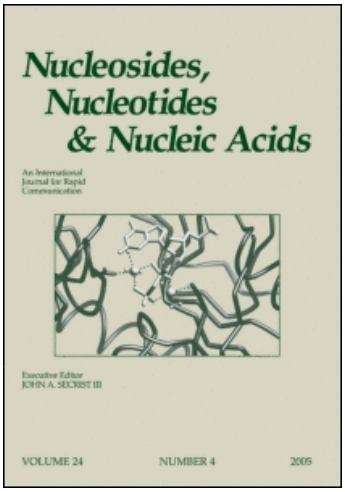

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Lars H. Dreioøe[a]; Jesper Wengel[a]
[a] Department of Chemistr, dense University, Odense M, Denmark

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SYNTHESIS AND EVALUATION OF OLIGODEOXYNUCLEOTIDES CONTAINING 3'-*O*-ETHYL-4'-*C*-(HYDROXYMETHYL)THYMIDINE: INTRODUCTION OF A NOVEL CLASS OF PHOSPHODIESTER INTERNUCLEOSIDE LINKAGES.

Lars H. Dreiøe and Jesper Wengel*

Department of Chemistry, Odense University, DK-5230 Odense M, Denmark.

Abstract: 3'-*O*-Ethyl-4'-*C*-(hydroxymethyl)thymidine (**5**) was synthesized and converted into the phosphoramidite building block **8**. Novel oligodeoxynucleotide analogues containing 4'-*C*-hydroxymethyl phosphodiester internucleoside linkages were synthesized on an automated DNA-synthesizer. The hybridization properties and enzymatic stability were studied on oligomers with one to four modifications. The 3'-end modified oligodeoxynucleotides were resistent towards 3'-exonuclease degradation and showed only moderate lowered affinity towards complementary DNA compared with oligodeoxynucleotides bearing modifications in the middle.

INTRODUCTION

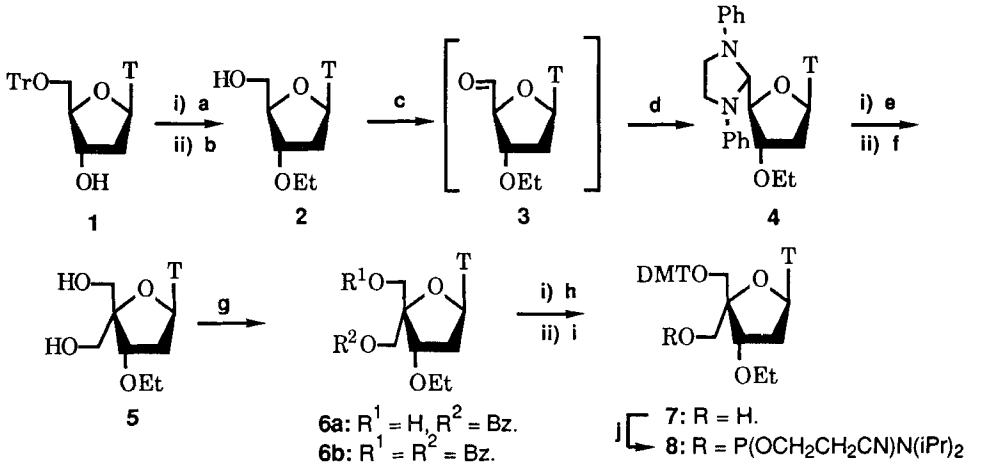
Since the introduction of oligonucleotide analogues as a powerful and revolutionary tool in manipulating gene expression, many attempts have been made to synthesize modified oligonucleotides fulfilling the critical parameters like stability towards nucleases, high affinity and specificity towards complementary unmodified sequences, and the ability to penetrate cell membranes.[1-3] The naturally occurring oligonucleotides lack the resistance towards (3'-exo)nucleolytic degradation which precludes their use as *in vivo* inhibitors of gene expression. Various modifications in the phosphate moiety (*e.g.* phosphorothioates,[4] phosphorodithioates,[5,6] methylphosphonates,[7] phosphate triesters[8]) have been studied. These modifications often lead to an increase in nuclease stability but except for the dithioates also result in chirality at phosphorus thus affording heterogeneous oligomers with lowered

affinity towards a target sequence. Oligodeoxynucleotide analogues containing monomers with modified five-membered carbohydrate 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] xylo-DNA,[16] and 3'-*C*-hydroxymethyl-DNA[17,18]), but hitherto unknown are analogues linked through the 4'-*C* of the pentofuranose ring. Incorporation of 3'-*O*-protected 4'-*C*-hydroxymethyl nucleosides results in analogues isostructural with natural oligodeoxynucleosides with respect to the number and kind of atoms connecting two adjacent phosphorus (-O-C-C-C-O-). This prompted us, as part of our ongoing research programme dealing with synthesis and evaluation of hydroxymethyl-DNA,[17,18] to investigate 4'-*C*-hydroxymethyl phosphodiester linked oligodeoxynucleotide analogues. This paper describes the synthesis of the novel 4'-*C*-(hydroxymethyl)thymidine phosphoramidite monomer **8** and its incorporation into oligodeoxynucleotides. These modified oligomers were studied with respect to their hybridization properties and stability towards snake venom phosphodiesterase (SV PDE).

RESULTS AND DISCUSSION

Thymidine was reacted with tritylchloride in dry pyridine to give 5'-*O*-tritylthymidine (**1**).[19] Using an earlier published method[20] **1** was reacted with ethyl iodide and potassium hydroxide in benzene/dioxane followed by detritylation in 80% acetic acid to give the 3'-*O*-alkylated nucleoside **2** in 56% yield. Pfitzner-Moffatt oxidation[21] of **2** was carried out in DMSO/DCC/TFA followed by *in situ* protection of the 5'-aldehyde **3** as its stable 1,3-diphenylimidazolidine upon reaction with *N,N'*-diphenylethylendiamine[22] thus affording **4** in 62% yield. The synthesis of 4'-*C*-hydroxymethyl nucleosides has been reported using mixed aldol condensations between properly protected carbohydrate or nucleoside aldehydes and formaldehyde.[23-26] The 5'-aldehyde **3** was regenerated with *p*-toluenesulfonic acid and condensed with formaldehyde in the presence of aqueous sodium hydroxide with concomitant Cannizzaro reduction to give the 4'-*C*-hydroxymethyl derivative **5** in 60% yield based on **4**. 4'-*C*-Benzoyloxymethyl nucleoside **6a** was obtained in 53% yield by reaction of **5** with benzoyl-

chloride in pyridine at 0 °C. Besides, the corresponding dibenzoylated nucleoside **6b** was isolated in 9% yield. The high regioselectivity might be a result of steric hindrance from the base moiety in consistence with similar results observed by O-Yang.[27] The structure of **6a** was confirmed by a NOE ^{1}H NMR experiment. Saturation of the signal at δ 3.81-3.92 ($HOC\underline{H}_2$-) gave enhancement of the signal from the 3'-proton (5.2%) and no effect on the signal from the 1'-proton. Additionally, no effect on the 3'-proton was observed when the signal at δ 4.44-4.58 ($BzOCH_2$-) was saturated, but enhancement of the 1'-proton at δ 6.19 (3.3%). 5'-*O*-Protection of **6a** by reaction with 4,4'-dimethoxytritylchloride in pyridine followed by debenzoylation in methanolic ammonia afforded the 5'-*O*-(4,4'-dimethoxytrityl)-4'-*C*-hydroxymethyl nucleoside **7** in 86% yield based on **6a**. The low reactivity of the 5'-OH necessitated the transient benzoylation of the 4'-*C*-hydroxymethyl substituent. The phosphoramidite building block **8** was synthesized from **7** using 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite in the presence of *N,N*-diisopropylethylamine[28,29] and isolated in 89% yield after precipitation in hexane.



T = thymin-1-yl. a) EtI, KOH, benzene, dioxane. b) 80% HOAc. c) DMSO, DCC, pyridine, TFA. d) *N,N'*-Diphenylethylenediamine, MeOH, 99% HOAc. e) *p*-Toluenesulfonic acid hydrate, CH_2Cl_2. f) *p*-Dioxane, 37% aq. formaldehyde, 2M NaOH. g) Benzoylchloride, pyridine. h) DMTCl, pyridine. i) NH_3/MeOH. j) 2-Cyanoethyl *N,N*-diisopropylphosphoramidochloridite, CH_2Cl_2, *N,N*-diisopropylethylamine.

TABLE 1

Sequence Synthesized		T_m (°C)	ΔT_m (°C)	H_m (%)
5' - CACCAACTTCTTCCACA - 3'	(A)	61.0	-	23
5' - CACCAACT*TCTTCCACA - 3'	(B)	52.5	8.5	16
5' - CACCAACT*TCTT*CCACA - 3'	(C)	45.0	8.0	16
5' - TTAACTTCTTCACATTC - 3'	(D)	52.0	-	23
5' - TTAACTTCTTCACATT*C - 3'	(E)	49.5	2.5	20
5' - TTAACTTCTTCACAT*T*C - 3'	(F)	46.0	3.0	21
5' - TT*AACT*TCTT*CACATT*C - 3'	(G)	26.0	6.5	17
5' - TTTTTTTTTTTTT - 3'	(H)	34.5	-	17
5' - TT*TTT*TTT*TTT*TT - 3'	(I)	<10	-	nd

ΔT_m = decrease in T_m per modification. nd = no cooperative melting observed above 10 °C. H_m = thermal hypochromicity. T* = modified monomer.

The oligodeoxynucleotides **A-I** (Table 1) were synthesized using standard phosphoramidite methodology on an automated DNA-synthesizer, building-blocks used were commercial 2'-deoxynucleoside ß-cyanoethylphosphoramidites and **8**. The coupling efficiency of the modified phosphoramidite **8** (app. 93%) was monitored by the release of the dimethoxytrityl cation after each coupling step. The oligodeoxynucleotides were released from the support and deblocked with concentrated ammonia at 20°C for 3 days. Purification of the oligomers was performed on Oligo-Pak™ purification cartridges. The composition of the oligodexynucleotide **C** was verified by matrix assisted laser desorption mass spectrometry which has become a powerful method for mass analysis of oligonucleotides.[30] The oligomer **C** with two modifications gave a relative molecular mass of 5148 Da corresponding well within experimental error with the calculated mass of 5150 Da. Because of the homogeneous results from the syntheses of all the modified oligomers **B**, **C**, **E**, **F**, **G** and **I** we consider their composition confirmed.

The hybridization properties of the modified oligomers were studied by mixing **A-I** with their complementary DNA-strands and determining the melting points of the DNA-DNA hybrids by UV measurements. The results are listed in Table 1. It can be seen that incorporation of one or two modifications in the middle of an oligodeoxynucleotide (**B** and **C**) leads to a decrease in melting temperature (ΔT_m) of app. 8°C per modification, whereas one or two modifications in the 3'-end region (**E** and **F**) leads to a decrease of 3°C and 2,5°C per modification, respectively. Considering the modified 13-mer **I**, no hyperchromicity was detected above 10 °C, compared with the T_m of 34 °C for the unmodified oligomer **H**. These results are confirmed by a decrease in T_m of 6.5 °C per modification with the four times modified oligomer **G**. The melting hypochromicity (Table 1) is lowered from 23% (unmodified **A**) to 16% for the oligomer **B** and **C** containing 4'-*C*-hydroxymethyl modifications in the middle, whereas incorporation of one or two modifications in the 3'-end only weakly decreases the melting hypochromicity from 23% (unmodified **D**) to 21% and 20% for the oligomers **E** and **F**, respectively. These data indicate that modifications in the middle cause appreciable distortion of the structure of the corresponding DNA-DNA duplex compared to the parent duplex consisting of natural deoxynucleosider while 3'-end modifications cause only minor distortion of the duplex structure.

Since it has been reported that 3'-exonucleases play a predominant role in the *in vivo* degradation of natural oligodeoxynucleotides[1,31] we have evaluated the stability towards SV PDE (3'-exonuclease). The increase in absorbance (hyperchromicity) at 260 nm was monitored during digestion with SV PDE.[16,32] The hyperchromicity arises as a consequence of the base stacking of oligodeoxynucleotides and consequent π-π orbital overlap[33] which is destroyed as the oligodeoxynucleotide is converted to its mononucleotide constituents during the enzymatic digestion. The results are summarized in Table 2. After digestion of one unmodified nucleoside from the 3'-end of oligonucleotide analogues **E**, **F**, **G**, and **I**, the resulting oligomers with one or two 4'-*C*-hydroxymethyl modifications in the 3'-end exhibit significant resistance towards further 3'-exonucleolytic degradation, whereas unmodified oligomer **D** is completely degradated after few minutes. Oligodeoxynucleotides **B** and **C** including one or two modifications in the middle are initially rapidly degradated showing

TABLE 2

Sequence Synthesized		$t_{1/2}$ (min)	H_e (%)[a]
5' - CACCAACTTCTTCCACA - 3'	(A)	< 1	22
5' - CACCAACT*TCTTCCACA - 3'	(B)	< 1[b]	9
5' - CACCAACT*TCTT*CCACA - 3'	(C)	< 1[b]	9
5' - TTAACTTCTTCACATTC - 3'	(D)	< 1	16
5' - TTAACTTCTTCACATT*C - 3'	(E)	> 60	2
5' - TTAACTTCTTCACAT*T*C - 3'	(F)	> 60	3
5' - TT*AACT*TCTT*CACATT*C - 3'	(G)	> 30	4
5' - TTTTTTTTTTTTT - 3'	(H)	< 1	8
5' - TT*TTT*TTT*TTT*TT - 3'	(I)	> 30	2

[a] H_e = enzymatic hypochromicity as calculated after 60 min digestion. [b] Initial hyperchromicity half-life as measured after 5 min digestion.

initial enzymatic hypochromicities of only 9% compared to 22% for unmodified **A**. These observations corresponds to earlier stability experiments on flexible oligomers contaning 3',4'-*seco*-thymidine monomers, where similar results from UV-experiments were supported by denaturing gel analyses of 5'-labelled material.[34] Therefore we interpret the enzymatic hypochromicity data on the mid-modified oligomers **B** and **C** as results of rapid degradation of the 17-mers to a 8-mer (from **B**) and a 12-mer (from **C**), both stabilized due to 3'-end protection by a 4'-*C*-hydroxymethyl monomer.

CONCLUSION

Oligodeoxynucleotide analogues containing one, two or four 3'-*O*-ethyl-4'-*C*-hydroxymethyl nucleosides have been synthesized. 3'-End modifications moderately weaken duplex stability while significantly improving the resistance towards 3'-exonucleolytic degradation. Therefore, the novel 4'-*C*-hydroxymethyl linked oligodeoxynucleotides described here may prove valuable as nuclease resistent probes or therapeutic agents. Additionally, they may

stimulate efforts towards synthesizing other 4'-*C*-hydroxymethyl linked or functionalized oligonucleotides.

EXPERIMENTAL

NMR spectra were recorded at 250 MHz for ^{1}H NMR and 62.9 MHz for ^{13}C NMR on a Bruker AC-250 spectrometer, and at 202.3 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, ^{13}C NMR) and relative to 85% H_3PO_4 as external standard (^{31}P NMR). 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. Solvent A: 10% MeOH in CH_2Cl_2. Solvent B: 5% MeOH in CH_2Cl_2. Snake venom phosphodiesterase (*Crotalus adamanteus*) was purchased from Pharmacia. Matrix assisted laser desorption mass spectrum was recorded on a prototype laser desorption mass spectrometer from Applied Biosystem Sweden AB, Uppsala, Sweden.

3'-*O*-Ethylthymidine (2)[20]

5'-*O*-Tritylthymidine (**1**) (15 g, 31 mmol) was dissolved in benzene (150 ml) and dioxane (50 ml) and ethyl iodide (5 ml, 62 mmol) and powdered potassium hydroxide (3.5 g, 62 mmol) was added. The reaction mixture was stirred at 70 °C for 10 h. After cooling the mixture to room temperature it was washed with a saturated aqueous solution of $NaHCO_3$ (3 x 50 ml) and evaporated. The residue was stirred in 80% acetic acid (200 ml) at 95 °C for 1 h. The mixture was neutralized with 2M NaOH and evaporated. The residue was dissolved in chloroform (100 ml) and washed with a saturated aqueous solution of $NaHCO_3$ (3 x 30 ml) and a saturated aqueous solution of NaCl (3 x 30 ml), dried (Na_2SO_4) and evaporated. Purification by silica gel column chromatography (1 - 5% MeOH in CH_2Cl_2) afforded **2** as a solid white material. Yield: 4.5 g (17.4 mmol, 56%). Litt.[20] 62%. Rf = 0.42 (solvent A). ^{1}H NMR (DMSO): δ 1.13 (t, 3H, J = 6.9 Hz, CH_2-$\underline{CH_3}$), 1.73 (s, 3H, CH_3), 2.06-2.24 (m, 2H, H-2'α, H-2'ß), 3.47 (q, 2H, J = 7.0 Hz, $\underline{CH_2}$-CH_3), 3.53-3.61 (m, 2H, H-5'a, H-5'b), 3.88-3.92 (m, 1H, H-4'), 4.04-4.08 (m, 1H, H-3'), 5.04 (t, 1H, J = 5.2 Hz,

OH), 6.11 (dd, 1H, J = 8.0 Hz, 6.2 Hz, H-1'), 7.68 (s, 1H, H-6), 11.25 (s, 1H, NH). ^{13}C NMR (DMSO): δ 12.07 (CH_3), 15.10 (CH_2-$\underline{C}H_3$), 36.24 (C-2'), 61.45 ($\underline{C}H_2$-CH_3), 63.61 (C-5'), 78.73 (C-3'), 83.73, 84.62 (C-1', C-4'), 109.35 (C-5), 135.84 (C-6), 150.34 (C-2), 163.55 (C-4).

5'-Deoxy-5',5'-(*N,N'*-diphenylethylenediamino)-3'-*O*-Ethylthymidine (4)

3'-*O*-Ethylthymidine (**2**) (1.0 g, 3.7 mmol) was coevaporated three times with dry pyridine. Trifluoroaceticacid (TFA) (0.18 ml, 2.35 mmol) was added dropwise to an ice-water cooled solution of **2**, dicyclohexylcarbodiimide (DCC) (2.3 g, 11.1 mmol) and pyridine (0.3 ml, 3.7 mmol) in DMSO (10 ml) and the resulting mixture was stirred at 20 °C for 10 h. After careful addition of a solution of oxalic acid dihydrate (0.9 g, 7.4 mmol) in methanol (3 ml), the reaction mixture was stirred at 20 °C for 1 h. *N,N'*-Dicyclohexylurea was filtered off and washed with methanol. *N,N'*-Diphenylethylenediamine (0.8 g, 3.7 mmol) and glacial acetic acid (0.44 ml) in methanol (3 ml) was added to the combined filtrates and stirring was continued at 20 °C for 20 h. After neutralization with a saturated aqueous solution of $NaHCO_3$ the resulting mixture was extracted with chloroform (3 x 50 ml), dried (Na_2SO_4) and evaporated to dryness. The residue was purified by silica gel column chromatography (1 - 4% MeOH in CH_2Cl_2) to give **4** as a white solid. Yield: 1.06 g (2.29 mmol, 62%). Rf = 0.67 (solvent A). ^{1}H NMR ($CDCl_3$): δ 1.19 (t, 3H, J = 7.0 Hz, CH_2-$\underline{CH}_3$), 1.66 (3H, s, CH_3), 1.92-2.02 (m, 1H, H-2'ß), 2.16-2.26 (m, 1H, H-2'α), 3.30-3.45 (m, 2H, $\underline{CH}_2$-CH_3), 3.61-3.81 (m, 4H, CH_2-CH_2), 4.08 (dd, 1H, J = 12.6 Hz, 5.9 Hz, H-3'), 4.37 (dd, 1H, J = 5.8 Hz, 1.0 Hz, H-4'), 5.76 (d, 1H, J = 0.8 Hz, H-5'), 6.25 (t, 1H, J = 6.1 Hz, H-1'), 6.78-6.91 (m, 7H, phenyl, H-6), 7.22-7.33 (m, 4H, phenyl), 9.12 (s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 12.37 (CH_3), 15.20 (CH_2-$\underline{C}H_3$), 37.64 (C-2'), 47.26, 47.32 ($\underline{C}H_2$-$\underline{C}H_2$), 65.15 ($\underline{C}H_2$-CH_3), 72.79 (C-5'), 79.05 (C-3'), 83.23, 84.08 (C-1', C-4'), 110.92 (C-5), 112.80, 113.72, 118.19, 118.91, 129.22, 129.38, 146.19, 146.57 (phenyl), 135.32 (C-6), 150.21 (C-2), 163.51 (C-4).

3'-*O*-Ethyl-4'-*C*-(hydroxymethyl)thymidine (5)

To a ice cooled solution of **4** (0.91 g, 1.95 mmol) in methylene chloride (65 ml) was added *p*-toluenesulfonic acid hydrate (0.90 g, 5.85 mmol) in acetone

(30 ml) and the mixture was stirred at 20 °C. After 1 h, and the white precipitate was filtered off and washed with methylene chloride (50 ml). The combined filtrates were washed with a saturated aqueous solution of $NaHCO_3$ (3 x 30 ml) and a saturated aqueous solution of NaCl (3 x 30 ml) and dried (Na_2SO_4). The solvent was removed under reduced pressure, and the residue was dissolved in *p*-dioxane (5.5 ml) and 37% aqueous formaldehyde (1.2 ml), and aqueous sodium hydroxide (3.0 ml, 2M) was added. The reaction mixture was stirred at 20 °C for 18 h, neutralized with glacial acetic acid in pyridine (1:1, v/v), diluted with methylene chloride (50 ml) and washed with a saturated aqueous solution of $NaHCO_3$ (3 x 10 ml) and a saturated aqueous solution of NaCl (3 x 10 ml) and dried (Na_2SO_4). The solvent was removed under reduced pressure and the residue purified by silica gel column chromatography (1 - 5% MeOH in CH_2Cl_2) affording **5** as a solid white material. Yield: 352 mg (1.17 mmol, 60%). Rf = 0.35 (solvent A). ^1H NMR ($CDCl_3$): δ 1.23 (t, 3H, J = 7.0 Hz, CH_2-$\underline{C}H_3$), 1.90 (s, 3H, CH_3), 2.43-2.48 (m, 2H, H-2'α, H-2'ß), 3.45-3.70 (m, 2H, $\underline{C}H_2$-CH_3), 3.72 (s, 2H, H-5"a, H-5"b), 3.78 (s, 2H, H-5'a, H-5'b), 4.38 (t, 1H, J = 5.5 Hz, H-3'), 6.16 (t, 1H, J = 6.7 Hz, H-1'), 7.35 (s, 1H, H-6). ^{13}C NMR ($CDCl_3$): δ 12.41 (CH_3), 15.30 (CH_2-$\underline{C}H_3$), 37.37 (C-2'), 63.63, 64.29, 66.17 (C-5', C-5", $\underline{C}H_2$-CH_3), 79.86 (C-3'), 86.75, 88.41 (C-1', C-4'), 111.16 (C-5), 137.15 (C-6), 150.49 (C-2), 163.84 (C-4). MS m/z = 300 (M^+, 5%). Anal. Calcd. for $C_{13}H_{20}O_6N_2 \cdot 0.5\ H_2O$: C, 50.48; H, 6.84; N, 9.06. Found: C, 50.44; H, 6.91; N, 8.73.

4'-*C*-(Benzoyloxymethyl)-3'-*O*-ethylthymidine (6a) and 5'-*O*-benzoyl-4'-*C*-(benzoyloxymethyl)-3'-*O*-ethylthymidine (6b)

Nucleoside **5** (0.3 g, 1.0 mmol) in dry pyridine (7 ml) was stirred at 0 °C and benzoylchloride (0.12 ml, 1.0 mmol) added dropwise. After 1 h the mixture was heated to room temperature and stirred for an additional 1 h. The reaction was quenched with *n*-butanol (0.2 ml), and after dilution with methylene chloride (30 ml), the reaction mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 x 10 ml) and a saturated aqueous solution of NaCl (2 x 10 ml), dried (Na_2SO_4) and evaporated to dryness under reduced pressure. After purification by silica gel column chromatography (0 - 2% MeOH in CH_2Cl_2) **6a** and **6b** were isolated as white solids.

Yield **6a**: 214 mg (0.53 mmol, 53%). Rf = 0.25 (solvent B). ^{1}H NMR ($CDCl_3$): δ 1.16 (t, 3H, J = 7.0 Hz, CH_2-$\underline{CH}_3$), 1.87 (s, 3H, CH_3), 2.41-2.49 (m, 2H, H-2'α, H-2'ß), 3.42-3.72 (m, 2H, $\underline{CH}_2$-CH_3), 3.81-3.92 (m, 2H, H-5'a, H-5'b), 4.39 (t, 1H, J = 5.6 Hz, H-3'), 4.44-4.58 (m, 2H, H-5"a, H-5"b), 6.19 (t, 1H, J = 6.7 Hz, H-1'), 7.40-8.07 (m, 6H, phenyl, H-6), 9.70 (s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 12.21 (CH_3), 15.03 (CH_2-$\underline{C}H_3$), 37.14 (C-2'), 63.58, 63.84, 65.96 (C-5', C-5", $\underline{C}H_2$-CH_3), 79.03 (C-3'), 86,52, 87.23 (C-1', C-4'), 110.37 (C-5), 128.27, 128.50, 129.43, 132.97 (phenyl), 137.09 (C-6), 150.49 (C-2), 164.60 (C-4), 166.38 (C=O). MS m/z = 404 (M^+, 1%). HRMS calcd.: 404.1583; found: 404.1586.

Yield **6b**: 46 mg (0.09 mmol, 9%). Rf = 0.40 (solvent B). ^{1}H NMR ($CDCl_3$): δ 1.17 (t, 3H, J = 7.0 Hz, CH_2-$\underline{CH}_3$), 1.71 (s, 3H, CH_3), 2.25-2.35 (m, 1H, H-2'ß), 2.56-2.66 (m, 1H, H-2'α), 3.42-3.66 (m, 2H, $\underline{CH}_2$-CH_3), 4.32 (dd, 1H, J = 6.6 Hz, 3.7 Hz, H-3'), 4.43-4.74 (m, 4H, H-5'a, H-5'b, H-5"a, H-5"b), 6.32 (t, 1H, J = 6.7 Hz, H-1'), 7.26-7.63 (m, 7H, phenyl, H-6), 8.02-8.07 (m, 4H, phenyl), 8.61 (s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 12.10 (CH_3), 15.06 (CH_2-$\underline{C}H_3$), 37.74 (C-2'), 63.40, 65.33, 66.33 (C-5', C-5", $\underline{C}H_2$-CH_3), 79.75 (C-3'), 85.39, 85.44 (C-1', C-4'), 111.10 (C-5), 128.40, 128.49, 129.56, 129.74, 131.80, 131.85, 133.09, 133.56 (phenyl), 135.22 (C-6), 149.89 (C-2), 163.33 (C-4), 165.95 (2 x C=O).

5'-*O*-(4,4'-Dimethoxytrityl)-3'-*O*-ethyl-4'-*C*-(hydroxymethyl)thymidine (7)

6a (156 mg, 0.38 mmol) was coevaporated with dry pyridine and dissolved in dry pyridine (5 ml), and 4,4'-dimethoxytritylchoride (158 mg, 0.47 mmol) was added. The resulting mixture was stirred at 20 °C for 4 h. After dilution with methylene chloride (35 ml), the reaction mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 x 10 ml) and a saturated aqueous solution of NaCl (2 x 10 ml), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was dissolved in methanolic ammonia (70 ml) and stirring was continued at 22 °C for 12 h. The solvent was removed under reduced pressure, and the residue purified by silica gel column chromatography (0 - 2% MeOH in CH_2Cl_2) to give **7** as a white solid material. Yield: 231 mg (0.33 mmol, 86%). Rf = 0.36 (solvent A). ^{1}H NMR ($CDCl_3$): δ 1.19 (t, 3H, J =

7.0 Hz, CH_2-$\underline{CH}_3$), 1.49 (s, 3H, CH_3), 2.26-2.33 (m, 1H, H-2'ß), 2.49-2.59 (m, 1H, H-2'α), 3.31-3.45 (m, 4H, $\underline{CH}_2$-CH_3, H-5'a, H-5'b), 3.58-3.76 (m, 2H, H-5"a, H-5"b), 3.78 (s, 6H, OCH_3), 4.33 (dd, 1H, J = 6.7 Hz, 3.5 Hz, H-3'), 6.35 (t, 1H, J = 6.7 Hz, H-1'), 6.81-6.85 (m, 4H, aryl), 7.23-7.56 (m, 10H, H-6, aryl), 9.03 (s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 11.71 (CH_3), 15.12 (CH_2-$\underline{C}H_3$), 38.05 (C-2'),55.11 (OCH_3), 63.86, 65.21, 65.88 (C-5', C-5", $\underline{C}H_2$-CH_3), 80.42 (C-3'), 84.39, 86.95, 87.77 (C-1', C-4', $C(Ar)_3$), 110.98 (C-5), 113.03, 113.16, 123.60, 127.01, 127.84, 128.00, 130.01, 135.50, 135.25, 144.21, 149.62, 158.64 (aryl), 135.86 (C-6), 150.20 (C-2), 163.64 (C-4). MS m/z = 602 (M^+, 1%). HRMS calcd.: 602.2628; found: 602.2637.

4'-*C*-[*O*-(2-Cyanoethoxy(diisopropylamino)phosphino)hydroxymethyl]-5'-*O*-(4,4'-dimethoxytrityl)-3'-*O*-ethylthymidine (8)

7 (121 mg, 0.2 mmol) was coevaporated three times with dry acetonitrile and dissolved under N_2 in anhydrous methylene chloride (0.7 ml). *N,N*-Diisopropylethylamine (0.18 ml) was added followed by dropwise addition of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.07 ml, 0.3 mmol). After 1 h analytical TLC showed no more starting material and the reaction was quenched with methanol (0.04 ml) and diluted with ethyl acetate (4 ml). The mixture was washed with a saturated aqueous solution of $NaHCO_3$ (2 x 3 ml) and a saturated aqueous solution of NaCl (2 x 3 ml), dried (Na_2SO_4) and evaporated under reduced pressure. The residual gum was dissolved in toluene (1 ml) and precipitated in hexane (150 ml) at 0 °C. The product was collected by filtration and dried under vacuum. Yield: 143 mg (0.18 mmol, 89%). Rf = 0.50 (solvent A). ^{31}P NMR ($CDCl_3$) δ 148.8, 149.1.

Synthesis of the oligodeoxynucleotides

The oligodeoxynucleotides (**A-I**) were synthesized on a Pharmacia Gene Assembler special® DNA-synthesizer in 0.2 µmol-scale (5 µmol amidite per cycle, Pharmacia primer support™) using commercial ß-cyanoethylphosphoramidites and compound **8**. The synthesis followed the regular protocol of the DNA-synthesizer for ß-cyanoethylphosphoramidites. The efficiency of each coupling step was monitored by release of the dimethoxytrityl cation. The

coupling efficiency of **8** was slightly lower (app. 93%) than for the unmodified phosphoramidites (app. 98%). Removal from the solid support and deprotection was carried out at 20 °C in concentrated ammonia for 72 hours. The oligomers were purified on Oligo-Pak™ cartridges.

Enzymatic stability of the oligodeoxynucleotides

A solution of the oligodeoxynucleotide (0.2-0.3 OD) in 2 ml of buffer (0.1 M Tris. HCl, 0.1 M NaCl, 14 mM $MgCl_2$, pH 8.6) was digested with 1.2 U SV PDE dissolved in: 5 mM Tris. HCl, 50% glycerol v/v, pH 7.5) at 25 °C. During digestion the increase in absorbance at 260 nm was monitored. The absorbance as a function of time curve of the digestion was plotted from which the hyperchromicity and $t_{1/2}$ were evaluated. The final hyperchromicity is defined as the value of the final absorbance divided with 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. Using the extinction coefficients for the constituent monomers (ε_{260}: A, 15400; C, 7300; G, 11700; T, 8800; T*, 8800) the hypochromicity values can be calculated as reported.[16]

Melting experiments

The melting experiments were carried out in medium salt buffer, 1 mM EDTA, 10 mM Na_2HPO_4, 140 mM NaCl, pH 7.2. The increase in absorbance at 260 nm was recorded as a function of time as the temperature was raised linearily from 10 to 75 °C. The melting hypochromicity values were calculated using the final and initial absorbance as described above.

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