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Oligodeoxynucleotide Analogues Containing 3'-Deoxy-3'-*C-threo*-hydroxymethylthymidine: Synthesis, Hybridization Properties and Enzymatic Stability

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Abstract: Novel Oligodeoxynucleotide analogues containing 3'-*C-threo*-methylene phosphodiester internucleoside linkages were synthesized on automated DNA-synthesizers using the phosphoramidite approach. The sugar modified phosphoramidite building block **5** was obtained by phosphitylation of 1-(2,3-dideoxy-5-*O*-(4,4'-dimethoxytrityl)-3-*C*-hydroxymethyl-β-D-*threo*-pentofuranosyl)thymine (**4**) which was synthesized in only three steps from 5'-*O*-(4,4'-dimethoxytrityl)thymidine (**1**). The hybridization properties and enzymatic stability of the oligonucleotide analogues were studied by UV experiments. 17-Mers having one or three modifications in the middle or two modifications in each end hybridized to DNA with moderate lowered affinity compared to unmodified 17-mers (ΔT_m 1-3°C per modification). Furthermore, the end-modified and all-modified oligonucleotides were stable towards snake venom phosphodiesterase.

INTRODUCTION

Since the introduction of the antisense approach as a new powerful therapeutic strategy much effort has been made towards the development of new stable oligonucleotides capable of hybridizing to complementary sequences.[1,2] To act as an effective inhibitor of gene expression (transcription, mRNA processing, RNA stability or translation) an oligonucleotide must be stable towards intra- and extracellular nucleases, hybridize with appropriate affinity and specificity to its target sequence, and penetrate cellular membranes.[1,3,4] Naturally occurring single stranded RNA and DNA do not fulfill these criteria, primarily because they suffer from rapid degradation by cellular nucleases, and therefore it is necessary to use chemically modified oligonucleotides. As modifications in the phosphate moiety (*e.g.* phosphorothioates,[3] phosphorodithioates,[5,6] methylphosphonates,[7] phosphate triesters[8]) have been intensively studied and often result in highly heterogeneous

oligomers because of the introduction of uncontrolled chirality at phosphorus, our research concentrates on synthesizing oligonucleotides modified in the sugar moiety or with neutral dephospho linkages.

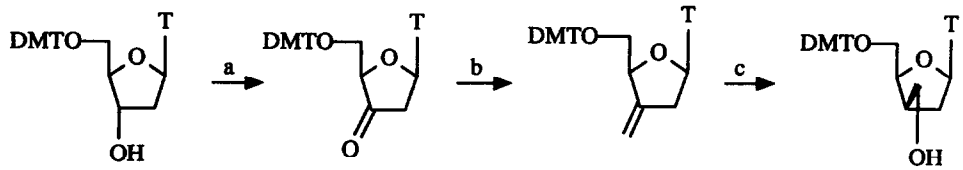
A number of oligonucleotides with modified five-membered sugar rings have been reported, *e.g.* α-DNA,[9] α-RNA,[10] 2'-*O*-methyl-RNA,[11] carbocyclic DNA,[12] 4'-thio-RNA,[13] L-DNA,[14,15] and xylo-DNA.[16] Incorporation of 1-(2-deoxy-β-D-*threo*-pentofuranosyl)thymine into mixed oligodeoxynucleotides (xylo-DNA) caused a significant increase in stability towards phosphodiesterases but concomitantly a large decrease in melting temperature (> 5°C/modification) compared to unmodified oligodeoxynucleotides.[16] Therefore, we decided to evaluate the novel and more flexible 3'-deoxy-3'-*C*-*threo*-methylene-DNA hoping thus to retain the enzymatic stability while improving base-pairing properties. This report describes the synthesis of the thymine phosphoramidite monomer **5** and its incorporation into mixed sequences of oligodeoxynucleotides. The modified oligomers were studied with respect to their hybridization properties and stability towards snake venom phosphodiesterase.

RESULTS AND DISCUSSION

The synthesis of 1-(2,3-dideoxy-5-*O*-(4,4'-dimethoxytrityl)-3-*C*-hydroxymethyl-β-D-*threo*-pentofuranosyl)thymine (**4**) was performed as follows: Thymidine was reacted with 4,4'-dimethoxytritylchloride in dry pyridine to give the 5'-*O*-protected nucleoside **1**.[17] Pyridinium dichromate (PDC) in dichloromethane in the presence of 3A molecular sieve powder was used for the oxidation of **1** to give 5'-*O*-(4,4'-dimethoxytrityl)-3'-ketothymidine (**2**) in 81% yield. This method was similar to a published procedure for the oxidation of 5'-*O*-tritylthymidine.[18] Wittig methylenation of **2** fails to give the desired 3'-methylene nucleoside **3** because of base induced β-elimination of the nucleobase.[19] However, **2** was methylenated using the electrophilic reagent $Zn/CH_2Br_2/TiCl_4$ in THF.[20,21] After column chromatographic purification the 2',3'-dideoxy-3'-*C*-methylene nucleoside **3** was obtained in 79% yield.

Borane:1,4-oxathiane was used for the hydroboration[22] of **3** and after oxidation with alkaline hydrogen peroxide and column chromatographic purification 1-(2,3-dideoxy-5-*O*-(4,4'-dimethoxytrityl)-3-*C*-hydroxymethyl-β-D-*threo*-pentofuranosyl)thymine (**4**) was isolated in 79% yield. The structural assignment of **4** was done by comparison of ^{1}H- and ^{13}C-NMR data of **4** with those of 1-(2,3-dideoxy-3-*C*-hydroxymethyl-5-*O*-trityl-β-D-*threo*-pentofuranosyl)thymine[23] where the configuration unambiguously was confirmed by a N.O.E. ^{1}H-NMR experiment, and with those of the corresponding 5'-*O*-monomethoxytrityl derivative which was recently synthesized in a mixture with its 3'-*C*-epimer from 3'-deoxy-3'-nitrothymidine.[24] The β-D-*threo* configuration of **4** is consistent with the syn-addition mechanism of hydroboration with attack from the less sterically hindered side, this being the α-face of the pentose ring. According to analytical TLC and ^{1}H-NMR of the crude product no other stereoisomer was formed so the hydroboration was regio- and stereospecific, and

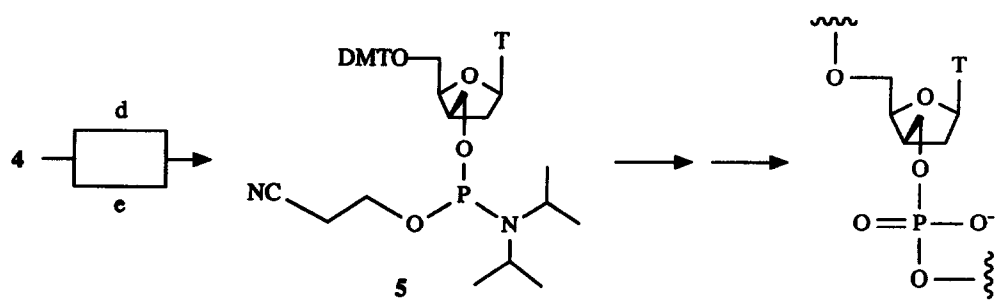
the procedure reported here constitutes an efficient and general route to 2',3'-dideoxy-3'-*C*-hydroxymethyl-β-D-*threo*-pentofuranosyl nucleosides in only 3 steps starting from 5'-*O*-protected 3'-deoxynucleosides.



a. PDC, 3A mol. sieve powder, CH_2Cl_2; b. Zn, CH_2Br_2, $TiCl_4$, THF, CH_2Cl_2; c. 1) BH_3: oxathiane, THF, 2) H_2O_2, NaOH.

Scheme 1

The phosphoramidite **5** to be used as monomeric building block for the preparation of the modified oligodeoxynucleotides was synthesized using two different methods. Reaction of *threo*-3'-*C*-hydroxymethyl nucleoside **4** with tetrazole-activated 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite ($NCCH_2CH_2OP(NPr^i_2)_2$) as described by Nielsen *et al.*[25] afforded the phosphoramidite **5** in 87% yield. Alternatively, **4** was reacted with 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite ($NCCH_2CH_2OP(Cl)NPr^i_2$) in the presence of *N,N*-diisopropylethylamine[26,27] thus giving **5** in 81% yield after precipitation with hexanes.



d. Tetrazole, $NCCH_2CH_2OP(NPr^i_2)_2$, CH_3CN; e. $EtN(Pr^i)_2$, $NCCH_2CH_2OP(Cl)NPr^i_2$, CH_2Cl_2.

Scheme 2

Oligodeoxynucleotides **A** to **G** (Table 1) were synthesized by standard phosphoramidite methodology on automated solid phase DNA synthesizers using the appropriate building blocks (**5** and commercial 2'-deoxynucleoside β-cyanoethylphosphoramidites). The coupling efficiency of the modified phosphoramidite **5**

was routinely >98% as monitored by the release of the dimethoxytrityl cation after each coupling coupling efficiency is similar to that usually observed in the condensation of unmodified 2'-deoxy β-cyanoethylphosphoramidites. The oligonucleotides were removed from the support and debl concentrated ammonia at 55°C. Purification of the oligos was performed either by re-precipitation or by filtration through a NAP-10^{R}-column.

Table 1. Oligodeoxynucleotides Synthesized and Melting Experiments.

Sequence	T_m (°C)	ΔT_m (°C)	Thermal hypochromicity (
5'-CACCAACTTCTTCCACA-3' (**A**)	62.0	-	23
5'-CACCAACT*TCTTCCACA-3' (**B**)	58.5	3.5	23
5'-CACCAAT*T*CT*TCCACA-3' (**C**)	53.0	3.0	23
[T_{13}] (**D**)	34.5	-	17
[(T^*_{12})T] (**E**)	<10	-	nd
5'-TTAACTTCTTCACATTC-3' (**F**)	52.0	-	17
5'-T*T*AACTTCTTCACAT*T*C-3' (**G**)	47.0	1.3	17

ΔT_m = Decrease in T_m per modification. nd = No cooperative melting observed above 10°C. A = 2'-deoxyadenosine, C cytidine, T = thymidine, T* = modified monomer **4**.

The composition of the oligodeoxynucleotides **A** and **C** was verified by matrix assisted laser mass spectrometry (Figure 1) which has become a powerful method for the mass a oligonucleotides.[28] Oligomer **C** with three modifications gave a relative molecular mass of 507 5077.3 Da, ~42 Da higher than the mass found for **A** as expected). Because of the homogeneous r the syntheses of all the modified oligodeoxynucleotides **B**, **C**, **E** and **G**, we consider this as a ver the composition of **B**, **C**, **E** and **G**.

The hybridization properties of the modified oligomers **B**, **C**, **E** and **G** towards their com DNA-strands were checked by UV melting point (T_m) measurements as previously described.[29] are given in Table 1. It can be seen that introduction of one or three modified nucleosides in th a sequence (**B** and **C**) results in a lowering of T_m by app. 3°C per modification, while substitution i with two modified nucleosides leads to a depression of T_m of only 1.3°C per modification. No hyp was detectable above 10°C in the case of the completely modified 13-mer $T^*_{12}T$ (**E**) whereas the unmodified 13-mer T_{13} (**D**) was 35°C. The melting hypochromicity (Table 1) for the mixed oli unchanged for the modified and unmodified sequences (for **A**, **B** and **C** 23% hypochromicity an **G** 17% hypochromicity) which indicates that incorporation of one or more β-D-*threo*-hyd nucleosides in mixed DNA-sequences causes little or no distortion of the secondary struc corresponding DNA-DNA duplex compared to the parent duplex consisting of natural deoxynuc

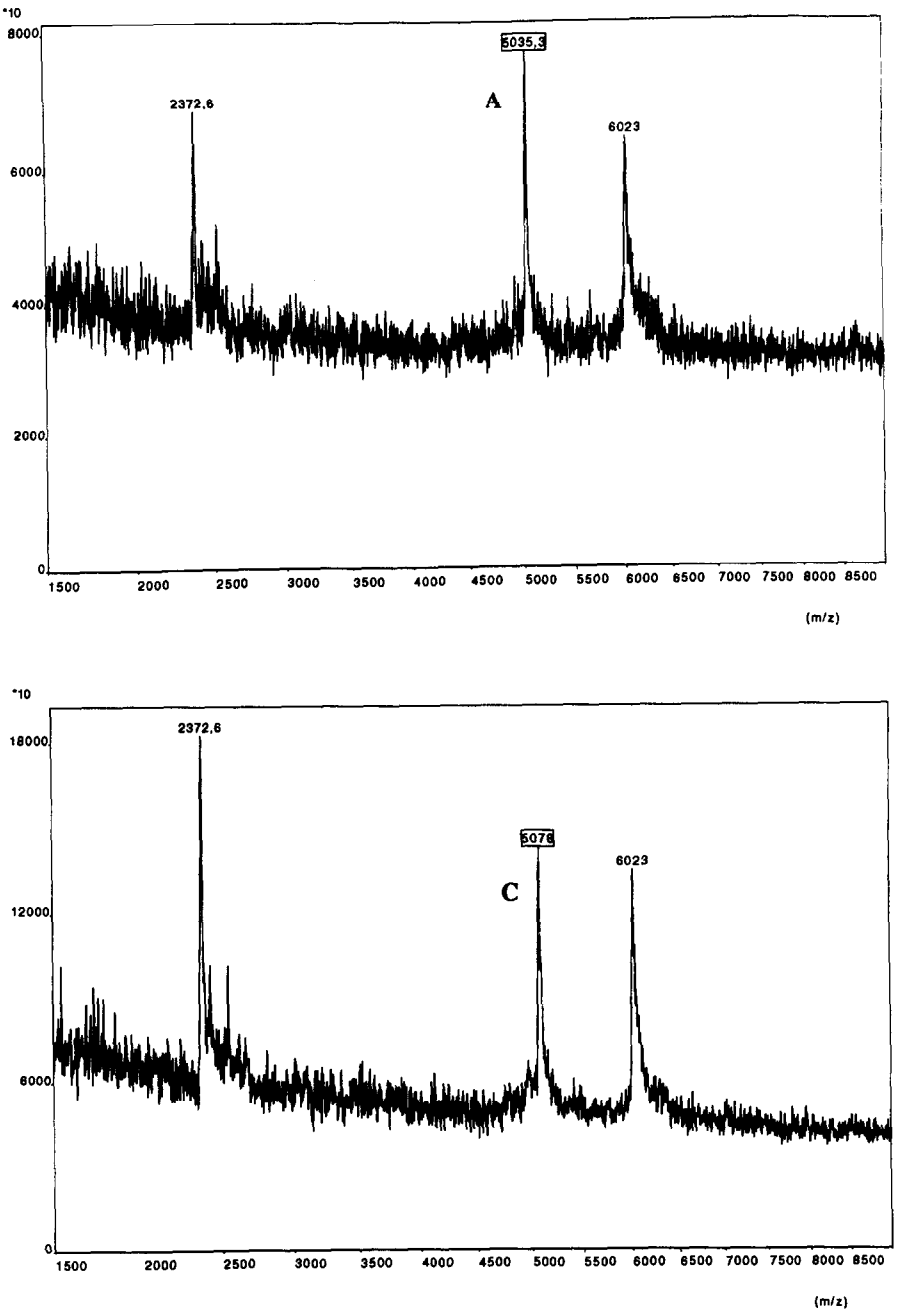


Figure 1. Matrix assisted laser desorption mass spectra of oligodeoxynucleotides A and C. The peaks at 2372.6 Da and 6023 Da are the internal calibrants T_8 and T_{20}.

The enzymatic stability of the modified oligonucleotides **B**, **C**, **E** and **G** was tested against snake v phosphodiesterase (SV PDE). Since it has been reported[1,30] that 3'-phosphodiesterases play a predominan in the *in vivo* degradation of natural oligonucleotides, we restricted our initial studies to the stability to the above mentioned 3'-exonuclease. The increase in absorbance (hyperchromicity) at 260 nm was foll during digestion with SV PDE.[16,31] The hyperchromicity arises as a consequence of the base stacki oligonucleotides and consequent π-π-orbital overlap[32] which is destroyed as the oligonucleotide is conv to its deoxynucleotide constituents during the enzymatic digestion. Figure 2 shows the time depe hydrolysis of **B** and **C** compared to the unmodified oligomer **A** while Figure 3 shows the hydrolyses of **E** and **G** compared to the unmodified oligomers **D** and **F**. The results are tabulated in Table 2.

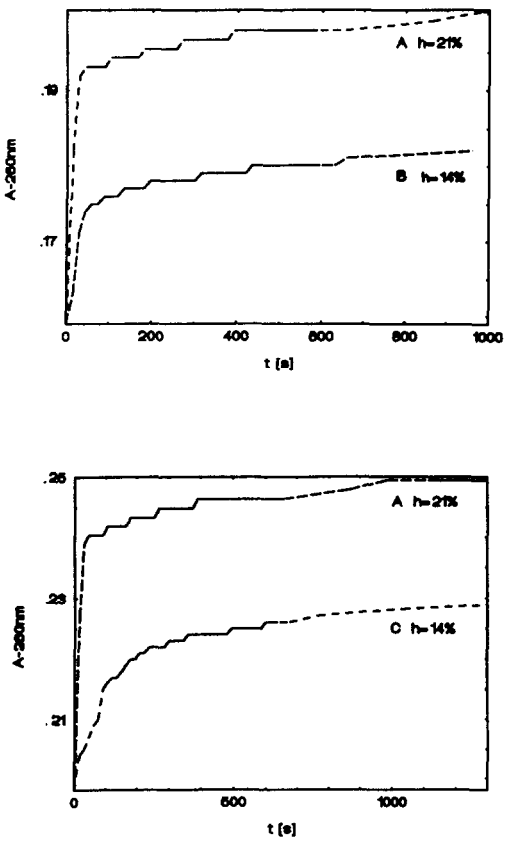


Figure 2. Time course of snake venom phosphodiesterase digestion of modified oligodeoxynucleotides with modification(s) in the middle.

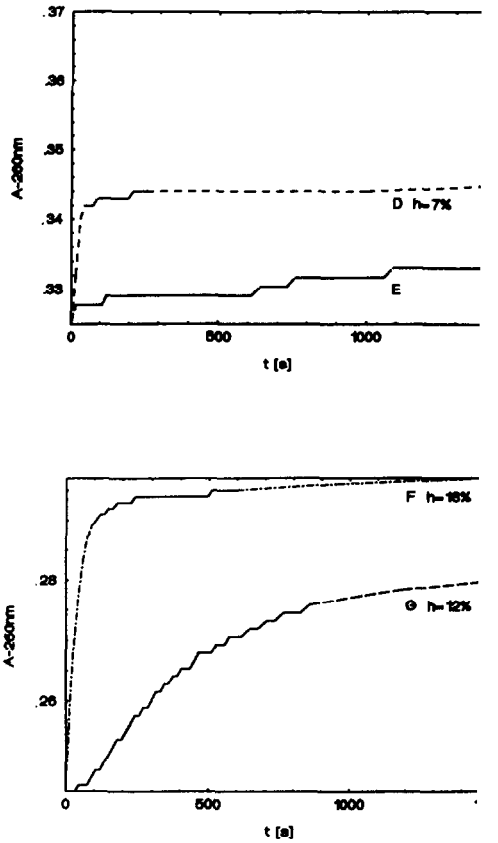


Figure 3. Time course of snake venom phc diesterase digestion of modified oligod nucleotides with modifications in the ends.

Table 2. Enzymatic Stability Experiments on the Oligodeoxynucleotides.

Sequence	$t_{1/2}$ (sec.)	Enzymatic hypochromicity (%)
A	24	21
B	36	14
C	120	14
D	10	7
E	*	-
F	30	18
G	250	12

*No hypochromicity detectable.

Incorporation of three modified nucleosides in the middle (oligonucleotide **C**) caused a 5-fold increase of the half-life ($t_{1/2}$) of the hypochromicity while one modification in the middle (**B**) had no significant effect. Protection with two modified nucleosides at each end (**G**) resulted in a large increase (app. 8-fold) of the half-life. Oligonucleotide $T^*_{12}T$ (**E**) seems to be resistant towards digestion with SV PDE. Thus, after one hour there was only a slight increase in the absorbance as compared to a hypochromicity of 7% during enzymatic digestion of unmodified T_{13} (**D**).

In conclusion, oligodeoxynucleotide analogues containing one or more 3'-*C-threo*-hydroxymethyl nucleosides **4** have been synthesized. These modifications only moderately weaken duplex stability and end-modifications significantly stabilize the oligomers towards the 3'-exonuclease snake venom phosphordiesterase. The results on the synthesis and properties of the novel 3'-deoxy-3'-*C-threo*-methylene-DNA described here open new possibilities for increasing the intracellular stability of antisense oligonucleotides.

EXPERIMENTAL

NMR spectra were recorded at 250 MHz for ^{1}H-NMR and 63 MHz for ^{13}C-NMR on a Bruker AC-250 spectrometer, and at 36.24 MHz on a Jeol FX 90Q spectrometer for ^{31}P-NMR; δ-values are in ppm relative to tetramethylsilane as internal standard (^{1}H-NMR and ^{13}C-NMR) and relative to 85% H_3PO_4 as external standard for ^{31}P-NMR; deuterium lock was established on DMSO-d_6 in an internal capillary tube in ^{31}P-NMR recordings. EI mass spectra were recorded on a Varian Mat 311A spectrometer. The silica gel (0.040-0.063 mm) used for column chromatography was purchased from Merck. Snake venom phosphodiesterase (*Crotalus adamanteus*) was obtained from Pharmacia.

5'-*O*-(4,4'-Dimethoxytrityl)-3'-ketothymidine (2)

5'-*O*-(Dimethoxytrityl)thymidine (**1**) (5.5 g; 10.1 mmol) was added in one portion to a slurry of 3A molecular sieve powder (4.3 g) and pyridinium dichromate (4.5 g; 12.0 mmol) in anhydrous CH_2Cl_2 (40 ml) (freed from acids by filtration through basic alumina). The sides of the flask were washed down with more CH_2Cl_2 (30 ml), and the mixture was stirred for 2 h at room temperature under N_2. The reaction mixture was filtered through 3A molecular sieve powder (deposited as a slurry with CH_2Cl_2) on a glass filter, the plug washed with more CH_2Cl_2 (75 ml) and the solvents evaporated. The resulting brown residue was suspended in EtOAc (1000 ml), sonicated for 5 min followed by filtration through 3A molecular sieve powder (deposited as a slurry with EtOAc) on a glass filter. The plug was washed with EtOAc (100 ml), and the solvent evaporated. The residue was triturated with dry Et_2O (150 ml), stirred overnight and filtered to give **2** as a white powder which was used without further purification in the next step. Yield: 3.45 g (81%). ^{13}C NMR ($CDCl_3$) δ: 11.34 (Me), 41.72 (C-2'), 55.09 (2×OCH_3), 62.84 (C-5'), 80.80 and 81.34 (C-1' and C-4'), 86.98 (CPh_3), 112.28 (C-5), 113.23, 127.07, 127.90, 129.87, 143.98 (DMT), 135.01 (C-6), 150.38 (C-2), 163.50 (C-4), 209.45 (C-3'). ^1H NMR ($CDCl_3$) δ: 1.34 (s, 3H, Me), 2.74 (dd, 1H, J = 19.3 Hz and 8.3 Hz, H-2'β), 3.08 (dd, 1H, J = 19.3 Hz and 6.6 Hz, H-2'α), 3.41-3.45 (m, 1H, H-5'a), 3.63 (dd, 1H, J = 10.4 Hz and 2.2 Hz, H-5'b), 3.78 (s, 6H, 2×OCH_3), 4.16 (br s, 1H, H-4'), 6.58 (t, 1H, J = 7.4 Hz, H-1'), 6.81-7.36 (m, 13H, DMT), 7.65 (s, 1H, H-6), 9.31 (s, 1H, NH). Rf = 0.33 (CH_3OH/CH_2Cl_2 0.5:9.5, v/v).

3'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)-3'-*C*-methylenethymidine (3)

To a stirred solution of **2** (2.0 g; 3.7 mmol) in anhydrous CH_2Cl_2 (40 ml) under N_2 at 0°C were added (at 10 min intervals; 3×10 ml) three portions of the slurry reagent prepared from Zn-dust (5.8 g; 89 mmol), CH_2Br_2 (2.02 ml; 29 mmol), and $TiCl_4$ (2.4 ml; 22 mmol) in anhydrous THF (50 ml)[20]. After 10 min the reaction mixture was allowed to warm to room temperature and after 2 h it was poured into ice-water saturated with $NaHCO_3$. This mixture was stirred with $CHCl_3$ (350 ml) and filtered through silica gel (deposited as a slurry with $CHCl_3$) on a glass filter. The separated water phase and the residue were washed with chloroform (3×50 ml), the organic phases combined, dried (Na_2SO_4) and evaporated. Purification by silica gel column chromatography (0-1% CH_3OH, 0.5% $(CH_3H_2)_3N$ in CH_2Cl_2) afforded **3** as a white solid. Yield 1.6 g (79%). ^{13}C NMR ($CDCl_3$) δ: 11.61 (Me), 38.89 (C-2'), 55.06 (2×OCH_3), 65.70 (C-5'), 80.86 (C-4'), 83.46 (C-1'), 86.44 (CPh_3), 107.96 (CH_2''), 111.16 (C-5), 113.07, 126.84, 129.95, 144.59, 158.52 (DMT), 135.31 (C-6), 144.37 (C-3'), 150.47 (C-2), 163.82 (C-4). ^1H NMR ($CDCl_3$) δ: 1.46 (s, 3H, Me), 2.81 (dd, 1H, J = 15.8 Hz and 6.2 Hz, H-2'β), 3.11 (dd, 1H, J = 16.0 Hz and 6.0 Hz, H-2'α), 3.38-3.40 (m, 2H, H-5'a), H-5'b), 3.78 (s, 6H, 2×OCH_3), 4.58 (br s, 1H, H-4'), 4.98 (br s, 1H, CH_2''a), 5.23 (br s, 1H, CH_2''b), 6.28 (t, 1H, J = 6.7 Hz, H-1'), 6.81-7.45 (m, 13H, DMT), 7.62 (s, 1H, H-6), 9.39 (s, 1H, NH). Rf = 0.5 (CH_3OH/CH_2Cl_2 0.5:9.5, v/v). MS m/z = 540 (M^+, 3.0%). Anal. calcd. for $C_{32}H_{32}O_6N_2 \cdot 0.75H_2O$: C, 69.36; H, 6.09; N, 5.06. Found: C, 69.24; H, 6.08; N, 4.97.

1-(2,3-Dideoxy 5-*O*-(4,4'-dimethoxytrityl)-3-*C*-hydroxymethyl-β-D-*threo*-pentofuranosyl)thymine (4)

A solution of **3** (1.0 g; 1.85 mmol) in anhydrous THF (4 ml) was added under N_2 to a stirred solution of BH_3:1,4-oxathiane (0.23 ml of a 7.8 M solution in oxathiane, 1.76 mmol) in anhydrous THF (4 ml) at room temperature. After 45 min a 2 M solution of NaOH (1.05 ml) was slowly added. After cooling of this mixture to 0°C 30% H_2O_2, aq. (0.25 ml) was added dropwise and stirring was continued for 60 min at room temperature. The reaction mixture was poured into ice-water (25 ml) and extracted with Et_2O (2×25 ml). The combined organic phase was washed with water (3×25 ml) and a saturated aqueous solution of $NaHCO_3$ (25 ml), dried (Na_2SO_4) and evaporated under reduced pressure. The crude product was purified on a silica gel column (0-2% CH_3OH, 1% C_5H_5N in CH_2Cl_2) to give **4** as a white solid. Yield 0.82 g (79%). ^{13}C NMR ($CDCl_3$) δ: 11.86 (Me), 33.93 (C-2'), 42.26 (C-3'), 55.10 (2×OCH_3), 61.28 (CH_2''), 62.67 (C-5'), 78.58 (C-4'), 84.08 (C-1'), 87.50 (CPh_3), 110.95 (C-5), 113.24, 126.11, 127.94, 129.89, 143.75, 158.68 (DMT), 134.84 (C-6), 150.53 (C-2), 163.83 (C-4). ^1H NMR ($CDCl_3$) δ: 1.57 (s, 3H, Me), 1.87-1.92 (m, 1H, H-2'β), 2.37-2.45 (m, 1H, 2'α), 2.65-2.70 (m, 1H, H-3'), 3.28 (dd, 1H, J = 10.4 Hz and 2.9 Hz, H-5'a), 3.45-3.76 (m, 3H, CH_2''a, CH_2''b, H-5'b), 3.78 (s, 6H, 2×OCH_3), 4.26-4.31 (m 1H, H-4'), 6.15 (dd, 1H, J = 8.2 Hz and 5.9 Hz, H-1'), 6.85-7.54 (m, 13H, DMT), 7.61 (s, 1H, H-6), 9.28 (s, 1H, NH). Rf = 0.32 (CH_3OH/CH_2Cl_2 0.5:9.5, v/v). MS m/z = 558 (m^+, 1.5%). HRMS: calc. 558.2366, found 558.2392.

1-[2,3-Dideoxy-5-*O*-(4,4'-dimethoxytrityl)-3-*C*-[*O*-(2-cyanoethyloxy(diisopropylamino)phosphino)hydroxymethyl]-β-D-*threo*-pentofuranosyl]thymine (5)

Method 1: **4** (168 mg; 0.30 mmol) was dried by coevaporation with dry CH_3CN (2 ml) and dissolved under N_2 in anhydrous CH_3CN (2 ml). 2-Cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite (162 mg, 80% pure due to thermal conversion into the phosphonic acid, $NCCH_2CH_2P(O)(NPr^i_2)_2$, detected by ^{31}P-NMR) was added, followed by tetrazole (22 mg; 0.32 mmol), and the reaction mixture stirred at room temperature for 60 min. On dilution with anhydrous CH_2Cl_2 (20 ml), the solution was washed with saturated $NaHCO_3$ (3×10 ml), dried (Na_2SO_4), filtered and evaporated to dryness. The resulting oil was redissolved in anhydrous CH_2Cl_2 (1.5 ml) and precipitated in anhydrous hexane (40 ml) at 0°C, using a dried, N_2-flushed Schlenk flask, giving 199 mg (87%) of **5**.

Method 2: **4** (142 mg; 0.25 mmol) was dried by coevaporation with dry CH_3CN (2 ml) and dissolved under N_2 in anhydrous CH_2Cl_2 (1.5 ml). *N,N*-diisopropylethylamine (0.23 ml) was added followed by dropwise addition of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.10 ml; 0.57 mmol). After 60 min when analytical TLC showed no more starting material, the reaction was quenched with CH_3OH (0.04 ml) and diluted with EtOAc (5.5 ml). This solution was washed with saturated aqueous solutions of $NaHCO_3$ (3×5 ml) and NaCl (3×5 ml), dried (Na_2SO_4) and evaporated. The residual gum was redissolved in anhydrous toluene (1.0 ml) and precipitated in ice-cold petroleum ether (200 ml). The product was collected by filtration and dried under vacuum to yield **5** as a white powder. Yield 157 mg (81%). ^1H NMR ($CDCl_3$) δ: 1.03-1.42 (m, $CH_3)_2C$), 1.45 (s, 3H, Me), 1.90-2.10 (m, H-2'β), 2.49-2.54 (m, CH_2CN, H-2'α), 2.75-2.92 (m, H-3'), 3.20-

3.78 (m, H-5'a, H-5'b, CH_2''a, CH_2''b, (CH)N, CH_2O, 2xOCH_3), 4.28-4.31 (m, 1H, H-4'), 6.20-6.23 (m, 1H, H-1'), 6.83-7.43 (m, 13H, DMT), 7.70 (s, 1H, H-6). ^{31}P-NMR ($CDCl_3$) δ: 147.00, 146.72. Rf = 0.54 (EtOAc/CH_2Cl_2/Et_3N 4.5:4.5:1, v/v/v).

Synthesis of T*-modified Oligodeoxynucleotides

The synthesis of oligodeoxynucleotides **B** and **C** was carried out on a Biosearch 8750 DNA synthesizer in a 1 μmol scale (20 μmol amidite per cycle, 500 Å CPG support) using commercial β-cyanoethylphosphoramidites as well as compound **5**. The synthesis followed the regular protocol of the DNA-synthesizer for β-cyanoethylphosphoramidites. The coupling efficiency of **5** was similar to those of the unmodified amidites (app. 99%). The oligodeoxynucleotides were removed from the solid support by treatment with concentrated ammonia at 55°C (this also removes the protecting groups) and reprecipitated in ethanol. The synthesis of the oligonucleotides **E** and **G** was carried out on a Pharmacia Gene Assembler^R Special DNA-synthesizer in a 0.2 μmol-scale (5 μmol amidite per cycle, Pharmacia primer support^{TM}) using commercial β-cyanoethylphosphoramidites as well as compound **5**. The synthesis followed the regular protocol of the DNA-synthesizer for β-cyanoethylphosphoramidites. Coupling efficiency, removal from the solid support and deprotection as described above. The ammonia solutions were desalted on a NAP-10^R-column (Pharmacia) eluting with water.

Matrix Assisted Laser Desorption Mass Spectrometry

The spectra were obtained on a prototype laser desorption mass spectrometer from Applied Biosystem Sweden AB, Uppsala, Sweden. The mass spectrometer is a linear time-of-flight instrument with a 0.7 m flight tube. The system is equipped with a nitrogen laser emitting 3 ns pulses at a wavelength of 337.1 nm. Positive ions are accelerated to an energy of 24 keV. The analogue signals are registered on a 300 MHz digitizer and further data processing is performed using producer supplied software. The spectra shown are raw data from the accumulation of 15 single shot spectra. The matrix used was a mixture of 50 g/l 3-hydroxypicolinic acid and 10 g/l nicotinamide in 20% acetonitrile. Prior to mass analysis, all oligonucleotides were purified on the Pharmacia SMART^{TM} FPLC system on a reversed phased column (C2/C18 SC2/10): Buffer A: 10 mM triethylammonium acetate (TEAA) in 5% acetonitrile. Buffer B: 10 mM TEAA in 30% acetonitrile. Gradient: 0-5 min: 100% A. 5-30 min: 0-100% B. 30-35 min: 100% B. 35-40 min: 100-0% B. 40-45 min: 100% A. Flow: 50 μl/min. The purified oligonucleotides were lyophilized and dissolved in ultra pure water to a concentration of 10-50 pmol/μl. To determine the mass of the oligonucleotides, 10 pmoles of the sample in question was mixed with equimolar amounts of two internal calibrants (an 8-mer and a 20-mer of oligodeoxythymidylic acid, molecular weight 2372.6 Da and 6023.0 Da, respectively, of the protonated forms). 1.5 μl of the matrix solution and the sample/calibrant mixture were mixed on the sample support and *in situ* ion-exchange, using NH_4^+-loaded polymer beads, was performed as described.[33]

Melting Experiments

The melting experiments were carried out as previously described in medium salt buffer, 2 mM EDTA, 20 mM Na_2HPO_4, 280 mM NaCl, pH 7.2.[29] The increase in absorbance at 260 nm as a function of time was recorded while the temperature was raised from 10 to 80°C in 0.5° steps. Melting hypochromicity values were calculated from the initial and final absorbance as described below.

Enzymatic Stability of the Oligodeoxynucleotides

A solution of the oligonucleotides (0.2-0.3 OD) in 2.0 ml of the following buffer (0.1 M Tris. HCl; pH 8.6; 0.1 M NaCl; 14 mM $MgCl_2$) was digested with 1.2 U snake venom phosphodiesterase (34 µl of a solution of the enzyme in the following buffer: 5 mM Tris. HCl; pH 7.5; 50% glycerol (v/v)) at 25°C. During digestion the increase in absorbance at 260 nm was followed. The absorption versus time curve of the digestion was plotted from which the hyperchromicity was evaluated. The final hyperchromicity is defined as the value of the final absorbance at 260 nm divided by the initial absorbance. The extinction coefficient of the oligonucleotide is the sum of the extinction coefficients of the constituent monomeric deoxynucleosides divided by the hyperchromicity. Then, the hypochromicity values can be calculated using the equation: $H = [(E_{monomer} - E_{oligo}) \cdot (E_{monomer})^{-1} \cdot 100\%$.[32] (Extinction coefficients for the constituent monomers: E_{260}: dA, 15400; dC, 7300; dG, 11700; T, T*, 8800.)

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