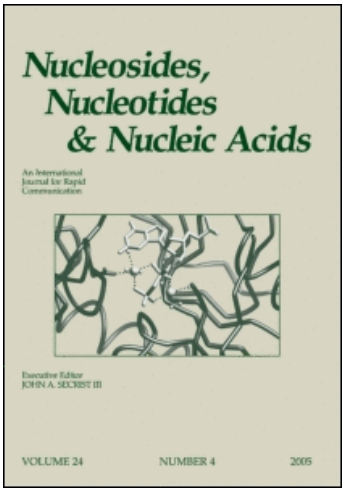

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Evaluation of Oligonucleotides Containing Two Novel 2'-*O*-Methyl Modified Nucleotide Monomers: A 3'-*C*-Allyl and a 2'-*O*-3'-*C*-Linked Bicyclic Derivative

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EVALUATION OF OLIGONUCLEOTIDES CONTAINING TWO NOVEL 2'-*O*-METHYL MODIFIED NUCLEOTIDE MONOMERS: A 3'-*C*-ALLYL AND A 2'-*O*,3'-*C*-LINKED BICYCLIC DERIVATIVE

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ABSTRACT. The two *ribo*-configured nucleosides 1-(3-*C*-allyl-2-*O*-methyl-β-D-*ribo*-pentofuranosyl)thymine **3** and (*1S,5R,6R,8R*)-5-hydroxy-6-(hydroxymethyl)-1-methoxy-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane **6** have been transformed into their corresponding phosphoramidites, **5** and **8** respectively, and used as building blocks for the synthesis of modified oligonucleotides. The oligonucleotides were shown to hybridize with decreased binding affinity towards complementary single stranded DNA and RNA.

INTRODUCTION

In the last decade much research has been focused on inhibiting protein expression through the sequence specific recognition of single stranded RNA by antisense oligonucleotides[2] and a number of promising, very efficient RNA-binding oligonucleotide mimics have been reported, *e.g.* PNA,[3] N3'-P5'-phosphoramidates,[4] and LNA[5]. Several oligodeoxynucleotides (ODNs) containing 3'-*C*-modified nucleotide monomers,[5e] *e.g.*, 3'-*C*-(hydroxymethyl)thymidine,[6] 3'-*C*-(aminomethyl)thymidine,[7] 3'-*C*-allylthymidine (**A**)[8] and

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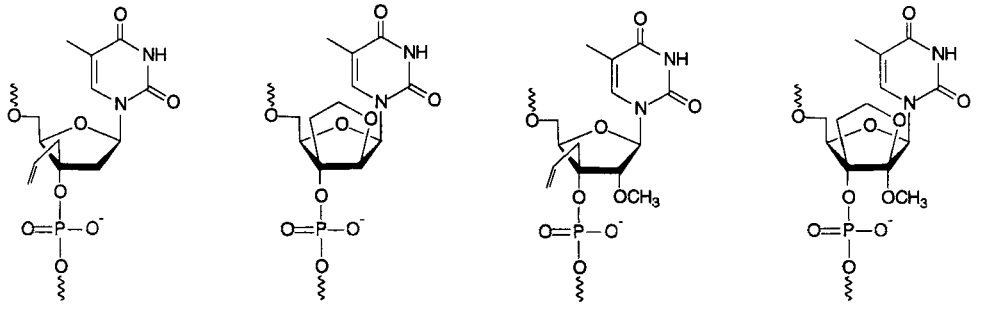
3'-*C*-(3-hydroxypropyl)thymidine,[9] have been synthesized and were shown to induce only minor decreases in the thermal affinity towards complementary single stranded DNA and to display improved 3'-exonucleolytic stability. The less convincing binding properties towards RNA obtained for oligonucleotides containing 3'-*C*-alkyl modified nucleotide monomers with more than one carbon atom in the branch[8,9] can be explained by a preference of the 3'-*C*-branched furanose ring to adopt an S-type conformation, with the 3'-*C* substituent in the equatorial position, in contrast to the N-type sugar conformation generally dominating in RNA-containing duplexes.[2c] We have recently reported strong binding towards complementary RNA when 2'-*O*,3'-*C*-linked bicyclonucleosides were incorporated 4 or 13 times consecutively in 14-mer oligothymidylates giving oligonucleotides of the type **B** whereas decreased binding towards RNA was obtained with oligonucleotides containing the bicyclic monomer once or twice or alternating with unmodified monomers.[10] The bicyclic monomeric nucleoside was later shown to exist in an S-type conformation (C1'-exo).[11] Several oligonucleotides containing 2'-*O*-alkyl modified nucleotide monomers[12] have shown favorable hybridization properties towards RNA because of the induction of an N-type conformational preference of the furanose sugars due to the *gauche* effect. We therefore decided to evaluate oligonucleotides with the 3'-*C*-branched and 2'-*O*,3'-*C*-linked bicyclic nucleotide monomers further derivatized with a 2'-*O*-methyl substituent (monomers **X** and **Y**) hoping thus to increase the binding affinity towards RNA.



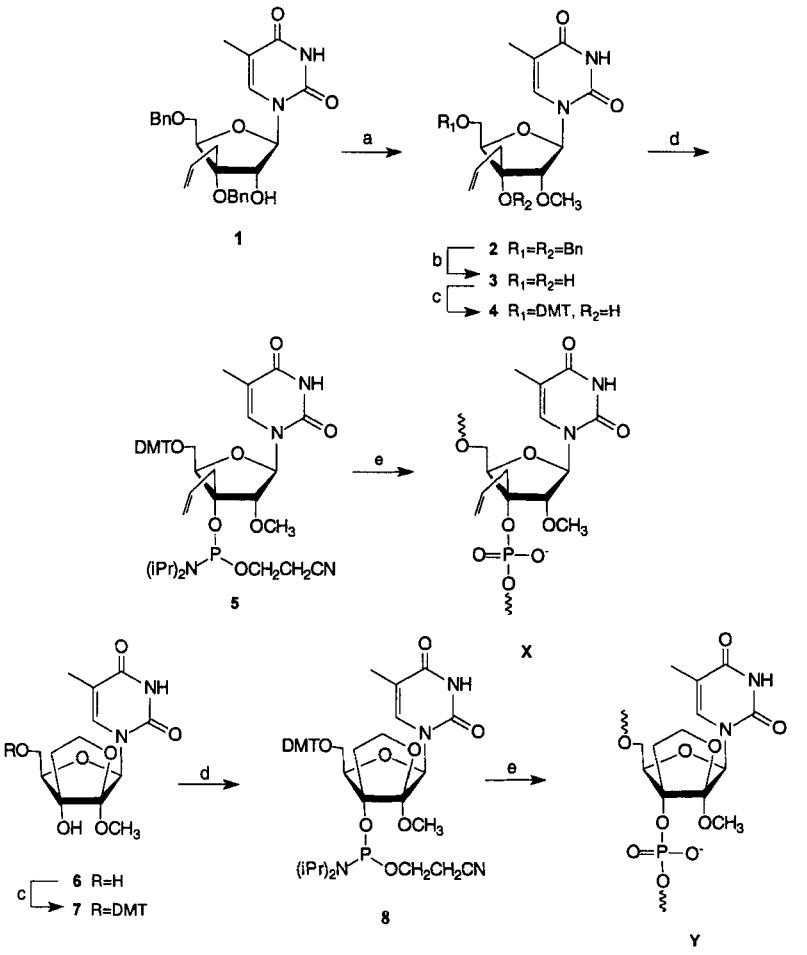
A[8] **B**[10] **X** **Y**

RESULTS AND DISCUSSION

Nucleoside chemistry. For the synthesis of oligonucleotides containing 1-(3-*C*-allyl-2-*O*-methyl-β-D-*ribo*-pentofuranosyl)thymine (monomer **X**), 1-(3-*C*-allyl-3,5-di-*O*-

benzyl-β-D-*ribo*-pentofuranosyl)thymine (**1**)[10] was methylated using 5 equivalents of CH_3I and 3 equivalents of NaH in anhydrous THF at 0 °C, as reported for the methylation of a 4'-*C*-hydroxymethyl modified thymidine derivative,[13] to give nucleoside derivative **2** in 84% yield. To avoid methylation in the base moiety it was necessary to keep the reaction temperature at 0 °C. The disappearance of the signal for the 2'-OH group and the presence of the signal for the NH (δ 11.4) in the ^1H NMR spectrum of compound **2** confirmed the assigned structure. Furthermore, the ^{13}C NMR of **2** showed a peak at ~59 ppm indicating an *O*-methyl group. To further confirm the structure of **2** a small amount of the *N*,*O*-dimethylated derivative was obtained (see experimental) showing no peak around δ 11.4 in ^1H NMR but an additional signal in ^{13}C NMR at ~27 ppm indicating an *N*-methyl group. The primary and tertiary benzyl protecting groups were removed using the Lewis acid BCl_3 and CH_2Cl_2 as solvent affording **3** in 80% yield. The debenzylated nucleoside **3** was then prepared for oligonucleotide synthesis by reaction with 4,4'-dimethoxytrityl chloride (DMTCl) in anhydrous pyridine affording the 5'-*O*-DMT protected nucleoside **4** in 75% yield. For the synthesis of the phosphoramidite building block **5**, nucleoside **4** was phosphitylated[14] in anhydrous CH_2Cl_2 by reaction with 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite in the presence of *N*,*N*-diisopropylethylamine affording amidite **5** in 34% yield after column chromatographic purification followed by precipitation in petroleum ether. In a similar manner, the bicyclic nucleoside **6**[15] was 5'-*O*-DMT protected in 93% yield to give compound **7** which was phosphitylated as described above affording the bicyclic phosphoramidite derivative **8** in 60% yield (Scheme).

Oligonucleotide synthesis and melting experiments. Oligonucleotides **C-L** (14-mer oligothymidylates; Table) were synthesized by the solid phase phosphoramidite method[16] on an automated DNA synthesizer using phosphoramidites **5** and **8** and commercial nucleoside phosphoramidites. The coupling efficiencies (Experimental) were 30-50% for amidite **5** and ~92% for amidite **8** using tetrazole as activator and iodine as oxidizing agent compared with approximately 99% for unmodified commercial amidites as evaluated by the release of the dimethoxytrityl cation after each coupling step.[17] The 5'-*O*-DMT-protected modified ONs were released from the solid support with simultaneous removal of the 2-cyanoethyl and nucleobase protecting groups by treatment with 32%



SCHEME. (a) CH_3I, NaH, THF; (b) BCl_3 in hexane, CH_2Cl_2; (c) DMTCl, pyridine; (d) 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite, *N,N*-diisopropylethylamine, CH_2Cl_2; (e) DNA synthesizer.

aqueous ammonia for 3 days at room temperature or 55 °C for 16 h. After reversed-phase purification and detritylation (see Experimental), the composition of ONs **D** and **G-L** was verified by MALDI-MS analysis (Table). The purity of oligomers **D-F** was analyzed by capillary gel electrophoresis, and of oligomers **G-L** by HPLC, to be >90%.

The hybridization properties of the modified ONs **D-L** towards complementary single stranded DNA and RNA were measured as described earlier[5b] and the results are outlined in the Table. Incorporation of the 3'-*C*-allyl monomer **X** once (**D**) or twice (**E**) in

TABLE. Sequences synthesized, melting temperatures towards complementary single stranded DNA[a] and RNA[b], and MALDI MS data.

ODN	Sequences	T_m (°C)[a]	ΔT_m °C[a]	T_m (°C)[b]	ΔT_m °C[b]	Mass Calc.	Mass Found
C	5'-TTTTTTTTTTTTTT-3'	34		35			
D	5'-TTTTTTTXTTTTTT-3'	24	-10	25	-10	4267	4267
E	5'-TTTTTTXTXTTTTT-3'	18	-8	18	-8.5		
F	5'-TTTTTTTTTTTTXT-3'	30	-4	32	-3		
G	5'-TTTTTTTYTTTTTT-3'	23	-11	23	-12	4269	4269
H	5'-TTTTTTYYTTTTTT-3'	19	-7.5	23	-6	4341	4340
I	5'-TTTTTTYTYTTTTT-3'	9	-12.5	15	-10	4341	4350
J	5'-TTTTTYYYYTTTTT-3'	5	-7	14	-5	4485	4485
K	5'-TTTYTYTYTYTTTT-3'	<5		<5		4485	4485
L	5'-YYYYYYYYYYYYT-3'	<5		<5		5134	5134

T = thymidine monomer; T_m = melting temperature (measured in medium salt buffer: 10 mM Na_2HPO_4, pH 7.0, 140 mM NaCl, 1 mM EDTA); ΔT_m = change in the T_m per modification compared with the unmodified control.

the middle of the sequence induces major decreases in the thermal stability of duplexes formed with complementary DNA and RNA when compared with the unmodified control (**C**). When **X** was incorporated once in the 3'-end (**F**), the thermal stability of the duplexes was less significantly reduced. Even more detrimental was the incorporation of 2'-*O*-methyl bicyclo[3.3.0]nucleoside monomer **Y** (ONs **G**-**L**) inducing strongly decreased melting temperatures of duplexes towards both DNA and RNA. These results should be compared with the results obtained with oligonucleotides containing 3'-*C*-allylthymidine (**A**)[8] where only minor decreases (ΔT_m ~ -1 °C/modification) in the thermal stability of duplexes with complementary DNA were seen and with the results of oligonucleotides containing 2'-*O*,3'-*C*-linked bicyclonucleosides (**B**) for which oligomers of the type **J** and **L** displayed *improved* binding affinity towards complementary RNA[10] (ΔT_m ~ +1 °C/modification).

The results shown in the Table involve exclusively oligothymidylate sequences. However, the bicyclic monomer **Y** was in addition incorporated in the mixed sequence 5'-d(GYGAYAYGC) for which no T_m above 5 °C could be detected against neither complementary single stranded DNA nor RNA. Likewise, as comparable T_m data were obtained by us[18a] for oligothymidylate and mixed base sequences containing a 3'-*C*-aminopropyl branched monomer and comparable T_m data were obtained by Schmit *et al.*[18b] for mixed base sequences containing 1-(2-*O*-methyl-3-*C*-methyl-β-D-*ribo*-pentofuranosyl)thymine, there is no reason to believe that incorporation of monomer **X** in mixed base sequences should give T_m data strongly contrasting the results shown in the Table. We and others[2d] have used the strategy to initially investigate the thymine analogue of a modification, and only when stimulated by encouraging T_m data has the synthesis of the other base analogues been undertaken. Nothing suggests this rationale not to be viable also for the two modifications examined herein.

In general, a 2'-*O*-methyl modification has shown affinity enhancing properties probably by constraining the furanose ring into an N-type sugar conformation (C-3'-endo).[12] In an attempt to explain the detrimental effect of incorporation of monomers **X** and **Y** we performed molecular modeling (HyperChem™ program; MM+ Molecular Mechanics Force Field) on pentanucleotides (T_5) containing **X** or **Y** as monomer number three. These studies suggest that both monomers cause a displacement of the thymine base in the following nucleoside (3'-end direction) resulting in distortion of the base stacking, however clearly more pronounced so for the bicyclic monomer **Y**. An explanation of the disappointing results obtained for the oligomers **D-F** could be that the allyl group dictates a sugar conformation of the S-type (C-2'-endo) as indicated by the coupling constant $J_{1',2'} = 7.2$ Hz of compound **3**.[19] Consequently, for ONs containing **X**, the 2'-*O*-methyl group is oriented in an equatorial position, possibly interacting with the nucleobase of the following nucleotide. From the melting results obtained with the bicyclic monomer **Y** it appears that the introduction of a 2'-*O*-methyl substituent does not induce an N-type sugar conformation favorable for duplex formation. In fact, molecular modeling (HyperChem™ program; MM+ Molecular Mechanics Force Field) on the 2'-*O*-methyl bicyclic nucleoside **6** resulted in an energy minimized structure with a furanose conformation closely resembling the S-type conformation (C1'-exo)[11] found for the corre-

sponding parent bicyclic nucleoside leading to monomer **B**. As for monomer **X**, a sterical conflict between the 2'-*O*-methyl group and the 3'-end following monomer may lead to the very disappointing binding properties of ODNs **G-L** strongly contrasting the promising results earlier obtained for ONs containing the 2'-hydrogen counterpart **B**.[10]

In conclusion, the 3'-*C*-branched and bicyclic 2'-*O*-methylated nucleosides **3** and **6**, respectively, have been converted into their corresponding phosphoramidite derivatives and incorporated into oligonucleotides. Compared with unmodified control sequences and the corresponding 2'-deoxy derivatives, oligonucleotides containing these modified nucleotide monomers displayed significantly decreased binding affinity towards complementary single stranded DNA and RNA. These results underline that although a certain modification, *i.e.* a 2'-*O*-methyl group, in the unmodified series induces increased binding affinity, the effect of the introduction of the same group into an already modified analogue can be entirely opposite.

EXPERIMENTAL

Reactions were conducted under an atmosphere of nitrogen or argon when anhydrous solvents were used. Petroleum ether used was of distillation range 60-80 °C. Chemical shifts are in ppm relative to tetramethylsilane as an internal standard (^{1}H and ^{13}C NMR) and relative to 85% H_3PO_4 as an external standard (^{31}P NMR). Nucleoside numbering is used for all compounds. FAB mass spectra were recorded on a Kratos MS 50 RF spectrometer. MALDI MS was performed using a Micromass TofSpec E mass spectrometer. Capillary gel electrophoresis was performed on a Beckman P/ACE System 5000. Microanalyses were performed at The Department of Chemistry, University of Copenhagen. The silica gel used for column chromatography (0.040-0.063 mm) was purchased from Merck. Silica gel HPLC was done by the use of a preparative Waters Delta Prep 4000 system with PrepPAK-500/silica cartridges; flow rate 60 ml/min. Oligoribonucleotide rA_{14} was purchased from T-A-G-Copenhagen ApS, Copenhagen, Denmark.

1-(3-*C*-Allyl-3,5-di-*O*-benzyl-2-*O*-methyl-β-D-*ribo*-pentofuranosyl)thymine (2). 1-(3-*C*-Allyl-3,5-di-*O*-benzyl-β-D-*ribo*-pentofuranosyl)thymine (**1**)[10] (2.02 g, 4.22 mmol) was coevaporated in anhydrous CH_3CN (3 x 20 ml) and redissolved in anhydrous THF (34 ml). The solution was cooled to 0 °C and NaH (0.507 g of a 60% dispersion in oil,

12.7 mmol) was added. The mixture was stirred for 25 min and CH_3I (1.33 ml, 21.1 mmol) was added dropwise over 30 min. After 6 h stirring at 0 °C ice cold water (15 ml) was added followed by dilution with EtOAc (75 ml). The mixture was washed with a saturated aqueous solution of $NaHCO_3$ (3 x 45 ml) and the combined aqueous phase was extracted with CH_2Cl_2 (45 ml). The combined organic phase was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Purification by silica gel column chromatography (1-3% CH_3OH in CH_2Cl_2, v/v) afforded nucleoside **2** as a white solid material. Yield 1.75 g (84%). ^{1}H NMR (DMSO-d_6) δ 1.44 (3 H, s, CH_3), 2.74 (1 H, dd, J = 6.8, 15.9 Hz, H-1"a), 2.82 (1 H, dd, J = 7.1, 16.0 Hz, H-1"b), 3.24 (3 H, s, OCH_3), 3.62 (1 H, m, H-5'a), 3.71 (1 H, dd, J = 2.8, 11.4 Hz, H-5'b), 4.01 (1 H, d, J = 8.1 Hz, H-2'), 4.25 (1 H, br s, H-4'), 4.60 (2H, s, Bn), 4.62 (1 H, d, J = 9.2 Hz, Bn), 4.73 (1 H, d, J = 11.4 Hz, Bn), 5.07-5.18 (2 H, m, H-3"), 5.93-5.98 (1 H, m, H-2"), 6.08 (1 H, d, J = 7.9 Hz, H-1'), 7.24-7.40 (10 H, m, Bn), 7.70 (1 H, s, H-6), 11.4 (1 H, s, NH). ^{13}C NMR (DMSO-d_6) δ 11.68 (CH_3), 33.72 (C-1"), 58.91 (OCH_3), 64.59, 69.19, 75.52 (2 x Bn, C-5'), 82.09, 82.86, 84.43, 86.44 (C-1', C-2', C-3', C-4'), 110.29 (C-5), 117.73 (C-3"), 127.33, 127.37, 127.49, 127.55, 127.70, 127.93, 128.28, 128.38, 128.62 (Bn), 133.40, 135.67, 137.73, 139.01 (Bn, C-6, C-2"), 150.96 (C-2), 163.61 (C-4). FAB-MS m/z = 493 $[M+H]^+$. Anal. calc. for $C_{28}H_{32}N_2O_6$: C, 68.28; H, 6.55; N, 5.69. Found: C, 68.22; H, 6.40; N, 5.59.

A minor amount of the dimethylated compound 1-(3-*C*-Allyl-3,5-di-*O*-benzyl-2-*O*-methyl-β-D-*ribo*-pentofuranosyl)-3-*N*-methylthymine was obtained with the following data: ^{1}H NMR (DMSO-d_6) δ 1.50 (3 H, s, CH_3), 2.74 (1 H, dd, J = 6.9, 15.8 Hz, H-1"a), 2.84 (1 H, dd, J = 6.5, 15.8 Hz, H-1"b), 3.15, 3.24 (2 x 3 H, 2 x s, NCH_3, OCH_3), 3.62-3.66 (1 H, m, H-5'a), 3.71 (1 H, dd, J = 2.7, 11.5 Hz, H-5'b), 4.04 (1 H, d, J = 8.0 Hz, H-2'), 4.25 (1 H, br s, H-4'), 4.61 (2H, s, Bn), 4.62 (1 H, d, J = 11.1 Hz, Bn), 4.76 (1 H, d, J = 11.3 Hz, Bn), 5.08-5.18 (2 H, m, H-3"), 5.93-6.02 (1 H, m, H-2"), 6.08 (1 H, d, J = 7.8 Hz, H-1'), 7.23-7.40 (10 H, m, Bn), 7.77 (1 H, s, H-6). ^{13}C NMR (DMSO-d_6) δ 12.38 (CH_3), 27.60 (NCH_3), 33.71 (C-1"), 59.04 (OCH_3), 64.68, 69.15, 72.53 (2 x Bn, C-5'), 82.30, 82.87, 85.52, 86.58 (C-1', C-2', C-3', C-4'), 109.29 (C-5), 117.78 (C-3"), 127.35, 127.72, 127.94, 128.29, 128.62 (Bn), 133.37, 134.18, 137.72, 139.04 (Bn, C-6, C-2"), 151.18 (C-2), 162.72 (C-4). FAB-MS m/z = 507 $[M+H]^+$.

1-(3-*C*-Allyl-2-*O*-methyl-β-D-*ribo*-pentofuranosyl)thymine (3). Nucleoside **2** (434 mg, 0.88 mmol) was coevaporated in anhydrous CH_3CN (3 x 5 ml) and redissolved in anhydrous CH_2Cl_2 (15 ml). The solution was cooled to –78 °C and BCl_3 (3.5 ml of a 1.0 M solution in hexane, 3.5 mmol) was added dropwise over 30 min. After stirring for 4 h, additional BCl_3 (0.9 ml) was added and the solution was allowed to reach -20 °C and was stirred for further 2 h. CH_3OH (10 ml) was added and the mixture was stirred for 12 h at room temperature followed by coevaporation with CH_3OH (3 x 5 ml). The residue was purified by silica gel column chromatography (0-4% CH_3OH in CH_2Cl_2, v/v) which afforded nucleoside **3** as a white solid material. Yield 218 mg (80%). ^1H NMR (CD_3OD) δ 1.89 (3 H, d, J = 1.1 Hz, CH_3), 2.58 (2 H, d, J = 6.9, H-1"), 3.38 (3 H, s, OCH_3), 3.75 (1 H, dd, J = 2.8, 12.1 Hz, H-5'a), 3.83 (1 H, dd, J = 2.7, 12.1 Hz, H-5'b), 3.89-3.92 (2 H, m, H-2', H-4'), 5.11-5.19 (2 H, m, 2 x H-3"), 5.96-6.03 (1 H, m, H-2"), 6.07 (1 H, d, J = 7.2 Hz, H-1'), 8.13 (1 H, d, J = 1.2 Hz, H-6). ^{13}C NMR (CD_3OD) δ 12.42 (CH_3), 39.59 (C-1"), 59.52, 61.77 (OCH_3, C-5'), 80.05, 87.22, 87.98, 88.19 (C-1', C-2', C-3', C-4'), 112.14 (C-5), 118.59 (C-3"), 134.70, 138.99 (C-6, C-2"), 152.90 (C-2), 166.45 (C-4). HRMS-FAB: Found 313.1435. Calc. for $C_{14}H_{21}N_2O_6$: 313.1400.

1-[3-*C*-Allyl-5-*O*-(4,4'-dimethoxytrityl)-2-*O*-methyl-β-D-*ribo*-pentofuranosyl]-thymine (4). Nucleoside **3** (188 mg, 0.60 mmol) was coevaporated in anhydrous pyridine (3 x 3 ml) and redissolved in anhydrous pyridine (1.5 ml). DMTCl (0.407 g, 1.2 mmol) was added and the mixture was stirred under argon at room temperature for 3 h. The mixture was evaporated to dryness under reduced pressure and the crude product was redissolved in CH_2Cl_2 (10 ml) and washed with brine (3 x 8 ml). The organic phase was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Purification by silica gel column chromatography (20-50% EtOAc in petroleum ether, 0.5% pyridine, v/v/v) followed by evaporation of the solvents and coevaporation from toluene (3 x 5 ml) afforded compound **4** as a white solid material. Yield 291 mg (75%). ^1H NMR ($CDCl_3$) δ 1.17 (3 H, s, CH_3), 2.30-2.33 (2 H, m, H-1"), 2.95 (1 H, br s, 3'-OH), 3.35 (1 H, dd, J = 2.7, 10.8 Hz, H-5'a), 3.52 (3 H, s, OCH_3), 3.67 (1 H, dd, J = 2.9, 10.7 Hz, H-5'b), 3.79 (6 H, s, 2 x OCH_3, (DMT)), 3.92 (1 H, d, J = 6.3 Hz, H-2'), 4.10 (1 H, d, J = 2.7 Hz, H-4'), 4.41 (1 H, d, J = 17.3 Hz, H-3"a), 4.89 (1 H, d, J = 10.3 Hz, H-3"b), 5.70-5.80 (1 H, m, H-2"), 6.18 (1 H, d, J = 6.3 Hz, H-1'), 6.84 (4 H, dd, J = 1.9, 9.0 Hz, DMT), 7.24-7.40 (9

H, m, DMT), 7.76 (1 H, s, H-6), 9.19 (1 H, s, NH). ^{13}C NMR ($CDCl_3$) δ 11.03 (CH_3), 38.26 (C-1"), 55.21 (2 x OCH_3, (DMT)), 59.09, 61.76 (OCH_3, C-5'), 78.17, 85.11, 85.38, 86.11, 87.47 (C-1', C-2', C-3', C-4', CAr_3), 111.67 (C-5), 113.23, 113.26 (DMT), 118.55 (C-3"), 127.53, 128.01, 128.76, 130.51, 130.54, 132.33, 134.68, 134.70, 135.99, 136.05, 143.53 (C-6, C-2", DMT), 150.69 (C-2), 159.04 (DMT), 166.45 (C-4). HRMS-FAB: Found 614.2689. Calc. for $C_{35}H_{38}N_2O_8$: 614.2628. Anal. calc. for $C_{35}H_{38}N_2O_8$,0.35 toluene: C, 69.53; H, 6.36; N, 4.33. Found, C: 69.84, H: 6.65, N: 4.15.

1-[3-*C*-Allyl-3-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-5-*O*-(4,4'-dimethoxytrityl)-2-*O*-methyl-β-D-*ribo*-pentofuranosyl]thymine (5). Nucleoside **4** (260 mg, 0.42 mmol) was coevaporated in a mixture of anhydrous CH_3CN (3 x 2 ml) and redissolved in a mixture of anhydrous CH_2Cl_2 (2 ml) and *N,N*-diisopropylethylamine (0.42 ml, 2.45 mmol). 2-Cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.2 ml, 0.84 mmol) was added during 10 min and the mixture was stirred for 4 h at room temperature. Additional 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.1 ml, 0.42 mmol) was added and the mixture was stirred for 12 h at room temperature. The reaction was quenched with CH_3OH (0.1 ml) and the mixture diluted with EtOAc (5 ml). The resulting mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 x 10 ml) and brine (2 x 10 ml), and the separated organic phase was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. After purification by silica gel column chromatography ((1) 0-2% triethylamine in CH_2Cl_2, v/v, (2) EtOAc:CH_2Cl_2:triethylamine:petroleum ether, 15:30:5:50, v/v/v/v), the crude product was redissolved in anhydrous toluene (1 ml) and precipitated by addition to petroleum ether (250 ml) at -30 °C affording amidite **5** as a white solid material after filtration. Yield 117 mg (34%). ^{31}P NMR ($CDCl_3$) δ 142.61, 142.90. FAB-MS *m/z* = 837 $[M+Na]^+$.

(*1S,5R,6R,8R*)-6-(4,4'-Dimethoxytrityloxymethyl)-5-hydroxy-1-methoxy-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (7). Same procedure as for compound **4**; used amounts: Nucleoside **6** (0.95 g, 3.03 mmol), DMTCl (1.54 g, 4.55 mmol), anhydrous pyridine (20 ml). Work-up as described for **4**. The residue was dissolved in CH_2Cl_2 containing 0.5% triethylamine and applied to a silica gel·HPLC column equilibrated with the same solvent. The column was washed with EtOAc:petroleum ether:triethylamine (15.0:84.5:0.5, v/v/v, 1000 ml), whereafter the product was eluted (0-2% CH_3OH in

CH_2Cl_2, 0.5% triethylamine, v/v/v) to give nucleoside **7** as a white solid material. Yield 1.71 (93%). ^1H NMR ($CDCl_3$) δ 1.93 (s, 3H, CH_3), 1.87-2.11 (m, 2H, H-1"), 3.32 - 3.45 (m, 2H, H-2"), 3.49 (s, 3H, OCH_3), 3.77 (s, 6H, 2 x OCH_3, (DMT)), 3.98-4.12 (m, 3H, H-5', H-4'), 6.04 (s, 1H, H-1'), 6.79-6.85 (m, 4H, DMT), 7.17-7.51 (m, 10H, DMT, H-6). ^{13}C NMR ($CDCl_3$) δ 12.7 (CH_3), 35.5 (C-1"), 51.7 (OCH_3), 55.2 (2 x OCH_3, (DMT)), 62.4, 69.2 (C-5', C-2"), 81.4, 85.0, 85.3, 86.5 (C-1', C-3', C-4', (CAr_3), 109.7, 109.8 (C-4, C-2'), 113.2, 126.8, 127.9, 128.2, 130.1, 135.7 (DMT), 137.7 (C-5), 144.7 (DMT), 150.7 (C-2), 158.6 (DMT), 164.2 (C-6). FAB-MS *m/z* 617.3 $[M+H]^+$. Anal. Calc. for $C_{34}H_{36}N_2O_9$: C, 66.22; H, 5.88; N, 4.54. Found: C, 66.44; H, 6.11; N, 4.23.

(*1S,5R,6R,8R*)-5-(2-Cyanoethoxy(diisopropylamino)phosphinoxy)-6-(4,4'-dimethoxytrityloxymethyl)-1-methoxy-8-(thymin-1-yl)-2,7-dioxabicyclo[3.3.0]octane (8). Same procedure as for compound **5**; used amounts: Nucleoside **7** (1.20 g, 1.95 mmol), *N,N*-diisopropylethylamine (1.35 ml, 7.8 mmol), 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.92 g, 3.9 mmol), anhydrous CH_2Cl_2 (10 ml). After 72 h, the mixture was diluted with CH_2Cl_2 (100 ml) and washed with a saturated aqueous solution of $NaHCO_3$. The organic phase was evaporated under reduced pressure and applied to silica gel HPLC in a gradient concentration of eluent B in eluent A (eluent A: petroleum ether:dichloromethane:pyridine, 50:50:0.5, v/v/v; eluent B: petroleum ether:dichloromethane:ethyl acetate:pyridine, 45:45:10:0.5, v/v/v/v). The residue was dissolved in anhydrous benzene (20 ml) and precipitated by addition to petroleum ether (400 ml) affording after filtration amidite **8** as a white solid material. Yield: 0.96 g (60%). ^{31}P NMR ($CDCl_3$) δ 142.52, 142.64. FAB-MS *m/z* 817.26 $[M+H]^+$. Anal. Calc. for $C_{43}H_{53}N_4O_{10}P$: C, 63.22; H, 6.54; N, 6.86. Found: C, 62.80; H, 6.40; N, 6.94.

Oligonucleotide synthesis and thermal denaturation studies. DMT-ON oligonucleotides (**D-L**) were synthesized on a Pharmacia Gene Assembler® Special DNA-synthesizer (0.2 μmol scale, 5 mol amidite per cycle, Pharmacia Primer Supports) using **5**, **8** and commercial 2-cyanoethyl phosphoramidites. The standard protocol of the synthesizer was used except for repeated and extended couplings (addition of amidite and tetrazole; addition of amidite and tetrazole; 2 x 24 min for **5** (30-50% coupling yield); 2 x 12 min for **8** (92% coupling yield); capping). The unmodified ON **C** was synthesized in the DMT-OFF mode and purified by ethanol precipitation. ONs **D-F** were purified using

disposable Oligopurification Cartridges (COP, Cruachem) following manufacturers protocol, whereas oligonucleotides **G-L** were purified by reversed-phase HPLC (gradient concentration 0-30% (during 40 min) of acetonitrile in 0.05 M triethylammonium acetate buffer (pH 7.0); Delta Pak C-18 column, 300A (3.9 x 30 cm.); flow rate: 1.5 ml/min.) Fractions of DMT-oligonucleotides (retention times 30-35 min) were collected and evaporated to dryness under reduced pressure. The residues were redissolved in 80% acetic acid and incubated for 1 h at room temperature. After evaporation, the DMT-OFF oligonucleotides were purified by the same procedure as described before and shown to be >90% pure. MALDI-MS analysis of ODNs **D** and **G-L** was performed as described earlier.[20] The purity of ODNs **D-F** was verified as >90% by capillary gel electrophoresis. Thermal denaturation curves were measured at 260 nm with a UV spectrophotometer equipped with a Peltier temperature controller. The melting temperatures (T_m values) were determined from the maxima of first derivative of the denaturation curves.

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