did not result in increased hepatocellular toxicity when the oligonculotides were dosed over 3 days. When the dosing was extended to 16 days, some of the short oligonuclotides showed increased levels of serum ALT. For one of these oligonucleotides we have shown that this increase can be revoked by the insertion of α-L-LNA units in the sequence without losing potency.

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SYNTHESIS OF PME DERIVATIVES OF NUCLEOBASES WITH CONFORMATION LOCKED VIA PYRROLIDINE RING

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Systematic biological investigation of the structurally diverse nucleoside phosphonic acids has led to potent antiviral drugs based on both the acyclic phosphonate nucleotides such as HPMPC (Cidofovir™), PMEA (Adefovir™), PMPA (Tenofovir™), used in the treatment of CMV-induced retinitis, hepatitis B and HIV, respectively. Here we present synthesis of such acyclic phosphonate nucleotides conformationaly locked *via* pyrrolidine ring.

INTRODUCTION

The azanucleosides represent an important group of antimetabolites that exhibit a variety of biological effects. In these nucleoside analogs, the furanose sugar is replaced by a nitrogen heterocycle. During our systematic study on synthesis and properties of pyrrolidine azanucleosides and azanucleotides we have described structures **1** and **2** (Fig. 1)[1–4]. Here we present the synthesis of diastereomeric azanucleoside phosphonic acids **3** and **4** that could be regarded as known antivirals phosphonomethoxyethyl derivatives of nucleobases[5,6] conformationaly locked *via* pyrrolidine ring.

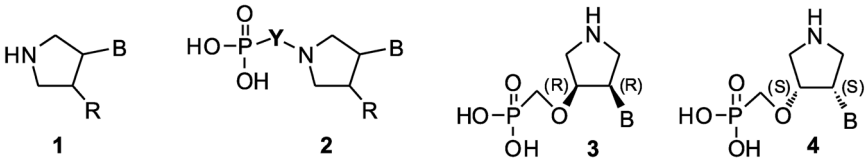
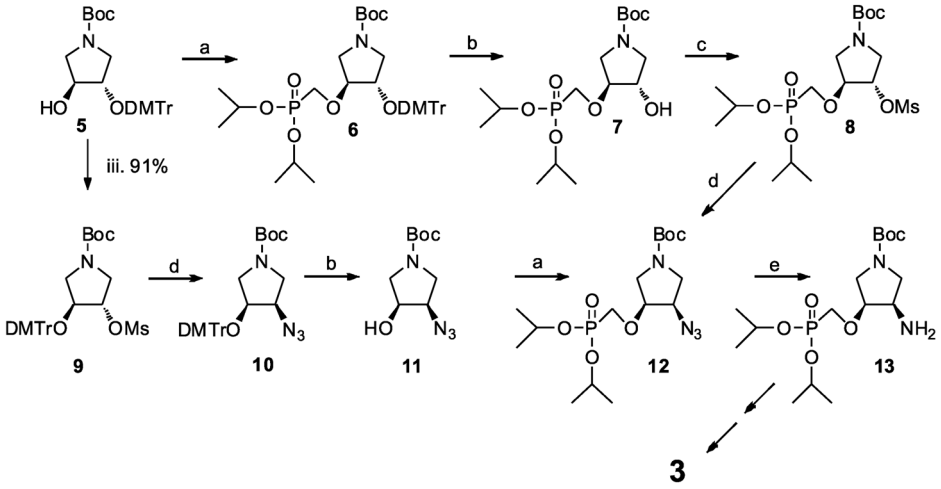


FIG. 1

RESULTS AND DISCUSSION

The synthesis of target compounds consisted of two parts: synthesis of intermediate containing primary amino group and construction of nucleobase on the amino moiety. Two routes to amino intermediate **13** were evaluated (Scheme 1). Monodimethoxytrityl derivative **5** reacted with diisopropyl tosyloxymethanphosphonate to afford phosphonate **6** that was treated with 1.5% TFA in DCM to afford compound **7**. Compound **7** was mesylated and treated with sodium azide giving azido derivative **12**. This reaction was accompanied by removal of isopropyl ester group decreasing yield of **12**. Thus different route to azido derivative **12** was developed.

Starting monodimethoxytrityl derivative **5** was first converted to azido derivative **11** that subsequently reacted with diisopropyl tosyloxymethanephosphonate. Obtained azido derivative **12** was finally converted to amino derivative **13** by catalytic hydrogenation over palladium catalyst. Nucleobases were constructed on the amino moiety of **13** (Scheme 1) using standard procedures. Enantiomeric phosphonic acids **4** were synthesized using the same procedure starting from enantiomeric 1-*N*-Boc-3-dimethoxytrityloxy-4-hydroxypyrrolidine.



SCHEME 1

a) $TsOCH_2P(O)(OiPr)_2$, NaH, THF; b) 1.5% TFA/DCM; c) MsCl, DMAP, DCM; d) NaN_3, DMF; e) H_2, Pd/C, EtOH

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TOWARDS THE EFFICIENT SYNTHESIS OF tRNA WITH SITE-SPECIFIC Cy3/Cy5 LABELS

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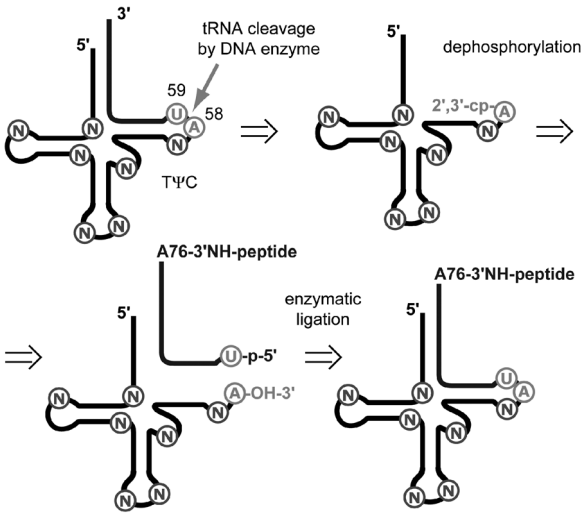
The synthesis of $tRNA^{Phe}$ with site-specifically Cy5 or Cy3 labels is presented based on a previously published combined synthetic and enzymatic approach. We aim at the investigation of ribosome kinetics during translation using fluorescence spectroscopic methods.

INTRODUCTION

The preparation of transfer RNA (tRNA) with site-specifically attached fluorescent dyes is challenging, in particular when all natural tRNA modifications should be available. Such conjugates are currently highly requested for the investigation of ribosome kinetics during the translational peptide synthesis cycle[1,2].



SCHEME 1
Concept for the semisynthesis of non-hydrolyzable 3′-peptidyl-tRNA conjugates. Modified nucleosides (N), cyclophosphate (cp), phosphate (p)

RESULTS AND DISCUSSION

Recently, we have presented a novel approach for the efficient synthesis of non-hydrolyzable 3′-peptidyl-tRNAs which contain all natural tRNA nucleoside modifications[3,4]. The basic concept is outlined in Fig. 1. In short, we started from natural tRNAs that were cleaved within the TΨC loop by DNA enzymes to obtain a 5′ tRNA fragment containing all modifications. After dephosphorylation of the 2′,3′-cyclophosphate moiety, this fragment fulfills the structural requirements for enzymatic ligation to RNA-peptide conjugates which have been prepared according to a solid-phase synthesis approach elaborated previously in our laboratory[5].

Ongoing efforts in our laboratory focus on the development of efficient labeling strategies for tRNA with Cy5- and Cy3-dyes. Our long-term aim is to contribute towards understanding of ribosome translational dynamics.

In this poster presentation, we will demonstrate site-specific labeling of tRNA with fluorescent dyes within the acceptor arm, starting from a previously introduced procedure for the semi-synthesis of 3′-peptidyl-tRNA[3].

Financial support from the Austrian Science Fund FWF (P21641) and from the bm:wf (GEN-AU programme 'Non-coding RNA', P7260-012-012) is acknowledged.

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TOWARDS THE EFFICIENT SYNTHESIS OF RNA WITH SITE-SPECIFIC ^{15}N-LABELS FOR NMR SPECTROSCOPIC APPLICATIONS

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The synthesis of ^{15}N-labeled uridine and adenosine building blocks for the incorporation into RNA by chemical solid-phase synthesis has been optimized following previously published procedures. We aim at the investigation of riboswitch folding and dynamics using NMR spectroscopic methods.

INTRODUCTION

Non-coding regions of mRNA which bind metabolites with high selectivity and specificity function as so-called riboswitches[1–3]. They represent gene regulation systems that are widespread among bacteria and importantly, they do not rely on the assistance of proteins. Riboswitches consist of a metabolite-sensitive aptamer and an adjoining expression platform. Although impressive progress has been made in revealing three-dimensional structures of metabolite-bound aptamer complexes of various riboswitch classes, less is known about how binding of the metabolite to the aptamer is communicated into a structural change of the expression platform which in turn signals "on" or "off" for gene expression. The simplified picture of bacterial transcription control is that upon metabolite binding either a mRNA terminator structure is formed which causes the RNA polymerase to stop synthesis ("off" regulation) or an existing terminator is disrupted which enables the polymerase to continue synthesis with the mRNA template ("on" regulation). In the case of translational control, accessibility versus sequestration of the Shine–Dalgarno sequence upon metabolite binding is the essence of the response mechanism for "on" versus "off" regulation, corresponding to hindrance or enabeling of bacterial ribosome translation initiation.

RESULTS AND DISCUSSION

Ongoing efforts in our laboratory focus on the synthesis of ^{15}N-labeled nucleoside phosphoramidites for the introduction into large RNAs by chemical solid-phase synthesis. Our long-term aim is to contribute to understanding of riboswitch folding and dynamics[4–6].

In this poster presentation, we will demonstrate the optimized synthesis of ^{15}N(3)-uridine and ^{15}N(1)-labeled adenosine derivatives as precursors for phosphoramidite building blocks, starting from published procedures by Pitsch[7] and Hrdlicka[8].

Financial support from the Austrian Science Fund FWF (I317) and from the bm:wf (GEN-AU programme "Non-coding RNA", P7260-012-012) is acknowledged.

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DNA GOLD NANOPARTICLE CONJUGATES INCORPORATING THIONUCLEOSIDES: 7-DEAZA-6-THIO-2′-DEOXYGUANOSINE AS GOLD SURFACE ANCHOR

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A new robust and efficient protocol for the modification of gold nanoparticles with oligonucleotides employing the thio group of 7-deaza-6-thio-2′-deoxyguanosine (**1**) as anchoring site is presented. Oligonucleotide AuNP conjugates were prepared and their hybridization properties were studied.

INTRODUCTION

DNA gold nanoparticles (DNA-AuNPs) have been widely employed in nucleic acid detection, labelling and the bottom-up assembly of nanoarchitectures[1,2]. DNA has to be covalently linked to the surface of AuNPs. Several strategies were developed for preparing DNA-AuNP conjugates[3,4]. Herein, an efficient procedure for DNA-AuNP conjugation was established using **1** as gold surface anchor. This synthetic strategy is convenient and works efficiently to prepare DNA-AuNPs with single or multiple anchoring sites within any oligonucleotide chain. Common protocols employ oligonucleotides with 3′- or 5′-terminal thiol groups introduced by linker units[3,4]. In this work, we selected 7-deaza-6-thio-2′-deoxyguanosine (**1**) as thionucleoside to be an integer part of a single-stranded oligonucleotide to function as anchoring site[5].

RESULTS AND DISCUSSION

7-Deaza-6-thio-2′-deoxyguanosine (**1**) was converted into its corresponding phosphoramidite building block **2** (Fig. 1). A series of oligonucleotides containing **1** at different positions were synthesized by solid-phase synthesis. After purification, modified oligonucleotides were directly mixed with AuNPs and stepwise adjusted to a final NaCl concentration of 0.1 M NaCl, 10 mM PO_4^{3-}, pH 7.0. During conjugation, the DNA gold nanoparticle solutions stayed deep red in colour. Moreover, the resulting DNA-AuNPs show the expected plasmon resonance at 524–526 nm indicating a non-aggregated state. Next, hybridization experiments were performed with

complementary DNA-AuNP conjugates. **Au3•Au4** and **Au5•Au6** containing **1** show sharp melting profiles at 520 nm indicating the 3-dimensional linked network of DNA-AuNPs assembled by the complementary duplexes (Fig. 2). The melting profiles are comparable to those obtained from the parent **Au7•Au8** employing the conventional alkylthiol linker.

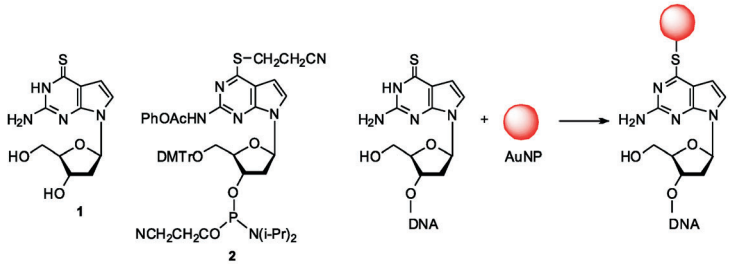
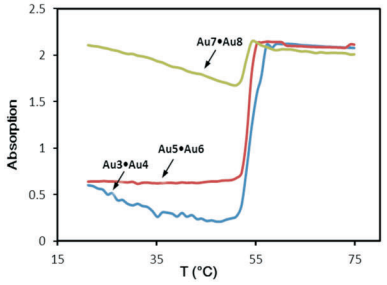


FIG. 1
Compounds used for DNA gold nanoparticle conjugation



DNA AuNP conjugates	T_m [°C]
5'-d(1 T_{10} TAG GTC AAT ACT) **(Au3)** 3'-d(ATC CAG TTA TGA T_{10} 1) **(Au4)**	54
5'-d(TA1 GTC AAT ACT TAG GTC AAT ACT) **(Au5)** 3'-d(ATC CAG TTA TGA ATC CAG TTA T1A) **(Au6)**	53
5'-d(**S**-$(CH_2)_6$ T_{10} TAG GTC AAT ACT) **(Au7)** 3'-d(ATC CAG TTA TGA T_{10}-$(CH_2)_6$-**S**) **(Au8)**	53

FIG. 2
Left: Melting profiles of DNA-AuNP assemblies measured at 520 nm in 0.1 M NaCl, 10 mM phosphate buffer (pH 7.0) with A_{520} = 2.1 for each DNA-AuNP conjugate solution. Right: T_m values of DNA duplex AuNP conjugates (= 15 nm diameter gold nanoparticle)

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SPATIALLY CONTROLLED DNA NANO-PATTERNS BY "CLICK" CHEMISTRY USING OLIGONUCLEOTIDES WITH DIFFERENT ANCHORING SITES

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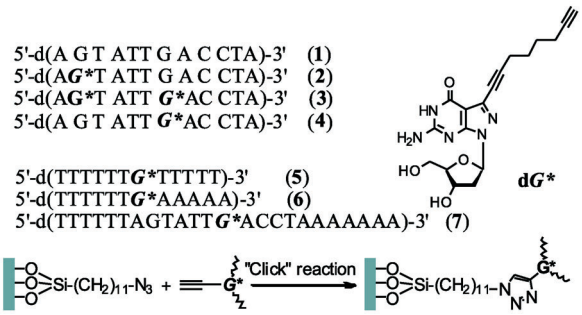
We report on a template-free strategy based on "click" chemistry to fabricate spatially controlled DNA nano-patterns immobilized on surfaces. DNA with different anchoring sites can be chemically immobilized on surfaces by forming 1,2,3-triazole linkages. The position of anchoring is of crucial importance for the self-assembly process of DNA and greatly influences the assembly of particular DNA nano-patterns.

INTRODUCTION

The fabrication of well-defined DNA nanostructures and patterns on solid surfaces based on the highly specific Watson–Crick or Hoogsteen base pairing has been described[1,2]. However, the obtained patterns usually lack chemical bonds with the substrate, which may be not stable enough for further manipulations. The "click" reaction has emerged as a convenient and effective protocol to immobilize DNA and other biomolecules onto surfaces[3–5]. Herein, we report a template-free strategy based on "click" chemistry to fabricate spatially controlled DNA nano-patterns immobilized on surfaces utilizing the self-assembly process of DNA with different anchoring sites[6]. A building block of 7-(octa-1,7-diynyl)-8-aza-7-deazapurine 2′-deoxyribonucleoside (d***G****) bearing a terminal triple bond can be introduced at any position of an oligonucleotide (ODN) chain[7]. Especially, a central d***G**** position can offer two flexible DNA arms, which allows DNA self-assembly by hybridization of the flanking elements. Nano-network structures with high regularity can be obtained. Due to the easy tuneability of the d***G**** position and the high stability of the DNA nano-patterns on surfaces, our method may help to extend applications of nano-architectures and to capture or deliver DNA for diagnostic purposes, DNA sequencing or nanomedical applications.

RESULTS AND DISCUSSION

Through substituting one or two dG nucleosides by d***G**** units, we introduced different anchoring sites in DNA strands (ODNs **2**–**7**). The 12-mer oligonucleotide 5′-d(AGTATTGACCTA) (**1**) was used as a reference (Scheme 1). ODN-**7** contains self-complementary elements at both sides of the modification. Both sides can form Watson–Crick base pairs resulting in double-stranded dA-dT regions (duplexes or hairpins).



SCHEME 1
The oligonucleotide sequences used in this study and the "click" reaction to anchor DNA onto the solid surface. d***G**** refers to the modified d*G* carrying a terminal triple bond

We were able to demonstrate that the position of such a d***G**** anchor containing a lipophilic side chain greatly influences the self-assembly process of DNA. Thus, it is of crucial importance for the generation of particular DNA nano-patterns. Figure 1a–1f shows that d***G**** in a central position of ODNs generates tuneable nano-networks with high regularity, compared to ODNs containing d***G**** in terminal or other positions.

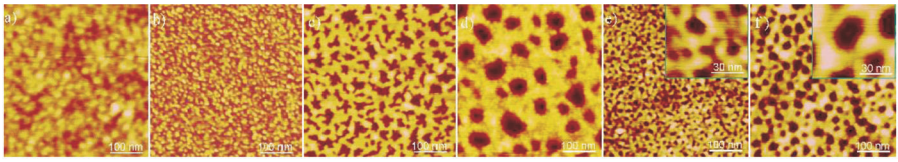


FIG. 1
AFM images of silicon wafers coated with DNA by "click" chemistry. (a) Densely packed particles of 20–30 nm diameter; (b) densely packed particles of 10–15 nm; (c) nano-network structures of 30–50 nm; (d) nano-hole network structure; (e) regular porous nano-network, pores about 20 nm, and (f) regular porous nano-network structure, pores about 40 nm

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SYNTHESIS AND SOME BIOLOGICAL PROPERTIES OF 5-ALKOXYMETHYL DERIVATIVES OF 2′-DEOXYURIDINE 5′-PHOSPHONATES

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New 5-dodecyloxymethyl-2′-deoxyuridine 5′-phosphonates were synthesized and their cytotoxicity in two cell cultures and stability at different pH values and in fetal calf serum were studied.

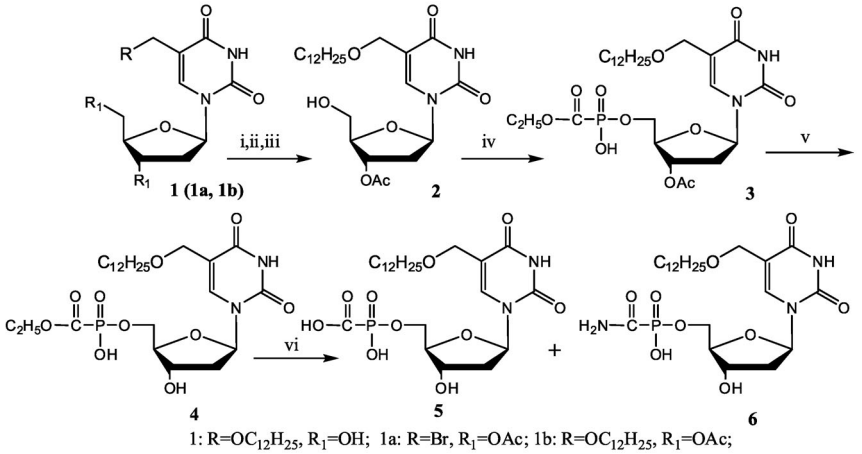
INTRODUCTION

Pyrimidine nucleosides bearing substituents in position 5 display various biological activities. Particularly, 5-modified nucleoside derivatives are widely used for therapy of viral infections[1]. Some 5-substituted pyrimidine nucleosides with lengthy 1-alkynyl radicals exhibited significant *in vitro* anti-tubercular activity[2]. Recently we reported that a series of 2′-deoxy-, 3′-azido-2′,3′-dideoxy-, and 3′-amino-2′,3′-dideoxypyrimidine nucleoside analogues bearing lengthy flexible alkyloxymethyl substituents demonstrated *in vitro* inhibitory properties towards *Mycobacterium tuberculosis*[3]. Nucleosides with large hydrophobic fragments at position 5 of the nucleic base are insoluble in water. One of the possibilities to increase their solubility is the synthesis of the corresponding 5′-monophosphates or 5′-phosphonate derivatives. However, 5′-monophosphates of the above mentioned nucleosides did not inhibit the growth of mycobacteria[3].

The goal of this work was the synthesis of 5-alkyloxymethyl-2′-deoxyuridine 5′-phosphonate derivatives as potential inhibitors of *M. tuberculosis* growth and evaluation of their stability and cytotoxicity.

Starting 5-dodecyloxymethyl-2′-deoxyuridine **1** was prepared by the known procedure[4] via radical bromination of 3′,5′-di-*O*-acetylthymidine followed by alkylation of**1a** with dodecanol and deprotection of resulting **1b**. The successive treatment of **1** with *t*-butyldimethylsilyl chloride, acetic anhydride and CH_3COOH led to the key dodecyloxymethyl-2′-deoxy-3′-*O*-acetyluridine **2**. The reaction of nucleoside **2** with ethoxycarbonylphosphonic acid in the presence of *N*,*N*′-dicyclohexylcarbodiimide similarly to the described procedure[5] resulted in 5′-ethoxycarbonyl phosphonate **3** in 73% yield. It was deprotected with 2% aqueous ammonia in EtOH

to give 65% of target 5′-ethoxycarbonyl phosphonate **4**. 5′-Aminocarbonyl phosphonate **5** and 5′-carboxyl phosphonate **6** were obtained from compound **4** by the reaction with 25% aqueous ammonia in 45 and 43% yields, respectively. UV, NMR and mass spectra confirmed the structures of the synthesized compounds.



SCHEME 1

Reagents: i) *t*-Bu$(CH_3)_2$SiCl, Py; ii) Ac_2O, Py; iii) 80% CH_3COOH, 37 °C; iv) $C_2H_5O(CO)$-$(PO)OH_2$, DMF, DCC; v) NH_4OH (2% aq. in EtOH); vi) NH_4OH (24% aq.)

Compounds **4–6** were stable at 37 °C in buffer solutions at pH 2, 7.4 and 9 and in fetal calf serum for more than 24 h. Phosphonates **4–6** were not cytotoxic at concentrations up to 300 μM in *Vero* and up to 200 μM in K562 cells. The anti-TB-activity will be reported.

This study was supported by the Fundamental Research Program of the Presidium of the Russian Academy of Sciences "Molecular and Cell Biology" and the Russian Foundation for Basic Research (grant No. 11-04-00603).

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SELECTIVE AND EFFICIENT LABELING OF OLIGONUCLEOTIDES BASED ON INVERSE ELECTRON-DEMAND DIELS-ALDER REACTION

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The attachment of fluorescent dyes and affinity tags (e.g. biotin) to biomolecules has become an indispensable tool in modern life sciences. Suitable labeling strategies should allow the site-specific incorporation of complex molecules in an efficient and selective way. Preferably they work under mild conditions at room temperature in aqueous media. Herein we report a new strategy for single and multiple labeling of oligonucleotides based on the inverse electron-demand Diels–Alder reaction.

INTRODUCTION

The harsh conditions of solid-phase oligonucleotide synthesis as well as the restricted tolerance of enzymes for modified triphosphates are limiting the variety of modifications that can be introduced into oligonucleotides. Therefore, there is an increasing interest in labeling strategies that introduce modifications in a mild and efficient way after synthesis or amplification of the oligonucleotide (post-synthetically)[1]. For many years, NHS-ester chemistry dominated post-synthetic DNA functionalization[2] though the need of high excess is limiting this strategy. Therefore, cycloadditions like the copper(I)-catalyzed azide alkyne "click" reaction have gained importance[3–5]. Recently Diels–Alder reactions with inverse electron-demand came to the fore and were successfully applied to modify peptides and small molecule drugs[6–9].

To apply this approach to oligonucleotides, we have introduced dienophiles (norbornene, *trans*-cyclooctene) into oligonucleotides and performed the inverse Diels–Alder reaction with water-stable tetrazine-dienes.

RESULTS AND DISCUSSION

We synthesized four different phosphoramidites and successfully incorporated them into DNA, both internally and terminally. The synthesized 19mer DNA-strands were then subjected to inverse electron-demand Diels–Alder reaction (Fig. 1). Therefore aqueous stock solutions of the oligonucleotides were mixed with a water stable tetrazine at RT. Analysis (HPLC and MS) of the crude reaction mixture showed conversions of up to

90% using only equimolar amounts of reactants. Diels–Alder reaction with an oligonucleotide bearing two dienophilic moieties yielded the double-labeled Diels–Alder product with a conversion of 65% [10]. This demonstrates that the cycloaddition efficiently works by only mixing the two reactants and without requirement of transition metals or any other additives. To straightforward analyze the crude reaction mixtures of the cycloadditions they can be directly subjected to LC-MS analysis.

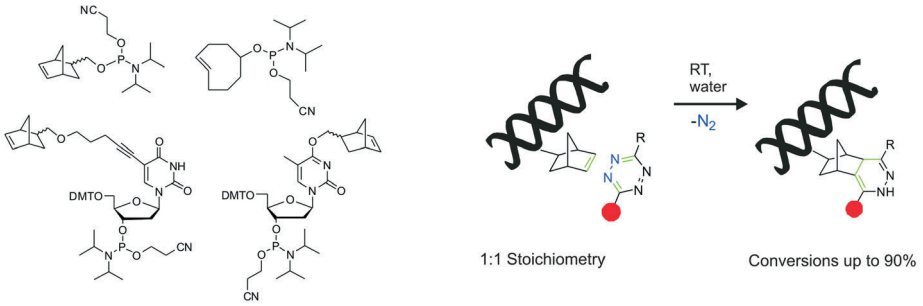

FIG. 1
Left: Synthesized building blocks for incorporating dienophilic moieties into oligonucleotides. Right: Diels–Alder reaction with inverse electron-demand between a norbornene-modified DNA and a tetrazine (R = aryl, heteroaryl)

As the utility of short oligonucleotides in molecular biology is limited, a double-stranded 109mer DNA was amplified using the 19mers as primers. Polymerase chain reaction (PCR) yielded a clean product which was then reacted with a biotin-modified tetrazine. Product formation of up to 75% was obtained by using less equivalents of tetrazine. Experiments on dsDNA bearing up to three norbornene moieties demonstrated the potential use of this strategy for multiple labeling[10]. To expand the applications of this approach, we are currently working on the enzymatically incorporation of modified building blocks into DNA as well as RNA.

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SYNTHESIS OF NEW POTENTIAL INHIBITORS OF 5′-NUCLEOTIDASES

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The work is aimed at the synthesis of potential selective inhibitors of 5′-nucleosidases. The phosphonobenzylidene ring was recognized as a target for proposed modifications. Substituents selected on the basis of a docking study, mostly the electronegative ones, should be suitable for the formation of additional hydrogen bonds, and thus the increased inhibition of the enzymes.

INTRODUCTION

The isopolar nucleoside phosphonic acids (NPAs) form a significant group of nucleotide analogues distinguished for their stability against phosphomonoesterases and nucleotidases due to the presence of bridging P–C–O linkage instead of the P–O one. It is obvious that the large family of nucleoside phosphonic acids represents a unique pool of potential antimetabolites which could act at various levels in the metabolism of living organisms. These compounds are known to act as substrates for nucleotide kinases, and the resulting triphosphate derivatives are incorporated into DNA by viral polymerases and terminate the growing chain. Less known, however, is the fact that NPAs can also act alone, without any additional transformation to secondary metabolites. Thus, several NPAs synthesized in our laboratory were found as potent bi-substrate inhibitors of thymidine phosphorylase[1] mimicking both nucleoside and phospho ester parts, and another NPAs exhibited selective inhibition of 5′-nucleotidases[2]. Since these enzymes play a regulatory role in the cellular metabolism of both nucleosides and nucleotides, they could be regarded as attractive targets for chemotherapeutics based on nucleoside phosphonic acids.

RESULTS AND DISCUSSION

As a lead structure we chose compound **1**[3] prepared in our laboratory which is known as the selective inhibitor of mitochondrial pyrimidine-specific 5′-nucleotidase (Fig. 1)[4,5]. From the crystallographic study[4] it follows that the phosphonate moiety of **1** does not interact with the nucleoside phosphoester binding site but with another site. The careful checking of the

crystal structure of the enzyme with phosphonate **1** and performed docking studies with several thousands compounds based on the lead structure **1** revealed two substituents of the benzene ring in the *para* position which could increase the interactions of **3** in the enzyme binding/catalytic site, and thus increase the inhibition effect.

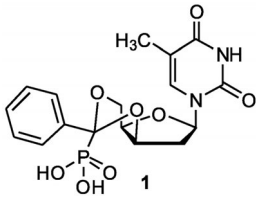
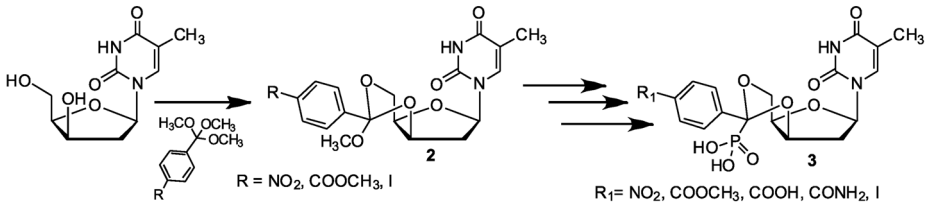


FIG. 1

Our effort was, therefore, focused on the synthesis of "calculated" compounds **3** with nitro and carboxyl functions in the *para* position of the benzylidene ring. Starting orthoesters were prepared from corresponding *N*,*N*-methylphenylbenzamides which can be converted in 3 steps into the trimethyl orthoesters.

The acid-catalyzed reactions of orthoesters with *xylo*T yielded nucleosides **2** which were transformed in the reaction with chloro diethyl phosphite, into the phosphonates **3** (Scheme 1).



SCHEME 1

Purified compounds were tested for inhibition activities at two forms of 5′(3′)-deoxyribonucleotidases. In vitro assay is based on hydrolysis of dUMP and separation of originated dU and substrate on reversed phase column using HPLC. First test of inhibition activity was with substance concentration ten times lower than substrate concentration. Concentration of the substrate was equal to K_m of used enzyme form. Inhibition constants of interesting compounds were measured using Williams–Morisson equation and nonlinear regression.

Support by the grant 203/09/0820 (Czech Science Foundation), Research Centres KAN200520801 (Academy of Sciences of the Czech Republic) and LC06077 (Ministry of Education, Youth and Sports of the Czech Republic) under the Institute research project Z40550506, is gratefully acknowledged.

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SYNTHESIS OF NOVEL *C*-(*o*-CARBORANYL)-2-DEOXY-D-RIBOSE CONJUGATES

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Carboranes are clusters consisting of carbon, boron and hydrogen and because they have high percent of boron by weight, they are used as ^{10}B carriers for cancer therapy (boron neutron capture therapy, BNCT). Therefore a series of novel *o*-carboranyl-deoxyribose conjugates as water soluble substances has been synthesized.

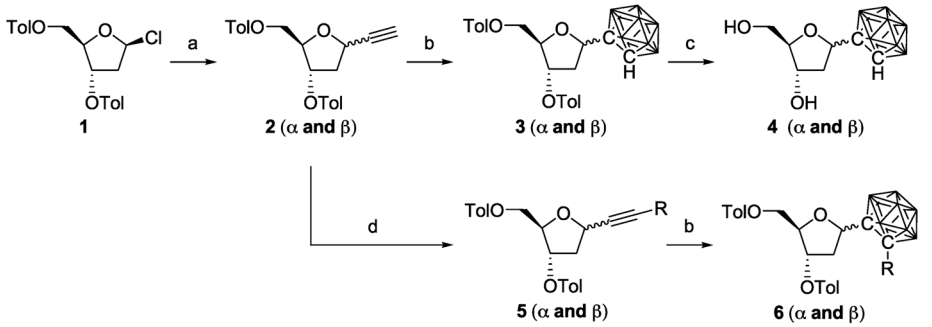
INTRODUCTION

Boron neutron capture therapy (BNCT) is a radiation-based method for treatment of various oncological diseases. Introduction of a stable ^{10}B isotope into tumor and its subsequent irradiation with a beam of slow (thermal) neutrons underlie the method. A nuclear reaction is initiated resulting in formation of high-energy fission products having quite short mean free path, comparable to a cell size. This, in principle, allows selective tumor cell destruction while leaving the surrounding healthy tissue intact[1]. Current generation of agents for BNCT usually contains boron polyhedral clusters and a biomolecular fragment, which is used as a carrier that ensures delivery of the drug to the malignant cells[2]. The use of carbohydrate containing multiple hydroxyl groups could help to compensate for the hydrofobicity of the carboranes, make them water soluble and thereby could enhance their uptake into tumor cells. Herein we would like to report a synthesis of new representant of carboranyl-saccharide conjugates: α- and β-*C*-carboranyl-deoxyribose.

RESULTS AND DISCUSSION

The reaction route to the carboranyl-saccharide conjugate comprises three main steps: introduction of a triple bond into a molecule of carbohydrate, formation of carborane framework and deprotection of carbohydrate molecule. By using this synthetic route a small series of various *C*-carboranyl-deoxyribose conjugates were prepared (Scheme 1).

Our synthesis started with ethynylation of 1-chloro-1,2-dideoxy-3,5-di-*O*-(4-toluoyl)-D-ribofuranose (**1**) giving rise to anomeric mixture of α- and β-1-ethynyl-1,2-dideoxy-3,5-di-*O*-(4-toluoyl)-D-ribofuranose (**2**), which was followed by the key step of the synthesis: reaction with bis(dimethyl sulfide)decaborane complex to yield anomeric mixture of 1-*o*-carboranyl-1,2-dideoxy-3,5-di-*O*-(4-toluoyl)-D-ribofuranose (**3**). The reaction sequence was finished by the selective removal of *p*-toluoyl groups furnishing the desired *o*-carboranyl-deoxyribose conjugates (**4**). In a similar manner the Sonogashira reaction of **2** with aryl halides provided alkynyl derivatives (**5**) that were converted to various subtituted *o*-carboranyl-deoxyribose conjugates. The prepared compounds were subjected various biological assays to evaluate their potential application in medicinal chemistry.



SCHEME 1

a) HC≡C-MgBr, THF; b) $B_{10}H_{12}(Me_2S)_2$, toluene, 120 °C; c) K_2CO_3; d) Sonogashira reaction

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EFFICIENT ONE-POT SYNTHESIS OF POLYSUBSTITUTED 6-[(1*H*-1,2,3-TRIAZOL-1-YL)METHYL]URACILS THROUGH THE "CLICK" PROTOCOL

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Preparation of several triazoloacyclic nucleosides and triazoloacyclic nucleoside phosphonates is described. The synthetic methodology was developed as the one-pot Cu(I)-catalyzed azide alkyne Huisgen click cycloaddition. A novel Cu(I)-catalyzed decarboxylation reaction of 1-substituted 1*H*-1,2,3-triazole-4-carboxylic acids at room temperature was observed and used for the preparation of 1-substituted 1*H*-1,2,3-triazoles. As congeners of TPI, the prepared compounds were screened as potential inhibitors of human thymidine phosphorylase but no inhibitory activity was observed.

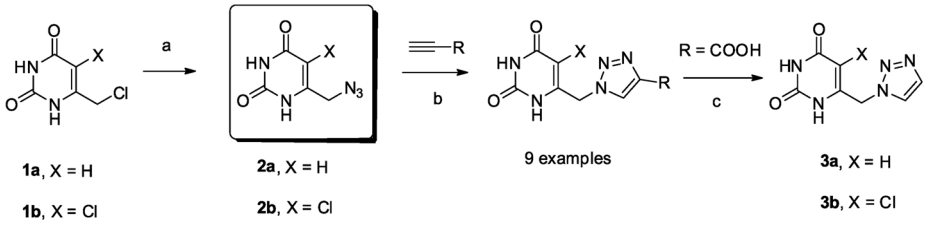
INTRODUCTION

Acyclic nucleoside phosphonates[1] (ANPs) are nucleotide analogues with a wide range of biological activities, especially antiviral[2], antiparasitic[3], and cytostatic[4]. Some initial studies describing the possibility to freeze the side-chain flexibility of acyclic nucleoside phosphonates with a triazole moiety have been reported[5]. Furthermore, substituted 6-[(1*H*-imidazol-1-yl)-methyl]uracils, as congeners of TPI, were identified as a promising inhibitors of human thymidine phosphorylase[6].

RESULTS AND DISCUSSION

The key intermediates **2a** and **2b** bearing azidomethyl group were prepared by the reaction of the chloromethyl derivatives **1a** and **1b** with sodium azide in DMF at room temperature (Scheme 1). This reaction gives quantitative yields of pure products **2** where sodium chloride is formed as the only by-product and thus the reaction mixture containing compounds **2** can be used directly in the next step. The intermediates **2** were reacted with wide range of commercially available terminal alkynes under the Cu(I)-catalyzed azide alkyne Huisgen click conditions to obtain substituted 6-[(1*H*-1,2,3-triazol-1-yl)methyl]uracils in nearly quantitative yields. Acyclic nucleoside phosphonate analogue was prepared using diisopropyl [(prop-2-yn-1-yloxy)-methyl]phosphonate[7]. These Cu(I)-"catalyzed" reactions work well (full conversion after 1 min at room temperature) only when at least one molar equivalent of Cu(I) is used. The prepared compounds, 6-[(1*H*-1,2,3-triazol-

1-yl)methyl]uracils, form stable complexes with Cu(I) and that is why at least one molar equivalent of Cu(I) has to be used.



SCHEME 1
a) NaN_3/DMF, r.t., 1 h; b) CuI, terminal alkyne/DMF, r.t., 1 min.; c) CuI/DMF, r.t., 72 h

Surprisingly, during the formation of 1-substituted 1*H*-1,2,3-triazole-4-carboxylic acids a novel Cu(I)-catalyzed decarboxylation reaction at room temperature was observed. Thus, prolonged reaction time yielded 1-substituted 1*H*-1,2,3-triazoles **3**.

This work is a part of the research project AVOZ40550506 of the Institute of Organic Chemistry and Biochemistry and was supported by Centre for New Antivirals and Antineoplastics (1M0508). This study was also supported by Gilead Sciences (Foster City, CA, U.S.A.).

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METAL ION CHELATES AS SURROGATES OF NUCLEOBASES FOR THE RECOGNITION OF NUCLEIC ACID SEQUENCES

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A 2,6-dihydrazinopurine ribonucleoside (**2**) has been synthesized and the affinity of its Pd^{2+} and Pt^{2+} complexes for the natural nucleobases studied with UV and NMR spectrometric titrations. Furthermore, for incorporation into oligonucleotides, this artificial nucleoside has also been converted to a conventionally protected phosphoramidite building block.

INTRODUCTION

The objective of the project is to develop a set of artificial pseudo-complementary nucleobases with an enhanced affinity towards the natural nucleobases while still retaining high selectivity. In this study Pd^{2+} and Pt^{2+} complexes of 2,6-dihydrazinopurine, were used as the artificial nucleobase. For high stability, the coordination of a ring nitrogen (N1 of purines and N3 of pyrimidines) of the natural nucleobase to a metal ion carried by the

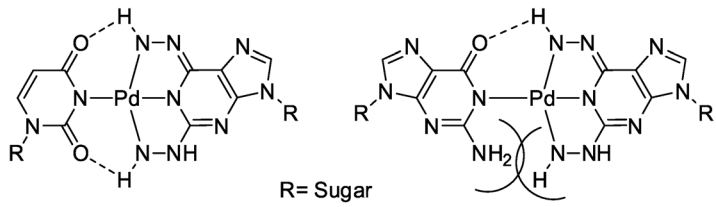

FIG. 1
Pd^{2+}-mediated base-pairs between natural nucleobases (uracil and guanine) and the 2,6-dihydrazinopurine derivative

artificial nucleobase is exploited. Discrimination between the four natural nucleobases, in turn, is achieved through a combination of additional destabilizing (steric) and stabilizing (hydrogen bonding) interactions (Fig. 1)[1]. A number of studies on metallo-base-pairs have been published, but the focus has generally been on expanding the genetic code by introducing a completely new artificial base-pair or impregnating DNA with metal ions for nanotechnolgical applications, rather than developing high-affinity complements for the natural nucleobases[2,3].

On a monomeric level, the binding affinity and selectivity of the artificial nucleobases may be studied by UV and NMR titrations. For a more realistic model, a series of short oligonucleotides each having one of the new artificial nucleosides in the middle of the sequence will be prepared and T_m values for the duplexes between these and complementary oligonucleotides having either A, C, G or T opposite to the artificial nucleoside will be measured.

RESULTS AND DISCUSSION

Synthesis strategy for the phosphoramidite building block (**6**) is presented in Fig. 2. First, the 2,6-dihydrazinopurine riboside (**2**) was prepared by treating 2′,3′,5′-tri-*O*-acetyl-6-chloro-2-iodopurine riboside (**1**) with hydrazine hydrate at room temperature[4]. After that the hydrazino groups were protected with trifluoroacetyl groups (**3**) and the 5′-OH as a dimethoxytrityl ether (**4**). Finally, the 2′-OH was silylated with TBDMS (**5**), and the 3′-OH phosphitylated by the conventional methods.

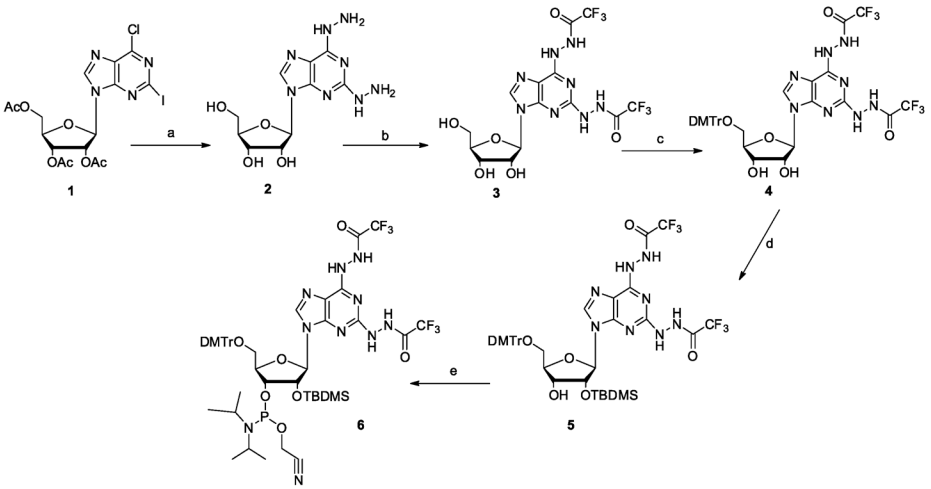

FIG. 2

a) $NH_2NH_2 \cdot H_2O$ (70 eq.); b) ethyl trifluoroacetate, MeOH; c) DMTrCl, dry pyridine; d) TBDMSCl, dry pyridine; e) chloro-(2-cyanoethyl)-*N*,*N*-diisopropylaminophosphine, triethylamine, CH_2Cl_2

The stability of Pd^{2+}-mediated base pairs between the natural nucleosides and the 2,6-dihydrazinopurine riboside (Fig. 1) was studied by ^1H NMR- and UV-spectrometric titrations and the preliminary results suggest that at least with uridine a stable base pair is formed.

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REACTIVITY OF CONJUGATED ALDEHYDES WITH DNA BASES: IDENTIFICATION AND QUANTIFICATION OF THE MAIN ADDUCTS

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The reactivity of 2,5-dioxo-pent-3-enyl acetate with DNA bases was studied. The different oxadiazabicyclo(3.3.0)octaimine adducts formed were fully characterized by NMR and quantified by HPLC-MS/MS.

Oxidative stress is known to be involved in several biological disorders and associated to several pathologies. Generated reactive oxygen species (ROS) are able to induce chemical modifications to biomolecules and in particular to DNA. Much attention has been focused on base damage, but recent observations suggest that sugar oxidative degradation pathways may also play an important role in DNA alteration. A common feature of sugar oxidation reactions is the transient generation of reactive aldehydes, especially α,β-unsaturated dicarbonyl species that, due to their electrophilic character, can react with nucleophiles moieties of DNA bases with the subsequent formation of adducts.

Examples include the formation of stable adducts in the reaction of cis- and trans-1,4-dioxo-2-butene with 2′-deoxycytidine (dCyd), 2′-deoxyguanosine (dGuo) and 2′-deoxyadenosine (dAdo)[1]. Also, trans-hydroxy-2-hexenal and trans-4-hydroxy-2-nonenal (products of lipid peroxidation) have been shown to add to dGuo [2] and dAdo [3]. Ravanat *et al.* have demonstrated that hydrogen abstraction at the 4′ position of the 2-deoxyribose moiety leads to the formation of dCyd adducts, following the transient formation of a conjugated aldehyde[4].

The proposed reaction mechanism involves initial reaction of the C1 atom of the unsaturated aldehyde with the exocyclic nitrogen atom of the nucleosides (N^4 of dCyd, N^2 of dGuo and N^6 of dAdo). This reaction is followed by 1,4-addition of the adjacent endocyclic nitrogen atom (N^3 of dCyd and N^1 of dGuo and dAdo) to the double bond, to form oxadiazabicyclo(3.3.0)octaimine adducts.

It was demonstrated that this reaction could lead to several oxidation products, namely substituted ethano and etheno adducts (Fig. 1). Regarding the ethano adducts it was shown that the ethano ring in the cis configuration exists mostly as a hemiaketal (II, Fig. 1), formed by the subsequent

attack of the alcohol onto the carbonyl group, while cyclisation is not possible for the corresponding trans isomers (I, Fig. 1). Finally the adducts can undergo dehydration to form an etheno derivative (III, Fig. 1). It was shown that this dehydration reaction was favoured by prolonged heating and treatment with acids or bases[5].

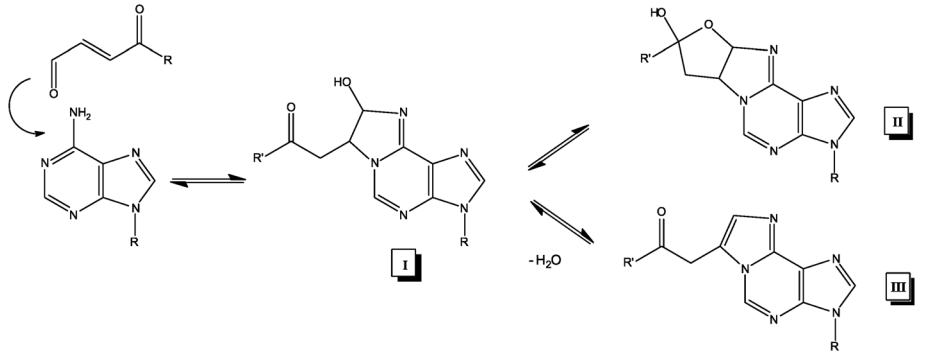

FIG. 1
Structures of the different possible 2′-deoxyadenosine adducts

To determine the structure and proportion of the different adducts, a chemical synthetic approach was designed to produce larger amounts of these DNA lesions. The strategy we have adopted consisted in the synthesis of the acetylated derivative of a ketoaldehyde that, upon incubation with the free nucleosides and bases, would give rise to the final products. For that purpose, we have developed a method based on the oxidation of furfuryl acetate to selectively generate 2,5-dioxo-pent-3-enyl acetate. The reaction mixture was then incubated with dCyd, dGuo, dAdo and 1-methylcytosine under different conditions of pH and temperature. In addition, in order to better study the relative reactivity of the conjugated keto-aldehyde with the different nucleobases we have performed the reaction directly with a mixture of nucleosides and with isolated DNA. Identification and quantification of the different adducts was performed by HPLC coupled with electrospray ionisation tandem mass spectrometry. In addition, attempts have been made to isolate the different possible isomers by HPLC in order to fully characterize them by NMR. In the near future efforts will be made to search for such DNA lesions in cells exposed to different conditions of oxidative stress.

During the last few years, several works have highlighted the fact that reactive aldehydes could be generated in double stranded DNA and induce

the formation of different types of DNA lesions, including inter-strand cross-links and DNA-protein cross-links[6]. Therefore, it is of primary importance to better understand the reactivity of these aldehydes with DNA bases.

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SYNTHESIS OF 4-(HET)ARYL PYRIMIDO[4,5-*b*]INDOLE RIBONUCLEOSIDES

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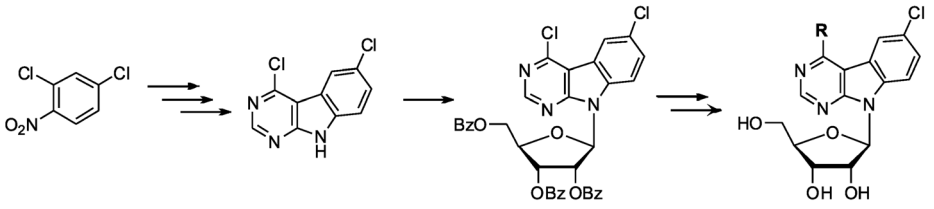
4,6-Dichloropyrimido[4,5-*b*]indole has been built-up from starting 2,4-dichloronitrobenzene in multigram scale and it has been used as key intermediate for synthesis of a series of 4-aryl or hetaryl ribonucleosides.

INTRODUCTION

7-Deazapurine nucleosides bearing hetaryl group in position 6 and H, F or Cl atom in position 7 showed cytostatic activity against multiple cell lines. Furyl and thienyl derivatives were the most active compounds[1]. Based on previous results, goal of this work was the synthesis of new type of annulated deazapurine derivatives bearing hetaryl group for cytotoxicity testing.

RESULTS AND DISCUSSION

4,6-Dichloropyrimido[4,5-*b*]indole was prepared as key intermediate. Synthesis started from 2,4-dichloronitrobenzene, which was transformed to 4,6-dichloropyrimidoindole in 4 steps (nucleofilic substitution, reduction, heterocyclization and substitution). First two steps were realized according to modified Gangjee conditions[2]. Benzoylated ribonucleoside was obtained by glycosylation of crude base, overall yield was about 30%. This synthesis has been optimized for tens gram scale with only one final column chromatography.



A series of target ribonucleosides bearing aryl or hetaryl group in position 4 of pyrimidine ring has been prepared by regioselective Pd-catalyzed Suzuki

or Stille cross-coupling reactions[1] of protected nucleoside in good yields. Final deprotection led to free nucleosides in 70–90% yields.

This work is a part of the research project from the Academy of Sciences of the Czech Republic Z4 055 0506. It was supported by the Ministry of Education, Youth and Sports of the Czech Republic (1M0508), Czech Science Foundation (P207/11/0344) and by Gilead Sciences, Inc.

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NEW AMPHIPHILIC PRODRUGS OF ADEFOVIR AND CIDOFOVIR

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New Adefovir prodrugs with an amphiphilic moiety consisting of decyl or decyloxyethyl chain bearing hydroxyl function(s), hexaethyleneglycol or a (5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl unit were prepared starting from the tetrabutylammonium salt of the phosphonate drug and an appropriate alkyl bromide or tosylate. Analogously, two esters of Cidofovir bearing a hexaethyleneglycol promoiety were prepared.

INTRODUCTION

The development of acyclic nucleosid phosphonates (ANPs) has resulted in three approved antiviral drugs and continues to provide new active compounds. The presence of the phosphonate group is responsible for their ionic character when subjected to physiological pH. The ionized molecule is not easily permeable through the gastrointestinal wall and biological membranes. In order to achieve better oral bioavailability, the phosphonate group of the drug can be transformed to a phosphonic ester or amidate, which is enzymatically cleaved to the parent drug after passing the intestinal barrier, or later inside the cells. The aim of this work is to apply several less common pro-moieties to PMEA (Adefovir) as a model drug. PMEA has demonstrated a broad spectrum of antiviral activity against human immunodeficiency virus (HIV) and other retroviruses and is active against various DNA viruses, including the hepatitis B virus and herpesviruses. The bis-(pivaoyloxymethyl)ester of PMEA (Adefovir Dipivoxyl) has been approved as an oral prodrug for the treatment of hepatitis B. The prodrug is cleaved in the serum generating pivalate and formaldehyde as its byproducts. Formaldehyde is the significant contributor to the overall cytotoxic effects of the prodrug. (*S*)-HPMPC (Cidofovir) exhibits broad-spectrum activity against a wide variety of DNA viruses including herpes-, adeno- and poxviruses. Despite of progressive investigation in the area of Cidofovir prodrugs, no FDA approved drug is so far available on the market.

RESULTS AND DISCUSSION

Routinely, we mask the phosphonate functions of ANPs as alkyloxyalkyl esters, however, their physical properties are far from being optimal. They suffer from low solubility in water. Additionally, an alkyl chain is subjected to a ω-oxidation process resulting in inactivation of the prodrug. We have focused on those promoieties consisting of the aliphatic chain modified by means of addition(s) of hydroxyl group(s) or insertions of oxygen atoms. A hydroxylated alkyl or alkyloxyalkyl unit (R^2) or a hexaethyleneglycol unit (R^1) was attached by ester linkage to the phosphonate group of PMEA. We also studied the effects of the addition of the hexaethylene glycol unit in (*S*)-HPMPC (**2**) as prodrugs closely related to Cidofovir hexadecyloxypropyl ester[1], currently developed for use in the prophylactic and preemptive therapy of dsDNA viral infections. The dioxolenone ester (**1**, R^3) has been prepared as a less toxic analogue of Adefovir Dipivoxyl. The prodrug is expected to be cleaved in serum yielding non-toxic CO_2 and diacetyl as by-products.

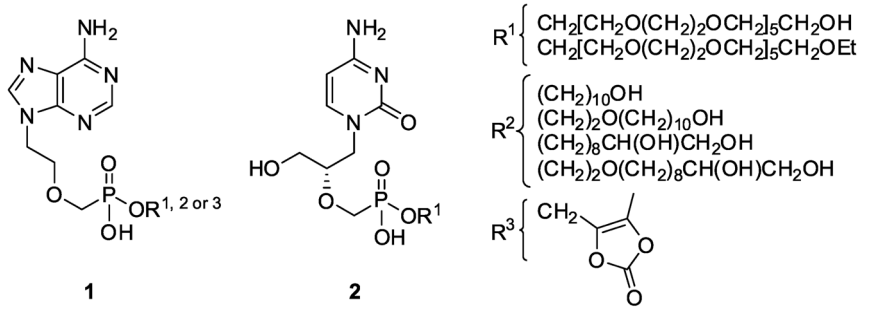

FIG. 1
The structures of the synthesized prodrugs

The activity of the prodrugs was evaluated *in vitro* against different virus families. A loss in the antiviral activities of the hydroxylated decyl or decyloxyethyl esters and hexaethyleneglycol esters of PMEA (**1**, $R^{1,2}$) against human immunodeficiency virus (HIV) and herpesviruses [including herpes simplex virus (HSV), varicella-zoster virus (VZV), and human cytomegalovirus (CMV)] occurred in comparison with PMEA. On the other hand, the (5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl ester of PMEA (**1**, R^3) showed significant activities against HIV and herpesviruses. (*S*)-HPMPC prodrugs (**2**) exhibited anti-cytomegalovirus activities in the same range as the parent drug, whereas the anti-HSV and anti-VZV activities were one- to seven-fold lower than that of (*S*)-HPMPC.

This work is a part of the research project of the institute AV0Z40550506 and Centre of New Antivirals and Antineoplastics 1M0508 supported by the Ministry of Education, Youth and Sports of the Czech Republic. It was also supported by Gilead Sciences, Inc. (Foster City, CA, USA) and the "Geconcerteerde Onderzoeksacties (GOA), Krediet nr. 10/014" of the K.U. Leuven.

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DIDEOXYAPIOSE NUCLEOSIDES REVISITED: SYNTHESES AND PROTIDE DERIVATIVES

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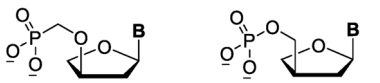
Almost two decades after the first synthesis of the dideoxy apiose nucleosides, we sought to revisit their synthesis and convert them to the corresponding phosphoramidate (ProTides).

INTRODUCTION

Dideoxyapiose nucleosides (ddAN) were synthesized in the early 90's as potential antivirals. However, the interest in this class of nucleosides faded away after they were reported to be inactive[1]. It might be expected that the inadequate cellular conversion to the corresponding triphosphate is responsible for their non-activity. Recently, Herdewijn and coworkers showed that the related L-2-deoxythreose nucleoside phosphonates (**I**; Fig. 1) selectively inhibit HIV without affecting human DNA synthesis[2]. A serious problem associated with phosphonates, however, is their low bioavailability. Inspired by the cellular activity of **I**, we decided to reinvestigate the synthesis of the ddANs. Note that the monophosphate forms of the envisaged L-ddANs (**II**), can be considered as the parent nucleotides from which the bioisosteric phosphonates **I** have been derived. Since cellular conversion to this monophosphate might form the bottleneck in the activation of the ddANs, we decided to convert them in to their masked monophosphates (ProTides)[3].



Ia B = thymin-1-yl (PMDTT)
Ib B = adenin-9-yl (PMDTA)

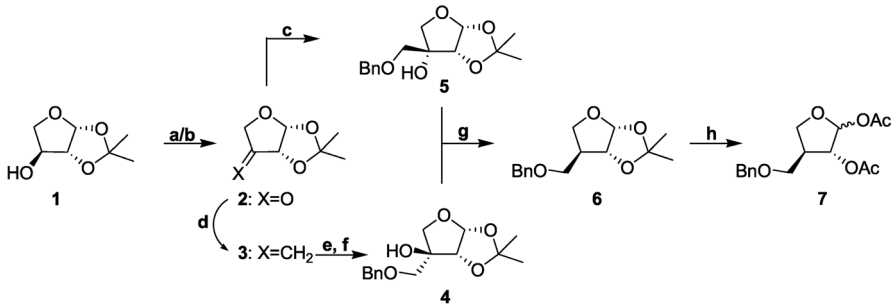
IIa B = thymin-1-yl
IIb B = adenin-9-yl

FIG. 1

RESULTS AND DISCUSSION

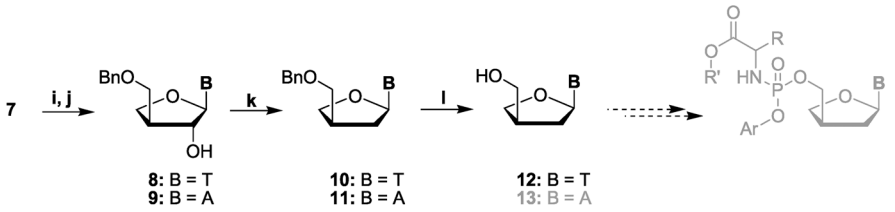
The threose compound **1** (Scheme 1) was synthesized following the reported procedure[4]. The oxidation of secondary hydroxyl group was performed using pyridinium chlorochromate (PCC). To avoid the toxicity

issues pertaining to PCC, we explored alternative oxidation conditions. Surprisingly, TMPO-BAIB oxidation, text book conditions to convert primary alcohol groups to the corresponding acid without affecting other hydroxyl groups, proved a valuable alternative for oxidation of **1** to **2**. Initially, the key intermediate **7** was produced via compound **5** [5], obtained by hydroxylation of exomethylene group of **3**, but afterword we followed a shorter and more consistent route through the apiose derivative **5**.



SCHEME 1

The nucleobases were installed by modified Vorbrüggen coupling of **7** with the appropriate silylated bases, followed by removal of the acetyl and benzoyl groups (Scheme 2). The compounds **10** and **11** were obtained by Barton–McCombie deoxygenation. The *dd*AN **12** was obtained after palladium catalysed debenzylation.



SCHEME 2

The synthesis of the nucleoside analogue **13** and the final ProTides is in progress. The ability of these phosphoramidates to inhibit various viral strains will be discussed.

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G-QUADRUPLEX FORMATION WITHIN PROXIMAL PROMOTER OF MYCN

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Oligonucleotide containing d[$G_3CG_3AG_3AG_3$] sequence originating from the MYCN proximal promoter in the presence of K^+ ions forms a stable G-quadruplex exhibiting characteristics of a parallel G-quadruplex folding topology.

INTRODUCTION

DNA containing guanine rich tracts can in the presence of cations, including biologically relevant K^+ and Na^+, form four stranded structures called G-quadruplexes. G-quadruplexes comprise of stacked G-quartets, each formed by assembly of four Hoogsteen hydrogen-bonded guanines in a coplanar arrangement. Stability of G-quadruplexes depends on a number of stacked G-quartets as well as on length and sequence of loops that connect guanines constituting G-quartets. Structures of the loops are tightly related to mutual orientations of the guanine rich strands of a G-quadruplex and therefore folding topology[1,2]. Evidence of G-quadruplex formation within genomes raises interest in their structures in relation to biological roles. In particular, G-quadruplexes formed within gene promoter regions received great attention as they were identified as potential targets of anticancer therapeutics.

MYCN proto-oncogene encodes a transcription factor which can promote or repress expression of various target genes through binding to their promoter regions. Additionally, MYCN is also involved in regulation of expression through mechanisms not including MYCN direct binding to promoters[3]. Aberrance in MYCN oncogene expression has been observed in variety of tumors including neuroblastoma[4].

We identified a putative G-quadruplex forming sequence in the MYCN proximal promoter at –273 to –287 region upstream of the transcription start site (TSS). With the use of CD, UV and NMR, which are well estab-

lished methods for studying G-quadruplexes, we set to probe G-quadruplex formation within MYCN promoter.

RESULTS AND DISCUSSION

In order to investigate G-quadruplex formation within MYCN promoter, we designed and synthesized oligonucleotide with d[$G_3CG_3AG_3AG_3$] sequence. ^{1}H NMR unequivocally established the presence of twelve imino resonances indicative of a G-quadruplex with three G-quartets (Fig. 1a). CD profile with maxima at around 260 nm and minima at 245 nm suggested formation of a parallel G-quadruplex (Fig. 1b). Preliminary NMR data analysis implied formation of intramolecular G-quadruplex, which was however involved in equilibrium between the two forms depending on concentrations of oligonucleotide and K^+ ions. Temperature-dependent melting profiles of samples with up to hundred times lower oligonucleotide concentration than in the case of NMR were consistent with intramolecular G-quadruplex formation.

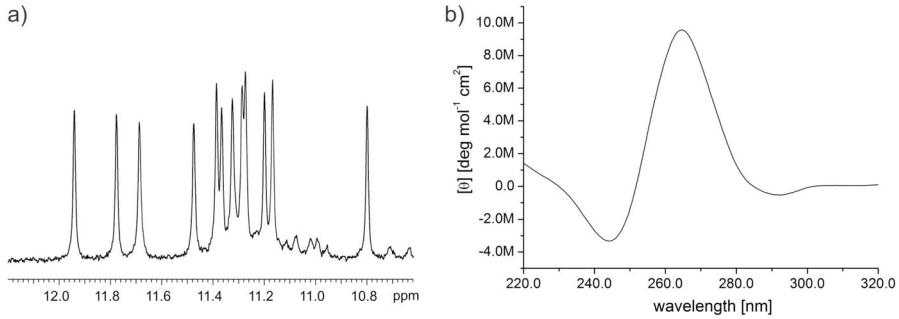

FIG. 1
a) Imino region of ^{1}H NMR spectrum b) CD spectrum reflecting a parallel fold

The authors acknowledge the financial support of Slovenian Research Agency (ARRS) and the Ministry of Higher Education, Science and Technology of the Republic of Slovenia (Grant Nos. P1-0242 and J1-0986) and COST MP0802.

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SYNTHESIS OF BASE-SUBSTITUTED URIDINE 5′-PHOSPHONATE ANALOGUES AS POTENTIAL $P2Y_2$ RECEPTOR LIGANDS

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A series of 5-modified 5′-phosphonate derivatives of UMP has been prepared in 6 steps from uridine. The analogs were evaluated for their affinity at the $P2Y_2$ receptor. Several derivatives showed partial agonistic activity at the $P2Y_2$ receptor.

INTRODUCTION

P2Y receptors are a family of eight class A GPCRs that respond to different nucleotides. The human $P2Y_2$ receptor is activated equipotently by UTP and ATP. This receptor subtype is distributed in a broad range of tissues[1] and plays important physiological roles in epithelial cells of the lung, gastrointestinal tract and the eye. $P2Y_2$ receptors are targets for several therapeutic approaches, some of which are currently being explored. Agonists are promising for cystic fibrosis, cancer and dry eye syndrome[1,2] while $P2Y_2$ antagonists exhibit anti-inflammatory[3] and neuroprotective effects[4].

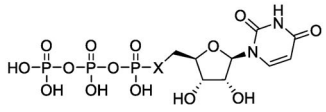
OBJECTIVES

When administered via inhalation, the natural ligand UTP (**1**), as well as most reported analogs thereof, are liable to enzymatic degradation at the surface of the airways. This results in a relative short time of action. In that context, we recently explored to what extent replacement of the α-phosphate group of UTP by an isosteric phosphonate affected $P2Y_2$ affinity and intrinsic activity[2].

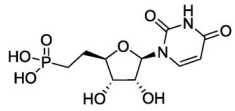
While we initially focused on a diphosphophosphonate mimic of UTP (**2**), it was accidently discovered that the synthetic precursor **3** was also capable of activating the $P2Y_2$ receptor, while being inactive at the $P2Y_4$ receptor.

From the growing series of UTP analogs that have been screened on P2Y receptors, it is known that incorporation of a large heterocyclic substituent

at position 5 of the base precludes receptor activation[5]. In this study, we decided to combine several heterocyclic 5-modifications with a 5′-methylene-phosphonate group (**4–11**).



X = O (UTP, **1**)
X = CH_2 (**2**)



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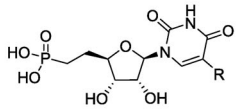

4: R = phenyl
5: R = naphthalen-2-yl
6: R = 4- fluorophenyl
7: R = *p*-tolyl
8: R = furan-2-yl
9: R = benzothiophen-3-yl
10: R = styryl
11: R = thiophen-2-yl

RESULTS AND DISCUSSION

The 5-modified 5′-phosphonate analogs were prepared in 6 steps. A Wittig reaction was used to introduce the 5′-methylene phosphonate group, while a Suzuki–Miyaura coupling allowed to introduce the 5-substituents on the 5-bromo precursor. Although it was earlier suggested that **3** might activate the $P2Y_2$ receptor via an allosteric mechanism, compounds **4–11** showed only marginal to no positive allosteric effect. However, derivatives **4–9** show partial agonist activity at the $P2Y_2$ receptors as indicated by activation of PLC in stably infected astrocytoma cells.

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INCORPORATION OF UNNATURAL AMINO ACIDS INTO PROTEINS FOR CLICK CHEMISTRY

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Using the pyrrolysine system two pyrrolysine analogs **1** and **2** has been incorporated into yellow fluorescent protein (YFP) at three defined positions and were further used for site specific glycosylation.

INTRODUCTION

The efficient genetic encoding of non-natural amino acids with a unique reactivity will allow the construction of novel modified proteins with useful physical and biological properties. More than 50 non-natural amino acids have been already incorporated into proteins *via* unique codon and an "orthogonal" *t*RNA-aminoacyl-*t*RNA synthetase pair[1]. Recently, a non-canonical lysine derivative containing methyl-pyrroline moiety was identified in the active site of monomethylamine methyltransferase from *Methanosarcina barkeri* [2]. This 22nd amino acid, named pyrrolysine, is encoded in *m*RNA by UAG amber stop codon. Its specific *t*RNA (*Pyl*T), which has the complementary CUA anticodon, is charged by the pyrrolysil-*t*RNA synthetase (*Pyl*S). The broad substrate specificity of *Pyl*S allows the incorporation of pyrrolysine analogs into proteins at defined positions. We have used this pyrrolysine system for incorporation of up to 3 non-natural amino acids **1** and **2** into YFP. Using the Cu(I)-catalyzed azide alkyne click chemistry we were able to further glycosylate the target protein[3].

RESULTS AND DISCUSSION

The pyrrolysine analog **1** was synthesized in six steps, while compound **2** was commercially available. We have used the *Methanosarcina mazei* pyrrolysyl-*t*RNA synthetase/$tRNA_{CUA}$ pair to incorporate these pyrrolysine analogs into YFP. By insertion of more than one *Amber*-codon into the open reading frame of YFP we were able to incorporate up to three artificial amino acids. We than used the alkyne functionalities in the protein to link various sugar moieties to the protein by Cu(I)-catalyzed azide-alkyne cycloaddition reaction (Fig. 1).

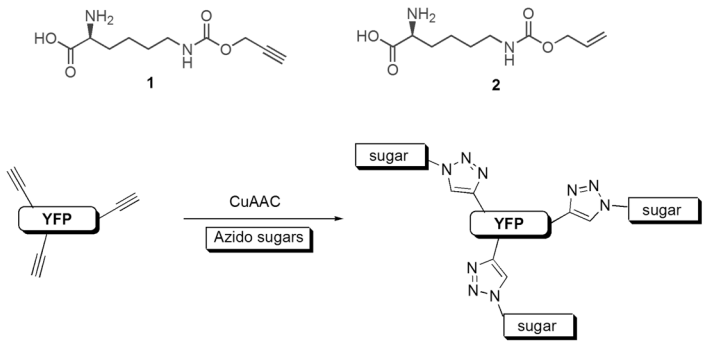

FIG. 1
Structures of alkyne and alkene amino acids **1** and **2**. Click reaction of alkyne containing modified YFP and azido sugars

Our goal is to modify proteins of interest under bioorthogonal and physiological conditions and use them for structural and biochemical studies.

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BENZOPHENONE MODIFIED DNA FOR PHOTOCATALYSIS

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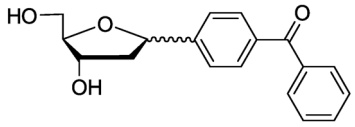
A Benzophenone – C-nucleoside was synthesized and incorporated into DNA for using oligonucleotides as photocatalysts. By modifying natural DNA we implemented the ability to use light for chemical reactions in structurally defined vicinity. Up to now we could use ss-DNA just as well as benzophenone as photocatalyst in our desired test reaction.

INTRODUCTION

Photocatalysts with high enantioselectivity combine demands of sustainable chemistry as well of modern catalysis. Benzophenone has already been applied successfully as a sensitizer for organocatalytic photoreactions by Bach and coworkers[1]. In order to gain enantioselectivity Feringa et al.[2] showed that DNA is highly suitable supramolecular framework. We currently work on functional DNA architecture by combining those two concepts. Accordingly we synthesized the benzophenone C-nucleoside **1** to modify synthetic DNA. After incorporation of **1**, ss- and ds-DNA showed the benzophenone phosphorescence pattern. That triplett energy could potentially be used to photocatalyse organic reactions, e.g. [2+2]-cycloadditions or electron transfer induced radical reactions.



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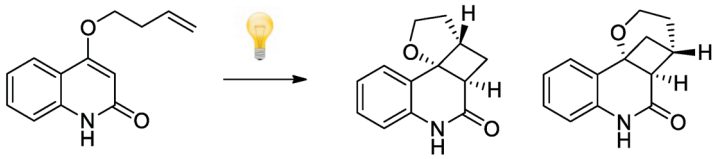
RESULTS AND DISCUSSION

During the synthetic route to **1** the key step is the C–C-coupling of a benzophenone derivative and Hoffer's chlorosugar, which was performed in a Grignard reaction. The following steps to the DNA building block were standard phosphoramidite chemistry.

By incorporating the C-nucleoside **1** into DNA, the oligonucleotide achieved the ability to act as a triplett-sensitizer. Phosphorescence measurements of **1** and different modified oligonucleotides gave the evidence for the benzophenone triplet state even in DNA. We chose the well-known

intramolecular [2+2]-cycloaddition of 4-(3-butenyloxy)quinolin-2(1*H*)-one **2** to try the photocatalysis[1,3,4].



2 **3** **4**

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SYNTHETIC GFP CHROMOPHORE IN DNA WITH LARGE APPARENT STOKES SHIFT

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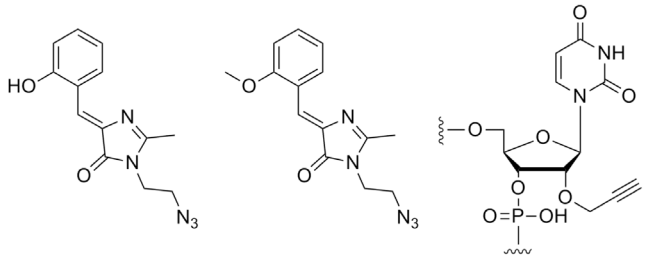
A synthetic GFP like chromophore bearing an o-phenol group instead of the *p*-phenol of natural GFP was synthesized. This arrangement enables an excited state proton transfer (ESPT) from the hydroxy group to the imidazolone. The chromophore was incorporated into DNA and characterized by optical spectroscopy. It became evident that the DNA environment controls the photophysical property in such a way, that almost solely the ESPT driven fluorescence is occurring. The apparent Stokes shift is larger than 200 nm (9000 cm^{-1}). Moreover, DNA increases the fluorescence intensity by constraining the internal conversion. The combination of both effects provides a new important concept for the design of fluorescent labels for nucleic acids[1].

INTRODUCTION

The application of the Green Fluorescence Protein in molecular biology and biochemistry is widely spread[2]. The green fluorescence of the protein results from a covalently and hydrogen-bond network that anchors the 4-(4-hydroxybenzylidene)-1,2-dimethyl-1*H*-imidazol-5(4*H*)-one. The chromophore undergoes excited state proton transfer (ESPT) to a remote residue and a very intense anion fluorescence appears[3]. The emission of the isolated molecule in solution is quenched by radiationless internal conversion due to its flexibility, and the absence of a proton transfer. The protein environment suppresses the fast internal conversion by restricting this flexibility[4]. Diederichsen et al. used a GFP chromophore analog as building block for PNA thus hindering the isomerization and rotation of the molecule due to the intercalation into the nucleobases[5]. But, the excited state proton transfer was not observed. Therefore, we decided to incorporate 1-(2-azidoethyl)-4-(2-hydroxy-benzylidene)-2-methyl-1*H*-imidazol-5(4*H*)-one (**1**) into DNA. In this chromophore an excited state proton transfer from the hydroxy group to the imidazolone takes place in an aprotic medium[6] and the interaction with the nucleobases should hinder the internal conversion. Hence, a DNA label with an enhancement of the emission and a large apparent Stokes shift is expected.

RESULTS AND DISCUSSION

Two different clickable GFP homologues were synthesized: The first one with a free hydroxy group (**1**) to enable an ESPT. In order to elucidate the effect of DNA on such proton transfer processes, the GFP chromophore in the second compound (**2**) bears a methyl group. The alkyne modified uridine **3** was incorporated into different oligo-nucleotides and the chromophores **1** and **2** were "clicked" postsynthetically to the DNA.



1 **2** **3**

SCHEME 1
GFP analogues and alkyne modified uridine

We observed an increase of the quantum yield up to 35-fold with the chromophore is tethered to DNA. This means that the nucleobases restrict the conformational flexibility of the GFP analog. While **1** shows two nearly equal fluorescence maxima at 500 and 600 nm in water, the modified oligonucleotides emit almost exclusively at 600 nm resulting in an apparent Stokes shift of 200 nm (9000 cm^{-1}). This behavior is due to the ESPT which is partial interrupted in aqueous solution and very efficient in the environment of DNA. As expected, **2** exhibits a normal Stokes shift in aqueous solution and in DNA.

By this work, we elucidated a new concept for the design of nucleic acid labels using the example of the well-known GFP chromophore. The combination of ESPT driven large apparent Stokes shifts and enhancement of fluorescence in DNA could be very useful for the design of future labels.

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VISIBLE DETECTION METHOD FOR PCR THROUGH UNNATURAL BASE PAIR SYSTEMS

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We report a novel PCR detection method by using an unnatural base pair between 7-(2-thienyl)imidazo[4,5-*b*]pyridine (**Ds**) and 2-nitro-4-propynylpyrrole (**Px**) along with another fluorescent unnatural base, 2-amino-6-(2-thienyl)purine (**s**). This method enables simple, visible detection of amplified target DNA.

INTRODUCTION

Real-time qPCR is widely used for detection of infectious agents because of its rapidity, sensitivity and reduced risk of carry-over contamination. However, for real-time PCR detection, an expensive machine is required, which limits practical clinical use. Therefore simple and less expensive end-point detection methods for diagnostic PCR are desired.

Here, we report a novel, visible detection method for PCR by using an unnatural base pair between 7-(2-thienyl)imidazo[4,5-*b*]pyridine (**Ds**) and Cy3-linked 2-nitro-4-propynylpyrrole (**Px**) and another fluorescent unnatural base, 2-amino-6-(2-thienyl)purine (**s**) (Fig. 1). The **Ds–Px** pair shows high amplification efficiency and pairing selectivity in PCR, and functional molecules such as the Cy3 fluorophore can be attached to the **Px** base moiety via its propynyl linker[1]. The s base exhibits intrinsic fluorescence (emission: 434 nm) with 365 nm excitation, but base stacking effects quench the

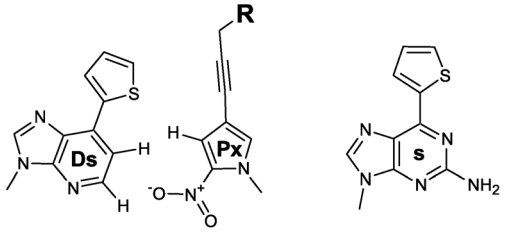

FIG. 1
The unnatural **Ds–Px** pair and fluorescent unnatural **s** base. A functional molecule (R) can be attached to the **Px** base

fluorescence when two s bases are located next to each other[2]. However, such self-quenched **s** bases can act as a FRET donor for a neighboring Cy3 dye linked to **Px**. We applied this FRET system to a simple, visible detection method for the amplified target DNA.

RESULTS AND DISCUSSION

We designed a PCR primer containing one **Ds** base and two adjacent **s** bases, which we call VisTag (Fig. 2). In VisTag PCR, we used a Cy3-linked **Px** substrate, Cy3-d**Px**TP. When Cy3-**Px** is incorporated opposite **Ds** in the primer tag, FRET occurs between **s** and Cy3 with 365 nm irradiation. The Cy3 dye was chosen because it exhibited little fluorescence with 365 nm irradiation. Thus, the amplified PCR products produce a large fluorescent signal, with little background fluorescence from unincorporated Cy3-d**Px**TP (Fig. 2).

We first performed VisTag PCR using different concentrations of 98-bp double-stranded DNA templates. As few as three copies of DNA can be visually detected with the naked eye when amplified by VisTag PCR. In addition, VisTag PCR can be used as quantitative amplification signal with a real-time PCR apparatus. Furthermore, the Cy3-**Px** incorporation into the amplified DNA allowed the site-specific fluorescent labeling of target DNA molecules. We demonstrated the detection of target DNA sequences with

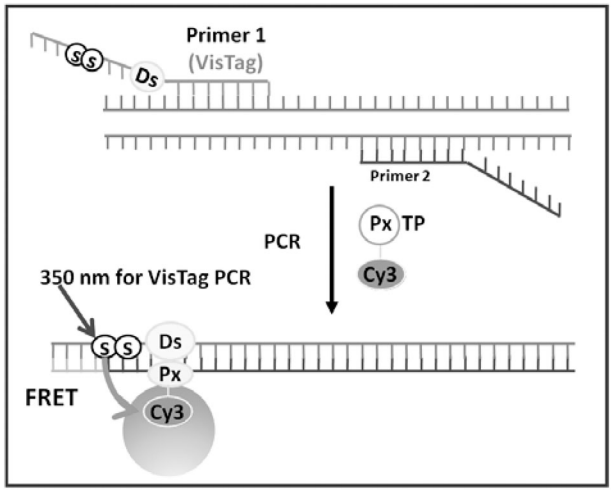

FIG. 2
The scheme of VisTag PCR amplification detection system

a single nucleotide polymorphism by VisTag PCR. We designed two types of VisTags, each primer targeting a mutated or wild-type base, and selectively identified a mutated or wild-type genome through this visual PCR amplification system.

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PHOSPHOMONOMORPHOLIDATES OF THE ACYCLIC NUCLEOSIDES BEARING A DOUBLE BOND CONJUGATED WITH THE PURINE BASE

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Acyclic nucleosides analogues bearing a double bond conjugated with the adenine and guanine were synthesized. Phosphomonomorpholidates of the correspondent 9-[(*E*)-4-hydroxybut-1-en-1-yl]purines (**6**) were obtained. The results show that aqueous alkaline is a convenient reagent for double bond isomerization in 9-[(*Z*)-4-hydroxybut-2-en-1-yl]purines (**4**).

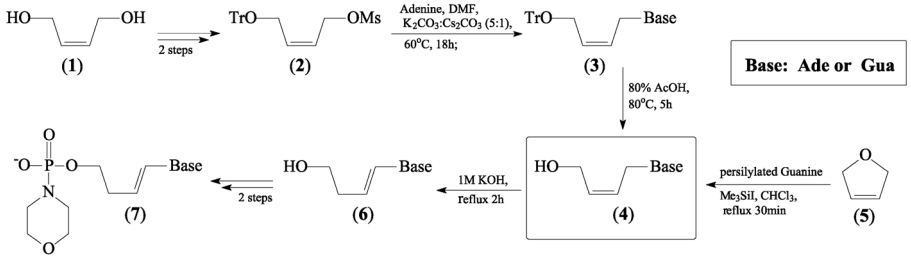
INTRODUCTION

Among various nucleoside analogues unsaturated compounds exhibit a wide spectrum of biological activities, e.g., stavudine (d4t), carbovir (carbocyclic analog of d4G) and abacavir display anti-HIV activity, whereas neplanocin A exemplifies antitumor agent[1]. The discovery of acyclovir[2] as an antiherpes agent stimulated the search for new antiviral nucleosides in which ribose or cyclopentane ring is substituted for acyclic residue[3]. As it was shown before, acyclic non-nucleoside analogues having elements of conformational inflexibility in the carbon fragment are good substrates for different DNA polymerases[4]. The mechanism of biological activity of those compounds involves intracellular phosphorylation resulting in the formation of the corresponding nucleoside triphosphate analogues that are terminators of the viral DNA synthesis catalyzed by DNA- and RNA-dependent DNA-polymerases. The efficacy of antiviral action directly depends on the first phosphorilation which occurs due to viral DNA-polymerases. To bypass the first phosphorylation polymerase-dependence there should be a suitable phosphate prodrug (ProTide approach)[5]. We describe herein the synthesis of acyclic nucleoside analogues bearing a double bond conjugated with the purine base and their nucleotide derivatives.

RESULTS AND DISCUSSION

The synthesis of 9-[(*Z*)-4-hydroxybut-1-en-1-yl]purines (**6**) and their phosphomonomorpholidates (**7**) is presented on the Scheme 1. 9-[(*Z*)-4-Hydroxybut-2-en-1-yl]adenine (**4**) was obtained from commercially available *cis*-2-buten-1,4-diol (**1**). Compound **1** was consecutively tritylated and

mesylated, and then intermediate product **2** alkylated adenine in the presence of K_2CO_3:Cs_2CO_3 (5:1) in DMF. After acid hydrolysis of 9-[(*Z*)-4-(trityloxy)but-2-en-1-yl]adenine (**3**) target product (**4**, Base = Ade) was obtained.



SCHEME 1
Approach to the synthesis of acyclic analogues **6** and **7**

The treatment of commercially available 2,5-dihydrofurane (**5**) with persilylated guanine in the presence of trimethyliodosilane[6] resulted in target 9-[(*Z*)-4-hydroxybut-2-en-1-yl]guanine (**4**).

The next stage of the research was to find the best way for the double bond isomerization to the position conjugated with the purine base. According to previous publications, this reaction requires *t*-BuOK in anhydrous conditions at room temperature[7]. However, we assume that 9-[(*E*)-4-hydroxybut-1-en-1-yl]purines (**6**) can be easier and cheaper obtained by compound **4** reflux in aqueous alkaline for 2 h.

Nucleoside phosphomonoamide is the last intermediate compound in the scheme of ProTide utilization that will result in monophosphate formation[8]. We assume that nucleotide phosphomonomorpholidates can be regarded as nucleotide depot-forms which may display antiviral activity. The last part of our research was the preparation of nucleotide derivatives **7**. The synthesis of this type compounds was performed by consecutive treatment of the nucleosides **6** by phosphorus oxychloride and amidation agent[9]. Antiviral properties of the obtained compounds **6** and **7** will be studied.

The research was supported by the Russian Foundation for Basic Research (project 10-04-00914-a) and by Presidium of RAS ("Molecular and Cellular Biology" Program).

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DINUCLEOTIDE CAP ANALOGS BEARING BRIDGING AND NON-BRIDGING MODIFICATIONS WITHIN TETRAPHOSPHATE CHAIN

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A series of dinucleotide cap analogues (**1**–**4**) containing various modifications in tetraphosphate chain has been synthesised. These modifications include replacement of non-bridging oxygen atom in α and δ positions by either sulfur atom (**1**, **2**) or borane moiety (**3**, **4**). Furthermore, compounds **2** and **4** contain methylene group which replaces oxygen between β and γ positions. Due to the presence of two new stereogenic centres in the tetraphosphate bridge and the fact that their structures indicated some elements of symmetry, they exist in three diastereomeric forms (RR, SS, RS). Diastereomers of all analogues were separated via RP-HPLC and association constants of the complexes with eIF4E protein were determined for analogue **2**. Furthermore, all diastereomers of **2** were found to increase translational efficiency of mRNA in comparison to mRNAs containing standard cap analogue.

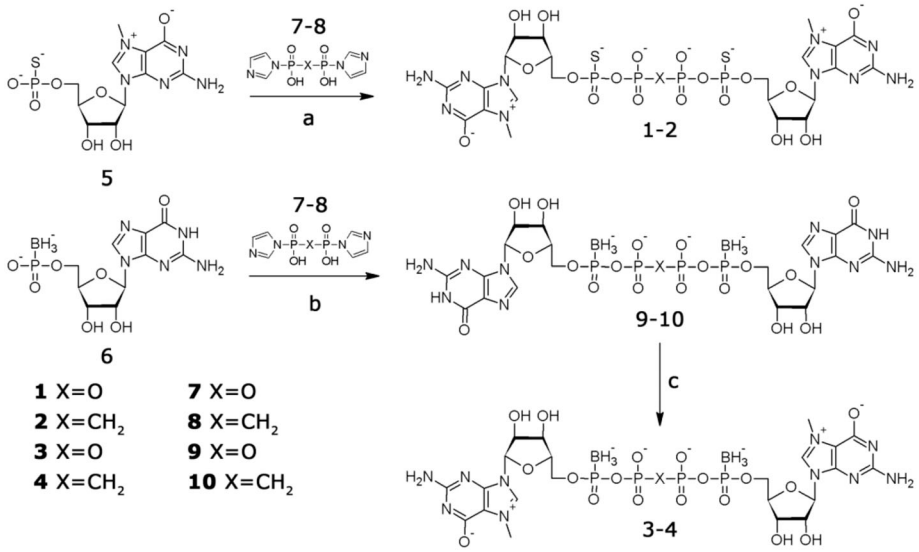
INTRODUCTION

A characteristic feature of 5′ terminus of all eukaryotic mRNAs is a specific cap residue composed of 7-methylguanosine attached by 5′–5′ triphosphate bridge to the first transcribed nucleoside[1]. This residue is involved in numerous events of mRNA metabolism, including its intracellular transport, translation and surveillance[1]. During the initiation of translation, the cap is specifically recognized by eukaryotic initiation factor 4E (eIF4E), which is a pivotal part of translation initiation complex. Cap is also a target for decapping enzymes Dcp1/2 and DcpS, which are involved in 5′ to 3′ and 3′ to 5′ mRNA degradation pathways, respectively. Moreover, it has been established that mRNAs capped with analogues with increased resistance to Dcp1/2 cleavage have prolonged half-time lives in cultured mammalian cells[2].

RESULTS AND DISCUSSION

The chemical synthesis and preliminary biochemical and biophysical studies on a series of cap analogues containing various modifications within polyphosphate bridge were performed (Scheme 1). The analogues share the same structural features, including 5′–5′ bridge elongated to tetraphosphate; presence of chemical modification (thiophosphate or borano-

phosphate moieties) both in α and δ position (moreover compounds **2** and **4** contain methylene group replacing oxygen between β and γ positions) and the presence of two 7-methylguanosine residues. The rationale for such modifications was to augment their affinity to eIF4E protein and make them more resistant towards enzymatic decapping. These cap analogues were prepared using synthetic strategy which relies on coupling reaction between two molecules of chemically modified NMP **5** or **6** and one molecule of *P1,P2*-diimidazolyl derivatives **7** or **8** [3]. This reaction is usually catalysed by $ZnCl_2$, however in the case of boranophosphate cap analogues $MgCl_2$ is more effective. The synthesis of all above-mentioned compounds lead to mixture of three diastereomers, which were separated using semi-preparative RP-HPLC. Such methodology leads to "two headed" cap analogues, which are similar to ARCA cap analogues since they always possess 7-methylguanosine in distal position after incorporation into mRNA[4]. The preliminary biochemical investigation have indicated that mRNAs capped with diastereomers of **2** are 2.0–1.1 times more effectively translated in a cell-free system than mRNAs containing standard cap analogue.



SCHEME 1
a) $ZnCl_2$, DMF; b) $MgCl_2$, DMF; c) CH_3I, DMSO

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THE FIRST EXAMPLES OF PHOSPHATE MODIFIED TRIMETHYLGUANOSINE CAP ANALOGUES

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TMG cap structure analogues modified in the 5′,5′-triphosphate bridge with methylenebis-(phosphonate) moiety at either α-β ($m_3^{2,2,7}GppCH_2pG$) or β-γ ($m_3^{2,2,7}GpCH_2ppG$) position have been synthesized. The aim of modification was to obtain TMG cap analogs resistance against decapping enzymes and increased stability *in vivo*, which could be useful for studying TMG related cellular processes and potential medicinal applications.

INTRODUCTION

Trimethylguanosine (TMG) cap structure ($m_3^{2,2,7}GpppG$) was found at 5′ end of mRNA's trans-splicing organisms including flatworms, nematodes and chordates[1]. Hypermetylated cap structure is also present at 5′ ends of some small nuclear RNAs (such as U1 RNA)[2] and is important factor for nucleo-membrane transport and splicing of pre-mRNA. Important feature of TMG capped snRNAs in higher organisms including humans is the possibility of their transport from the cytoplasm to the nucleus. This transport is mediated by TMG-snurportin interaction and has been recently shown to be potentially useful for therapeutic purposes[3]. Despite the fact that $m_3^{2,2,7}GpppG$ and $m_3^{2,2,7}GpppA$ were chemically synthesized two decades ago[4], in this report we describe for the first time TMG cap analogues with modification in the 5′,5′-triphosphate bridge. We expect these analogues to be resistant against enzymatic degradation by decapping proteins: Dcp1/Dcp2 or DcpS.

RESULTS AND CONCLUSION

The synthesis of two TMG cap analogues, $m_3^{2,2,7}GpCH_2ppG$ and ($\mathbf{7}$) $m_3^{2,2,7}GppCH_2pG$ ($\mathbf{8}$), is depicted in Fig. 1. A tree-steps reaction involving reductive methylation was used to obtain dimethylguanosine ($\mathbf{2}$) starting from guanosine ($\mathbf{1}$)[5]. Dimethylguanosine monophosphate was synthesized using Yoshikawa's method, then methylated at *N7* position with MeI in DMSO, and converted into P-imidazolide ($\mathbf{4}$) using 2,2′-dithiodipyridine and triphenylphosphine system.

Reacting guanosine with methylenebis(posphonic chloride) afforded guanosine 5′-methylenebis(phosphonate) (**3**)[6], which was then coupled with **4**, in DMF in the presence of $ZnCl_2$ excess, to give TMG cap analog **8** with ~60% isolated yield.

TMG cap analog **7** was obtained by a generally similar route with comparable yield.

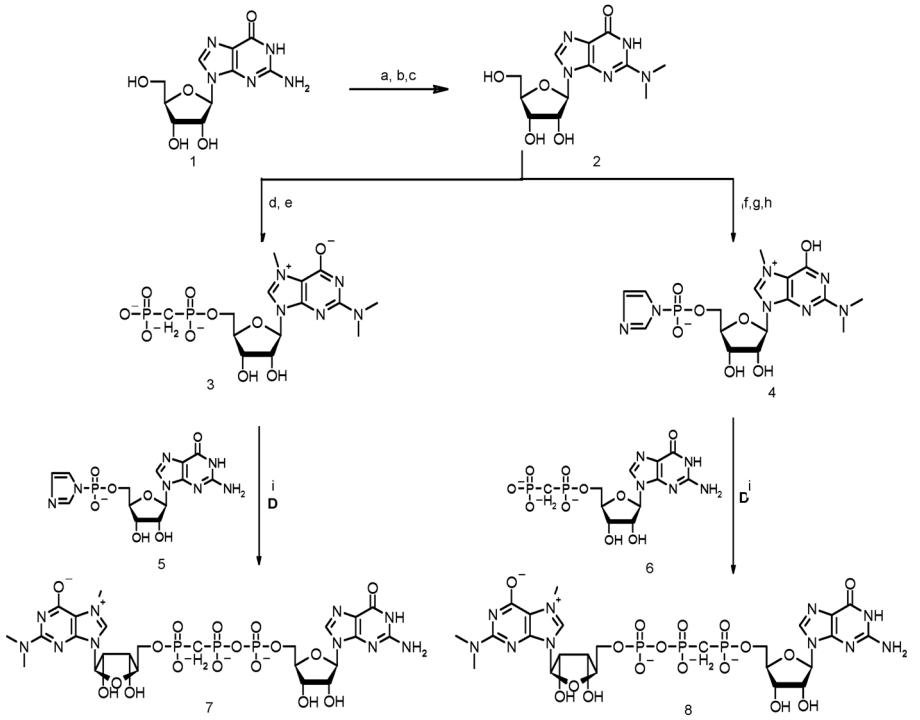

FIG. 1

Synthesis of $m_3^{2,2,7}GpCH_2ppG$ and (**7**) $m_3^{2,2,7}GppCH_2pG$ (**8**). a) $(CH_3CO)_2O$, DMAP, TEA, CH_3CN; b) $(HCOH)_n$, $NaBH_3CN$, CH_3COOH, 40 °C; c) NH_3, 50 °C; d) $CH_2(POCl_2)_2$, $(MeO)_3PO$; e) CH_3I, DMSO; f) $POCl_3$, $(MeO)_3PO$; g) CH_3I, DMSO; h) imidazole, 2,2-DTDP, $(CH_3CH_2)_3N$, DMF, PPh_3; i) $ZnCl_2$, DMF

Financial support from Polish Ministry of Science and Higher Education (NN 301096339) is gratefully acknowledged. M. Z. is grateful to the Foundation for Polish Science for financial support.

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