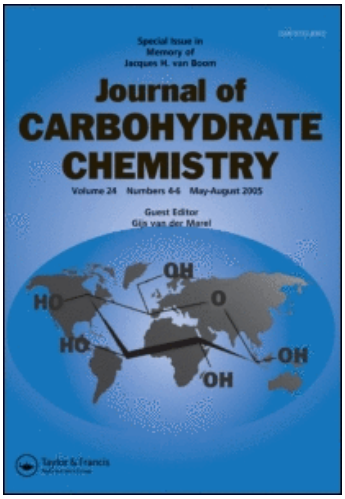


Journal of Carbohydrate Chemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

Oligodeoxynucleotides Containing Diastereomeric O2',C3'-linked Bicyclic Nucleotide Units for Functionalization of the Major Groove of Nucleic Acid Duplexes: A Summary and Novel Derivatives

Michael Raunkjær[a]; Kim F. Haselmann[b]; Jesper Wengel[a]

[a] Nucleic Acid Center, Department of Chemistry, University of Southern Denmark, Odense M, Denmark [b] Mass Spectrometry Laboratory, Department of Chemistry, University of Southern Denmark, Odense M, Denmark

To cite this Article Raunkjær, Michael , Haselmann, Kim F. and Wengel, Jesper(2005) 'Oligodeoxynucleotides Containing Diastereomeric O2',C3'-linked Bicyclic Nucleotide Units for Functionalization of the Major Groove of Nucleic Acid Duplexes: A Summary and Novel Derivatives', Journal of Carbohydrate Chemistry, 24: 4, 475 — 502

To link to this Article: DOI: 10.1081/CAR-200067044

URL: http://dx.doi.org/10.1081/CAR-200067044

Journal of Carbohydrate Chemistry, 24:475–502, 2005

ISSN: 0732-8303 print 1532-2327 online
DOI: 10.1081/CAR-200067044

Oligodeoxynucleotides Containing Diastereomeric O2′,C3′-linked Bicyclic Nucleotide Units for Functionalization of the Major Groove of Nucleic Acid Duplexes: A Summary and Novel Derivatives

Michael Raunkjær

Nucleic Acid Center, Department of Chemistry, University of Southern Denmark, Odense M, Denmark

Kim F. Haselmann

Mass Spectrometry Laboratory, Department of Chemistry, University of Southern Denmark, Odense M, Denmark

Jesper Wengel

Nucleic Acid Center, Department of Chemistry, University of Southern Denmark, Odense M, Denmark

The synthesis and evaluation of a range of piperazino-derivatized diastereomeric O2′,C3′-linked bicyclic nucleotides are described. A new and optimized protocol is presented for the synthesis of the bicyclic scaffold on which the piperazino moiety is

Received March 29, 2005; accepted April 25, 2005.
Dedicated to the memory of professor Jacques H. van Boom.
Address correspondence to Jesper Wengel, Nucleic Acid Center, Department of Chemistry, University of Southern Denmark, Campusvej 55, DK-5230, Odense M, Denmark. E-mail: jwe@chem.sdu.dk

appended. At low salt concentration, the C2″-*S*-configured piperazino-modified oligonucleotides display significantly enhanced hybridization affinity toward complementary DNA and RNA targets relative to the unmodified oligonucleotide control, whereas no melting transition is observed for hybrids formed with the C2″-*R*-configured piperazino-modified oligonucleotides. Upon derivatization of the piperazino moiety with a 1-pyrenebutanoyl group, all modified oligonucleotides display strong DNA binding and profound DNA hybridization selectivity.

Keywords Oligonucleotide, Bicyclic nucleotide, Fluorescence, Pyrene, Low salt hybridization, DNA selectivity, RNA selectivity

INTRODUCTION

During the past two decades, chemically modified oligonucleotides (ONs) have been intensively investigated toward the development of novel therapeutic and diagnostic agents. The desire to create ONs with enhanced nuclease stability, cell penetration ability, or increased binding affinity toward complementary nucleic acids has motivated the discovery of, for example, radically modified analogs as peptide nucleic acid (PNA, Fig. 1),[1] bicyclic ONs as locked nucleic acid (LNA, Fig. 1),[2,3] or six-membered analoges like hexitol nucleic acid (HNA, Fig. 1).[4] The hybridization properties of modified ONs have also

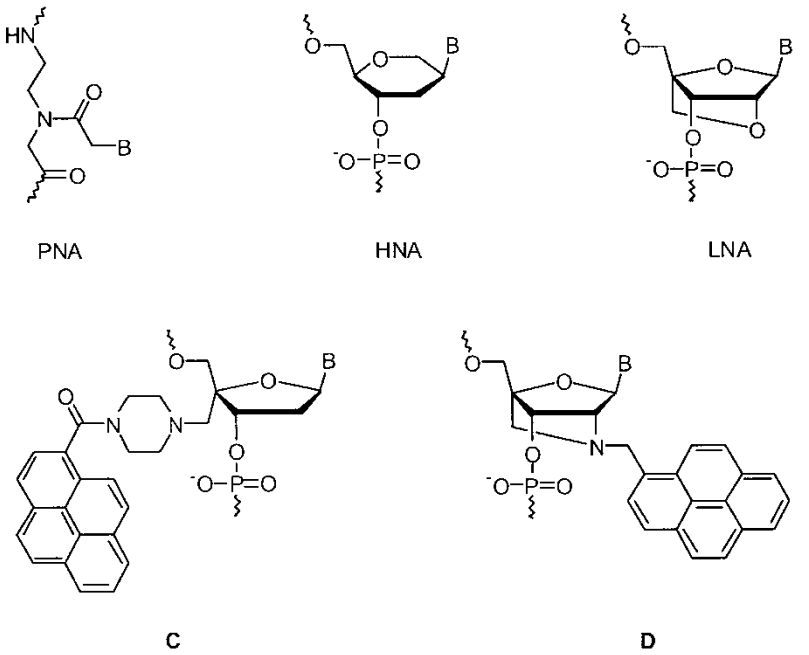


Figure 1: **C** = C4′-(pyren-1-yl)carbonylpiperazinomethyl-DNA and **D** = N2′-(pyren-1-yl) methyl-2′-amino-LNA.

been modulated by introduction of analogs with cationic groups embarking on a gain in duplex stability by overall net charge reduction. This has been studied by modifying the base,[5–11] phosphate,[12–14] or sugar[15–21] moieties of nucleotides.

The scope of ON applications can be broadened considerably by conjugation of an ON with additional moieties.[22] For synthetic convenience, the position most frequently used for conjugation is the 5′-end of an ON. Nevertheless, it is desirable if conjugation can also be performed internally in the sequence. Recently, we have demonstrated the utility of 2′-amino-LNA nucleotides[23] and piperazino-derivatized nucleotides[24–26] for this purpose, thereby extending the scope of piperazino-derivatized ONs previously investigated by our group.[27] Thus, a pyrene-functionalized 4′-*C*-piperazinomethyl nucleotide (**C**, Fig. 1), when incorporated into an ON, induces DNA-selective hybridization with the pyrene moiety facing the minor groove of the duplex.[25] Likewise, pyrene-derivatives of 2′-amino-LNA nucleotides **D** (Fig. 1) induced enhanced thermal stabilities (T_m values) when placed in one strand, or in both strands in a zipperlike constitution, in all cases with the pyrene moiety facing the minor groove.[23]

In 1997, our group reported the synthesis of the O2′,C3′-linked bicyclic nucleotide **P** (Fig. 2) in the search for novel bicyclic nucleotide analogs.[28] Incorporation of monomer **P** in 14-mer thymidylate sequences resulted in a decrease in affinity toward the complementary DNA targets when compared to the unmodified reference duplex. Overall, the affinity was observed to decrease significantly when several monomers **P** were incorporated into the ON. Furthermore, it was not possible to detect any melting transition for 5′-$\mathbf{P}_{13}$T:DNA. In general, the modified sequences displayed a decreased affinity toward complementary RNA targets, except for a slight increase of 1°C in T_m value observed when investigating 5′-$T_5\mathbf{P}_4T_5$:RNA. Interestingly, a large increase in thermal stability ($\Delta T_m = 14$°C) was obtained for 5′-$\mathbf{P}_{13}$T:RNA. This result suggests the helical structure of 5′-$\mathbf{P}_{13}$T:RNA to be different from 5′-T_{14}:RNA, since sequences having a single or several interdispersed monomers **P** display destabilizing behavior when targeting complementary RNA.

With the observed RNA selectivity in mind, we decided to study further the applicability of the bicyclic monomer **P**. Appending a conjugation point to the bicyclic scaffold would allow attachment of different moieties such as alkylamines, oligopeptides, oligonucleotides, or pyrene groups to the modified ON. The position of attachment(s) in the ON could be decided simply by choosing the order of incorporation of unmodified and modified monomers. Also, the position of conjugation to the bicyclic frame was of importance, and we decided to install the conjugation moiety on C2″. In this way, a conjugated group was enforced to face the major groove of the helical structure obtained when hybridizing toward a complementary strand.

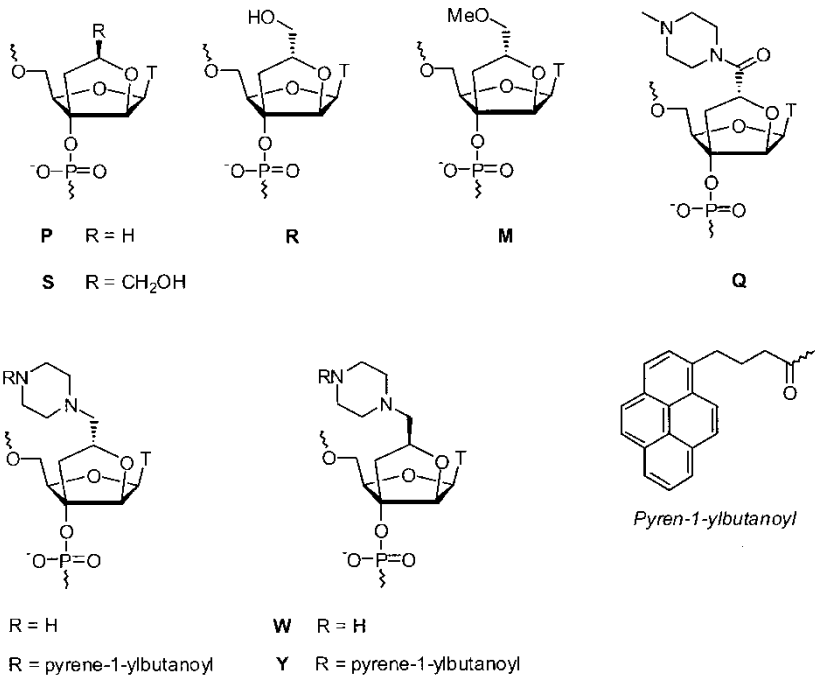
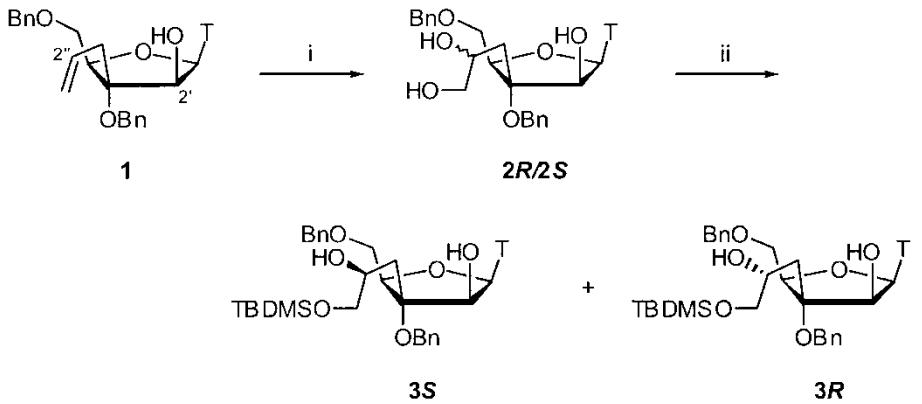


Figure 2: Structures of the O2′, O3′-linked bicyclic nucleotide monomers.

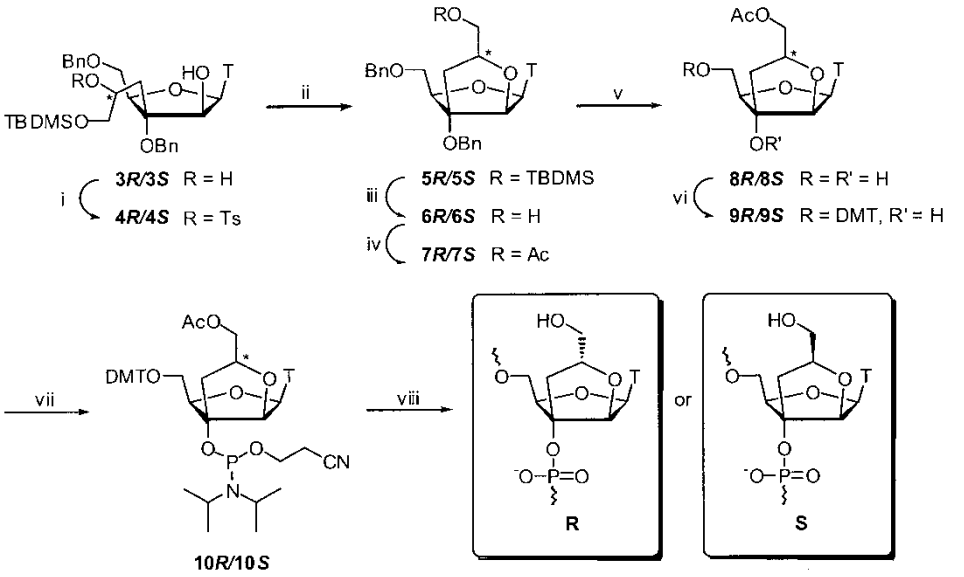
The initial work of constructing a bicyclic scaffold with a conjugation point attached was carried out as shown in Scheme 1.[29] Starting from nucleoside **1**,[28] a dihydroxylation reaction was performed using OsO_4 and as co-oxidant NMO. In this way, triol **2** was provided in 88% yield as an inseparable



Scheme 1: Reagents and conditions: i) OsO_4, NMO, THF, H_2O, rt, 15 hr, 88%; ii) TBDMSCl, pyridine, rt, 15 hr, ***3S***: 53%, ***3R***: 31%, mixture: 13%. T = thymin-1-yl.

mixture of diastereomers (ratio 1 : 2, *R* : *S*). Regioselective TBDMS protection of the primary hydroxyl group allowed separation of the diastereomers providing nucleoside **3*R*** (31% yield) and **3*S*** (53% yield) as well as a mixture of the two diastereomers (14%).[29]

Regioselective tosylation followed by a base-mediated intramolecular ring closure afforded the O2′,C3′-linked bicyclic nucleoside **5*R*** (from **3*S***) and **5*S*** (from **3*R***) in yields of 45% and 52%, respectively (Sch. 2). For convenience of ON synthetic protocols, the TBDMS group was exchanged with an acetyl group providing nucleoside **7*R*** in 76% yield from **5*R***. Hydrogenolysis using H_2 and $Pd(OH)_2$ on carbon as catalyst afforded the debenzylated nucleoside **8*R*** in 94% yield. The monomer building block for ON synthesis was obtained by regioselective O5′-DMT protection and O3′ phosphitylation affording phosphoramidite **10*R*** in 58% yield from **8*R***. Overall, phosphoramidite **10*R*** was provided in 19% yield from **3*S***. The same synthetic transformations were carried out for the C2″-*S*-configured nucleosides, thereby providing a synthetic route yielding phosphoramidite **10*S*** in an overall yield of 20% from **3*R*** (the use of "*" indicates that reactions are independently carried out on each diastereomer).[29]



Scheme 2: Reagents and conditions: i) TsCl, pyridine, rt, 72 hr; ii) K_2CO_3, 18-crown-6, DMF, 70°C, 24 hr, **5*R***: 45% (from **3*S***) and **5*S***: 52% (from **3*R***); iii) TBAF, THF, 3 hr, rt, **6*R***: 80% and **6*S***: 94%; iv) Ac_2O, pyridine, rt, 15 hr; **7*R***: 96% and **7*S***: 92%; v) H_2, $Pd(OH)_2$/C, EtOH, rt, 96 hr, **8*R***: 94% and **8*S***: 95%; vi) DMTCl, pyridine, rt, 15 hr, **9*R***: 92% and **9*S***: 96%; vii) 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite, DIPEA, DCM, rt, 18 hr, **10*R***: 64% and **10*S***: 50%; viii) DNA-synthesizer. T = thymin-1- yl. The use of "*" indicates that reactions are independently carried out on each diastereomer.

Phosphoramidites **10*R*** and **10*S*** were used as building blocks for incorporation of monomers **R** and **S**, respectively (Sch. 2). Thermal denaturation studies revealed monomer **S** to behave like parent monomer **P** when incorporated into a 14-mer homothymidylate sequence (Table 1). An increase in thermal stability (ΔT_m of $+14^\circ C$) was observed for 5′-$\mathbf{S}_{13}$T:RNA, whereas no thermal transition was detectable for 5′-$\mathbf{S}_{13}$T:DNA. Hence, monomer **S** seems a promising candidate for attachment of different moieties to duplexes formed with RNA complements since the properties of monomer **P** are preserved. Interestingly, monomer **R** behaves differently from monomer **S** since T_m values of $61^\circ C$ were observed for both 5′-$\mathbf{R}_{13}$T:RNA, 5′-$\mathbf{R}_{13}$T:DNA (**ON3** in Table 1) and for 5′-$\mathbf{R}_{13}$T alone. Accordingly, we rationalized that monomer **R** induces a strong self-complexation in the almost fully modified sequence, suggesting 5′-$\mathbf{R}_{13}$T to be organized in a complex based on T:T basepairing.[29] CD experiments showed neither an A-type nor a B-type helical structure of the homo-complex. The introduction of a C2″ hydroxymethyl group on the bicyclic scaffold was clearly influencing the properties of the corresponding ONs. In the case of the **R** monomer, one could easily imagine the C2″ hydroxymethyl group to interact with the O2 carbonyl group in the thyminyl moiety via an intramolecular hydrogen bond. This suggested the thymine moiety to be preorganized in a *syn*-conformation allowing the formation of a homocomplex.[29]

We have recently introduced monomer **M** (Fig. 2), a methylated derivative of monomer **R**.[26] This monomer was synthesized as a variant of monomer **R** that lacks the hydroxyl group needed to form the intramolecular hydrogen

Table 1: Thermal denaturation studies of known ON derivatives at 110 mM (Na^+) and pH 7.

Entry	ON Sequence	DNA target T_m	DNA target ΔT_m	RNA target T_m	RNA target ΔT_m
Ref A	5′-T_{14}	**31**		**29**	
ON1	5′-T_7**R**T_6	**27**[a]	*−4*	**25**[a]	*−4*
ON2	5′- T_5**R**$_4T_5$	**10**[a]	*−21*	**25**[a]	*−4*
ON3	5′- $\mathbf{R}_{13}$T	**60**[a]	[b]	**61**[a]	[b]
ON4	5′-T_7**M**T_6	**27**	*−4*	**24**	*−5*
ON5	5′- T_5**M**$_4T_5$	**10**	*−21*	**25**	*−4*
ON6	5′- $\mathbf{M}_{13}$T	**<10**		**38**	+9

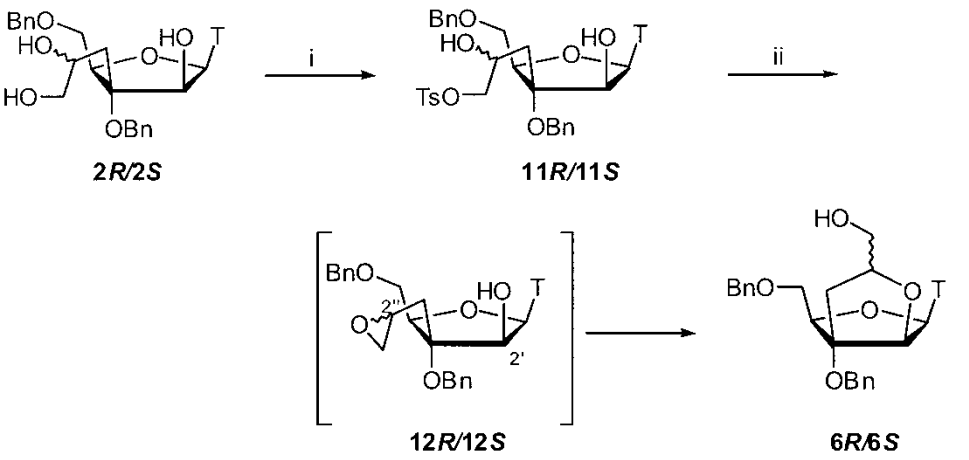
Thermal denaturation experiments were performed with 1.5 μM of the two complementary strands in a medium salt buffer: 0.1 mM EDTA, 100 mM sodium chloride, 10 mM sodium phosphate, pH 7.0. The melting temperatures (T_m (°C)) were determined as the local maximum of the first derivative of the melting curve (A_{260} vs. temperature). ΔT_m values are calculated relative to the relevant unmodified reference. [a]T_m values are found in ref. 29. [b]A value of 61°C was obtained in an analogous thermal denaturation experiment without the addition of complementary strand.

bond with the base suggested in the case of monomer **R**. Accordingly, no self-complexation was observed for 5′-$\mathbf{M}_{13}$T (**ON6**, Table 1). In fact, **ON6** displays similar hybridization properties to those of 5′-$\mathbf{P}_{13}$T and 5′-$\mathbf{S}_{13}$T with increased affinity toward complementary RNA ($\Delta T_m = +9^\circ C$) and RNA selective hybridization. One or four consecutive modifications of **M** induce a moderate decrease in thermal stability toward RNA (**ON4** and **ON5**, Table 1), also in analogy to the results obtained for ONs containing modification **R** (**ON1** and **ON2**, Table 1) and corresponding well with the behavior of ONs modified with the parent compound **P**. The thermal stability studies of ONs modified with monomer **M** showed the RNA binding capability to be preserved when the C2″ hydroxymethyl moiety was chemically functionalized. This result reveals the structural design of monomer **R** to be a promising candidate for further conjugation that apparently is compatible with both stereochemical configurations of C2″.

These promising results prompted us to design monomer **Q** (Fig. 2) for studying the hybridization properties at various pH values and salt concentrations of modified ONs carrying the basic piperazino functionality.[26] Hybridization experiments were carried out on partly modified mixed 9-mer sequences and showed increased affinity toward RNA targets, relative to the unmodified duplex, at pH 5 in a 40 mM Na^+ buffer. At pH 7, there are no increases in duplex stability. The thermal stability studies demonstrate the applicability of the piperazino-moiety for enhancement of duplex stability under selected conditions. This motivated further use of the distal nitrogen atom of the piperazino group as a conjugation site enabling further molecular designs, and we herein report our studies in this direction.

RESULTS AND DISCUSSION

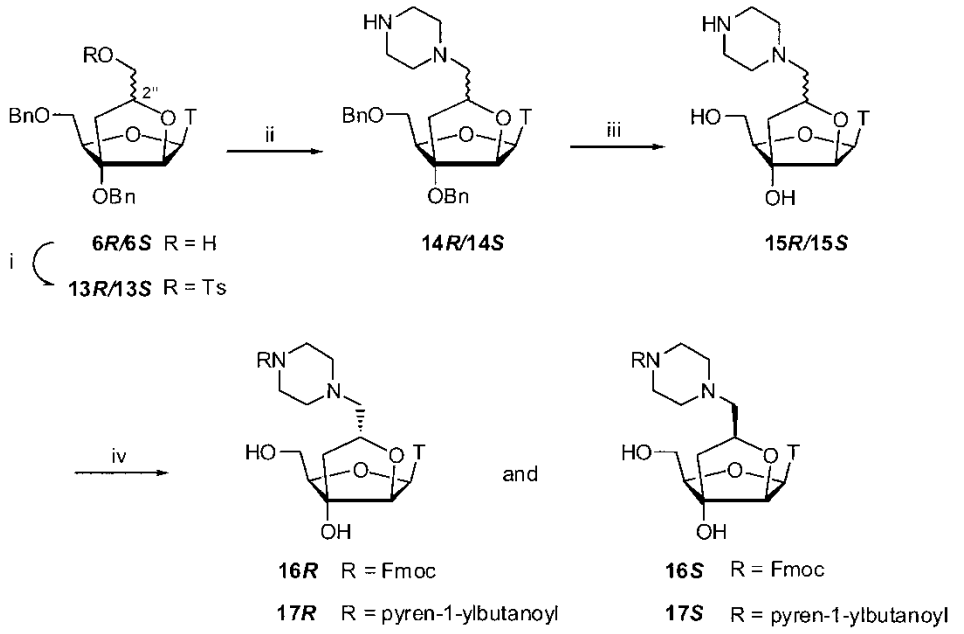
In addition to involving many synthetic steps, the original synthesis of monomers **R** and **S** (Sch. 2) had another drawback: the regioselective tosylation of the mixture **3*R***/**3*S*** and the base mediated ring closure of the mixture **4*R***/**4*S*** were both low yielding. This prompted us to search for a new synthetic route toward the ring-closed key nucleosides **6*R*** and **6*S***. The initial silyl protection of the primary hydroxyl group of nucleosides **2*R***/**2*S*** (Sch. 1) was omitted. Instead, the primary hydroxyl group was regioselectively tosylated using tosyl chloride in anhydrous pyridine (Sch. 3). The crude tosylated nucleoside mixture **11*R***/**11*S*** was, upon treatment with saturated methanolic ammonia, converted into an inseparable mixture of the desired ring-closed nucleosides **6*S*** and **6*R*** (in a 1:2 ratio). The overall yield calculated from starting nucleosides **2*R***/**2*S*** was 81%. We argue the reaction to take place via an intramolecular nucleophilic attack of the 2′-OH on the C2″ of the in situ formed epoxide **12*R***/**12*S***, thereby forming the kinetically favored ring-closed products **6*R***/**6*S***. The overall isolated yield was somewhat lower (55%) when the



Scheme 3: Reagents and conditions: i) TsCl, pyridine, rt, 12 hr; ii) sat. NH_3 in MeOH, MeOH, 0°C, 1 hr, (82%) from **2*R*/2*S***. T = thymin-1- yl.

ring-closing step was performed with NaH as base and DMF as solvent. Although the products were obtained as inseparable mixtures, the new synthetic protocol offers fewer synthetic steps and is highly rewarding with respect to yield and ease of the work-up procedures.

Alternative ways of synthesizing nucleoside mixture **12*R*/12*S*** were investigated in an attempt to influence the diastereochemical outcome of the reaction. Treatment of alkene **1**[28] (Sch. 1) with *m*-CPBA resulted in an 80% yield of diastereomeric product mixture **12*R*/12*S*** in a 1:1 ratio (100 mg reaction scale). Unfortunately, the yield was significantly reduced (40–61% isolated of epoxide mixture **12*R*/12*S***) upon scaling up the reaction. Employing 1,1,1-trifluoroacetone and Oxone for the in situ formation of methyl(trifluoromethyl)dioxirane[30] as well as use of acetonitrile or benzonitrile in the presence of H_2O_2 (the *Payne epoxidation*)[31,32] failed to give the desired epoxide. However, with easy access to nucleoside mixture **6*R*/6*S***, it was now possible to investigate several ways of introducing the piperazino moiety. Attachment via amide bond formation, as in monomer **Q**,[26] was abandoned due to instability (slow amide bond cleavage) observed during deprotection after ON synthesis. Oxidation of the primary alcohol to an aldehyde, a substrate for reductive amination,[33] proved troublesome. Instead, we attempted to install the piperazino group by a nucleophilic attack of piperazine on a triflated nucleoside target. The triflation of mixture **6*R*/6*S*** in DCM or anhydrous pyridine was inefficient as byproducts were formed during the reaction even at low temperature (−20°C). Analytical data suggested these byproducts to be formed via an elimination reaction of the triflated product. Alternatively, the use of TsCl in anhydrous pyridine afforded tosylated nucleoside mixture **13*R*/13*S*** (Sch. 4) as an inseparable mixture of diastereomers without the



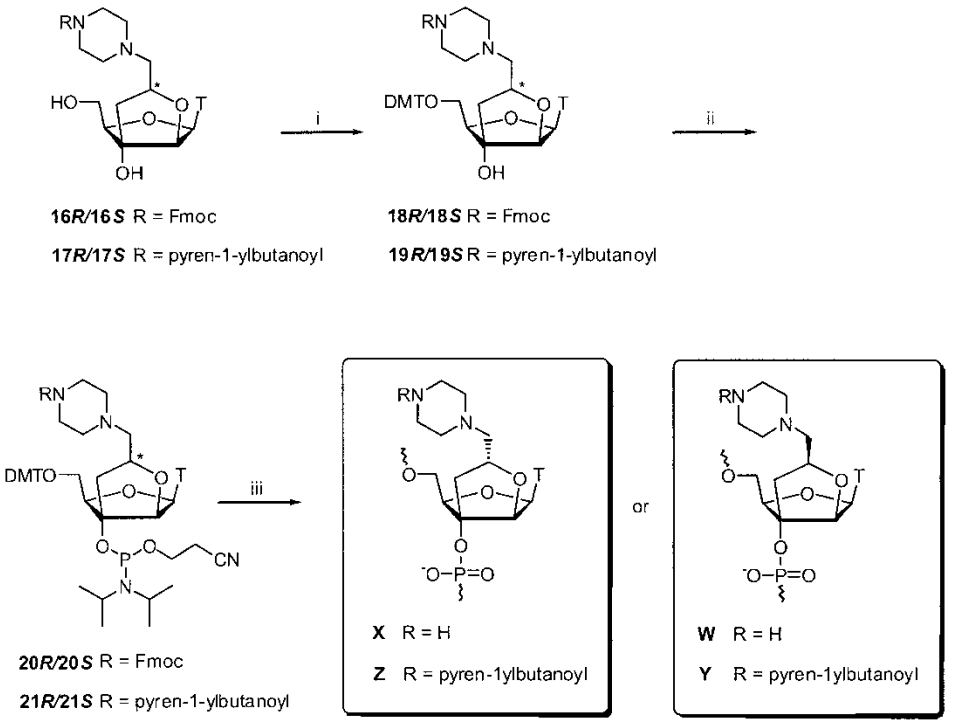
Scheme 4: i) TsCl, pyridine, rt, 12 hr, 85%; ii) piperazine, DMF, 70°C, 4 hr, 74%; iii) H_2, $Pd(OH)_2/C$, MeOH, rt, 144 hr, 90%; iv) FmocCl, MgO, THF, H_2O, 0°C, 30 min, **16*R***: 65%, **16*S***: 32%, **17*R***: 49%, and **17*S***: 20%. T = thymin-1-yl.

formation of byproducts. The crude mixture **13*R*/13*S*** was treated with piperazine (10 eq.) in DMF at elevated temperature affording amine mixture **14*R*/14*S*** in 74% yield from the mixture **6*R*/6*S***. Hydrogenolysis of the benzyl groups using H_2 and $Pd(OH)_2$ on carbon in MeOH as solvent provided the deprotected nucleoside mixture **15*R*/15*S*** in 90% yield. Importantly, the 1 : 2 ratio of the inseparable diastereomeric products was maintained during the high-yielding (68%) sequence including tosylation, introduction of the piperazino group, and debenzylation. In this way, the diastereomeric assignment of the relative ratio between nucleosides **15*R*** and **15*S*** was determined according to the known diasteromeric ratio known of diol mixture **2*R*/2*S***.[34]

The secondary amine in nucleoside mixture **15*R*/15*S*** was Fmoc protected by application of regioselective conditions (Sch. 4) : FmocCl was added to a solution of nucleosides **15*R*/15*S*** and MgO in a mixture of H_2O and THF at 0°C, thereby avoiding additional reaction with the hydroxyl groups.[35] The two diastereomeric products were separated by use of column chromatography, affording nucleosides **16*R*** and **16*S*** in yields of 65% and 32%, respectively. Furthermore, nucleoside mixture **15*R*/15*S*** was treated with 1-pyrenebutyric acid in an EDC-mediated coupling reaction in MeOH as solvent providing nucleosides **17*R*** and **17*S*** in yields of 49% and 20%, respectively,[36] after separation by column chromatography. The four diastereomerically pure nucleosides

thus obtained were each treated with DMTCl and DIPEA in a mixture of DCM and CH_3CN as solvent (1 : 1 ratio) affording nucleosides **18*R*** and **18*S*** (Fmoc protected) and nucleosides **19*R*** and **19*S*** (pyrene-derivatized) in yields of 85%, 88%, 77%, and 75%, respectively (Sch. 5). Finally, the four O5′-DMT protected nucleosides were each reacted with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite and DIPEA in anhydrous DCM, yielding the target amidites **20*R***, **20*S***, **21*R***, and **21*S*** in yields of 60%, 68%, 61%, and 24%, respectively.

Amidites **20*R*** and **20*S*** were used as building blocks for incorporation of monomers **X** and **W** in mixed 9-mer ONs (Table 2) by use of standard coupling procedures (see the Experimental section for details). Furthermore, one **W** monomer was built into a 14-mer oligothymidylate (**ON7**, Table 2). The Fmoc moieties were removed during base-mediated deprotection of the phosphate and nucleobase protecting groups. Caution had to be exercized as intramolecular strand cleavage was observed when using prolonged



Scheme 5: Reagents and conditions: i) DMTCl, DIPEA, DCM, CH_3CN, rt, 30 min, **18*R***: 84%, **18*S***: 88%, **19*R***: 77%, and **19*S***: 75%; ii) 2-cyanoethyl *N*,*N* diisopropylphosphoramidochloridite, DIPEA, anhydrous DCM, rt, 1 hr, **20*R***: 60%, **20*S***: 68%, **21*R***: 61%, and **21*S***: 24%; iii) DNA-synthesizer. T = thymin-1-yl. The use of "*" indicates that reactions are independently carried out on each diastereomer.

Table 2: Thermal denaturation studies of piperazino-modified ONs

		$(Na^+) = 110\,mM$[a]				$(Na^+) = 10\,mM$[b]			
		DNA target		RNA target		DNA target		RNA target	
Entry	**Sequence**	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m	T_m	ΔT_m
Ref A	5′-T_{14}	31		28		16		<10	
ON7	5′-T_6**W**T_7	32	*+1*	28	*0*	17	*+1*	<10	
Ref B	5′-GTG-ATA-TGC	29		27		<10		<10	
ON8	5′-GTG-A**W**A-TGC	29	*0*	28	*+1*	17	*>+7*	16	*>+6*
ON9	5′-G**W**G-A**W**A-**W**GC	27	*−2*	28	*+1*	21	*>+11*	21	*>+11*
ON10	5′-GTG-A**X**A-TGC	27	*−2*	26	*−2*	<10		<10	
ON11	5′-G**X**G-A**X**A-**X**GC	17	*−12*	19	*−8*	<10		<10	

See caption below Table 1. [a]0.1 mM EDTA, 100 mM NaCl, 10 mM sodium phosphate, pH 7.0. [b]0.1 mM EDTA, 10 mM sodium phosphate, pH 7.0.

deprotection time. MS analysis suggested a hydrolysis of the O3′ phosphate bond of the modified monomers to occur (data not shown).

The hybridization affinity of **ON7** toward complementary DNA and RNA targets did not decrease in a medium salt buffer (110 mM $[Na^+]$, Table 2), relative to the unmodified reference **Ref A**. Similarly, comparable hybridization affinity relative to **Ref A** was observed toward complementary DNA in the low salt buffer (10 mM $[Na^+]$, Table 2). However, no melting transition was detected with RNA as complementary target. Relative to **Ref B**, the mixed 9-mer ON with one **W** monomer incorporated showed comparable affinity toward DNA and RNA targets in the medium salt buffer (**ON8**, Table 2). The same tendency was observed for **ON9**, an ON with three **W** monomers incorporated. Apparently, the hybridization affinity toward complementary targets is not influenced when the piperazino moiety is pointing away from the nucleobase, that is, with *S*-configuration of C2″. Furthermore, complexes formed between **ON8** or **ON9** and complementary strands displayed a significant increase in T_m value, relative to the reference duplexes, when the salt concentration was lowered to 10 mM $[Na^+]$. This enhancement in hybridization affinity is probably explained by a net charge reduction of the duplex, because the amino functionality of the piperazino moiety of a monomer **W**, anticipated to be at least partially protonated at pH 7,[37] compensates for the loss of positively charged counterions at reduced salt concentration.

Interestingly, ONs with monomer **X** incorporated displayed different hybridization properties than ONs with monomer **W** incorporated. Thus, **ON10** showed a small decrease in duplex stability toward complementary DNA and RNA targets, and the binding affinity of **ON11** was significantly reduced (Table 2). Hence, in the low salt buffer (10 mM $[Na^+]$), neither **ON10**

nor **ON11** showed any thermal transition. This indicates that the protonated piperazino moiety of monomer **X** is disturbing Watson-Crick base-pairing. Furthermore, with these experiments we have gained information on how to covalently attach the piperazino moiety to the C2″-*R*-configured nucleoside. The choice of an amide bond linkage, as seen for monomer **Q**, resulted in a small decrease in T_m toward DNA and RNA targets in a medium salt buffer at pH 7 (one monomer incorporated). Like **ON11**, a large decrease in T_m value was observed in a low salt buffer with three **Q** monomers incorporated. Based on these results it seems of less importance whether the piperazino group is appended to the nucleotide via an amide bond or via an alkylamino-linkage. The similar hybridization properties observed indicate that it is the distal amino group in the piperazino moieties that is at least partially protonated at pH 7.

The pyrenyl-derivatized monomers **Y** and **Z** were incorporated in mixed 9-mer ONs, and their influence on hybridization properties was investigated by thermal denaturation studies (Table 3). ONs with one monomer incorporated, **Y** in **ON12** and **Z** in **ON13**, showed a significant increase in hybridization affinity toward complementary DNA targets with a ΔT_m of +11 and +9°C relative to **Ref B**, respectively. Duplexes of **ON12** and **ON13** with complementary RNA also displayed increased T_m values, although not to the same extent as seen with the DNA target. The same tendency was observed at low salt concentration.

An ON with two incorporations of either monomer **Y** or monomer **Z** (**ON14** and **ON15**, Table 3) was furthermore studied. **ON14** displayed the same tendency as **ON12**, that is, an enhanced T_m value toward both the DNA ($\Delta T_m = +10$°C) and the RNA ($\Delta T_m = +2$°C) target in a medium salt buffer.

Table 3: Thermal denaturation studies of pyrenebutanoyl piperazino-modified ONs.

		$(Na^+) = 110$ mM[a]				**$(Na^+) = 10$ mM[b]**			
		DNA target		**RNA target**		**DNA target**		**RNA target**	
Entry	**Sequence**	**T_m**	**ΔT_m**	**T_m**	**ΔT_m**	**T_m**	**ΔT_m**	**T_m**	**ΔT_m**
Ref B	5′-GTG-ATA-TGC	29		27		<10		<10	
ON12	5′-GTG-A**Y**A-TGC	40	*+11*	33	+6	25	*>+15*	19	*>+9*
ON13	5′-GTG-A**Z**A-TGC	38	+9	30	+3	25	*>+15*	17	*>+7*
Ref C	5′-GCA-ATA-CAC	29		28		<10		<10	
ON14	5′-GCA-**Y**A**Y**-CAC	39	*+10*	30	*+2*	23	>+13	<10	
ON15	5′-GCA-**Z**A**Z**-CAC	38	*+9*	29	*+2*	25	*>+15*	<10	

See caption below Table 1. [a]0.1 mM EDTA, 100 mM NaCl, 10 mM sodium phosphate, pH 7.0. [b]0.1 mM EDTA, 10 mM sodium phosphate, pH 7.0.

ON15 displayed the same characteristics as **ON14** in a medium salt buffer. The DNA selectivity of **ON14** and **ON15** was even more pronounced in the low salt buffer. Thus, increases in ΔT_m values of $>+13^\circ C$ and $>+15^\circ C$ relative to **Ref C**, respectively, were observed against complementary DNA, whereas no melting transitions were observed with RNA as complementary target. Thus, while monomers **X** or **W** induce preferential hybridization toward RNA, monomers **Y** or **Z** clearly induce preferential hybridization toward DNA. No difference in hybridization properties for the pyrenyl-derivatized ONs was observed for the two different C2″ configurations. This observation suggests that the pyrene moieties are interacting with the duplex, for example, by intercalation or by groove binding, and that this effect is dominating the diastereoselective effect of the piperazino moiety. Alternatively, the conversion of the distal amine functionality of the piperazino derivatives into the neutral amide functionality of the pyrenyl derivatives may suggest that a positive charge is playing a role for the observed diastereoselective hybridization characteristics of the piperazino derivatives.

Thermal denaturation studies of duplexes with modified building blocks incorporated in both strands were also carried out (Table 4). In general, high T_m values were obtained in these experiments, with the combination **ON13**:**ON15** forming the most thermally stable complex both in medium and low salt buffer. This confirms the duplex stabilizing behavior of ONs with monomer **Y** or monomer **Z** incorporated, and convincingly demonstrates the applicability of the piperazino-functionalized ONs for display of additional functionalities in the major groove.

In light of the enhanced hybridization properties induced by incorporation of monomer **Y** or **Z** into ONs, their base-discriminating properties were evaluated (Table 5). Satisfactory Watson-Crick selectivity was observed for both **ON12** and **ON13** as the enhanced T_m values, for the A:T matched base pair significantly differed from the T_m values measured with the corresponding mismatch base pairs. The ability of a DNA nucleotide placed between two monomers of **Y** (or **Z**) to discriminate mismatched complements was investigated as well. As depicted in Table 5, incorporation of the pyrene-derivatized monomers into **ON14** and **ON15** resulted in very satisfactory

Table 4: Thermal denaturation studies of complexes formed between two modified strands.

110 mM (Na^+)	ON14	ON15	10 mM (Na^+)	ON14	ON15
ON12	42	44	**ON12**	26	31
ON13	43	46	**ON13**	31	35

See caption below Table 1. Measurements performed in pH 7.0 buffers.

Table 5: Base pairing selectivity studies, $(Na^+) = 110\,mM$, pH = 7.0.

		Target 5′-GCA-TBT-CAC			
Entry	**Sequence**	**B = A (T_m)**	**B = C (T_m)**	**B = T (T_m)**	**B = G (T_m)**
Ref B	5′-GTG-ATA-TGC	29	13	18	18
ON12	5′-GTG-A**Y**A-TGC	40	<10	27	28
ON13	5′-GTG-A**Z**A-TGC	38	21	23	30
		Target 5′-GTG-A**B**A-TGC			
Ref C	5′-GCA-TAT-CAC	<10	12	29	20
ON14	5′-GCA-**Y**A**Y-**CAC	22	23	39	26
ON15	5′-GCA-**Z**A**Z-**CAC	20	26	38	23

See caption below Table 1.

base-discriminating behavior of the central DNA nucleotide, notably with improved discrimination between the T (matched) and G (mismatched) nucleotides relative to the unmodified reference **Ref C**.

The fluorescence properties of the pyrene-modified ONs were investigated using steady-state fluorescence emission spectroscopy. The experiments were conducted for the single-stranded ONs and for the duplexes formed with complementary DNA or RNA. Low monomer fluorescence emission bands at $\lambda = 378\,nm$ and $\lambda = 398\,nm$ were observed for the singly modified **ON12** and **ON13** upon excitation at $\lambda_{max} = 340\,nm$. The low level of excimer fluorescence can be explained by a low concentration of ONs, for example, the unlikely event of having two pyrene units from two different ONs in proximity. Furthermore, the nucleobases can function as monomer fluorescence quenchers, and the monomer emission is expected to be reduced by the presence of nearby nucleobases in the flexible ON.[38–42] The fluorescence experiments showed the fluorescence monomer emission of the modified ONs to decrease upon duplex formation with a complementary sequence. This can be explained if the pyrene units align in the major groove in proximal distance to the nucleobases. Also, intercalation of the complex by the pyrene unit(s) is a possibility. Okamoto et al. have investigated the temperature dependence of λ_{max} of an intercalating pyrene moiety.[43] Upon heating, the pyrenyl-modified ON and the complementary target dissociated, leading to a red shift of λ_{max} by 6 nm. Employment of the same strategy on **ON12** furnished a λ_{max} red shift of 3 nm, neither ruling out nor confirming intercalation as the mechanism of action.

The reduced fluorescence emission could also be described by the fluorescence quantum yield ϕ. The fluorescence quantum yields were calculated for the modified ONs alone and for the complexes formed with complementary targets (Table 6).[44] Clearly, the value of ϕ is reduced when the modified ON is

Table 6: Fluorescence quantum yields.

Quantum yield ϕ	ON14	ON15	Target DNA	Target RNA	Without target
ON12	0.014	0.018	0.012	0.022	0.026
ON13	0.012	0.019	0.032	0.034	0.047
ON14			0.022	0.024	0.037
ON15			0.024	0.030	0.045

Fluorescence quantum yields (ϕ) determined in a 110 mM (Na^+) buffer at pH 7.0.

targeting complementary DNA or RNA. In general, the most significant decrease in ϕ was observed for the complexes formed with monomers incorporated in each strand.

CONCLUSION

The synthetic route toward diastereomeric O2′,C3′-linked bicyclic nucleotides with a hydroxymethyl handle has been successfully optimized. In each of the two diastereomeric structures, the conjugation handle was derivatized with a piperazino moiety, and the corresponding phosphoramidites were used for incorporation of piperazino-modified nucleotides into modified ONs. The C2″-*S*-configured piperazino-modified ONs displayed significantly enhanced hybridization affinity toward complementary DNA and RNA targets in a low salt buffer. No thermal transition was detected when applying the same conditions to C2″-*R*-configured piperazino-modified ONs. This observation suggests the protonated piperazino moiety of this monomer to disturb Watson-Crick base-pairing. We have demonstrated the applicability of both diastereomeric piperazino-derivatized structures to function as attachment points by introducing a 1-pyrenebutyric acid moiety by amide bond formation. A preference for DNA hybridization was revealed for all ONs modified with pyrene-derivatized nucleotides due to a significant increase in DNA binding relative to that obtained with the reference ON. Notably, the DNA preference and excellent base-pairing selectivity were preserved after incorporation of more than one pyrene-functionalized monomer in an ON. The results obtained testify to the utility of ONs containing diastereomeric O2′,C3′-linked bicylic nucleotide units for functionalization of the major groove of nucleic acid duplexes.

EXPERIMENTAL

The atom numbering of compounds follows the conventional nucleoside style, for example, furanose atoms are numbered with a prime and atoms originating

from the allyl-moiety (structure **1**[28]) are numbered with a double prime. All compounds are named according to the von Baeyer nomenclature.

General

All reagents were purchased from commercial suppliers and used without further purification. THF was distilled from sodium and benzophenone ketyl. Reactions were carried out under an atmosphere of nitrogen when anhydrous solvents were used. Organic phases were dried using Na_2SO_4 followed by filtration and evaporation under reduced pressure to achieve crude product residues. Column chromatography was performed using Silica gel 60 (0.040–0.063 mm), and fractions containing the product were pooled and evaporated to dryness. Dichloromethane (DCM) used for column chromatography was distilled prior to use. TLC analysis was conducted with Merck silica gel plates (60 F_{254}) with UV absorption (254 nm); with spraying (ammonium molybdate (25 g/L), ceric ammonium sulfate (10 g/L), and charring at 150°C; or with H_2SO_4 (5 vol%) in EtOH and charring at 150°C as methods applied for detection. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded with a Bruker WM-300 (300/75.1 MHz) spectrometer; chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard and relative to 85% H_3PO_4 as external reference (^{31}P NMR). When given, assignments of ^1H and ^{13}C peaks are based on ^1H-^1H COSY and ^1H-^{13}C HMQC spectra. HRMS (high resolution mass spectrometry) was performed by matrix-assisted laser-desorption ionization-mass spectrometry (MALDI-MS) on a 4.7 Tesla Ultima (IonSpec, Irvine, CA) Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. Nano-electrospray mass spectrometry was performed on the same instrument. Fluorescence measurements were performed on a Perkin-Elmer LS 55 luminescence spectrometer equipped with a Peltier temperature controller.

(1*S*,3*R*,5*R*,6*R*,8*R*)-5-Benzyloxy-6-benzyloxymethyl-3-hydroxymethyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (6*R*)[29] **and (1*S*,3*S*,5*R*,-6*R*,8*R*)-5-Benzyloxy-6-benzyloxymethyl-3-hydroxymethyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (6*S*).**[29] To a solution of compounds **2*R*/2*S*** (4.01 g, 7.83 mmol) in anhydrous pyridine (50 mL) was added TsCl (2.23 g, 11.72 mmol). The reaction mixture was stirred at rt for 12 hr before addition of a sat. aq. solution of $NaHCO_3$ (10 mL). The resulting mixture was evaporated to dryness under reduced pressure and the residue was dissolved in a mixture of EtOAc (100 mL) and H_2O (50 mL). The organic phase was separated and washed successively with a sat. aq. solution of $NaHCO_3$ (3 × 50 mL) and H_2O (50 mL), and subsequently dried and evaporated to dryness under reduced pressure. The residue was dissolved in a precooled mixture of MeOH and sat. methanolic NH_3 (50 mL, 1 : 1 v/v) and stirred at 0°C for 1 hr. The reaction mixture was evaporated to dryness under reduced pressure and the residue

was purified by silica gel column chromatography with MeOH in DCM [0–4% (v/v)] as eluent. An inseparable mixture of diastereomers **6*R*/6*S*** (ratio 1 : 2; *S* : *R*) was obtained in yield of 3.17 g (82%). Analytical data were consistent with the data reported in literature.

(1*S*,3*R*,5*R*,6*R*,8*R*)-5-Benzyloxy-6-benzyloxymethyl-3-piperazinometyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (14*R*) and (1*S*,3*S*,5*R*,-6*R*,8*R*)-5-Benzyloxy-6-benzyloxymethyl-3-piperazinomethyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (14*S*). TsCl (2.24 g, 11.74 mmol) was added to a solution of compound **6*R*/6*S*** (2.90 g, 5.87 mmol) in anhydrous pyridine. The reaction mixture was stirred at rt for 12 hr before addition of a sat. aq. solution of $NaHCO_3$ (10 mL). The resulting mixture was evaporated to dryness under reduced pressure and the residue was dissolved in a mixture of EtOAc (100 mL) and H_2O (50 mL). The organic phase was washed successively with a sat. aq. solution of $NaHCO_3$ (3×50 mL) and H_2O (50 mL), and was dried and evaporated to dryness under reduced pressure. The residue was dissolved in anhydrous DMF, and piperazine (5.22 g, 60.7 mmol) was added. The reaction mixture was stirred at 70°C for 4 hr and then evaporated to dryness under reduced pressure. The product was purified by silica gel column chromatography with MeOH in DCM [2–6% (v/v)] as eluent. The products were obtained as an inseparable mixture of diastereomers **14*R*/14*S*** (ratio 1 : 2; *S* : *R*) in a yield of 2.4 g (74%). ^{13}C NMR δ_C ($CDCl_3$) (*major diastereomer*) 163.8, 150.7, 137.6, 137.5, 137.4, 128.5, 128.4, 127.9, 127.8 127.1, 108.9, 93.2, 85.2, 82.6, 79.9, 73.5, 68.7, 67.2, 62.7, 54.8, 46.6, 45.6, 45.3, 37.3, and 12.4; ^{13}C NMR δ_C ($CDCl_3$) (*minor diastereoisomer*) 163.7, 150.1, 137.6, 137.5, 137.4, 128.5, 128.4, 127.9, 127.8, 127.1, 109.2, 92.6, 84.3, 81.8, 79.7, 73.7, 68.0, 67.6, 63.3, 54.6, 46.7, 45.6, 45.3, 41.1, and 12.7; HRMS (**14*R*/14*S***) $[M + Na]^+$ m/z 585.2661 (calcd. 585.2611).

(1*S*,3*R*,5*R*,6*R*,8*R*)-5-Hydroxy-6-hydroxymethyl-3-piperazinomethyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (15*R*) and (1*S*,3*S*,5*R*,6*R*,-8*R*)-5-hydroxy-6-hydroxymethyl-3-piperazinomethyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (15*S*). 20% Pd(OH)2/C (850 mg) was added to a solution of nucleosides **14*R*/14*S*** (2.10 g, 3.70 mmol) in MeOH (15 mL) and the reaction mixture was degassed with hydrogen gas and stirred in an atmosphere of hydrogen at rt for 168 hr. The reaction mixture was filtered and the filtrate was evaporated to dryness affording a mixture of nucleosides **15*R*/15*S*** (diastereomeric ratio of 2 : 1) as a white foam (563 mg, 90%). ^{13}C NMR δ_C (DMSO-d_6) (*major diastereomer*) 166.4, 151.9, 139.8, 109.5, 90.0, 88.1, 84.7, 84.2, 81.6, 62.4, 61.2, 51.4, 44.7, 40.8 and 12.8; ^{13}C NMR δ_C (DMSO-d_6) (*minor diastereomer*) 166.4, 151.9, 139.1, 109.5, 86.5, 85.8, 84.6, 81.7, 81.5, 62.6, 62.4, 51.4, 44.7, 40.4, and 12.4. HRMS $[M + H]^+$ m/z 383.1925 (calcd. 383.1931).

(1*S*,3*R*,5*R*,6*R*,8*R*)-5-Hydroxy-6-hydroxymethyl-3-(*N*-[9-fluorenylmethoxycarbonyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]-octane (16*R*) and (1*S*,3*S*,5*R*,6*R*,8*R*)-5-Hydroxy-6-hydroxymethyl-3-(*N*-[9-fluorenylmethoxycarbonyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (16*S*). To a stirred solution of nucleosides **15*R*/15*S*** (418 mg, 1.09 mmol) in a mixture of THF (15 mL) and H_2O (5 mL) was added MgO (306 mg, 7.59 mmol), and the mixture was cooled to 0°C. A solution of FmocCl (312 mg, 1.21 mmol) in THF (5 mL) was added dropwise to the reaction mixture and stirring was continued at 0°C for 30 min before addition of EtOAc (20 mL). The resulting mixture was filtered and the residue was washed with EtAOc (2 × 10 mL). The combined filtrate was evaporated to dryness under reduced pressure and coevaporated with abs. EtOH (2 × 20 mL). The residue was purified by silica gel column chromatography with MeOH in DCM [5 : 95 (v/v)] as eluent to afford as white foams the separated diastereomers **16*R*** and **16*S*** in yields of 394 mg (65%) and 195 mg (32%), respectively. Analytical data for **16*R***: ^{1}H NMR δ_H ($CDCl_3$) 7.75 (d, $J = 7.3$ Hz, 2H, Ar^{Fmoc}), 7.54 (d, $J = 7.3$ Hz, 2H, Ar^{Fmoc}), 7.39 (t, $J = 7.3$ Hz, 2H, Ar^{Fmoc}), 7.30 (t, $J = 7.3$ Hz, 2H, Ar^{Fmoc}), 7.20 (s, 1H, H-6), 6.05 (br s, 1H, H-1′), 4.53–4.19 (m, 5H, H-2′, H-2″, $CHCH_2^{Fmoc}$ and $CHCH_2^{Fmoc}$), 3.86 (m, 3H, H-4′, H^a-5′ and H^b-5′), 3.43 (m, 4H, 2 × CH_2^{pip}), 2.77–2.65 (m, 3H, H^a-3″ and CH_2^{pip}), 2.63–2.45 (m, 4H, H^a-1″, H^b-3″ and CH_2^{pip}), 1.89 (s, 3H, CH_3), 1.72 (m, 1H, H^b-1″); ^{13}C NMR δ_C ($CDCl_3$) 163.9, 155.1 and 150.8 (CO), 143.9, 143.8, 141.2, 127.7, 127.5, 127.0, 124.9 and 120.0 (Ar^{Fmoc}), 137.8 (C6), 109.6 (C5), 87.5 (C3′), 88.1, 83.2, 82.0, 78.5, 77.2 and 47.2 (CH), 67.3, 62.0, 60.7, 53.4, 43.4, 39.7 and 29.6 (CH_2) and 12.5 (CH_3); MALDI-MS $[M + Na]^+$ m/z 627.2 (calcd. 627.3). Analytical data for **16*S***: ^{1}H NMR δ_H ($CDCl_3$) 8.72 (1H, s, NH), 7.76 (d, $J = 7.7$ Hz, 2H, Ar^{Fmoc}), 7.55 (d, $J = 7.4$ Hz, 2H, Ar^{Fmoc}), 7.41 (t, $J = 7.0$ Hz, 2H, Ar^{Fmoc}), 7.32 (t, $J = 7.6$ Hz, 2H, Ar^{Fmoc}), 7.16 (d, $J = 0.9$ Hz, 1H, H-6), 5.97 (d, $J = 5.0$ Hz, 1H, H-1′), 4.45 (m, 4H, H-2′, H-2″ and $CHCH_2^{Fmoc}$), 4.23 (t, $J = 6.6$ Hz, $CHCH_2^{Fmoc}$), 4.08–3.47 (m, 3H, H-4′, H^a-5′ and H^b-5′), 3.47 (m, 4H, 2 × CH_2^{pip}), 2.77–2.65 (m, 3H, H^a-3″ and CH_2^{pip}), 2.48–2.34 (m, 4H, H^a-1″, H^b-3″ and CH_2^{pip}), 1.92 (d, $J = 0.8$ Hz, 3H, CH_3), 1.87 (m, 1H, H^b-1″); ^{13}C NMR δ_C ($CDCl_3$) 164.0, 155.0, 150.4 (CO), 143.8, 141.3, 127.7, 127.0, 124.9 and 120.0 (Ar^{Fmoc}), 136.8 (C6), 109.7 (C5), 86.7 (C3′), 88.1, 83.8, 82.3, 81.1, 77.2, and 47.2 (CH), 67.3, 62.2, 60.8, 54.2, 43.3, 39.2, and 29.6 (CH_2) and 12.6 (CH_3); MALDI-MS $[M + Na]^+$ m/z 627.2 (calcd. 627.3).

(1*S*,3*R*,5*R*,6*R*,8*R*)-5-Hydroxy-6-hydroxymethyl-3-(*N*-[pyren-1-ylbutanoyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (17*R*) and (1*S*,3*S*,5*R*,6*R*,8*R*)-5-Hydroxy-6-hydroxymethyl-3-(*N*-[pyren-1-ylbutanoyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]-octane (17*S*). EDC hydrochloride (230 mg, 1.2 mmol) and DIPEA (210 μL, 1.2 mmol)

were added to a stirred solution of nucleosides **15*R*/15*S*** (399 mg, 1.04 mmol) and 1-pyrenebutyric acid (346 mg, 1.2 mmol) in MeOH (15 mL). The reaction mixture was stirred at rt for 12 hr and then evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of EtOAc (20 mL) and H_2O (5 mL), and the separated organic phase was washed successively with brine (2 × 10 mL) and H_2O (10 mL) and was dried and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using MeOH in DCM [5 : 95 (v/v)] as eluent to give as white foams the separated diastereomers **17*R*** and **17*S*** in yields of 332 mg (49%) and 136 mg (20%), respectively. Analytical data for **17*R***: ^{1}H NMR δ_H ($CDCl_3$) 8.30 (d, $J = 9.4$ Hz, 1H, Ar^{pyr}), 8.15–7.94 (m, 7H, Ar^{pyr}), 7.83 (d, $J = 7.7$ Hz, 1H, Ar^{pyr}), 7.12 (d, $J = 1.3$ Hz, 1H, H-6), 5.97 (d, J 4.3 Hz, 1H, H-1′), 4.45 (m, 1H, H-2″), 4.29 (d, $J = 4.5$ Hz, 1H, H-2′), 3.85–3.70 (m, 3H, H-4′, H^a-5′ and H^b-5′), 3.41–3.21 (m, 5H, $CH^aCH^{b(pip)}$ and 2 × CH_2^{pip}), 2.71 (m, 1H, $CH^aCH^{b(pip)}$), 2.42–2.13 (m, 2H, CH_2^{pip}), 2.63–2.13 (m, 10H, H^a-3″, H^b-3″, CH_2^{pip} and 3 × $CH_2^{butanoyl}$), 1.83 (m, 3H, H^a-1″ and CH_3), 1.63 (m, 1H, H^b-1″); ^{13}C NMR δ_C ($CDCl_3$) 171.2, 163.7, and 150.6 (CO), 137.8, 131.3, 130.8, 129.8, 128.7, 127.4, 127.3, 127.2, 127.1, 126.6, 125.8, 124.9, 124.8, 124.7 (2 peaks) and 123.3 (Ar^{pyr}), 135.9 (C6), 109.4 (C5), 88.0, 87.4, 83.1, 81.8, 81.7, 61.8, 60.5, 53.9, 53.4, 44.8, 41.1, 39.6, 32.7, 32.3, 26.8, and 12.4; HRMS $[M + Na]^+$ m/z 675.2761 (calcd. 675.2789). Analytical data for **17*S***: ^{1}H NMR δ_H ($CDCl_3$) 8.31 (d, $J = 9.1$ Hz, 1H, Ar^{pyr}), 8.17–7.95 (m, 7H, Ar^{pyr}), 7.84 (d, $J = 7.8$ Hz, 1H, Ar^{pyr}), 7.14 (d, $J = 1.4$ Hz, 1H, H-6), 5.93 (d, $J = 4.7$ Hz, 1H, H-1′), 4.39 (d, $J = 5.0$ Hz, 1H, H-2′), 4.35 (m, 1H, H-2″), 4.04–3.89 (m, 3H, H-4′, H^a-5′ and H^b-5′), 3.74 (m, 1H, $CH^{\alpha}CH^{b(pip)}$), 3.53 (m, 1H, $CH^{\alpha}CH^{b(pip)}$), 3.43–3.31 (m, 2H, CH_2^{pip}), 2.63–2.13 (m, 13H, H^a-1″,H^a-3″, H^b-3″, 2 × CH_2^{pip} and 3 × $CH_2^{butanoyl}$), 1.92 (d, $J = 1.3$ Hz, 3H, CH_3), 1.79 (dd, $J = 2.0$ and 13.6 Hz, 1H, H^b-1″); ^{13}C NMR δ_C ($CDCl_3$) 171.1, 164.0, 150.3 (CO), 136.9, 131.3, 130.8, 129.8, 128.7, 127.4, 127.3, 127.1, 126.7, 126.6, 125.8, 124.9, 124.8, 124.7, 123.4, and 123.3 (Ar^{pyr}), 135.9 (C6), 109.4 (C5), 87.9, 86.7, 83.8, 82.5, 80.7, 61.9, 60.7, 54.2, 54.0, 44.8, 41.1, 39.2, 32.7, 32.2, 26.8, and 12.5; HRMS $[M + Na]^+$ m/z 675.2782 (calcd. 675.2789).

(1*S*,3*R*,5*R*,6*R*,8*R*)-6-((4,4′-Dimethoxytrityl)oxymethyl)-5-hydroxy-3-(*N*-[9-fluorenylmethoxycarbonyl]piperazino)methyl-8-(thymin-1-yl)-2,-7-dioxabicyclo[3.3.0]octane (18*R*). DMTCl (769 mg, 2.28 mmol) and DIPEA (660 μL, 3.79 mmol) were added to a stirred solution of nucleoside **16*R*** (458 mg, 0.758 mmol) in a mixture of anhydrous DCM (10 mL) and anhydrous CH_3CN (10 mL). The reaction mixture was stirred for 30 min at rt whereupon DCM (20 mL) and H_2O (5 mL) were added. The organic phase was washed successively with a sat. aq. solution of $NaHCO_3$ (3 × 5 mL) and H_2O (10 mL) and subsequently dried and evaporated to dryness under reduced pressure. The residue was purified by column chromatography using sat. methanolic

ammonia and MeOH [0.5% sat. methanolic ammonia, 0–3% MeOH/(v/v/v)] in DCM as eluent to give nucleoside **18*R*** (580 mg, 84%) as a white foam. ^{1}H NMR δ_H (DMSO-d_6) 11.4 (s, 1 H, NH) 7.87 (d, $J = 7.4$ Hz, 2H, Ar^{Fmoc}), 7.56 (d, $J = 7.2$ Hz, 2H, Ar^{Fmoc}), 7.43–7.20 (m, 14H, Ar^{DMT}, Ar^{Fmoc} and H-6), 6.88 (d, $J = 7.8$ Hz, 4H, Ar^{DMT}), 5.91 (d, $J = 4.5$ Hz, 1H, H-1′), 5.75 and 5.70 (s, 2H, NH and 3′-OH), 4.35 (d, $J = 6.6$ Hz, 2H, $CHCH_2^{Fmoc}$), 4.23 (t, $J = 6.4$ Hz, 1H, $CHCH_2^{Fmoc}$), 4.15 (m, 2H, H-2′ and H-2″), 4.00 (m, 1H, H-4′), 3.74 (s, 6H, 2 × OCH_3), 3.32 (m, 6H, H^a-3″, H^b-3″, H^a-5′, H^b-5 and CH_2^{pip}), 2.46 (m, 2H, CH_2^{pip}), 2.24–2.07 (m, 5H, H^a-1″ and 2 × CH_2^{pip}), 1.81 (s, 3H, CH_3), 1.61 (m, 1H. H^b-1″); ^{13}C NMR δ_C (DMSO-d_6) 163.7, 154.2 and 150.0 (CO), 158.1, 154.2, 144.8, 143.8, 140.7, 137.5, 135.5, 135.4, 129.7, 127.8, 127.7, 127.6, 127.1, 126.7, 125.0, 120.0, 113.2 and 107.4 (C5, C6 and Ar), 87.6, 86.6, 85.7, 82.3, 81.0, 79.8, 66.4, 62.2, 60.7, 55.0, 54.9, 52.7, 46.8, 43.3 and 12.1; HRMS $[M + Na]^+$ m/z 929.3755 (calcd. 929.3732).

(1*S*,3*S*,5*R*,6*R*,8*R*)-6-((4,4′-Dimethoxytrityl)oxymethyl)-5-hydroxy-3-(*N*-[9-fluorenylmethoxycarbonyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (18*S*). The procedure for preparation of compound **18*R*** was used. Compound **16*S*** (282 mg, 0.47 mmol) was reacted with DMTCl (473 mg, 1.40 mmol) in a mixture of anhydrous DCM (10 mL) and CH_3CN (10 mL) in the presence of DIPEA (325 μL, 0.742 mmol). Nucleoside **18*S*** (373 mg, 88%) was obtained as a white foam. ^{1}H NMR δ_H (DMSO-d_6) 11.5 (s, 1 H, NH) 7.87 (d, $J = 7.8$ Hz, 2H, Ar^{Fmoc}), 7.60 (d, $J = 7.1$ Hz, 2H, Ar^{Fmoc}), 7.44–7.21 (m, 14H, Ar^{DMT}, Ar^{Fmoc} and H-6), 6.89 (d, $J = 9.0$ Hz, 4H, Ar^{DMT}), 5.87 (d, $J = 3.6$ Hz, 1H, H-1′), 5.80 (s, 2H, NH and 3′-OH), 4.36 (d, $J = 6.3$ Hz, 2H, $CHCH_2^{Fmoc}$), 4.25 (t, $J = 6.1$ Hz, 1H, $CHCH_2^{Fmoc}$), 4.18 (d, 1H, H-2′), 4.06 (m, 1H, H-4′), 3.92 (m, 1H, H-2″), 3.74 (s, 6H, 2 × OCH_3), 3.40–3.32 (m, 6H, H^a-3″, H^b-3″, H^a-5′, H^b-5 and CH_2^{pip}), 2.48 (m, 2H, CH_2^{pip}), 2.27–2.09 (m, 4H, 2 × CH_2^{pip}), 1.96 (dd, $J = 5.4$ and 12.8, 1H, H^a-1″), 1.80 (s, 3H, CH_3), 1.60 (dd, 1H, $J = 9.4$ and 12.4, 1H H^b-1″); ^{13}C NMR δ_C (DMSO-d_6) 163.7, 158.1, 158.0, 149.9, 144.7, 142.5, 139.4, 137.4, 136.5, 135.4, 135.3, 129.8, 128.9, 127.8, 127.7, 127.2, 126.7, 121.4, 120.0, 113.2 and 109.7 (CO, C5, C6 and Ar), 86.5, 86.4, 86.1, 85.8, 84.2, 79.1, 61.8, 55.0, 54.9, 54.5, 45.4, 43.3 and 12.1; HRMS $[M + Na]^+$ m/z 929.3755 (calcd. 929.3732).

(1*S*,3*R*,5*R*,6*R*,8*R*)-6-((4,4′-Dimethoxytrityl)oxymethyl)-5-hydroxy-3-(*N*-[pyren-1-ylbutanoyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (19*R*). The procedure for preparation of compound **18*R*** was used. Compound **17*R*** (120 mg, 0.18 mmol) was reacted with DMTCl (186 mg, 0.55 mmol) in a mixture of anhydrous DCM (3 mL) and CH_3CN (3 mL) in the presence of DIPEA (160 μL, 0.92 mmol). Nucleoside **19*R*** (135 mg, 77%) was obtained as a white foam. ^{1}H NMR δ_H ($CDCl_3$) 8.30 (d, $J = 9.2$ Hz, 1H, Ar^{pyr}), 8.16–7.96 (m, 7H, Ar^{pyr}), 7.89 (d, $J = 7.8$ Hz, 1H, Ar^{pyr}), 7.55–7.21 (m, 9H, H-6

and Ar^{DMT}), 6.83 (d, $J = 8.5$ Hz, 4H, Ar^{DMT}), 6.05 (br s, 1H, H-1′), 4.35 (d, $J = 4.0$ Hz, 1H, H-2′), 4.00 (m, 1H, H-2″), 4.80–3.05 (m, 15H-4′, H^a-5′, H^b-5′, $2 \times OCH_3$, H^a-3″, H^b-3″ and $2 \times CH_2^{pip}$), 2.60–2.10 (m, 5H, H^a-1″ and $2 \times CH_2^{pip}$), 2.05–1.95 (m, 7H, $3 \times CH_2^{butanoyl}$), 1.90 (d, $J = 0.9$ Hz, 3H, CH_3), 1.61 (m, 1H, H^b-1″); ^{13}C NMR δ_C ($CDCl_3$) 170.9, 163.1, 158.7, 150.8, 144.7, 137.8, 136.3, 135.7, 135.7, 131.4, 130.9, 130.0, 129.9, 129.7, 128.8, 128.1, 127.9, 127.8, 127.4, 127.3, 127.1, 126.7, 126.6, 125.7, 125.0, 124.9, 124.8, 124.7, 123.6, 113.3 and 109.4 (CO, C6, C5 and Ar), 88.6, 88.5, 86.6, 82.7, 82.5, 77.1, 62.4, 62.0, 55.2, 53.9, 53.7, 53.6, 44.8, 41.1, 40.3, 40.2, 32.7, 32.2, 26.8 and 12.5; HRMS $[M + Na]^+$ m/z 977.4104 (calcd. 977.4096).

(1*S*,3*S*,5*R*,6*R*,8*R*)-6-((4,4′-Dimethoxytrityl)oxymethyl)-5-hydroxy-3-(*N*-[pyren-1-ylbutanoyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (19*S*). The procedure for preparation of compound **18*R*** was used. Compound **17*S*** (100 mg, 0.15 mmol) was reacted with DMTCl (155 mg, 0.46 mmol) in a mixture of anhydrous DCM (2 mL) and CH_3CN (2 mL) in the presence of DIPEA (133 μL, 0.74 mmol). Nucleoside **19*S*** (110 mg, 75%) was obtained as a white foam. ^{1}H NMR δ_H ($CDCl_3$) 8.31 (d, $J = 9.3$ Hz, 1H, Ar^{pyr}), 8.17–7.95 (m, 7H, Ar^{pyr}), 7.85 (d, $J = 7.7$ Hz, 1H, Ar^{pyr}), 7.51–7.20 (m, 9H, H-6 and Ar^{DMT}), 6.83 (d, $J = 8.4$ Hz, 4H, Ar^{DMT}), 5.98 (d, $J = 4.7$ Hz, 1H, H-1′), 4.41 (d, $J = 5.0$ Hz, 1H, H-2′), 4.27 (m, 1H, H-2″), 4.20 (dd, $J = 3.8$ and 6.5 Hz, 1H, H-4′), 3.79 (m, 7H, H^a-5′, H^b-5′ and $2 \times OCH_3$), 3.47–3.40 (m, 6H, H^a-3″, H^b-3″ and $2 \times CH_2^{pip}$), 2.58–2.45 (m, 4H, $2 \times CH_2^{pip}$), 2.40–2.17 (m, 7H, H^a-1″ and $3 \times CH_2^{butanoyl}$), 1.90 (d, $J = 0.9$ Hz, 3H, CH_3), 1.60 (m, 1H, H^b-1″); ^{13}C NMR δC ($CDCl_3$) 171.0, 163.0, 158.5, 150.3, 144.6, 136.9, 135.9, 135.7, 135.6, 131.3, 130.1, 130.0, 129.9, 128.8, 128.1, 127.9, 127.4, 127.3, 127.1, 126.7, 126.6, 125.0, 124.9, 124.8, 124.7, 123.4, 113.2 and 109.5 (CO, C6, C5 and Ar), 88.8, 86.6, 86.5, 84.0, 81.8, 80.1, 62.2, 62.0, 55.2, 55.1, 55.0, 54.9, 44.8, 41.1, 39.2, 32.7, 32.2, 26.8 and 12.5; HRMS $[M + Na]^+$ m/z 977.4085 (calcd. 977.4096).

(1*S*,3*R*,5*R*,6*R*,8*R*)-5-(2-Cyanoethoxy(diisopropylamino)phosphinoxy)-6-((4,4′-dimethoxytrityl)oxymethyl)-3-(*N*-[9-fluorenylmethoxycarbonyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (20*R*). 2-Cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (188 μL, 0.86 mmol) was added dropwise to a stirred solution of nucleoside **18*R*** (494 mg, 0.55 mmol) dissolved in a mixture of anhydrous DCM (6 mL) and DIPEA (1.2 mL). The reaction mixture was stirred for 1 hr at rt and subsequently evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using EtOAc in petroleum ether [1 : 1 (v/v)] as eluent to give amidite **20*R*** (363 mg, 60%) as a white foam. ^{31}P NMR δ_P (DMSO-d_6) 142.7 and 142.6; HRMS $[M + Na]^+$ m/z 1129.4815 (calcd. 1129.4811).

(1*S*,3*S*,5*R*,6*R*,8*R*)-5-(2-Cyanoethoxy(diisopropylamino)phosphinoxy)-6-((4,4′-dimethoxytrityl)oxymethyl)-3-(*N*-[9-fluorenylmethoxycarbonyl]-piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (20*S*). The procedure for preparation of amidite **20*R*** was used. Compound **18*S*** (260 mg, 0.29 mmol) dissolved in a mixture of anhydrous DCM (2 mL) and DIPEA (0.4 mL) was reacted with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (70 μL, 0.32 mmol). The residue was purified by silica gel column chromatography using EtOAc in petroleum ether [1 : 1 (v/v)] as eluent to give amidite **20*S*** (217 mg, 68%) as a white foam. ^{31}P NMR δ_P (DMSO-d_6) 143.3 and 143.1; HRMS $[M + Na]^+$ m/z 1129.4837 (calcd. 1129.4811).

(1*S*,3*R*,5*R*,6*R*,8*R*)-5-(2-Cyanoethoxy(diisopropylamino)phosphinoxy)-6-((4,4′-dimethoxytrityl)oxymethyl)-3-(*N*-[pyren-1-ylbutanoyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (21*R*). The procedure for preparation of amidite **20*R*** was used. Compound **19*R*** (110 mg, 0.12 mmol) dissolved in a mixture of anhydrous DCM (1 mL) and DIPEA (0.2 mL) was reacted with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (40 μL, 0.18 mmol). The residue was purified by silica gel column chromatography using EtOAc in petroleum ether [1 : 1 (v/v)] as eluent to give amidite **21*R*** (82 mg, 61%) as a white foam. ^{31}P NMR δ_P (DMSO-d_6) 142.7 and 142.6; HRMS $[M + Na]^+$ m/z 1177.5159 (calcd. 1177.5175).

(1*S*,3*S*,5*R*,6*R*,8*R*)-5-(2-Cyanoethoxy(diisopropylamino)phosphinoxy)-6-((4,4′-dimethoxytrityl)oxymethyl)-3-(*N*-[pyren-1-ylbutanoyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (21*S*). The procedure for preparation of amidite **20*R*** was used. Compound **19*S*** (100 mg, 0.10 mmol) dissolved in a mixture of anhydrous DCM (1 mL) and DIPEA (0.2 mL) was reacted with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (36 μL, 0.16 mmol). The residue was purified by silica gel column chromatography using EtOAc in petroleum ether [1 : 1 (v/v)] as eluent to give amidite **21*S*** (30 mg, 24%) as a white foam. ^{31}P NMR δ_P (DMSO-d_6) 143.4 and 143.2; HRMS $[M + Na]^+$ m/z 1177.5180 (calcd. 1177.5175).

Oligonucleotide Synthesis

All ONs were prepared on a Biosearch 8700 Synthesizer using the phosphoramidite approach. Phosphoramidites **20*R***, **20*S***, **21*R***, and **21*S*** were used as building blocks using 10 min coupling time with pyridinium hydrochloride as activator and *tert*-butylhydroperoxide as oxidant (stepwise coupling yield of 98%). The unmodified DNA nucleotides were incorporated using 2 min coupling time (stepwise coupling yields of >99%). The O5′-DMT-*ON* ONs were deprotected and cleaved form the solid support by treatment with sat. methanolic NH_3 for 2 hr at 55°C and were purified by RP-HPLC. Detritylation

was performed using 80% AcOH for 20 min, and the O5′-DMT-*OFF* ONs were repurified by RP-HPLC to yield ONs of a satisfactory purity (>80% as evaluated by gel capillary electrophoresis). Prior to thermal denaturation studies, the ONs were desalted using NAP^{TM} columns (Amersham Pharmacia AB, Uppsala, SE).

Mass Spectrometric Analysis of Oligonucleotides

ON7-ON15 were analyzed by nanoelectrospray mass spectrometry using the following procedure: 5-μL aliquot of SPE purified sample (using micro columns with NAP^{TM} resin, Amersham Pharmacia AB, Uppsala, SE) was loaded onto a pulled glass capillary (Proxeon, Odense, DK). The glass capillary was placed in a nanospray micromanipulator and a potential of approximately −800 V was applied for negative ion mode. The electrospray source was a standard hexapole based for external accumulation and with heated metal capillary introduction. Due to the nature of the samples, only external calibration was done, but with careful control of the signal magnitude. **ON7** 1096.00 (calcd. 1095.45 for $[C_{147}H_{203}N_{30}O_{97}P_{13}K]^{4-}$), **ON8** 721.19 (calcd. 722.14 for $[C_{96}H_{120}N_{36}O_{54}P_8]^{4-}$), **ON9** 1056.68 (calcd. 1056.59 for $[C_{110}H_{145}N_{40}O_{56}P_8]^{3-}$), **ON10** 963.23 (calcd. 963.19 for $[C_{96}H_{121}N_{36}O_{54}P_8]^{3-}$), **ON11** 1056.62 (calcd. 1056.59 for $[C_{110}H_{145}N_{40}O_{56}P_8]^{3-}$), **ON12** 394.85 (calcd. 394.33 for $[C_{116}H_{130}N_{36}O_{55}P_8]^{8-}$), **ON13** 397.59 (calcd. 397.08 for $[C_{116}H_{129}N_{36}O_{55}P_8Na]^{8-}$), **ON14** 874.51 (calcd. 874.47 for $[C_{141}H_{159}N_{37}O_{55}P_8]^{4-}$) and **ON15** 351.89 (calcd. 351.38 for $[C_{141}H_{152}N_{37}O_{55}P_8Na]^{10-}$).

Thermal Denaturation Studies

The melting temperature experiments were performed on a Perkin-Elmer lambda 20 spectrometer using a 1.0-mL cuvette with 1.5 μM of the two complementary strands in either **buffer 1** (100 mM sodium chloride, 10 mM sodium phosphate, and 0.1 mM EDTA, pH 7.0) or **buffer 2** (10 mM sodium phosphate and 0.1 mM EDTA, pH 7.0). The following example illustrates a typical procedure of sample preparation (in **buffer 1**): the two complementary strands (dissolved in distilled H_2O) were added to 500 μL of **buffer A** (200 mM sodium chloride, 20 mM sodium phosphate, and 0.2 mM EDTA). Distilled H_2O was added until a total volume of 1000 μL was reached. **Buffer A** was prepared by mixing appropriate volumes of **buffer B** (200 mM sodium chloride, 20 mM NaH_2PO_4, and 0.2 mM EDTA) and **buffer C** (200 mM sodium chloride, 10 mM Na_2HPO_4, and 0.2 mM EDTA) until a pH value of 7.0 was obtained (using a pH-meter for determination of the pH value). The samples were heated to 70°C and cooled to 5°C before initiating the experiment with a ramp of 1°C/min. The melting temperature T_m was determined as the local maximum of the first derivative of the melting curve (A_{260} vs. temperature).[45]

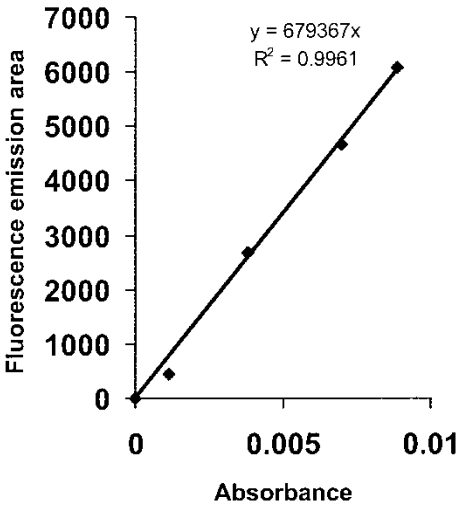


Figure 3: Liner relationship of fluorescence emission area vs. absorbance.

Fluorescence Experiments

Steady-state fluorescence emission spectra (360–600 nm) were obtained using quartz cuvettes with a pathlength of 1.0 cm and a concentration of 0.15 μM of strands in T_m buffer. Spectra were recorded at 20°C with an average of five scans using an excitation wavelength of 340 nm, excitation slit of 4.0 nm, emission slit of 2.5 nm, and scan speed of 120 nm/min. The quantum yield ϕ_S was determined using 1-pyrenebutyric acid as a reference. The quantum yield is calculated according to the equation:

$$\phi_S = \phi_P \times [\text{Area(S)}/A_{340}(S)]/[\text{Area(P)}/A_{340}(P)] \times [n^2(S)/n^2(P)]$$

where the quantum yield ϕ_P of 1-pyrenebutyric acid is 0.063 (measured relative to known fluorescence quantum yield for 9,10-diphenylanthracene in cyclohexane, $\phi_F = 0.95$)[46] in MeOH. The value used for [Area(P)/A_{340}(P)] is 290838 (slope of the linear relationship of fluorescence emission area vs. absorbance). Area (S) is the area under the fluorescence curve (360–600 nm) and A_{340} is the absorbance at 340 nm. The values of refractive index *n(S)* and *n(P)* equal 1.3328 (H_2O) and 1.3288 (MeOH), respectively. To confirm the applicability of the method, the linear correlation of absorbance and concentration was verified for monomer **17*R*** (fluorescence emission area vs. absorbance) at low concentration (Fig. 3).

ACKNOWLEDGMENTS

Ms. Britta M. Dahl (University of Copenhagen), Ms. Kirsten Østergaard, and Ms. Suzy W. Lena are thanked for oligonucleotide synthesis and

oligonucleotide purification. The Danish National Research Foundation and the Danish Research Agency are thanked for financial support.

REFERENCES AND NOTES

[1] Wittung, P.; Nielsen, P.; Buchardt, O.; Egholm, M.; Norden, B. DNA-like double helix formed by peptide nucleic-acid. Nature **1994**, *368*, 561.

[2] Koshkin, A.A.; Singh, S.K.; Nielsen, P.; Rajwanshi, V.K.; Kumar, R.; Meldgaard, M.; Olsen, C.E.; Wengel, J. LNA (Locked nucleic acids): synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. Tetrahedron **1998**, *54*, 3607.

[3] Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.-I.; Morio, K.-I.; Doi, T.; Imanishi, T. Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2′-O,4′-C-methyleneribonucleosides. Tetrahedron Lett. **1998**, *39*, 5401.

[4] Van Aerschot, A.; Verheggen, I.; Hendrix, C.; Herdewijn, P. 1,5-Anhydrohexitol nucleic acids, a new promising antisense construct. Angew. Chem. Int. Ed. Engl. **1995**, *34*, 1338.

[5] Heystek, L.E.; Zhou, H.Q.; Dande, P.; Gold, B. Control over the localization of positive charge in DNA: the effect on duplex DNA and RNA stability. J. Am. Chem. Soc. **1998**, *120*, 12165.

[6] Prakash, T.P.; Barawkar, D.A.; Kumar, V.; Ganesh, K.N. Synthesis of site-specific oligonucleotide-polyamine conjugates. Bioorg. Med. Chem. Lett. **1994**, *4*, 1733.

[7] Ramasamy, K.S.; Zounes, M.; Gonzales, C.; Freier, S.M.; Lesnik, E.A.; Cummins, L.L.; Griffey, R.H.; Monia, B.P.; Cook, D.P. Remarkable enhancement of binding-affinity of heterocycle-modified DNA to DNA and RNA — synthesis, characterization and biophysical evaluation of N-2-imidazolylpropylguanine and N-2-imidazolylpropyl-2-aminoadenine modified oligonucleotides. Tetrahedron Lett. **1994**, *35*, 215.

[8] Maier, M.A.; Barber-Peoc'h, I.; Manoharan, M. Postsynthetic guanidinylation of primary amino groups in the minor and major grooves of oligonucleotides. Tetrahedron Lett. **2002**, *43*, 7613.

[9] Hashimoto, H.; Nelson, M.G.; Switser, C. Zwitterionic DNA. J. Am. Chem. Soc. **1993**, *115*, 7128.

[10] Seela, F.; Zulauf, M. Oligonucleotides containing 7-deazaadenines : the influence of the 7-substituent chain length and charge on the duplex stability. Helv. Chim. Acta **1999**, *82*, 1878.

[11] Lin, K.-I.; Matteucci, M.D. A cytosine analogue capable of clamp-like binding to a guanine in helical nucleic acids. J. Am. Chem. Soc. **1998**, *120*, 8531.

[12] Letsinger, R.L.; Singman, C.N.; Histand, G.; Salunkhe, M. Cationic oligonucleotides. J. Am. Chem. Soc. **1988**, *110*, 4470.

[13] Fathi, R.; Huang, Q.; Coppola, G.; Delaney, W.; Teasdale, R.; Krieg, A.M.; Cook, A.F. Oligonucleotides with novel, cationic backbone substituents—aminoethylphosphonates. Nucleic Acids Res. **1994**, *22*, 5416.

[14] Michel, T.; Debart, F.; Vasseur, J.-J. Efficient guanidination of the phosphate linkage towards cationic phosphoramidate oligonucleotides. Tetrahedron Lett. **2003**, *44*, 6579.

[15] Hickman, D.T.; Tan, T.H.S.; Morral, J.; King, P.M.; Cooper, M.A.; Micklefield, J. Design, synthesis, conformational analysis and nucleic acid hybridisation properties of thymidyl pyrrolidine-amide oligonucleotide mimics (POM). Org. Biomol. Chem. **2003**, *1*, 3277.

[16] Meier, R.; Grüschow, S.; Leumann, C. Nucleic-acid analogs with restricted conformational flexibility in the sugar-phosphate backbone ('bicyclo-DNA') - part 7—synthesis and properties of oligodeoxynucleotides containing [(3′*S*,5′*S*,6′*R*)-6′-amino-2′-deoxy-3′,5′-ethano-β-D-ribofuranosyl]thymine (=(6′*R*)-6′-amino-bicyclothymidine). Helv. Chim. Acta **1999**, *82*, 1813.

[17] Pfundheller, H.M.; Bryld, T.; Olsen, C.E.; Wengel, J. Oligonucleotides containing novel 4′-*C*- or 3′-*C*-(aminoalkyl)-branched thymidines. Helv. Chim. Acta **2000**, *83*, 128.

[18] Aurup, H.; Tuschl, T.; Benseler, F.; Ludwig, J.; Eckstein, F. Oligonucleotide duplexes containing 2′-amino-2′-deoxycytidines—thermal-stability and chemical-reactivity. Nucleic Acids Res. **1994**, *22*, 20.

[19] Kanazaki, M.; Ueno, Y.; Shuto, S.; Matsuda, A. Highly nuclease-resistant phosphodiester-type oligodeoxynucleotides containing 4′-α-C-aminoalkylthymidines form thermally stable duplexes with DNA and RNA. A candidate for potent antisense molecules. J. Am. Chem. Soc. **2000**, *122*, 2422.

[20] Griffey, R.H.; Monia, B.P.; Cummins, L.L.; Freier, S.; Greig, M.J.; Guinosso, C.J.; Lesnik, E.; Manalili, S.M.; Mohan, V.; Owens, S.; Ross, B.R.; Sasmor, H.; Wancewicz, E.; Weiler, K.; Wheeler, P.D.; Cook, P.D. 2′-*O*-aminopropyl ribonucleotides : a zwitterionic modification that enhances the exonuclease resistance and biological activity of antisense oligonucleotides. J. Med. Chem. **1996**, *39*, 5100.

[21] Wang, G.; Seifert, W.E. Synthesis and evaluation of oligodeoxynucleotides containing 4′-*C*-substituted thymidines. Tetrahedron Lett. **1996**, *37*, 6515.

[22] Goodchild, J. Conjugates of oligonucleotides and modified oligonucleotides : a review of their synthesis and properties. Bioconjugate Chem. **1990**, *1*, 165.

[23] Hrdlicka, P.J.; Babu, B.R.; Sørensen, M.D.; Wengel, J. Interstrand communication between 2′-*N*-(pyren-1-yl)methyl-2′-amino-LNA monomers in nucleic acid duplexes : directional control and signalling of full complementarity. Chem. Commun. **2004**, 1478.

[24] Raunkjær, M.; Bryld, T.; Wengel, J. *N*-Methylpiperazinocarbonyl-2′,3′-BcNA and 4′-*C*-(*N*-methylpiperazino)methyl-DNA : introduction of basic functionalities facing the major groove and the minor groove of a DNA : DNA duplex. Chem. Commun. **2003**, 1604.

[25] Bryld, T.; Højland, T.; Wengel, J. DNA-selective hybridization and dual strand invasion of short double-stranded DNA using pyren-1-ylcarbonyl-functionalized 4′-*C*-piperazinomethyl-DNA. Chem. Commun. **2004**, 1064.

[26] Raunkjær, M.; Sørensen, M.D.; Wengel, J. Synthesis and thermal denaturation studies of novel 2′-*O*,3′-*C*-linked bicyclic oligonucleotides with a methoxy or a piperazino group facing the major groove of nucleic acid duplexes. Org. Biomol. Chem. **2005**, *3*, 130.

[27] Petersen, G.V.; Wengel, J. Synthesis and characterization of short oligonucleotide segments containing nonnatural internucleoside amine and amide linkages. Nucleosides Nucleotides **1995**, *14*, 925.

[28] Nielsen, P.; Pfundheller, H.M.; Olsen, C.E.; Wengel, J. Synthesis of 2′-*O*,3′-*C*-linked bicyclic nucleosides and bicyclic oligonucleotides. J. Chem. Soc. Perkin Trans. 1 **1997**, 3423.

[29] Raunkjær, M.; Olsen, C.E.; Wengel, J. Oligonucleotide analogues containing (2″*S*)- and (2″*R*)-2′-*O*,3′-*C*-((2′-*C*-hydroxymethyl)ethylene)-linked bicyclic nucleoside monomers : synthesis, RNA-selective binding, and diastereoselective formation of a very stable homocomplex based on T : T base pairing. J. Chem. Soc. Perkin. Trans. 1 **1999**, 2543.

[30] Wang, D.; Wong, M.-K.; Yip, Y.-C. Epoxidation of olefins using methyl(trifluoromethyl)dioxirane generated in-situ. J. Org. Chem. **1995**, *60*, 3887.

[31] Payne, G.B. A simplified procedure for epoxidation by benzonitrile-hydrogen peroxide — selective oxidation of 2-allylcyclohexanone. Tetrahedron **1962**, *18*, 763.

[32] Payne, G.B.; Deming, P.H.; Williams, P.H. Reactions of hydrogen peroxide. 7. Alkali-catalyzed epoxidation and oxidation using a nitrile as co-reactant. J. Org. Chem. **1961**, *26*, 659.

[33] Borch, R.F.; Bernstein, M.D.; Durst, H.D. Cyanohydridoborate anion as a selective reducing agent. J. Am. Chem. Soc. **1971**, *93*, 2897.

[34] NOE experiments did not reveal a significant difference between the diastereomers. It was not possible to obtain a crystal of the separated piperazino-modified nucleosides for use in X-ray analysis. The stereochemical assignment of structures was based on the known 1 : 2 ratio of diol **2**. Phosphoramidites derived from C2″-*R*-configured compounds all displayed values below 143 ppm (^{31}P NMR). Phosphoramidites derived from C2″-*S*-configured compounds all displayed values above 143 ppm (see the Experimental section).

[35] Kim, D.-H.; Rho, H.-S.; You, J.W.; Lee, J.C. Chemoselective *N*-acylation of amino alcohols promoted by magnesium oxide in aqueous organic solution. Tetrahedron Lett. **2002**, *43*, 277.

[36] The highest yield was achieved by use of EDC in MeOH. Other coupling reagents (DIC or DCC) with or without activators (HOBt or DMAP) and in different solvents such as DMF or DCM were lower yielding.

[37] Hall, H.K., Jr. Field and inductive effects on the base strengths of amines. J. Am. Chem. Soc. **1956**, *78*, 2570.

[38] Shafirovich, V.V.; Courtney, S.H.; Ya, N.Q.; Geacintov, N.E. Proton-coupled photoinduced electron-transfer, deuterium-isotope effects, and fluorescence quenching in noncovalent benzo[a]pyrenetetraol-nucleoside complexes in aqueous solutions. J. Am. Chem. Soc. **1995**, *117*, 4920.

[39] Geacintov, N.E.; Zhao, R.; Kuzmin, V.A.; Kim, S.K.; Pecora, L. Mechanisms of quenching of the fluorescence of a benzo[a]pyrenetetraol metabolite model-compound by 2′-deoxynucleosides. J. Photochem. Photobiol. **1993**, *58*, 185.

[40] Margulis, L.; Pluzhnikov, P.F.; Mao, B.; Kuzmin, V.A.; Chang, Y.J.; Scott, T.W.; Geacintov, N.E. Photoinduced electron-transfer in a covalent benzo[a]pyrene diol epoxide-2′-deoxyguanosine complex. Chem. Phys. Lett. **1991**, *187*, 597.

[41] Zahavy, E.; Fox, M.A. Photophysical quenching mediated by guanine groups in pyrenyl-*N*-alkylbutanoamide end-labeled oligo nucleotides. J. Phys. Chem. B **1999**, *103*, 9321.

[42] Steenken, S.; Jovanovic, S.V. How easily oxidizable is DNA? One-electron reduction potentials of adenosine and guanosine radicals in aqueous solution. J. Am. Chem. Soc. **1997**, *119*, 617.

[43] Okamoto, A.; Ichiba, T.; Saito, I. Pyrene-labeled oligodeoxynucleotide probe for detecting base insertion by excimer fluorescence emission. J. Am. Chem. Soc. **2004**, *126*, 8364.

[44] The quantum yield, or quantum efficiency is defined as the number of photo physical events occuring (*e.g.*, fluorescence emission) per photon absorbed. The calculations are described in the Experimental section.

[45] Mergny, J.-L.; Lacroix, L. Analysis of thermal melting curves. Oligonucleotides **2003**, *13*, 515.

[46] Morris, J.V.; Mahaney, M.A.; Huber, J.R. Fluorescence quantum yield determinations — 9,10-diphenylanthracene as a reference-standard in different solvents. J. Phys. Chem. **1976**, *80*, 969.