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Incorporation of hexose nucleoside analogues into oligonucleotides: synthesis, base-pairing properties and enzymatic stability

Koen Augustyns, Frank Vandendriessche, Arthur Van Aerschot, Roger Busson, Claus Urbanke[1] and Piet Herdewijn*

Laboratory of Pharmaceutical Chemistry, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven and [1]Medizinische Hochschule, Konstanty Gutschow Straße 8, 3000 Hannover, Germany

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ABSTRACT

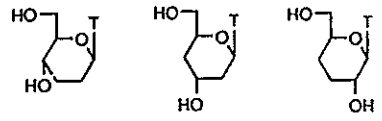
Oligonucleotides containing 1-(2,4-dideoxy-β-D-*erythro*-hexo-pyranosyl)thymine (2) and 1-(3,4-dideoxy-β-D-*erythro*-hexopyranosyl) thymine (3) were synthesized on a solid support using the phosphoramidite approach. The properties of these oligonucleotides were compared with the earlier reported characteristics of oligonucleotides containing 1-(2,3-dideoxy-β-D-*erythro*-hexopyranosyl) thymine (1). The order in enzymatic stability of end-substituted oligonucleotides is 3>1>>2. The hybridization properties of the modified oligonucleotides are in reverse order: 2>>1>3.

INTRODUCTION

Oligonucleotides of a defined sequence, complementary to a target mRNA or DNA, are valuable tools to disrupt genetic expression[1] (antisense or antigene approach). As all viral infections and most of the cancers involve undesirable expression of genes, the interest in the therapeutic applications of this antisense approach is justified. A major problem however is the rapid enzymatic degradation of natural oligodeoxynucleotides. The synthetic challenge lies therefore in the development of enzymatically stable oligonucleotide analogues, without disrupting the ability to form a stable duplex with its natural complement. Besides modification of the phosphate backbone, alteration of the sugar moiety of nucleosides can also be considered to reach this goal. Most of the reported sugar-modified oligonucleotides still contain a five-membered sugar ring, closely resembling the natural deoxyribose (α-DNA[2], α-RNA[3], 2′-*O*-methyl RNA[4], 2′-*O*-allyl RNA[5], carbocyclic DNA[6], xylo-DNA[7]). Recently oligonucleotides containing a completely altered backbone (acyclic polyamide[8], morpholino-nucleosides with carbamate linkages[9]) were reported to have good hybridizing properties.

Little is known, however, about the synthesis of oligonucleotides containing hexopyranose instead of pentofuranose sugars. Therefore we were interested in the synthesis of oligonucleotides containing dideoxy-β-D-*erythro*-hexopyranosyl nucleoside analogues (**1**–**3**) (scheme I). Dimers and oligomers containing **1** were reported earlier[10] and were also synthesized by Eschenmoser[11] in an effort to answer the question why nature chose pentose and not hexose nucleic acids.

SCHEME I



T = thymin-1-yl

These analogues have an increased enzymatic stability but suffer from a reduced duplex forming capability with the natural complement. We expected that we could improve the hybridization properties, while retaining the enzymatic stability, with the synthesis of oligonucleotides containing the monomers: 1-(2,4-dideoxy-β-D-*erythro*-hexopyranosyl)thymine (**2**) and 1-(3,4-dideoxy-β-D-*erythro*-hexopyranosyl)thymine (**3**).

For this purpose, building blocks for automated solid-phase synthesis were prepared which allow the incorporation of these nucleosides into any position of an oligonucleotide chain. Furthermore, we studied the behaviour of these oligonucleotides with respect to their enzymatic stability and base-pairing properties with complementary oligo(dA). The properties of these three analogues (**1**–**3**) are compared.

RESULTS AND DISCUSSION

Synthesis of the building blocks of 2 and 3 and incorporation into oligonucleotides

The synthesis of **2** starting from either 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose or from tri-*O*-acetyl-D-glucal is described separately[12]. Protection of the primary hydroxyl function of **2** with dimethoxytrityl chloride afforded **4** (scheme

* To whom correspondence should be addressed

can be calculated that ΔG(AA/TT) = 5.54 kJ/mol under our conditions, which is slightly lower than the reported values[21,22] because of the lower salt concentration used. Therefore the destabilization caused by a substitution with **2** in the middle of a sequence (**20**) (ΔG=8.2 kJ/mol) corresponds to 1.5 times the nearest-neighbour interaction. This is slightly lower than what would be expected for an internal loop containing two unpaired bases. (The difference in ΔG equals then two times the nearest-neighbour interaction). The destabilization is also of the same magnitude as the least stable base-base mismatch[22]. All this indicates that the effect of one substitution in the middle is limited to one base pair. A completely modified 13-mer (**21**) is not capable of base pairing with an unmodified $(dA)_{13}$.

The effect on duplex stability of a substitution with **3** is more dramatic. Comparing **22** with **16** and **24** with **25** indicates that one modified nucleoside at either end gave no important change in melting properties. Adding a second one at either end (**23**) resulted in a large reduction of the melting temperature. The ΔG value of **23** (30.2 kJ/mol) corresponds well to a calculated value for an inner 11-mer with two dangling ends (ΔG = −25.1 + 10×(5.54) = 30.3 kJ/mol), indicating that one modified nucleoside is base paired and the other one not. Comparing **25** with **16** and **24** with **22** indicates a drop of the melting temperature of around 16°C. The destabilization caused by a substitution with **3** in the middle of a sequence (**25**) (ΔG = 18.2 kJ/mol) corresponds to 3.3 times the nearest-neighbour interaction, indicating an internal loop of at least four unpaired bases. Unlike **2**, substitution with **3** in the middle, results in a destabilization which is not just limited to the modified base pair. The results obtained with **3** are very much like the results obtained with **1**.

Enzymatic hydrolysis

It was reported[1] that a 3′-exonuclease activity was the major cause of the degradation of natural oligonucleotides in serum. This was supported by the results of several authors who reported that 3′,5′-end modified oligonucleotides had an increased half-life. Therefore it seemed suitable to study the enzymatic stability of end-protected oligonucleotides with a 3′-exonuclease (snake venom phosphodiesterase, SV PDE). During digestion with SV PDE, the increase in absorbance at 260 nm was followed. Results of enzymatic stability are indicated in Table I. End-protection with **2** gave only a slight increase of the half-life (e.g. two hexoses at either end (**18**): $t_{1/2}$×4.5). This is in contrast with end protection with **3** and **1**. Substitution with one hexose at either end likewise gave only a slight increase in protection, but substitution with two hexoses at either end resulted in a large increase of the half-life.

CONCLUSION

Several oligomers have been synthesized containing one or more hexose nucleosides (**1**−**3**). Substitution with these nucleoside analogues in the middle of a 13-mer had a more pronounced effect on duplex stability than end-substitution. The magnitude of destabilization of the duplex was largely dependent on the hexose analogue used: **3**>**1**>>**2** (figure 1 and 2). Unfortunately, the enzymatic stability of these oligonucleotides (a necessary property for their use as antisense constructs) was in an identical order: **3**>**1**>>**2**.

Apparently, when the hexose nucleoside is able to substitute the natural deoxyribose giving good hybridization properties, the modified oligonucleotide is also recognized by destructive exonucleases. Nevertheless, we are currently further investigating the properties of 3′-end substituted antisense constructs containing **2**.

EXPERIMENTAL SECTION

The general procedures used in the experimental part of this work are the same as in ref. 10.

1-(2,4-dideoxy-6-*O*-dimethoxytrityl-β-D-*erythro*-hexopyranosyl)thymine (4)

A solution of 314 mg (1.23 mmol) of 1-(2,4-dideoxy-β-D-*erythro*-hexopyranosyl)thymine[12] (**2**) and 500 mg (1.48 mmol) of dimethoxytrityl chloride in pyridine dry was stirred for 24 h at room temperature. The reaction mixture was diluted with CH_2Cl_2 and washed twice with a saturated sodium bicarbonate solution. The aqueous layer was extracted with CH_2Cl_2 and the combined organic layer was dried, evaporated and purified by column chromatography [(1) CH_2Cl_2-Et_3N 99:1, (2) CH_2Cl_2-Et_3N-MeOH 98:1:1, (3) CH_2Cl_2-Et_3N-MeOH 97:1:2]

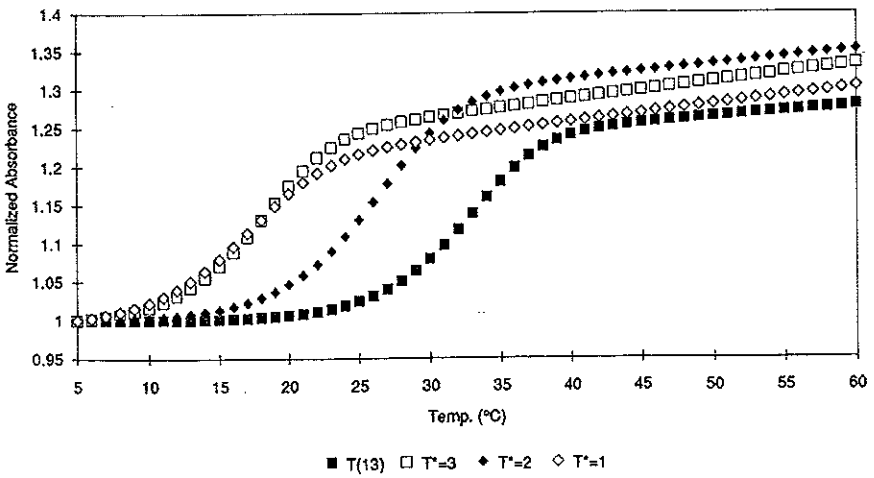


Figure 1. Normalized melting curves of T(6)T*T(6).dA(13).

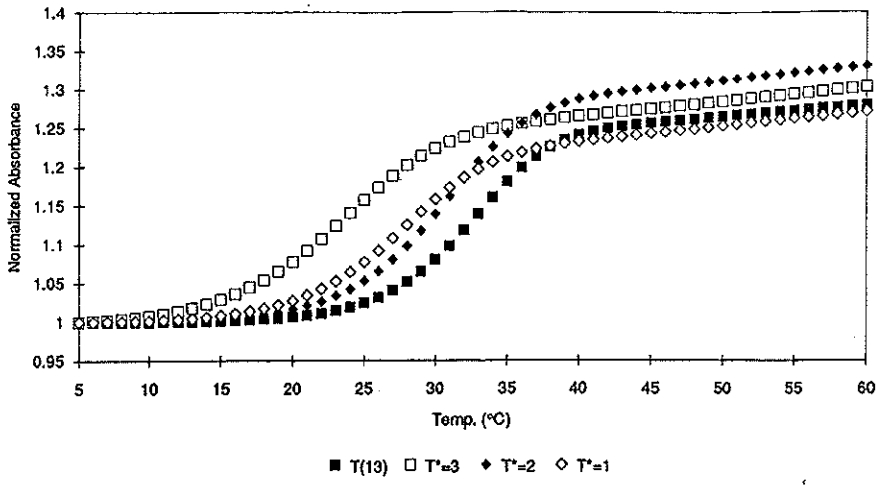


Figure 2. Normalized melting curves of T*T*T(9)T*T*.dA(13).

affording 680 mg (1.22 mmol, 99%) of the title compound.
UV (MeOH) λ_{max} = 267 nm (log ϵ = 3.97)
^{1}H NMR ($CDCl_3$ + D_2O) δ 1.50–2.25 (m, 4H, H-2′, H-4′), 1.94 (s, 3H, CH_3), 2.95–3.30 (m, 2H, H-6′), 3.77 (s, 6H, OCH_3), 4.20–4.60 (m, 2H, H-3′, H-5′), 6.14 (dd, 1H, J=10.1 Hz, H-1′), 6.80 (d, 4H, aromatic H), 7.15–7.50 (m, 1OH, H-6, aromatic H)ppm.
^{13}C NMR ($CDCl_3$) δ 12.4 (CH_3), 34.2, 37.8 (C-2′, C-4′), 55.0 (OCH_3), 63.9 (C-3′), 66.3 (C-6′), 72.7 (C-5′), 78.3 (C-1′), 85.7 (Ph_3C), 110.7 (C-5), 136.0 (C-6), 150.0 (C-2), 163.6 (C-4)ppm + trityl signals.

[1-(2,4-dideoxy-6-*O*-dimethoxytrityl-β-D-*erythro*-hexopyranosyl)thymine]-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (5)

To a solution of 279 mg (0.5 mmol) of **4** in CH_2Cl_2 dry (2.5 mL) was added 265 μL (1.52 mmol) of *N*,*N*-diisopropylethylamine and 170 μL (0.76 mmol) of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite. After stirring this mixture for 4 h at room temperature under argon, it was diluted with CH_2Cl_2 and washed once with a sodium bicarbonate solution (5%) and three times with brine. The organic layer was dried, evaporated and purified by flash column chromatography (hexane-EtOAc-Et_3N 29:69:2) affording 255 mg (0.34 mmol, 68%) of the title compound as a white foam.
UV (MeOH) λ_{max} = 267 nm
^{1}H NMR ($CDCl_3$) δ 1.10–1.35 (m, 12H, $NCCH_3$), 1.45–2.20 (m, 4H, H-2′, H-4′), 1.95 (s, 3H, CH_3), 2.45–2.85 (m, 2H, CH_2CN), 3.05–3.30 (m, 2H, H-6′), 3.45–4.00 (m, 4H, NCH, $POCH_2$), 3.78 (s, 6H, OCH_3), 4.10–4.65 (m, 2H, H-3′, H-5′), 6.11 (dd, 1H, J=10.1 Hz, H-1′), 6.70–6.95 (m, 4H, aromatic H), 7.05–7.70 (m, 10H, H-6, aromatic H), 8.90 (br s, 1H, NH)ppm.
^{13}C NMR ($CDCl_3$) δ 12.4 (CH_3), 20.2 (d, J=7.3 Hz, $\underline{CH_2}CN$), 24.5 (d, J=7.3Hz, $NC\underline{CH_3}$), 33.6, 37.0 (C-2′, C-4′), 43.3 (d, J=13.5 Hz, $N\underline{CH}$), 55.0 (OCH_3), 58.2 (m, $PO\underline{CH_2}$), 66.2 (C-6′), 67.2 (d, J=18.3 Hz, C-3′), 72.9 (C-5′), 78.3 (C-1′), 85.8 (Ph_3C), 110.6 (C-5), 117.3, 117.7 (CN), 135.4 (C-6), 149.7 (d, C-2), 163.4 (C-4)ppm + trityl signals.
^{31}P NMR ($CDCl_3$) δ 147.1 and 147.4 (2×s)

1-(2,4-dideoxy-6-*O*-dimethoxytrityl-3-*O*-succinyl-β-D-*erythro*-hexopyranosyl)thymine (6)

A mixture of 112 mg (200 μmol) of **4**, 12 mg (100 μmol) of 4-dimethylaminopyridine (DMAP) and 60 mg (600 μmol) of succinic anhydride in pyridine dry (2 mL) was stirred at room temperature for one week, followed by evaporation and coevaporation with toluene (three times). The resulting residue was dissolved in CH_2Cl_2 and washed with brine (3×) and water. The organic layer was dried and evaporated affording 96 mg (146 μmol, 73%) of the title compound as a white foam.
UV (MeOH) λ_{max} = 267 nm (log ϵ = 4.04)
^{1}H NMR ($CDCl_3$) δ 1.55–2.45 (m, 4H, H-2′, H-4′), 1.95 (s, 3H, CH_3), 2.70 (s, 4H, CH_2CH_2C=O), 3.05–3.30 (m, 2H, H-6′), 3.78 (s, 6H, OCH_3), 4.00–4.35 (m, 1H, H-5′), 5.40 (m, 1H, H-3′), 5.96 (dd, 1H, J=2.0 and 10.8 Hz, H-1′), 6.80 (d, 4H, aromatic H), 7.10–7.55 (m, 10H, H-6, aromatic H)ppm.
^{13}C NMR ($CDCl_3$) δ 12.5 (CH_3), 29.1, 29.9 ($\underline{CH_2}\underline{CH_2}C$=O), 31.5, 34.5 (C-2′,C-4′), 55.0 (OCH_3), 65.9 (C-6′), 67.3 (C-3′), 73.2 (C-5′), 78.4 (C-1′), 85.9 (Ph_3C), 111.5 (C-5), 135.2 (C-6), 150.7 (C-2), 163.8 (C-4), 170.8, 174.4 (C=O)ppm + trityl signals.

1-*O*-Acetyl-2,6-di-*O*-benzoyl-3,4-dideoxy-D-*erythro*-hexopyranose (α and β anomer) (10)

3.15 g (8.51 mmol) of methyl 2,6-di-*O*-benzoyl-3,4-dideoxy-α-D-*erythro*-hexopyranoside (**9**) was dissolved in a mixture of acetic acid (7 mL) and acetic anhydride (1.7 mL). The resulting solution was cooled in an ice-bath and 1.1 mL of concentrated sulfuric acid was added dropwise. After stirring for 24 h at room temperature 3.38 g of sodium acetate was added. This mixture was kept for 0.5 h at room temperature, evaporated and dissolved in diethyl ether (200 mL). The solution was washed with H_2O (200 mL) and saturated sodium bicarbonate solution (2×200 mL). The combined aqueous layer was extracted with diethyl ether. The combined organic layer was dried, evaporated and purified by column chromatography (hexane—EtOAc 8:2) yielding 2.20 g (5.53 mmol, 65%) of the title compound as a mixture of the two anomers.
^{1}H NMR ($CDCl_3$) δ 1.60–2.60 (m, 7H, CH_3, H-3, H-4), 3.90–4.55 (m, 3H, H-5, H-6), 4.85–5.35 (m, 1H, H-2), 5.96

(m, 0.35H, H-1), 6.43 (m, 0.65H, H-1), 7.25–7.75 (m, 6H, aromatic H), 7.90–8.20 (m, 4H, aromatic H)ppm.
^{13}C NMR ($CDCl_3$) δ 20.8, 22.9 (CH_3), 25.7, 26.1, 27.0 (C-3, C-4), 65.8, 66.1, 68.5, 69.0, 70.1, 74.3 (C-2, C-5, C-6), 89.7, 93.6 (C-1), 128.2, 129.5, 133.0 (aromatic), 165.3, 166.1, 168.8, 169.0 (C=O)ppm.

1-(2,6-di-*O*-benzoyl-3,4-dideoxy-β-D-*erythro*-hexopyranosyl) thymine (11)

A mixture of 1.59 g (4 mmol) of **10**, silylated thymine (4.8 mmol) and 655 μL (5.6 mmol) of $SnCl_4$ in 40 mL of acetonitrile dry was stirred at 40°C for 1.5 h. The resulting mixture was diluted with CH_2Cl_2 (50 mL) and washed with saturated sodium bicarbonate solution (100 mL). The organic layer was dried, evaporated and purified by column chromatography [(1) CH_2Cl_2, (2) CH_2Cl_2-MeOH 99:1] affording 1.63 g (3.51 mmol, 88%) of the title compound.
UV (MeOH) λ_{max} = 266 nm (log ε = 4.02)
CIMS (NH_3): 465 (MH^+), 482 (MNH_4^+)
^{1}H NMR ($CDCl_3$) δ 1.50–2.65 (m, 4H, H-3′, H-4′), 1.88 (s, 3H, CH_3), 4.00–4.30 (m, 1H, H-5′), 4.42 (d, 2H, J=4.8 Hz, H-6′), 4.85–5.20 (m, 1H, H-2′), 5.98 (d, 1H, J=9.2 Hz, H-1′), 7.19 (s, 1H, H-6), 7.30–7.70 (m, 6H, aromatic H), 7.85–8.15 (m, 4H, aromatic H), 9.15 (br s, 1H, NH)ppm.
^{13}C NMR ($CDCl_3$) δ 12.2 (CH_3), 26.5, 28.2 (C-3′, C-4′), 65.8 (C-6′), 69.6 (C-2′), 76.3 (C-5′), 82.2 (C-1′), 111.5 (C-5), 128.3, 129.6, 133.1, 133.3 (aromatic), 134.8 (C-6), 150.5 (C-2), 163.1 (C-4), 165.4, 166.1 (C=O)ppm.

1-(3,4-dideoxy-β-D-*erythro*-hexopyranosyl)thymine (3)

A solution of 1.63 g (3.51 mmol) of **11** in methanolic ammonia (100 mL) was kept for 3 days at room temperature. After evaporation and purification by column chromatography (CH_2Cl_2-MeOH 90:10) 0.90 g (3.51 mmol, 100%) of the title compound was obtained.
mp: 136-140°C
UV (MeOH) λ_{max} = 266 nm (log ε = 3.96); (0.01N NaOH) λ_{max} = 266 nm (log 1 = 3.78)
CIMS (NH_3): 257 (MH^+), 274 (MNH_4^+)
^{1}H NMR (DMSO-d_6) δ 1.15–2.20 (m, 4H, H-3′, H-4′), 1.80 (s, 3H, CH_3), 3.25–3.80 (m, 4H, H-2′, H-5′, H-6′), 4.66 (t, 1H, J=5.3 Hz, 6′OH), 5.10 (d, 1H, J=4.9 Hz, 2′OH), 5.26 (d, 1H, J=8.8 Hz, H-1′), 7.51 (s, 1H, H-6), 11.20 (br s, 1H, NH)ppm.
^{13}C NMR (DMSO-d_6) δ 12.0 (CH_3), 26.5, 31.4 (C-3′, C-4′), 63.7 (C-6′), 66.2 (C-2′), 78.7 (C-5′), 84.9 (C-1′), 109.2 (C-5), 136.8 (C-6), 151.0 (C-2), 163.7 (C-4)ppm.
Anal. Calcd. for $C_{11}H_{16}N_2O_5$. 1/4 H_2O: C, 50.67; H, 6.38; N, 10.74.
Found: C, 50.67; H, 6.36; N, 10.48.

1-(3,4-dideoxy-6-*O*-dimethoxytrityl-β-D-*erythro*-hexopyranosyl)thymine (12)

Starting with 512 mg (2 mmol) of **3**, the title compound (967 mg, 1.73 mmol, 87%) was obtained following the same procedure as for **4**.
UV λ_{max} = 267 nm (log ε = 4.04)
^{1}H NMR ($CDCl_3$ + D_2O) δ 1.45–2.40 (m, 4H, H-3′, H-4′), 1.86 (s, 3H, CH_3), 2.85–3.35 (m, 2H, H-6′), 3.65–4.20 (m, 2H, H-2′, H-5′), 3.74 (s, 6H, OCH_3), 5.61 (d, 1H, J=8.8 Hz, H-1′), 6.78 (d, 4H, aromatic H), 7.05–7.60 (m, 10H, H-6, aromatic H)ppm.
^{13}C NMR ($CDCl_3$) δ 12.2 (CH_3), 27.6, 31.0 (C-3′, C-4′), 54.9 (OCH_3), 65.7 (C-6′), 69.0 (C-2′), 77.3 (C-5′), 85.1 (C-1′), 85.7 (Ph_3C), 110.6 (C-5), 135.8 (C-6), 151.4 (C-2), 164.0 (C-4)ppm + trityl signals.

[1-(3,4-dideoxy-6-*O*-dimethoxytrityl-β-D-*erythro*-hexopyranosyl)thymine]-2′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (13)

Starting with 279 mg (0.5 mmol) of **12**, the title compound (314 mg, 0.41 mmol, 82%) was obtained following the same procedure as for **5**.
UV (MeOH) λ_{max} = 266 nm
^{1}H NMR ($CDCl_3$) δ 1.00–1.30 (m, 12H, $NCCH_3$), 1.50–2.10 (m, 4H, H-3′, H-4′), 1.96 (s, 3H, CH_3), 2.45–2.70 (m, 2H, CH_2CN), 2.90–4.00 (m, 8H, H-2′, H-5′, H-6′, NCH, $POCH_2$), 3.77 (s, 6H, OCH_3), 5.63 (d, 1H, J=7.9 Hz, H-1′), 6.70–6.95 (m, 4H, aromatic H), 7.10–7.55 (m, 10H, H-6, aromatic H), 8.75 (br s, 1H, NH)ppm.
^{13}C NMR ($CDCl_3$) δ 12.2 (CH_3), 20.1, 20.4 ($\underline{CH_2}CN$), 24.4 ($NC\underline{CH_3}$), 27.5, 30.8 (C-3′, C-4′), 43.0, 43.2 (2×d, J=12.2 Hz, $N\underline{CH}$), 55.0 (OCH_3), 57.2, 58.1 (2×d, J=14.7 Hz, $PO\underline{CH_2}$), 65.7 (C-6′), 70.0 (d, J=11.0 Hz) and 71.2 (d, J=14.6 Hz) (C-2′), 77.5 (C-5′), 83.9 (C-1′), 85.9 (Ph_3C), 110.6 (C-5), 117.5 (CN), 135.6 (C-6), 150.5 (C-2), 163.3 (C-4)ppm + trityl signals.
^{31}P NMR ($CDCl_3$) δ 147.1 and 148.9 (2×s)

1-(3,4-dideoxy-6-*O*-dimethoxytrityl-2-*O*-succinyl-β-D-*erythro*-hexo-pyranosyl)thymine (14)

Starting with 112 mg (0.2 mmol) of **12**, the title compound (94 mg, 0.14 mmol, 70%) was obtained following the same procedure as for **6**.
UV λ_{max} = 266 nm (log ε = 4.04)
^{1}H NMR ($CDCl_3$) δ 1.50–2.35, (m, 4H, H-3′, H-4′), 1.93 (s, 3H, CH_3), 2.97 (s, 4H, $CH_2\underline{CH_2}C{=}O$), 3.00–3.35 (m, 2H, H-6′), 3.70–3.95 (m, 1H, H-5′), 3.78 (s, 6H, OCH_3), 4.70–5.00 (m, 1H, H-2′), 5.73 (d, 1H, J=9.3 Hz, H-1′), 6.79 (d, 4H, aromatic H), 7.15–7.50 (m, 10H, H-6, aromatic H)ppm.
^{13}C NMR ($CDCl_3$) δ 12.4 (CH_3), 27.1, 28.3, 28.5, 29.1 (C-3′, C-4′, $\underline{CH_2CH_2}C{=}O$), 55.0 ($OCH_3$), 65.5 (C-6′), 69.2 (C-2′), 77.6 (C-5′), 82.1 (C-1′), 85.9 (Ph_3C), 111.2 (C-5), 135.6 (C-6), 151.1 (C-2), 164.6 (C-4), 171.0, 175.5 (C=O)ppm + trityl signals.

Preparation of the solid supports (*7* and *15*)

A mixture of 53 mg (80 μmol) of the succinates (**6** or **14**), 400 mg of pre-activated LCAA-CPG[13], 5 mg of DMAP, 32 μl of Et_3N and 153 mg (800 μmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide. HCl in 4 mL of pyridine dry was sonicated for a few minutes, and shaken at room temperature for 16 h. After shaking, the solid support was filtered off and washed successively with pyridine, methanol and CH_2Cl_2 followed by drying under vacuum. The unreacted sites on the surface of the support were capped using 1.5 mL of 1-methylimidazole in THF (Applied Biosystems) and 1.5 mL of acetic anhydride in lutidine—THF 1:1:8 (Applied Biosystems). After shaking for 4 h at room temperature, the solid support was filtered off, washed with CH_2Cl_2 and dried under vacuum. Colorimetric dimethoxytrityl analysis[14] indicated a loading of 26.2 μmol/g for **7** and 28.8 μmol/g for **15**.

DNA synthesis

Oligonucleotide synthesis was performed on an Applied Biosystems DNA synthesizer model 381A using the phosphoramidite approach (1 μmol scale, end dimethoxytrityl off). The obtained sequences were deprotected and cleaved from the solid support by treatment with concentrated ammonia (3×30min) at room temperature. After prepurification on a NAP-10^R column (Sephadex G25-DNA grade, Pharmacia), eluted with buffer A (see below), purification was done on a Mono Q^R HR 5/5 anion exchange column (Pharmacia) with the following gradient system [A:10 mM NaOH pH 12.0; 0.1 M NaCl−B:10 mM NaOH pH 12.0; 0.9 M NaCl—60 till 80% in 40 min, (except for **21**, 30 till 60% B in 40 min, and for **22**, **24**, **25**, 60 till 75% B in 40 min) flow rate = 1 mL/min]. Then the solution was desalted on a NAP-10^R column (Pharmacia), followed by lyophilisation of the solvent.

Enzymatic degradation of the oligonucleotides (17-20, 22-25)

A solution of the oligonucleotide (0.2 OD) in 400 μL of the following buffer [50 mM Tris.HCl pH = 8.6; 50 mM sodium chloride; 7 mM $MgCl_2$] was digested with 1.2 U snake venom phosphodiesterase (Pharmacia) [=2.4 μl of a solution of the enzyme in the following buffer: 5 mM Tris.HCl pH = 7.5; 50% glycerol (v/v)] at 37°C for 16 h. This digestion was followed by treatment with 1.2 U alkaline phosphatase (Boehringer Mannheim) for 4 h at 37°C. Aliquots of this solution (=30 μL) were analyzed by HPLC. All oligonucleotides showed the correct ratio of unmodified nucleoside over modified nucleoside.

HPLC analysis. Reverse phase HPLC on an analytical Zorbax C-8 column was performed using a Merck-Hitachi L-6200 intelligent pump. For **17**−**20** isocratic elution was performed with 5% MeOH in 0.1 M triethylammonium acetate (flow rate = 1 mL/min). The elution time was 6.5 min and 10.0 min for thymidine and **2**, respectively. For **22**−**25** the column was eluted with 0.5% MeOH in 0.1 M triethylammonium acetate (flow rate = 1 mL/min). The elution time was 8.4 min and 9.6 min for **3** and thymidine respectively. The signals were integrated with a Hewlett-Packard 3390A integrator.

Melting temperatures

Melting curves were measured and evaluated in exactly the same way as described in ref. 10.

Enzymatic stability of the oligonucleotides

A solution of the oligonucleotide (1 OD) in 2.5 mL of the following buffer [0.1 M Tris.HCl pH = 8.6; 0.1 M sodium chloride; 14 mM $MgCl_2$] was digested with 0.1 U snake venom phosphodiesterase [= 2 μL of a solution of the enzyme in the following buffer: 5 mM Tris.HCl pH = 7.5; 50% glycerol (v/v)] at 37°C. During digestion the increase in absorbance at 260 nm was followed. The absorption versus time curve of the digestion was fitted to a simple exponential. This resulted in a half-life of around 2 min for the unmodified T_{13}. Digestion of T_{13} was repeated every time as a control, and the half-lifes indicated in Table 1 are ratios of modified oligonucleotide over T_{13}.

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REFERENCES

1. a) Uhlmann, E. and Peyman, A. (1990) *Chemical Reviews*, **90**, 543−584. b) Cohen, J.S. (1989) Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. The Macmillan Press LTD, London.
2. Rayner, B., Malvy, C., Paoletti, J., Lebleu, B., Paoletti, C. and Imbach, J.-L. (1989) Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression. The Macmillan Press LTD, London, pp. 119−136.
3. Debart, F., Rayner, B., Degols, G. and Imbach, J.-L. (1992) *Nucleic Acids Res.*, **20**, 1193−1200.
4. Sproat, B.S., Beijer, B. and Iribarren, A. (1990) *Nucleic Acids Res.*, **18**, 41−49.
5. Iribarren, A.M., Sproat, B.S., Neuner, P., Sulston, I., Ryder, U. and Lamond, A.I. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7747−7751.
6. a) Perbost, M., Lucas, M., Chavis, C., Pompon, A., Baumgartner, H., Rayner, B., Griengl, H. and Imbach, J.-L. (1989) *Biochem. Biophys. Res. Comm.*, **165**, 742−747. b) Sagi, J., Szemzo, A., Szecsi, J. and Otvos, L. (1990) *Nucleic Acids Res*, **18**, 2133−2140.
7. Rosemeyer, H., Krecmerova, M. and Seela, F. (1991) *Helv. Chim. Acta*, **74**, 2054−2067.
8. a) Weller, D.D., Daly, D.T., Olson, W.K. and Summerton, J.E. (1991) *J. Org. Chem.*, **56**, 6000−6006. b) Nielsen, P.E., Egholm, M., Berg, R.H. and Buchardt, O. (1991) *Science*, **254**, 1497−1500.
9. Stirchak, E.P., Summerton, J.E. and Weller, D.D. (1989) *Nucleic Acids Res.*, **17**, 6129−6141.
10. Augustyns, K., Van Aerschot, A., Urbanke, C. and Herdewijn, P. (1992) *Bull. Soc. Chim. Belg.*, **101**, 119−130.
11. a) Eschenmoser, A. (1991) *Nachr. Chem. Tech. Lab.*, **39**, 795−807. b) Eschenmoser, A. and Dobler, M. (1992) *Helv. Chim. Acta*, **75**, 218−259.
12. Augustyns, K., Van Aerschot, A. and Herdewijn, P., *Bioorg. Med. Chem. Letters*, in press.
13. Pon, R.T., Usman, N. and Ogilvie, K.K. (1988) *BioTechniques*, **6**, 768−775.
14. User Bulletin, Applied Biosystems, 1 April 1987, issue no. 13, revised.
15. Ogawa, T. and Matsui, M. (1981) *Tetrahedron*, **37**, 2363−2369.
16. Garegg, P.J. and Samuelsson, B. (1979) *Synthesis*, 813−814.
17. Garegg, P.J., Johansson, R. and Samuelsson, B. (1984) *J. Carbohydr. Chem.*, **3**, 189−195.
18. Vorbrüggen, H., Krolikiewicz, K., Bennua, B. (1981) *Chem. Ber.*, **114**, 1234−1255.
19. a) Beaucage, S.L. and Caruthers, M.H. (1981) *Tetrahedron Lett.*, **22**, 1859−1862. b) Sinha, N.D., Biernat, J. and Köster, H. (1983) *Tetrahedron Lett.*, **24**, 5843−5846.
20. Riesner, D. and Römer, R. (1973) Duchesne, J. (ed.), Physicochemical Properties of Nucleic Acids. Academic Press, London, Vol. II, pp. 237−318.
21. Breslauer, K.J., Frank, R., Blöcker, H. and Marky, L.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3746−3750.
22. Aboul-ela, F., Koh, D. and Tinoco, Jr. I. (1985) *Nucleic Acids Res.*, **13**, 4811−4824.