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Journal of Carbohydrate Chemistry Publication details, including instructions for authors and subscription information:

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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 Published online: 16 Aug 2006.

To cite this article: Michael Raunkjær, Kim F. Haselmann & Jesper Wengel (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, 24:4-6, 475-502

To link to this article: <u>http://dx.doi.org/10.1081/CAR-200067044</u>

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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'-**P**₁₃T:DNA. In general, the modified sequences displayed a decrease of 1°C in T_m value observed when investigating 5'-T₅**P**₄T₅:RNA. Interestingly, a large increase in thermal stability ($\Delta T_m = 14^{\circ}C$) was obtained for 5'-**P**₁₃T:RNA. This result suggests the helical structure of 5'-**P**₁₃T:RNA to be different from 5'-T₁₄: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 OsO₄ 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, H₂O, rt, 15hr, 88%; ii) TBDMSCI, pyridine, rt, 15hr, 35:53%, 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 **3R** (31% yield) and **3S** (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 H₂ 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) Ac₂O, pyridine, rt, 15 hr; 7*R*: 96% and 7*S*: 92%; v) H₂, 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 \mathbf{R} and \mathbf{S} , respectively (Sch. 2). Thermal denaturation studies revealed monomer \mathbf{S} to behave like parent monomer \mathbf{P} when incorporated into a 14-mer homothymidylate sequence (Table 1). An increase in thermal stability (ΔT_m of +14°C) was observed for 5'-S₁₃T:RNA, whereas no thermal transition was detectable for 5'- $S_{13}T$:DNA. Hence, monomer S seems a promising candidate for attachment of different moieties to duplexes formed with RNA complements since the properties of monomer \mathbf{P} are preserved. Interestingly, monomer \mathbf{R} behaves differently from monomer \mathbf{S} since T_m values of 61°C were observed for both 5′- $R_{13}T$: RNA, 5′- $R_{13}T$: 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 \mathbf{M} (Fig. 2), a methylated derivative of monomer \mathbf{R} .^[26] This monomer was synthesized as a variant of monomer \mathbf{R} that lacks the hydroxyl group needed to form the intramolecular hydrogen

Entry	ON Sequence	DNA	arget	RNA target	
		Tm	ΔT_m	Tm	$\Delta \mathbf{T_m}$
Ref A	5′-T ₁₄	31		29	
ON1 ON2 ON3	5′-T7 R T6 5′- T5 R 4T5 5′- R 13T	27° 10° 60°	-4 -21 b	25° 25° 61°	-4 -4 b
ON4 ON5 ON6	5′-T7 M T6 5′- T5 M 4T5 5′- M 13T	27 10 <10	-4 -21	24 25 38	-5 -4 +9

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

Thermal denaturation experiments were performed with 1.5 μ M 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 (A₂₆₀ vs. temperature). ΔT_m values are calculated relative to the relevant unmodified reference. $^{\circ}T_m$ values are found in ref. 29. ^bA 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 selfcomplexation 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 \mathbf{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 3R/3S and the base mediated ring closure of the mixture 4R/4S were both low yielding. This prompted us to search for a new synthetic route toward the ring-closed key nucleosides 6R and 6S. The initial silvl protection of the primary hydroxyl group of nucleosides 2R/2S (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 11R/11S was, upon treatment with saturated methanolic ammonia, converted into an inseparable mixture of the desired ring-closed nucleosides 6S and 6R (in a 1:2 ratio). The overall yield calculated from starting nucleosides 2R/2S 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 12R/12S, thereby forming the kinetically favored ring-closed products 6R/6S. The overall isolated yield was somewhat lower (55%) when the



Scheme 3: Reagents and conditions: i) TsCl, pyridine, rt, 12 hr; ii) sat. NH₃ in MeOH, MeOH, 0°C, 1 hr, (82%) from 2R/2S. 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 12R/12S were investigated in an attempt to influence the diastereochemical outcome of the reaction. Treatment of alkene $\mathbf{1}^{[28]}$ (Sch. 1) with *m*-CPBA resulted in an 80% yield of diastereomeric product mixture 12R/12S in a 1:1 ratio (100 mg reaction scale). Unfortunately, the yield was significantly reduced (40-61%)isolated of epoxide mixture 12R/12S) 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 6R/6S, it was now possible to investigate several ways of introducing the piperazino moiety. Attachment via amide bond formation, as in monomer \mathbf{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 6R/6S in DCM or anhydrous pyridine was inefficient as byproducts were formed during the reaction even at low temperature $(-20^{\circ}C)$. 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 13R/13S (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) H₂, Pd(OH)₂/C, MeOH, rt, 144 hr, 90%; iv) FmocCl, MgO, THF, H₂O, 0°C, 30 min, **16R**: 65%, **16S**: 32%, **17R**: 49%, and **17S**: 20%. T = thymin-1-yl.

formation of byproducts. The crude mixture 13R/13S was treated with piperazine (10 eq.) in DMF at elevated temperature affording amine mixture 14R/14S in 74% yield from the mixture 6R/6S. Hydrogenolysis of the benzyl groups using H₂ and Pd(OH)₂ on carbon in MeOH as solvent provided the deprotected nucleoside mixture 15R/15S in 90% yield. Importantly, the 1:2 ratio of the inseparable diastereomeric products was maintained during the highyielding (68%) sequence including tosylation, introduction of the piperazino group, and debenzylation. In this way, the diastereomeric assignment of the relative ratio between nucleosides 15R and 15S was determined according to the known diasteromeric ratio known of diol mixture 2R/2S.^[34]

The secondary amine in nucleoside mixture 15R/15S was Fmoc protected by application of regioselective conditions (Sch. 4): FmocCl was added to a solution of nucleosides 15R/15S and MgO in a mixture of H₂O 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 16R and 16S in yields of 65% and 32%, respectively. Furthermore, nucleoside mixture 15R/15S was treated with 1-pyrenebutyric acid in an EDC-mediated coupling reaction in MeOH as solvent providing nucleosides 17R and 17S in yields of 49% and 20%, respectively,^[36] after separation by column chromatography. The four diastereomerically pure nucleosides

21R/21S R = pyren-1-ylbutanoyl

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 20R and 20S 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 exercised as intramolecular strand cleavage was observed when using prolonged



Scheme 5: Reagents and conditions: i) DMTCI, 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.

		(Na ⁺) = 110 mM ^a				(Na ⁺) = 10 mM ^b			
		D ta	NA rget	R ta	NA rget	D ta	NA rget	R ta	NA rget
Entry	Sequence	Tm	ΔT_m	Tm	ΔT_m	Tm	$\Delta \mathbf{T_m}$	T _m	ΔT_m
Ref A ON7	5′-T ₁₄ 5′-T ₆ W T ₇	31 32	+1	28 28	0	16 17	+1	<10 <10	
Ref B ON8 ON9 ON10 ON11	5'-GTG-ATA-TGC 5'-GTG-AWA-TGC 5'-GWG-AWA-WGC 5'-GTG-AXA-TGC 5'-GXG-AXA-XGC	29 29 27 27 17	0 -2 -2 -12	27 28 28 26 19	+1 + 1 - 2 - 8	<10 17 21 <10 <10	>+7 >+11	<10 16 21 <10 <10	>+6 >+11

 Table 2: Thermal denaturation studies of piperazino-modified ONs

See caption below Table 1. °0.1 mM EDTA, 100 mM NaCl, 10 mM sodium phosphate, pH 7.0. °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 $(10 \text{ mM} [\text{Na}^+])$, 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 $(\Delta T_m = +10^{\circ}C)$ and the RNA $(\Delta T_m = +2^{\circ}C)$ target in a medium salt buffer.

		(Na $^+$) = 110 mM a				(Na ⁺) = 10 mM ^b			
		D ta	NA rget	R ta	NA rget	D ta	NA Irget	RI tar	NA get
Entry	Sequence	Tm	$\Delta \mathbf{T_m}$	Tm	$\Delta \mathbf{T_m}$	Tm	ΔT_m	Tm	$\Delta \mathbf{T_m}$
Ref B	5'-GTG-ATA-TGC	29		27		<10		<10	
ON12 ON13	5'-GTG-A Y A-TGC 5'-GTG-A Z A-TGC	40 38	+11 +9	33 30	+6 +3	25 25	>+15 >+15	19 17	>+9 >+7
Ref C ON14 ON15	5'-GCA-ATA-CAC 5'-GCA -Y AY-CAC 5'-GCA -ZAZ -CAC	29 39 38	+10 +9	28 30 29	+2 +2	<10 23 25	> + 13 >+ 15	<10 <10 <10	

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

See caption below Table 1. ^a0.1 mM EDTA, 100 mM NaCl, 10 mM sodium phosphate, pH 7.0. ^b0.1 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 \mathbf{X} or \mathbf{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 pyrenederivatized monomers into **ON14** and **ON15** resulted in very satisfactory

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

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

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

Entry		Target 5'-GCA-TBT-CAC						
	Sequence	B = A (T _m)	B = C (T _m)	B = T (T _m)	B = G (T _m)			
Ref B ON12 ON13	5'-GTG-ATA-TGC 5'-GTG-A Y A-TGC 5'-GTG-A Z A-TGC	29 40 38	13 <10 21	18 27 23	18 28 30			
			Target 5'-GT	g-ABA-TGC				
Ref C ON14 ON15	5′-GCA-TAT-CAC 5′-GCA- Y A Y- CAC 5′-GCA- Z A Z- CAC	<10 22 20	12 23 26	29 39 38	20 26 23			

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

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

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

Table 6: Fluorescence quantum yields.

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 $\mathbf{1}^{[28]}$) 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, ¹³C, and ³¹P 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 $85\%~{
m H_3PO_4}$ as external reference (³¹P NMR). When given, assignments of ¹H and ¹³C peaks are based on ¹H-¹H COSY and ¹H-¹³C 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.

(1S,3R,5R,6R,8R)-5-Benzyloxy-6-benzyloxymethyl-3-hydroxymethyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (6R)^[29] and (1S,3S,5R,-6R,8R)-5-Benzyloxy-6-benzyloxymethyl-3-hydroxymethyl-8-(thymin-1yl)-2,7-dioxabicyclo[3.3.0]octane (6S).^[29] To a solution of compounds 2R/2S (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 NaHCO₃ (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 H₂O (50 mL). The organic phase was separated and washed successively with a sat. aq. solution of NaHCO₃ (3 × 50 mL) and H₂O (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 NH₃ (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.

(1S,3R,5R,6R,8R)-5-Benzyloxy-6-benzyloxymethyl-3-piperazinometyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (14R) and (1S,3S,5R,-6R,8R)-5-Benzyloxy-6-benzyloxymethyl-3-piperazinomethyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (14S). TsCl (2.24g, 11.74 mmol) was added to a solution of compound 6R/6S (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 H₂O (50 mL). The organic phase was washed successively with a sat. aq. solution of NaHCO₃ $(3 \times 50 \text{ mL})$ and H₂O (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/**14S** (ratio 1:2; S:R) in a yield of 2.4 g (74%). ¹³C NMR δ_{C} (CDCl₃) (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; ¹³C NMR $\delta_{\rm C}$ (CDCl₃) (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 (14R/14S) [M + Na]⁺ m/z 585.2661 (calcd. 585.2611).

(1S,3R,5R,6R,8R)-5-Hydroxy-6-hydroxymethyl-3-piperazinomethyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (15R) and (1S,3S,5R,6R,-8R)-5-hydroxy-6-hydroxymethyl-3-piperazinomethyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (15S). 20% Pd(OH)2/C (850 mg) was added to a solution of nucleosides 14R/14S (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 15R/15S (diastereomeric ratio of 2:1) as a white foam (563 mg, 90%). ¹³C NMR $\delta_{\rm C}$ (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; ¹³C NMR $\delta_{\rm C}$ (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).

(1S,3R,5R,6R,8R)-5-Hydroxy-6-hydroxymethyl-3-(N-[9-fluorenylmethoxycarbonyl]piperazino)methyl-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (16R) and (1S,3S,5R,6R,8R)-5-Hydroxy-6-hydroxymethyl-3-(N-[9-fluorenylmethoxycarbonyl]piperazino)methyl-8-(thymin-1-yl)-2,7dioxabicyclo[3.3.0]octane (16S). To a stirred solution of nucleosides 15R/ 15S (418 mg, 1.09 mmol) in a mixture of THF (15 mL) and H₂O (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 ($2 \times 10 \text{ mL}$). The combined filtrate was evaporated to dryness under reduced pressure and coevaporated with abs. EtOH $(2 \times 20 \text{ mL})$. 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 16R and 16S in yields of 394 mg (65%) and 195 mg (32%), respectively. Analytical data for 16R: ¹H NMR $\delta_{\rm H}$ (CDCl₃) 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", CHCH2^{Fmoc} and CHCH2^{Fmoc}), 3.86 (m, 3H, H-4', H^a-5' and H^b-5'), 3.43 (m, 4H, 2 × CH₂^{pip}), 2.77-2.65 (m, 3H, H^a-3" and CH₂^{pip}), 2.63-2.45 (m, 4H, Ha-1", Hb-3" and CH2pip), 1.89 (s, 3H, CH3), 1.72 (m, 1H, H^b-1"); ¹³C NMR $\delta_{\rm C}$ (CDCl₃) 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 (CH₂) and 12.5 (CH₃); MALDI-MS $[M + Na]^+ m/z$ 627.2 (calcd. 627.3). Analytical data for 16S: ¹H NMR $\delta_{\rm H}$ (CDCl₃) 8.72 (1H, s, NH), 7.76 (d, $J = 7.7 \,\text{Hz}$, 2H, Ar^{Fmoc}), 7.55 (d, $J = 7.4 \,\text{Hz}$, 2H, Ar^{Fmoc}), 7.41 (t, $J = 7.0 \,\text{Hz}$, 2H, Ar^{Fmoc}), 7.32 (t, $J = 7.6 \,\text{Hz}$, 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 CHCH₂^{Fmoc}), 4.23 (t, J = 6.6 Hz, CHCH₂^{Fmoc}), 4.08–3.47 (m, 3H, H-4', H^a-5' and H^b-5'), 3.47 (m, 4H, $2 \times CH_{2}^{pip}$), 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, CH₃), 1.87 (m, 1H, H^b-1"); ¹³C NMR $\delta_{\rm C}$ (CDCl₃) 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 (CH₂) and 12.6 (CH₃); MALDI-MS $[M + Na]^+ m/z$ 627.2 (calcd. 627.3).

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

were added to a stirred solution of nucleosides 15R/15S (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 $(2 \times 10 \text{ mL})$ and H₂O (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 17R:¹H NMR $\delta_{\rm H}$ (CDCl_3) 8.30 (d, J = 9.4 Hz, 1H, Ar^{pyr}), 8.15–7.94 (m, 7H, Ar^{pyr}), 7.83 $(d, J = 7.7 \text{ Hz}, 1\text{H}, \text{Ar}^{\text{pyr}}), 7.12 (d, J = 1.3 \text{ Hz}, 1\text{H}, \text{H-6}), 5.97 (d, J 4.3 \text{ Hz}, 1\text{H}, 1\text{H})$ 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 $2 \times CH_2^{pip}$), 2.71(m, 1H, CH^aCH^{b(pip)}), 2.42-2.13 (m, 2H, CH^{pip}₂), 2.63-2.13 (m, 10H, H^a-3", $H^{b}-3''$, CH_{2}^{pip} and $3 \times CH_{2}^{butanoyl}$), 1.83 (m, 3H, $H^{a}-1''$ and CH_{3}), 1.63 (m, 1H, H^b-1"); ¹³C NMR δ_{C} (CDCl₃) 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 17S: ¹H NMR $\delta_{\rm H}$ (CDCl₃) 8.31 (d, $J = 9.1 \,{\rm Hz}$, 1H, Ar^{pyr}), 8.17–7.95 (m, 7H, Ar^{pyr}), 7.84 $(d, J = 7.8 \text{ Hz}, 1\text{H}, \text{Ar}^{\text{pyr}}), 7.14 (d, J = 1.4 \text{ Hz}, 1\text{H}, \text{H-6}), 5.93 (d, J = 4.7 \text{ Hz}, 1\text{H}, 1\text{H})$ 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^aCH^{b(pip)}), 3.53 (m, 1H, CH^aCH^{b(pip)}), 3.43–3.31 (m, 2H, CH₂^{pip}), 2.63–2.13 (m, 13H, H^a-1", H^a-3", H^b-3", $2 \times CH_2^{pip}$ and $3 \times \text{CH}_2^{\text{butanoyl}}$, 1.92 (d, J = 1.3 Hz, 3H, CH_3), 1.79 (dd, J = 2.0 and 13.6 Hz, 1H, H^b-1"); ¹³C NMR $\delta_{\rm C}$ (CDCl₃) 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).

(1S,3R,5R,6R,8R)-6-((4,4'-Dimethoxytrityl)oxymethyl)-5-hydroxy-3-(N-[9-fluorenylmethoxycarbonyl]piperazino)methyl-8-(thymin-1-yl)-2,-7-dioxabicyclo[3.3.0]octane (18R). DMTCl (769 mg, 2.28 mmol) and DIPEA (660 μ L, 3.79 mmol) were added to a stirred solution of nucleoside 16R (458 mg, 0.758 mmol) in a mixture of anhydrous DCM (10 mL) and anhydrous CH₃CN (10 mL). The reaction mixture was stirred for 30 min at rt whereupon DCM (20 mL) and H₂O (5 mL) were added. The organic phase was washed successively with a sat. aq. solution of NaHCO₃ (3 × 5 mL) and H₂O (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. ¹H NMR $\delta_{\rm H}$ (DMSO-*d*₆) 11.4 (s, 1 H, NH) 7.87 (d, $J = 7.4 \,{\rm Hz}$, 2H, Ar^{Fmoc}), 7.56 (d, $J = 7.2 \,{\rm Hz}$, 2H, Ar^{Fmoc}), 7.43–7.20 (m, 14H, Ar^{DMT}, Ar^{Fmoc} and H-6), 6.88 (d, $J = 7.8 \,{\rm Hz}$, 4H, Ar^{DMT}), 5.91 (d, $J = 4.5 \,{\rm Hz}$, 1H, H-1'), 5.75 and 5.70 (s, 2H, NH and 3'-OH), 4.35 (d, $J = 6.6 \,{\rm Hz}$, 2H, CHCH^{Fmoc}), 4.23 (t, $J = 6.4 \,{\rm Hz}$, 1H, CHCH^{Fmoc}), 4.15 (m, 2H, H-2' and H-2''), 4.00 (m, 1H, H-4'), 3.74 (s, 6H, $2 \times {\rm OCH}_3$), 3.32 (m, 6H, H^a-3", H^b-3", H^a-5', H^b-5 and CH^{pip}), 2.46 (m, 2H, CH^{pip}), 2.24–2.07 (m, 5H, H^a-1" and $2 \times {\rm CH}^{pip}$), 1.81 (s, 3H, CH₃), 1.61 (m, 1H. H^b-1"); ¹³C NMR $\delta_{\rm C}$ (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).

(1S,3S,5R,6R,8R)-6-((4,4'-Dimethoxytrityl)oxymethyl)-5-hydroxy-3-(N-[9-fluorenylmethoxycarbonyl]piperazino)methyl-8-(thymin-1-yl)-2,7dioxabicyclo[3.3.0]octane (18S). The procedure for preparation of compound 18R was used. Compound 16S (282 mg, 0.47 mmol) was reacted with DMTCl (473 mg, 1.40 mmol) in a mixture of anhydrous DCM (10 mL) and CH₃CN (10 mL) in the presence of DIPEA (325 µL, 0.742 mmol). Nucleoside 18S (373 mg, 88%) was obtained as a white foam. ¹H NMR $\delta_{\rm H}$ (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, CHCH₂^{Fmoc}), 4.25 (t, J = 6.1 Hz, 1H, CHCH₂^{Fmoc}), 4.18 (d, 1H, H-2'), 4.06 (m, 1H, H-4'), 3.92 (m, 1H, H-2"), 3.74 (s, 6H, $2 \times OCH_3$), 3.40-3.32 (m, 6H, H^a-3", H^b-3", H^a-5', H^b-5 and CH₂^{pip}), 2.48 (m, 2H, CH₂^{pip}), 2.27-2.09 (m, 4H, $2 \times CH_2^{pip}$), 1.96 (dd, J = 5.4 and 12.8, 1H, H^a-1"), 1.80 (s, 3H, CH₃), 1.60 (dd, 1H, J = 9.4 and 12.4, 1H H^b-1"); ¹³C NMR $\delta_{\rm C}$ (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 CH₃CN (3 mL) in the presence of DIPEA (160 μ L, 0.92 mmol). Nucleoside 19*R* (135 mg, 77%) was obtained as a white foam. ¹H NMR $\delta_{\rm H}$ (CDCl₃) 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', $2 \times \text{OCH}_3$, H^a-3", H^b-3" and $2 \times \text{CH}_2^{\text{pip}}$), 2.60–2.10 (m, 5H, H^a-1" and $2 \times \text{CH}_2^{\text{pip}}$), 2.05–1.95 (m, 7H, $3 \times \text{CH}_2^{\text{butanoyl}}$), 1.90 (d, J = 0.9 Hz, 3H, CH₃), 1.61 (m, 1H, H^b-1"); ¹³C NMR δ_{C} (CDCl₃) 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).

(1S,3S,5R,6R,8R)-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 (19S). The procedure for preparation of compound 18R was used. Compound 17S (100 mg, 0.15 mmol) was reacted with DMTCl (155 mg, 0.46 mmol) in a mixture of anhydrous DCM (2 mL) and CH₃CN (2 mL) in the presence of DIPEA (133 µL, 0.74 mmol). Nucleoside 19S (110 mg, 75%) was obtained as a white foam. ¹H NMR $\delta_{\rm H}$ (CDCl₃) 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 = 3.8and 6.5 Hz, 1H, H-4'), 3.79 (m, 7H, H^a-5', H^b-5' and $2 \times \text{OCH}_3$), 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, CH₃), 1.60 (m, 1H, $H^{b}-1''$); ¹³C NMR δ C (CDCl₃) 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).

(1S,3R,5R,6R,8R)-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 (20R). 2-Cyanoethyl N,N-diisopropylphosphoramidochloridite (188 μ L, 0.86 mmol) was added dropwise to a stirred solution of nucleoside 18R (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 20R (363 mg, 60%) as a white foam. ³¹P NMR δ_P (DMSO- d_6) 142.7 and 142.6; HRMS [M + Na]⁺ m/z 1129.4815 (calcd. 1129.4811).

(1S,3S,5R,6R,8R)-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 (20S). The procedure for preparation of amidite 20R was used. Compound 18S (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 μ L, 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 20S (217 mg, 68%) as a white foam. ³¹P NMR δ_P (DMSO- d_6) 143.3 and 143.1; HRMS [M + Na]⁺ m/z 1129.4837 (calcd. 1129.4811).

(1S,3R,5R,6R,8R)-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 (21R). The procedure for preparation of amidite 20R was used. Compound 19R (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 μ L, 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 21R (82 mg, 61%) as a white foam. ³¹P NMR $\delta_{\rm P}$ (DMSO- d_6) 142.7 and 142.6; HRMS [M + Na]⁺ m/z 1177.5159 (calcd. 1177.5175).

(1S,3S,5R,6R,8R)-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 (21S). The procedure for preparation of amidite 20R was used. Compound 19S (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 μ L, 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 21S (30 mg, 24%) as a white foam. ³¹P NMR δ_P (DMSO-d₆) 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 NH₃ 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 NAPTM 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 NAPTM 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 $-800\,\mathrm{V}$ 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. $[C_{96}H_{120}N_{36}O_{54}P_8]^{4-}), \quad \textbf{ON9} \quad 1056.68 \quad (calcd.$ 722.14 for 1056.59 for $[C_{110}H_{145}N_{40}O_{56}P_8]^{3-}), \text{ ON10 } 963.23 \text{ (calcd. } 963.19 \text{ for } [C_{96}H_{121}N_{36}O_{54}P_8]^{3-}),$ $\textbf{ON11} \hspace{0.1 in} 1056.62 \hspace{0.1 in} (calcd. \hspace{0.1 in} 1056.59 \hspace{0.1 in} for \hspace{0.1 in} \left[C_{110}H_{145}N_{40}O_{56}P_{8}\right]^{3-}), \hspace{0.1 in} \textbf{ON12} \hspace{0.1 in} 394.85$ $H_{129}N_{36}O_{55}P_8Na]^{8-}), \ \textbf{ON14} \ 874.51 \ (calcd. \ 874.47 \ for \ [C_{141}H_{159}N_{37}O_{55}P_8]^{4-})$ 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 μ L of **buffer A** (200 mM sodium chloride, 20 mM sodium phosphate, and 0.2 mM EDTA). Distilled H₂O was added until a total volume of $1000 \,\mu$ L 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 Na₂HPO₄, 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 (A₂₆₀ 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 $0.15 \,\mu\text{M}$ of strands in $T_{\rm m}$ 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 $\phi_{\rm S}$ was determined using 1-pyrenebutyric acid as a reference. The quantum yield is calculated according to the equation:

$$\phi_{\rm S} = \phi_{\rm P} \times [{\rm Area}({\rm S})/{\rm A}_{340}({\rm S})]/[{\rm Area}({\rm P})/{\rm A}_{340}({\rm P})] \times [n^2({\rm S})/n^2({\rm P})]$$

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)/A₃₄₀(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 A₃₄₀ is the absorbance at 340 nm. The values of refractive index n(S) and n(P) equal 1.3328 (H₂O) and 1.3288 (MeOH), respectively. To confirm the applicability of the method, the linear correlation of absorbance and concentration was verified for monomer **17R** (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.

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