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Novel Linear and Branched Oligodeoxynucleotide Analogues Containing 4'-*C*-(Hydroxymethyl)thymidine

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Abstract: 4'-*C*-(Hydroxymethyl)thymidine has been incorporated into novel linear and branched oligodeoxynucleotide (ODN) analogues by use of the phosphoramidite synthons **4**, **7** and **11**. ODNs containing modification **X** (from amidite **4**, unnatural 5'-hydroxy to 4'-*C*-hydroxymethyl backbone) displayed significantly decreased thermal stabilities towards complementary DNA compared to unmodified controls. On the contrary, ODNs containing modification **Y** (from amidite **7**, natural 5'-hydroxyl to 3'-hydroxyl backbone) formed highly stable duplexes towards both complementary DNA and RNA. Phosphoramidite **11** containing two dimethoxytrityl protective groups was used for fully automated synthesis of a branched ODN analogue containing identical sequences in two out of the three branches originating from the branching point (modification **Z**). Branched ODN **S** exhibited only moderately decreased thermal stability (towards complementary linear DNA) compared to the linear reference (ODN **R**). This demonstrates the versatility of the additional 4'-*C*-hydroxymethyl functionality (in modification **Y**) as an attachment point for introduction of molecular entities pointing into the minor groove of a DNA:DNA duplex.

INTRODUCTION

Recently, we have communicated preliminary results showing that oligodeoxynucleotide (ODN) analogues containing 4'-*C*-(hydroxymethyl)thymidine (modification **Y**) are able to form stable duplexes towards complementary DNA.[1] This, together with the observed increased resistance towards 3'-exonucleolytic degradation,[1] has stimulated us to further evaluate 4'-*C*-(hydroxymethyl)thymidine as a monomeric substitute in antisense molecules. This modification contains an additional 4'-*C*-hydroxymethyl functionality allowing attachment of *e.g.* intercalators, lipophilic groups, positively charged alkyl amines or a third strand to an ODN, which may contribute to solve the problems of obtaining *e.g.* sufficient target strand affinity or membrane permeability often encountered for antisense molecules.[2,3] Figure 1 shows the structure of the 4'-*C*-hydroxymethyl modified monomer (**Y**) together with 3'-*C*-hydroxymethyl[4,5] (**A**) and 5'-*C*-hydroxymethyl[6] (**B**) modifications. Common to these three modifications is excellent hybridization properties and increased resistance towards exonucleolytic degradation, but molecular modelling reveals that whereas the *C*-

hydroxymethyl groups in **Y** is facing the minor groove in a DNA:DNA duplex, the *C*-hydroxymethyl groups in **A** and **B** are oriented into the major groove. Thus, we have now on hand *C*-hydroxymethyl substituted monomers suitable for covalent attachment of additional groups facing either the minor or the major groove. Besides, 1'-*C*-hydroxymethylthymidine has been applied by others in syntheses of 1'-tethered oligonucleotides.[7]

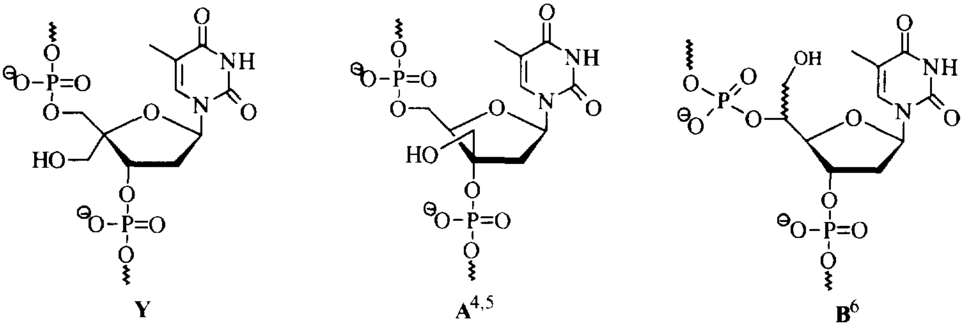


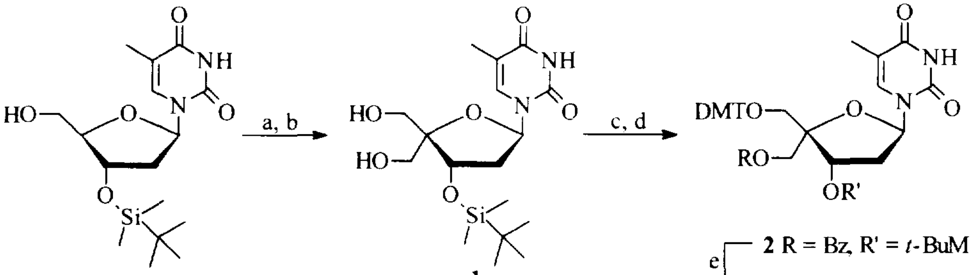
Figure 1. Structures of three *C*-hydroxymethyl substituted monomers incorporated into oligodeoxynucleotides showing excellent hybridization properties.

In this paper, we report synthesis and evaluation of ODNs containing 4'-*C*-(hydroxymethyl)thymidine a) with an unnatural 5'-hydroxyl to 4'-*C*-hydroxymethyl backbone (monomer **X**), b) with a natural 5'-hydroxyl to 3'-hydroxyl backbone (monomer **Y**) and c) as branching point (monomer **Z**) in a branched ODN. These modified ODNs have been synthesized on an automated DNA-synthesizer by use of the phosphoramidite building blocks **4**, **7** and **11** and commercial unmodified phosphoramidites.

RESULTS AND DISCUSSION

Synthesis of phosphoramidite building blocks 4, 7 and 11

Crossed aldol condensations of different protected carbohydrate and nucleoside 5'-aldehydes with formaldehyde followed by sodium borohydride reduction or *in situ* Cannizzaro reduction have been key steps applied in earlier syntheses of 4'-*C*-hydroxymethyl nucleosides[8-12] and in syntheses of ODNs containing 3'-*O*-ethyl-4'-*C*-(hydroxymethyl)thymidine,[13] a 4'-*C*-linked biotinylated thymidine monomer[14] or 4'-*C*-(*tert*-butylcarbonyl)thymidine.[15] Analogously, we obtained 4'-hydroxymethyl nucleoside **1** in 52% yield from 3'-*O*-(*tert*-butyldimethylsilyl)thymidine[10] by Swern oxidation[16] followed by crossed aldol condensation with formaldehyde and sodium borohydride reduction. In an earlier communication,[10] nucleoside **1** was obtained in 50% yield from the same starting material by Moffatt oxidation and subsequent aldol condensation and Cannizzaro reduction. However, we obtained much lower and more variable yields when using this route instead of the one described above involving Swern oxidation. As expected, no signal from a 4'-proton was present in the ^{1}H NMR spectrum of compound **1**. Selective acylation of the more reactive 4'-*C*-hydroxymethyl functionality was accomplished with benzoyl chloride in anhydrous pyridine to give derivative **2** in 52% yield after column chromatographic purification. A downfield shift for the two 4'-*C*-hydroxymethyl protons of approximately 0.7 ppm after benzoylation confirmed the regioselective acylation. Besides, the structure of **2** and the assignment of the signals in the ^{1}H NMR spectrum was verified by ^{1}H-^{1}H COSY and ^{1}H NOE NMR experiments. Thus, *e.g.* saturation of the signals from the 5'-protons (3.71-3.87 ppm) gave enhancement of the signal at 4.72-4.79 ppm (3'-proton) but not of the signal at 6.16-6.21 ppm (1'-proton). Protection of the free 5'-hydroxy group (4,4'-dimethoxytrityl chloride in anhydrous pyridine) followed by debenzoylation (NH$_3$, MeOH) afforded 5'-*O*-trityl nucleoside **3** in 42% yield. Subsequent reaction of **3** with 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite and *N,N*-diisopropylethylamine in anhydrous methylene chloride gave the phosphoramidite **4** in 79% yield after precipitation from petroleum ether (Scheme 1). This amidite was used for automated synthesis of ODNs containing monomer **X** linked through the 5'-hydroxyl and the 4'-*C*-hydroxymethyl functionalities.



1

2 R = Bz, R' = t-BuMe$_2$Si
e → 3 R = H, R' = t-BuMe$_2$Si
f → 4 R = P(OCH$_2$CH$_2$CN)N(iPr)$_2$
R' = t-BuMe$_2$Si

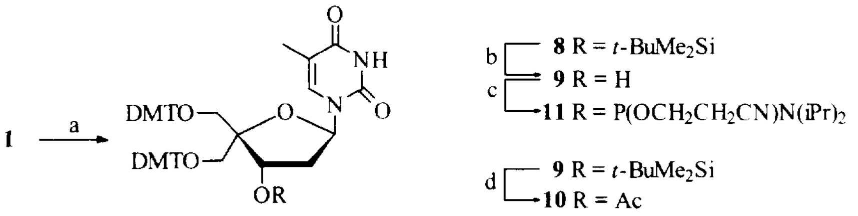
3 R = H, R' = t-BuMe$_2$Si
g → 5 R = R' = H
h → 6 R = t-BuMe$_2$Si, R' = H
f → 7 R = t-BuMe$_2$Si
R' = P(OCH$_2$CH$_2$CN)N(iPr)$_2$

a) Oxalyl chloride, DMSO, EtN(iPr)$_2$; b) NaOH, 37% aqueous formaldehyde, NaBH$_4$, *o*-dioxane; c) benzoyl chloride, pyridine; d) DMT-Cl, pyridine; e) NH$_3$, MeOH; f) NCCH$_2$CH$_2$OP(Cl)N(iPr)$_2$, EtN(iPr)$_2$, CH$_2$Cl$_2$; g) Bu$_4$NF, THF; h) t-BuMe$_2$SiCl, imidazole, DMF.
DMT = 4,4'-dimethoxytrityl.

Scheme 1

For incorporation of 4'-*C*-(hydroxymethyl)thymidine into ODNs with a natural 5'-hydroxyl to 3'-hydroxyl linked backbone (monomer **Y**), amidite **7** was synthesized as outlined in Scheme 1. Nucleoside diol **5** was obtained in 89% yield by desilylation of compound **3** with Bu$_4$NF in THF. Subsequent monosilylation of the more reactive 5'-hydroxyl afforded nucleoside **6** in 77% yield after purification on preparative TLC. The desired phosphoramidite **7** was prepared as described above for **4** and was isolated in 86% yield after precipitation from petroleum ether.

To evaluate the influence of an additional ODN-strand linked to the 4'-*C*-hydroxymethyl functionality on the DNA:DNA duplex stability, we prepared the branching amidite **11** (Scheme 2). Protection of the two primary hydroxyl groups in diol **1** by reaction with 2.5 equivalents of 4,4'-dimethyoxytrityl chloride in anhydrous pyridine afforded the fully protected nucleoside **8** in 69% yield after column chromatographic purification. To facilitate purification after desilylation, **8** was desilylated by use of KF, H_2O[17] to give derivative **9** in 89% yield. Acetylation of **9** (acetic anhydride, triethylamine and a catalytic amount of dimethylaminopyridine) yielded compound **10**. From the ^1H NMR spectrum of **10** it was clear that the 3'-hydroxy group was acetylated (the H-3' signal shifts from 4.58 ppm (in **9**) to 5.68 ppm (in **10**)) which verifies the structure of compounds **8**, **9** and **11**. Phosphitylation of the 3'-hydroxyl group of **9** was performed as described above to give the branching amidite **11** in 89% yield after precipitation from petroleum ether.



a) DMTCl, pyridine; b) KF, H_2O; c) $NCCH_2CH_2OP(Cl)N(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2; d) Ac_2O, pyridine. DMT = 4,4'-dimethoxytrityl.

Scheme 2

Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides **A-S** (Tables 1-3) were synthesized by the solid phase phosphoramidite method[18] on a automated DNA synthesizer using **4**, **7** and **11** and commercial 2'-deoxynucleoside-β-cyanoethylphosphor-amidites. The coupling efficiency (12 min couplings) of the modified phosphoramidites was approximately 70% for amidite **4**, approximately 90% for amidite **7** and approximately 70% for amidite **11**, compared to approximately 99% for standard phosphoramidites (2 min couplings). As the branching amidite **11** contains two DMT-groups, the sequences of two out of the three branches will be identical and synthesized simultaneously after detritylation. Accordingly, the "DMT-yields" observed spectroscopically after detritylation (standard detritylation conditions used) were twice the yield obtained for the branching amidite **11**. As branching is performed by elongation at two primary hydroxyl groups, the divergent approach described here should enable straight-forward synthesis of dendrimeric ODN analogues. This class of molecules have earlier been synthesized by use of a convergent strategy where coupling of two adjacent identical solid phase bound ODNs was performed with a 2',3'-di(phosphoramidite) derivative.[19] If the 4'-*C*-hydroxymethyl group in compound **1** *e.g.* is levulinated[20] and the 5'-hydroxyl group subsequently DMT-protected before phosphitylation, synthesis of analogous branched ODNs with three different branches around monomer **Z** should be possible. The dimethoxytrityl protected oligodeoxynucleotides were removed from the solid support by treatment with concentrated ammonia at room temperature for 3 days which also removed the phosphate and nucleobase protecting groups. Desilylation (Bu_4NF, ODNs containing **X** or **Y**), desalting (NAP columns for ODNs **A**-**S**), and purification on disposable reversed phase chromatography cartridges (COP columns, includes detritylation) was done as previously described.[4] The composition of the synthesized oligodeoxynucloetides was verified by matrix laser desorption mass spectrometry (MALDI-MS), which has proven useful as an analytical tool for mass analysis of oligodeoxynucloetides.[21,22] The calculated masses for ODNs **E** (5100.4 Da) and **K** (5095.4 Da) containing **X** correspond with the measured masses (5101.7 Da and 5095.6 Da, respectively). As earlier reported,[1] the composition of ODNs **K** and **M** were likewise confirmed. The MALDI-MS spectrum of the branched ODN **S** is depicted in Figure 2. The calculated mass for **S** (10772.0 Da) corresponds excellently with the measured (10771.9 Da).

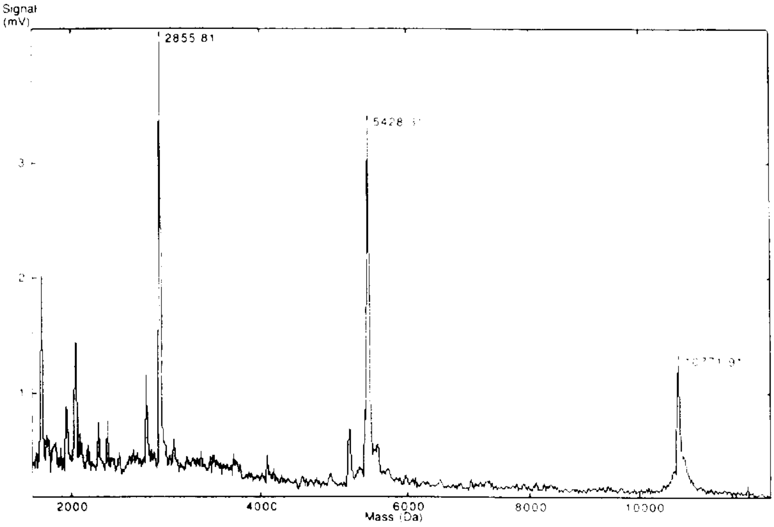


Figure 2. MALDI-MS spectrum of branched ODN **S**. The peaks at 2855.8 and 5428.3 are internal references.

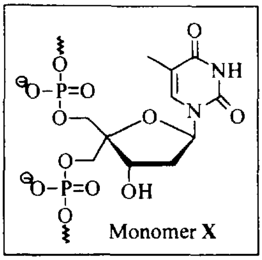
Hybridization properties

Incorporation of the building block **4** (Table 1, monomer **X**, 5'-hydroxyl to 4'-*C*-hydroxymethyl backbone) induced significant decreases (between 3 °C and 13 °C per modification) in the melting points towards complementary DNA when compared to unmodified controls **A**, **D** and **G**. More promising results were obtained for ODNs containing monomer **Y** (Table 2, 5'-hydroxyl to 3'-hydroxyl backbone). Thus, both towards complementary DNA (ODNs **J**, **K**, **M**, **N** and **P**)[1] and complementary RNA (ODNs **P** and **Q**) only very small decreases in thermal stabilities were observed. Especially, the satisfactory hybridization properties towards complementary RNA, together with the earlier reported conclusive stabilization towards 3'-exonucleolytic degradation induced by monomer **Y**,[1] indicate that ODNs containing this monomer deserves further evaluation for interesting biological activities (*e.g.* as antisense molecules). These and earlier results[5,13,23-27] on 2'-deoxy carbohydrate modified ODNs suggest that the probability of obtaining satisfactory hybridization properties is conclusively larger when the pentofuranose ring and the natural 5'-hydroxyl to 3'-hydroxyl backbone is preserved.

A decrease in T_m of 4 °C was observed when comparing the branched analogue **S** to the linear reference **R** (Table 3). This relatively small decrease shows that formation of a stable duplex is possible despite the attachment of an additional ODN-strand in the minor groove. This result, together with the recently reported excellent hybridization properties for 4'-*C*-linked biotinylated ODNs,[14] shows that 4'-*C*-functionalized ODNs are capable of binding effectively with complementary nucleic acids.

Table 1. Structure of monomer **X**, sequences synthesized and hybridization data towards complementary DNA

Sequences		T_m (°C)	ΔT_m(°C)
A	5'-CACCAACTTCTTCCACA-3'	60	-
B	5'-CACCAA**X**TTCTTCCACA-3'	51	-9
C	5'-CACCAAC**X**TCT**X**CCACA-3'	44	-8
D	5'-TTAACTTCTTCACATTC-3'	51	-
E	5'-TTAACTTCTTCACAT**X**C-3'	48	-3
F	5'-TTAACTTCTTCACA**XX**C-3'	39	-6
G	5'-TTTTTTTTTTTTTT-3'	36	-
H	5'-TTTTTT**X**TTTTTTT-3'	23	-13

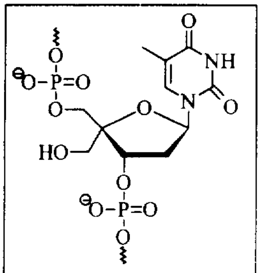

Monomer **X**

A = 2'-deoxyadenosine; C = 2'-deoxycytidine; T = thymidine; T_m = melting temperature; ΔT_m = change in T_m / modification.

Table 2. Structure of monomer **Y**, sequences synthesized and hybridization data towards complementary DNA[a] and RNA.[b]

	Sequences	T_m (°C)[a]	ΔT_m(°C)[a]	T_m (°C)[b]	ΔT_m(°C)[b]
I[1]	5'-CACCAACTTCTTCCACA-3'	60	-		
J[1]	5'-CACCAA**Y**TTCTTCCACA-3'	59	-1		
K[1]	5'-CACCAAC**Y**TCT**Y**CCACA-3'	58	-1		
L[1]	5'-TTAACTTCTTCACATTC-3'	51	-		
M[1]	5'-TTAACTTCTTCACAT**Y**C-3'	51	0		
N[1]	5'-TTAACTTCTTCACA**YY**C-3'	47	-2		
O	5'-TTTTTTTTTTTTTT-3'	36	-	32	-
P	5'-TTTTT**Y**T**Y**T**Y**TTTT-3'	35	-0 3	28	-1 3
Q	5'-TTTTTTTT**Y**TTTTT-3'	-	-	30	-2

A = 2'-deoxyadenosine; C = 2'-deoxycytidine; T = thymidine; T_m = melting temperature; ΔT_m = change in T_m / modification

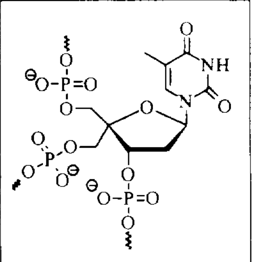


Monomer **Y**

Table 3. Linear and branched sequence synthesized and hybridization data towards complementary DNA

	Sequences	T_m (°C)	ΔT_m (°C)
R	5'-CCCCCCCCCCTTTTTCCCCCCCCCC-3'	62	-
S	5'-CCCCCCCCCCTT-**Z**-TTCCCCCCCCCC-3' 5'-CCCCCCCCCCTT┘	58	- 4

C = 2'-deoxycytidine; T = thymidine; T_m = melting temperature; ΔT_m = change in T_m.



Monomer **Z**

CONCLUSION

Three different phosphoramidite derivatives have been synthesized for incorporation of 4'-C-(hydroxymethyl)thymidine into ODNs. While incorporation of 5'-hydroxyl to 4'-*C*-hydroxymethyl linked monomer **X** induced large decreases in DNA:DNA-duplex stabilities, incorporation of 5'-hydroxyl to 3'-hydroxyl linked monomer **Y** had no significant influence on neither DNA:DNA-duplex nor DNA:RNA-duplex stabilities. This, combined with the reported resistance towards nucleolytic degradation and the possibility of attaching *e.g.* fluorescent probes, intercalating agents or additional ODN-strands at the 4'-*C*-functionality, may stimulate continued interest in this novel class of molecules.

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 202.33 MHz for ^{31}P NMR on a Varian Unity 500 spectrometer. 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 (^{31}P NMR). FAB mass spectra were recorded on a Kratos MS 50 RF spectrometer. Micro analyses were performed at The H. C. Ørsted Institute, University of Copenhagen. The silica gel (0.040-0.063 mm) used for column chromatography was purchased from Merck. Oligodeoxynucleotides were synthezied on a Pharmacia Gene Assambler® Special DNA-Synthesizer. Purification of 5'-O-DMT-ON oligodeoxynucleotides was accomplished using disposable Oligopurification Cartridges (COP, Cruachem) and desalting using NAP-10 columns (Pharmacia). Complementary oligonucleotide (rA_{14}) was purchased from DNA technology ApS, Aarhus, Denmark.

3'-*O*-*tert*-Butyldimethylsilyl-4'-*C*-(hydroxymethyl)thymidine (1)[10]

To a well stirred solution of anhydrous dichloromethane (100 mL) at -78 °C was added oxalyl chloride (3.91g, 30.8 mmol). After 15 min, anhydrous DMSO (4.0 mL, 56.4 mmol) was added dropwise. After stirring for 10 min, nucleoside **1** (3.99 g, 11.0 mmol) in anhydrous dichloromethane (11 mL) was added followed by diisopropylethylamine (9.52 mL, 55.0 mmol, after additional 10 min). The reaction mixture was allowed to warm to room temperature, H_2O (50 mL) was added and the organic phase was washed with H_2O (3 x 35 mL). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure. To the crude aldehyde was added 37% aqueous formaldehyde (0.75 mL, 27.1 mmol), NaOH (3.65 mL, 2 M solution) and dioxane (25 mL), and the mixture was stirred for 4 h at room temperature. The mixture was cooled to 5 °C, $NaBH_4$ (0.5 g, 13.22 mmol) was added, and stirring was continued for 30 min at room temperature. After addition of a 4:1 mixture of pyridine/acetic acid (5 mL) and evaporation of the solvents, the product was isolated as a white solid material after column chromatographic purification (0-10% MeOH in CH_2Cl_2, v/v). Yield 1.98 g (52%). ^{1}H NMR ($CDCl_3$) δ: 0.12 (6H, s, $Si(CH_3)_2$), 0.91 (9H, s, *tert*-butyl), 1.89 (3H d, J = 0.8 Hz, CH_3), 2.36-2.46 (2H, m, H-2'a, H-2'b), 3.17-3.30 (2H, br s, 2 x OH), 3.63 (1H, d, J = 12.6 Hz, H-5'a), 3.68 (1H, d, J = 13.0 Hz, H-5'b), 3.79-3.86 (2H, m, 4'-*C*-CH_2a, 4'-*C*-CH_2b), 4.73 (1H, dd, J = 7.2, 5.5 Hz, H-3'), 6.24 (1H, t, J = 6.6 Hz, H-1'), 7.32 (1H, d, J = 1.1 Hz, H-6). ^{13}C NMR ($CDCl_3$) δ: -5.28 ($Si(CH_3)_2$), -4.86 ($Si(CH_3)_2$), 12.31 (CH_3), 17.76 ($\underline{C}(CH_3)_3$), 25.55 ($C(\underline{C}H_3)_3$), 40.58 (C-2'), 63.13 (C-5'), 63.46 (4'-*C*-CH_2), 72.46 (C-3'), 85.84 (C-1'), 88.78 (C-4'), 111.06 (C-5), 136.99 (C-6), 150.44 (C-2), 163.87 (C-4). Anal. Calcd. for $C_{17}H_{30}N_2O_6Si$, 0.25H_2O: C, 52.22; H, 7.86; N, 7.16; found: C, 51.90; H, 7.86; N, 7.49.

4'-*O*-Benzoyloxymethyl-3'-*O*-(*tert*-butyldimethylsilyl)thymidine (2)

To nucleoside **1** (508 mg, 1.31 mmol), dissolved in anhydrous pyridine (5 mL) at 5 °C under argon, was dropwise added benzoyl chloride (0.16 mL, 1.38 mmol). After 1 h at 5 °C, the reaction mixture was allowed to warm to room temperature and stirring was continued for 1 h. The reaction was quenched with *n*-butanol (0.28 mL, 3.1 mmol) and dichloromethane (40.0 mL) was added. The mixture was washed with saturated aqueous solutions of $NaHCO_3$ (2 x 5 mL) and NaCl (2 x 5 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. After coloum chromotographic purification (0-1% $MeOH/CH_2Cl_2$, v/v), **2** was isolated as a white solid matrial. Yield 0.34g (52%). ^1H NMR ($CDCl_3$) δ: 0.10 (6H, s, $Si(CH_3)_2$), 0.89 (9H, s, *tert*-butyl), 1.88 (3H, s, CH_3), 2.37 (1H, ddd, J = 13.4, 6.7, 4.4 Hz, H-2'a) 2.44-2.57 (1H, m, H-2'b), 3.74 (1H, d, J = 11.8 Hz, H-5'a), 3.85 (1H, d, J = 11.8 Hz, H-5'b), 4.40 (1H, d, J = 12.1 Hz, 4'-*C*-CH_2a), 4.59 (1H, d, J = 12.1 Hz, 4'-*C*-CH_2b), 4.76 (1H, dd, J = 6.5, 4.3 Hz, H-3'), 6.19 (1H, t, J = 6.7 Hz, H-1'), 7.36 (1H, d, J = 0.8 Hz, H-6), 7.40-7.48 (3H, m, Ar), 8.01-8.05 (2H, m, Ar), 9.26 (1H, br s, NH). ^{13}C NMR ($CDCl_3$) δ: -5.21 ($Si(CH_3)_2$), -4.77 ($Si(CH_3)_2$), 12.40 (CH_3), 17.85 ($\underline{C}(CH_3)_3$), 25.60 ($C(\underline{C}H_3)_3$), 40.49 (C-2'), 63.20 (C-5'), 64.29 (4'-*C*-CH_2), 72.47 (C-3'), 86.96 (C-1'), 87.93 (C-4'), 110.93 (C-5), 128.41, 129.63, 129.75, 131.92, 132.08, 133.16 (Ar), 137.20 (C-6), 150.33 (C-2), 163.90 (C-4), 166.67 (C=O). MS (FAB) m/z = 491 ($M+H^+$, 19%). Anal. calcd. for $C_{24}H_{34}N_2O_7Si$: C: 58.75, H: 6.99, N: 5.71; found: C: 58.65, H: 6.92, N: 5.79.

3'-*O*-*tert*-Butyldimethylsilyl-5'-*O*-(4,4'-dimethoxytrityl)-4'-*C*-(hydroxymethyl)thymidine (3)

Nucleoside **2** (489 mg, 1.0 mmol) was dissolved in anhydrous pyridine (1.0 mL) at room temperature and 4,4'-dimethoxytrityl chloride (685 mg, 2.02 mmol) was added. After stirring for 2 h, the mixture was diluted with dichloromethane (20 mL) and washed with saturated aqueous solutions of $NaHCO_3$ (2 x 20 mL) and NaCl (2 x 20 mL). The organic phase was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. MeOH saturated with NH_3 (5.8 mL) was added and stirring at room temperature was continued for 96 h after which the mixture was evaporated under reduced pressure. Purification of the residue by preparative TLC (4% $MeOH/CH_2Cl_2$, v/v) afforded compound **3** as a white solid matrial. Yield 105 mg (42%). ^1H NMR ($CDCl_3$) δ: 0.06 (6H, s, $Si(CH_3)_2$), 0.85 (9H, s, *tert*-butyl), 1.48 (3H, d, J = 0.8 Hz, CH_3), 2.24-2.41 (2H, m, H-2'a, H-2'b), 3.31 (1H, d, J = 10.2 Hz, H-5'a), 3.39 (1H, d, J = 10.2 Hz, H-5'b), 3.62-3.79 (8H, m, 4'-*C*-CH_2a, 4'-*C*-CH_2b, OCH_3), 4.70 (1H, dd, J = 7.1, 4.3 Hz, H-3'), 6.38 (1H, t, J = 6.6 Hz, H-1'), 6.81-7.44 (13H, m, Ar), 7.56 (1H, d, J = 1.1 Hz, H-6). ^{13}C NMR ($CDCl_3$) δ: -5.26 ($Si(CH_3)_2$), -4.71 ($Si(CH_3)_2$), 11.80 (CH_3), 17.85 ($\underline{C}(CH_3)_3$), 25.63 ($C(\underline{C}H_3)_3$), 41.82 (C-2'), 55.23 (OCH_3), 64.22 (C-5'), 64.32 (4'-*C*-CH_2), 73.40 (C-3'), 84.41 (C-1'), 86.96 (C-4'), 88.52 (CAr_3), 111.07 (C-5), 113.26, 127.16, 127.95, 128.19, 130.02, 130.11, 135.28 (Ar), 135.54 (C-6), 144.19 (Ar), 150.16 (C-2), 158.76 (Ar), 163.61 (C-4). MS (FAB) m/z = 689 ($M+H^+$, 2%).

3'-*O*-*tert*-Butyldimethylsilyl-4'-O-(2-cyanoethoxy(diisopropylamino)phosphinoxymethyl)-5'-O-(4,4'-dimethoxytrityl)thymidine (4)

Nucleoside **3** (127 mg, 0.184 mmol) was dissolved under nitrogen in a mixture of anhydrous dichloromethane (0.65 mL) and *N*,*N*-diisopropylethylamine (0.16 mL, 0.92 mmol), and 2-cyanoethyl *N*,*N*-diisopropylaminophosphoramidochloridite (0.08 mL, 0.36 mmol) was added. After 1 h at room temperature, the reaction was quenched with MeOH (0.15 mL) and the mixture was diluted with EtOAc (4 mL). The mixture was washed with saturated aqueous solutions of $NaHCO_3$ (3 x 15 mL) and NaCl (3 x 15 mL). The organic layer was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The obtained foam was dissolved in toluene (0.2 ml) and precipitated from petroleum ether (45 mL) at -40 °C. The product was filtrered and redissolved in anhydrous acetronitrile (5 mL) and evaporated under reduced pressure to give amidite **4** as a white solid matrial. Yield 129 mg (79%). ^{31}P NMR ($CDCl_3$) δ: 149.5, 149.9. MS (FAB) m/z = 889 (M+H$^+$, 3%).

5'-*O*-(4,4'-Dimethoxytrityl)-4'-*C*-(hydroxymethyl)thymidine (5)

To a solution of nucleoside **3** (200 mg; 0.29 mmol) in anhydrous THF (4 mL) was added tetra-*n*-butylammonium fluoride (0.4 mL of a 1.1 M solution in THF, 0.44 mmol). After stirring at room temperature for 3 h, the reaction mixture was evaporated under reduced pressure. The product was obtained as a white solid material after coloum chromatographic purification (0-2% MeOH/CH_2Cl_2, v/v). Yield 148 mg (89%). ^{1}H NMR ($CDCl_3$) δ: 1.45 (3H, s, CH_3), 2.38-2.47 (2H, m, H-2'a, H-2'b), 3.24 (1H, d, J = 9.9 Hz, H-5'a), 3.36-3.40 (2H, m, H-5'b, 4'-*C*-CH_2a), 3.77 (m, 7H, 4'-*C*-CH_2b, OCH_3), 4.68 (1H, dd, J = 6.0, 2.8 Hz, H-3'), 6.49 (1H, t, J = 6.2 Hz, H-1'), 6.81-7.39 (13H, m, Ar), 7.40 (1H, s, H-6). ^{13}C NMR ($CDCl_3$) δ: 11.74 (CH_3), 41.35 (C-2'), 55.23 (OCH_3), 54.10 (C-5'), 65.90 (4'-*C*-CH_2), 74.30 (C-3'), 84.61, 87.18, 88.21 (C-1', C-4', CAr_3), 111.47 (C-5), 113.30, 127.16, 127.86, 127.99, 128.16, 130.12, 135.15 (Ar), 135.26 (C-6), 135.67, 144.19 (Ar), 150.73 (C-2), 158.74 (Ar), 163.87 (C-4). Anal. Calcd. for $C_{32}H_{34}N_2O_8$, $0.5H_2O$: C: 71.85, H: 6.03, N: 3.66; found: C: 71.88, H: 6.22, N: 3.12.

4'-*C*-(*tert*-Butyldimethylsilyloxymethyl)-5'-*O*-(4,4'-dimethoxytrityl)thymidine (6)

To a solution of nucleoside **5** (145 mg, 0.25 mmol) in anhydrous DMF (2 mL) was added imidazole (52 mg, 0.76 mmol) and *tert*-butyldimethylsilyl chloride (53 mg, 0.35 mmol). After stirring for 1.5 h at 36 °C under argon, the reaction mixture was evaporated under reduced pressure and purified on preparative TLC (5% MeOH/CH_2Cl_2, v/v) to give **6** as a white solid material. Yield 134 mg (77%). ^{1}H NMR ($CDCl_3$) δ: 0.04 (6H, s, $Si(CH_3)_2$), 0.82 (9H, s, *tert*-butyl, 2.43-2.47 (2H, m, H-2'a, H-2'b), 3.25 (1H, d, J = 9.9 Hz, H-5'a), 3.39 (1H, d, J = 9.9 Hz, H-5'b), 3.57 (1H, d, J = 10.5 Hz, 4'-*C*-CH_2a), 3.79 (6H, s, OCH_3), 3.82 (1H, d, J = 10.6 Hz, 4'-*C*-CH_2b), 4.62-4.69 (1H, m, H-3'), 6.51 (1H, t, J = 6.3 Hz, H-1'), 6.81-7.41 (13H, m, Ar), 7.60 (1H, d, J = 0.8 Hz, H-6). ^{13}C NMR ($CDCl_3$) δ: -5.80 ($Si(CH_3)_2$), -5.66 ($Si(CH_3)_2$), 11.70 (CH_3), 17.87 ($\underline{C}(CH_3)_3$), 25.64 ($C(\underline{C}H_3)_3$, 41.14 (C-2'), 55.23 (OCH_3), 64.82 (C-5'), 66.28 (4'-*C*-CH_2), 74.26 (C-3'), 84.42 (CAr_3), 87.21, 88.05 (C-1', C-4'), 111.25 (C-5), 113.29, 127.17, 127.99, 128.16, 130.12, 135.25 (Ar), 135.29 (C-6), 135.76, 144.22 (Ar), 150.21 (C-2), 158.78 (Ar), 163.52 (C-4). Anal. Calcd. for $C_{38}H_{48}N_2O_8Si$, $0.25H_2O$: C: 65.82, H: 6.98, N: 4.04; found: C: 65.38, H: 7.18, N: 4.49.

4'-*C*-(*tert*-Butyldimethylsilyloxymethyl)-3'-*O*-(2-cyanoethoxy(diisopropylamino)phosphino)-5'-*O*-(4,4'-dimethoxytrityl)thymidine (7)

This reaction was done as described above for synthesis of **4**. Amounts of reagents used: Nucleoside **6**: 100 mg (0.15 mmol); *N*,*N*-diisopropylethylamine: 0.15 mL (0.86 mmol); 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite: 0.07 mL (0.31 mmol). After precipitation, the product was isolated as a white solid material. Yield 111 mg (86%). ^{31}P NMR ($CDCl_3$) δ: 150.6, 150.2. MS (FAB) m/z = 889 ($M+H^+$, 11%).

3'-*O*-*tert*-Butyldimethylsilyl-5'-*O*-(4,4'-dimethoxytrityl)-4'-*C*-(4,4'-dimethoxytrityloxymethyl)thymidine (8)

Nucleoside **3** (146 mg, 0.37 mmol) was dissolved in anhydrous pyridine (4 mL) at room temperature and 4,4'-dimethoxytrityl chloride (312 mg, 0.92 mmol) was added. After stirring for 12 h, the mixture was diluted with dichloromethane (10 mL) and washed with saturated aqueous solutions of $NaHCO_3$ (2 x 10 mL) and NaCl (2 x 10 mL). The organic phase was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. Column chromatographic purification (10-50% EtOAc/petroleum ether, v/v) afforded **8** as a pale yellow solid material. Yield 255 mg (69%). ^{1}H NMR ($CDCl_3$) δ: -0.24 (3H, s, $Si(CH_3)_2$), -0.12 (3H, s, $Si(CH_3)_2$), 0.68 (9H, s, *tert*-butyl), 1.60 (3H, s, CH_3), 1.87-1.98 (1H, m, H-2'a), 2.18 (1H, ddd, J = 13.3, 5.9, 3.4 Hz, H-2'b), 3.05 (1H, d, J = 10.5 Hz, H-5'a), 3.25 (1H, d, J = 9.7 Hz, H-5'b), 3.47 (1H, d, J = 10.5 Hz, 4'-*C*-CH_2a), 3.53 (1H, d, J = 9.6 Hz, 4'-*C*-CH_2a), 3.77 (6H, s, OCH_3), 3.78 (6H, s OCH_3), 4.40 (1H, dd, J = 6.2, 3.4 Hz, H-3'), 6.34 (1H, t, J = 6.6 Hz, H-1'), 6.73-7.44 (27H, m, Ar, H-6), 8.67 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ: -5.38 ($Si(CH_3)_2$), -4.91 ($Si(CH_3)_2$), 12.07 (CH_3), 17.79 ($\underline{C}(CH_3)_3$), 25,60 ($C(\underline{C}H_3)_3$), 40.95 (C-2'), 55.13 (OCH_3), 55.18 (OCH_3), 63.81, 64.00 (4'-*C*-CH_2, C-5'), 73.18 (C-3'), 84.09 (C-1'), 86.11 (C-4'), 86.75 (CAr_3), 88.67 (CAr_3), 110.87 (C-5), 113.15, 126.53, 126.95, 127.80, 128.21, 128.23, 130.01, 130.09, 130.18, 135,42, 135.55, 135.63, 135.91 (Ar), 136.35 (C-6), 144.56, 144.97 (Ar), 150.19 (C-2), 158.33, 158.56, 158.64 (Ar), 163.69 (C-4). MS (FAB) m/z = 990 ($M+H^+$, 7%).

5'-*O*-(4,4'-Dimethoxytrityl)-4'-*C*-(4,4'-dimethoxytrityloxymethyl)thymidine (9)

Nucleoside **8** (238 mg, 0.24 mmol) was dissolved in DMF (8 mL) at room temperature and KF, $2H_2O$ (128 mg, 1.36 mmol) and 18-crown-6 (21 mg, 0.08 mmol) was added. After stirring for 48 h, the precipitate was filtred off and washed with DMF (3 mL). The combined filtrate was evaporated under reduced pressure and EtOAc (50 mL) was added. The organic phase was washed with H_2O (2 x 50 mL) and a saturated aqueous solution of NaCl (3 x 50 mL), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. After purification by coloum chromatography (20-50% EtOAc/petroleum ether, v/v), **9** was isolated as a white solid material. Yield 188 mg (89%). ^{1}H NMR ($CDCl_3$) δ: 1.51 (3H, s, CH_3), 2.38-2.44 (2H, m, H-2'a, H-2'b), 2.79-2.81 (1H, d, J = 4.1 Hz, OH), 3.20-3.33 (3H, m, H-5'a, H-5'b, 4'-*C*-CH_2a), 3.66 (1H, d, J = 9.9 Hz, 4'-*C*-CH_2b), 3.77 (6H, s, OCH_3), 3.78 (6H, s, OCH_3), 4.58-4.63 (1H, m, H-3'), 6.43 (1H, t, J = 6.9, H-1'), 6.75-7.41

(26H, m, DMT), 7.53 (1H, d, J = 1.0 Hz, H-6), 8.74 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ: 11.83 (CH_3), 40.64 (C-2'), 55.15 (OCH_3), 55.19 (OCH_3), 63.43 (C-5'), 66.78 (4'-*C*-CH_2), 74.09 (C-3'), 84.41 (C-1'), 87.02 (C-4'), 87.28 (CAr_3), 88.12 (CAr_3), 111.15 (C-5), 113.30, 113.36, 126.98, 127.11, 127.78, 127.98, 128.15, 129.85, 130.12, 134.82, 134.94, 135.22, 135.36 (Ar), 135.68 (C-6), 143.99, 144.29 (Ar), 150.27 (C-2), 158.67, 158.75 (Ar), 163.64 (C-4). MS (FAB) m/z = 876 (M^+, 1%). Anal. Calcd. for $C_{53}H_{52}N_2O_{10}$, $0.5H_2O$: C: 71.85, H: 6.03, N: 3.16; found: C: 71.88, H: 6.22, N: 3.12.

3'-O-Acetyl-5'-*O*-(4,4'-dimethoxytrityl)-4'-*C*-(4,4'-dimethoxytrityloxymethyl)thymidine (10)

To a solution of **9** (10 mg, 0.01 mmol) in anhydrous dichloromethane (2.5 mL) at room temperature was added Ac_2O (0.01 mL, 0.11 mmol), triethylamine (0.03 mL, 0.22 mmol) and a catalytic amount of dimethylaminopyridine. After stirring for 5 h, the reaction mixture was diluted with CH_2Cl_2 (10 mL) and washed with saturated aqueous solutions of $NaHCO_3$ (2 x 10 mL) and NaCl (2 x 10 mL), dried (Na_2SO_4) and evaporated under reduced pressure. After purification by preparative TLC (8% MeOH/CH_2Cl_2, v/v), **10** was isolated as a white solid matrial in sufficient amount (7.1 mg, 68% yield) for ^{1}H NMR analysis (one and two dimensional spectras recorded). Selected ^{1}H NMR resonances ($CDCl_3$) δ: 1.42 (3H, s, CH_3), 1.84 (3H, s, $CH_3C(O)$), 2.35-2.61 (2H, m, H-2'a, H-2'b), 5.68-5.70 (1H, m, H-3'), 6.35-6.36 (1H, m, H-1'), 7.52 (1H, s, H-6).

3'-*O*-(2-Cyanoethoxy(diisopropylaminophosphino))-5'-*O*-(4,4'-dimethoxytrityl)-4'-*C*-(4,4'-dimethoxytrityloxymethyl)thymidine (11)

This reaction was done as described above for synthesis of **4**. Amounts of reagents used: Nucleoside **9**: 145 mg (0.16 mmol); *N,N*-diisopropylethylamine: 0.15 mL (0.86 mmol); 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite: 0.08 mL (0.36 mmol). After precipitation, the product was isolated as a white solid material. Yield 158 mg (89%). ^{31}P NMR ($CDCl_3$) δ: 149.8, 149.29.

Synthesis of oligodeoxynucleotides

Syntheses of oligodeoxynuccleotides were carried out in 0.2 μmol scale (5 μmol amidite per cycle, Pharmacia Primer Supports) using **4**, **7**, **11** and commercial β-cyanoethylphosphoramidites. The regular protocol of the DNA-synthesizer for β-cyanoethylphosphoramidites was followed with the only exception that the coupling time for amidites **4**, **7** and **11** was extended to 12 min. Cleavage from the solid support, deblocking, desilylation (except for unmodified ODNs and ODN S) desalting and purification was performed as described.[4]

Melting experiments

The melting experiments were carried out as previously described.[28]

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