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Bioorganic & Medicinal Chemistry Letters
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Bioorganic & Medicinal Chemistry Letters 14 (2004) 581–584

Synthesis and incorporation of an α-hexofuranosyl thymidine into oligodeoxynucleotides via its two exocyclic OH-groups

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Received 3 October 2003; revised 14 November 2003; accepted 28 November 2003

Abstract—1-(2,3-Dideoxy-3-amino-α-D-*arabino*-hexofuranosyl)thymine is considered as a conformationally restricted acyclic nucleoside using the furanose ring to link the diol backbone to the nucleobase. The appropriately substituted phosphoramidites were synthesised via 1-(5,6-di-*O*-acetyl-2,3-dideoxy-3-phthalimido-α-D-*arabino*-hexofuranosyl)thymine and used in oligodeoxynucleotide (ODN) synthesis. However, the binding affinity of the mixed ODNs towards complementary DNA and RNA was decreased compared to the wild-type oligos. The decrease was smaller when the monomer was inserted near the end of the sequence. The insertions into an α T sequence or in a β T sequence gave nearly the same dropping in melting temperature per modification which indicates that the new nucleotide modifications behave both as α and β nucleotides.

In recent years, much effort has been put into the design and synthesis of new oligodeoxynucleotides (ODNs) in order to improve the hybridisation affinity towards complementary DNA or RNA sequences.[1] Since the discovery of PNA (peptide nucleic acid, Fig. 1), constructed from 2-aminoethyl glycine units to which nucleobases are attached by methylenecarbonyl linkers,[2] a considerable number of ODNs having acyclic DNA backbone have been synthesised.[3] Usually ODNs possessing an acyclic moiety bind poorly to DNA or RNA due to loss in entropy upon duplex formation. However, rigid carboxamide groups in PNA improve the hybridisation properties. Hexofuranosyl nucleotides, by insertions into ODNs via the two exocyclic OH-groups, are here considered as conformationally restricted acyclonucleotides constrained by the furanose ring between the nucleobase and the ethyleneglycol backbone. Previously, this type of nucleosides have been synthesised and investigated as potential anticancer and anti-viral agents.[4] In oligonucleotide chemistry hexofuranosyl analogues were regarded as branched ODNs possessing free 5′-C-hydroxymethyl group that could be used for conjugation reactions.[5]

In this investigation we decided to incorporate 1-(3-amino-2,3-dideoxy-α-D-*arabino*-hexofuranosyl)thymine into oligonucleotide sequences in order to evaluate the thermal stability of ODN/DNA and ODN/RNA duplexes. The positively charged amino group is expected to neutralise the electrostatic repulsion between phosphodiester backbones and thus lead to better binding properties. Due to free rotation around C-4′–C-5′ bond, the hexofuranosyl nucleosides can exist in two conformations **1** and **1′** corresponding to α and β anomers, respectively, of natural nucleosides (Fig. 2). Therefore, oligos from this type of nucleosides may have the advantage like PNA in forming duplexes

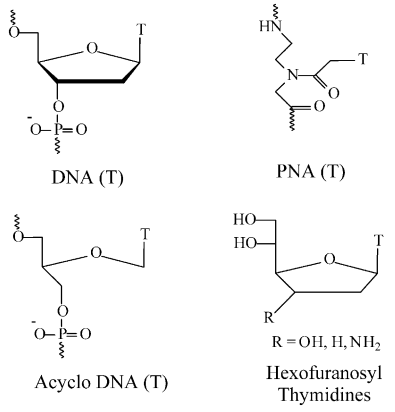

Figure 1.

Keywords: Hexofuranosyl nucleoside; DNA; Backbone modification; Duplex stability; Exocyclic DNA backbone.
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† A research center funded by The Danish National Research Foundation for studies on nucleic acid chemical biology.

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doi:10.1016/j.bmcl.2003.11.075

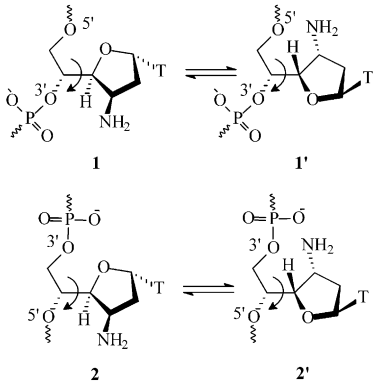


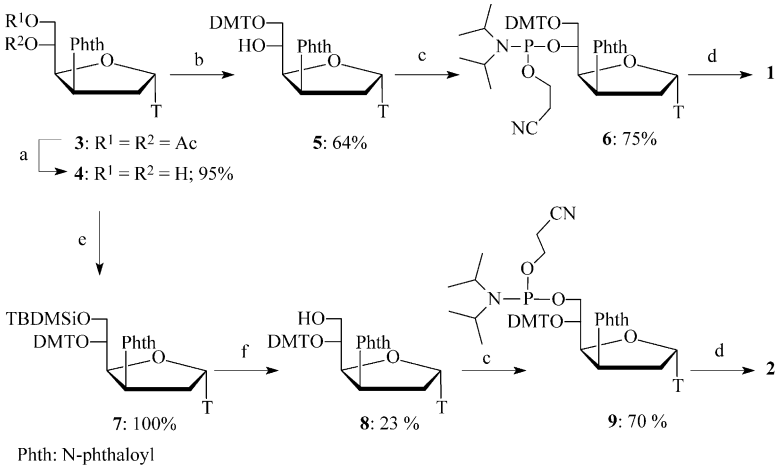
Figure 2.

simulating both β and α anomers. To put it another way, this type of oligos may hybridise like PNA, both in a parallel and an antiparallel fashion. This property seems to be a prerequisite for strand invasion.[2b] Another feature, the primary OH-group of the hexofuranosyl nucleoside can be considered as the 5′-OH group of natural occurring nucleosides as in **1** or the nucleoside can be reverted so the same OH-group is considered as the 3′-OH as in **2** (Fig. 2 and Scheme 1).

The synthesis of target building blocks **6** and **9** started from previously published 1-(5,6-di-*O*-acetyl-2,3-dideoxy-3-phtalimido-α-D-*arabino*-hexofuranosyl)thymine (**3**)[4a] that was selectively deprotected in the refluxing mixture of Et_3N/MeOH to give **4** in 95% yield.[6] The primary alcohol **4** was treated with an excess of DMTCl in pyridine with further purification on a silica gel column affording compound **5** in 64% yield. The final phosphoramidite **6** was obtained as a mixture of two diastereoisomers (3:4) by treatment with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite in the presence of *N*,*N*-diisopropylammonium tetrazolide in 75% yield. To incorporate compound **4** into ODN in the opposite direction, nucleoside **4** was reacted with *tert*-butyldimethylsilyl chloride (TBDMSiCl) in pyridine followed by treatment with DMTCl in the presence of $AgNO_3$ and pyridine in anhydrous THF for 2 days. The latter procedure was used because the standard DMTCl/pyridine procedure failed. Desilylation using tetra-*n*-butylammonium fluoride (TBAF) in THF gave compound **8** in 23% yield after 2 days of stirring at room temperature and at 50 °C overnight. The low yield observed here is probably due to competitive partial deprotection of *N*-phtaloyl group by the nucleophilic fluoride ion. Phosphitylation of **8** afforded a diastereomeric mixture (1:1) of the phosphoramidite **9** in 70% yield after silica gel column purification.

Oligonucleotides were synthesised by the solid phase phosphoramidite method on an automated Pharmacia Gene Assembler™ Special synthesiser in 0.2 μmol-scale (7.5 μmol embedded per cycle, Pharmacia primer support™) using an increased coupling time (10 min) for compound **6** and **9** compared to 2 min couplings for commercial phosphoramidites. The coupling efficiency for both monomers using 3,5-dicyanoimidazole as activator was only 70–80% and was not improved by prolonged or repeated couplings. The 5′-DMT-on derivatives were cleaved off from the solid support (rt, 3 h) and deprotected (60 °C, overnight) using 32% aqueous ammonia for standard support or the solution of 2% LiCl in 32% aqueous ammonia (w/w) when using universal support for α nucleotides. The purification of ODNs was performed on a preparative HPLC (Waters Delta Prep 4000) using an Xterra2 column,[7] followed by removal of DMT (80% AcOH) with further precipitation from ethanol. However for **ODN12**–**ODN15** (Table 1) a second HPLC purification was needed due to failure of the precipitation procedure. In this case the volatile salts were removed by several co-evaporations with water.[7] The removal of *N*-phthaloyl group during standard oligonucleotide deprotection was confirmed by MALDI-TOF analysis for the final ODNs (Table 1).

The hybridisation properties of the modified ODNs towards complementary DNA and RNA single strands were evaluated by thermal denaturation studies. The melting temperature (T_m) as a first derivative of the melting curve and the differences between modified and unmodified oligomers as the change in melting



Scheme 1. Reagents and conditions: (a) Et_3N, MeOH, reflux, 4 h; (b) DMTCl, Py, rt, overnight; (c) $NC(CH_2)_2OP(NPr^i_2)_2$, *N*,*N*-diisopropylammonium tetrazolide, CH_2Cl_2; (d) DNA synthesizer; (e) (i) TBDMSiCl, Py, 0 °C, overnight; (ii) DMTCl, $AgNO_3$, Py, THF, rt, 2 days; (f) Bu_4NF, THF, rt, 2 days, then 50 °C, overnight.

Table 1. Oligodeoxynucleotides synthesised, melting experiments, calculated and experimental masses of ODNs from MALDI-TOF MS[a]

No.	Sequence	T_m (°C) (ODN/DNA)	ΔT_m (C)	T_m (°C) (ODN/RNA)	ΔT_m (°C)	m/z, calc. (Da)	m/z, found (Da)
ODN1	5′-GCTTCTCTAGAATG	42.6[b]		43.8[c]			
ODN2	5′-GCTTC**1**CTAGAATG	33.7	−8.9	36.9	−6.9	4281.03	4280.59
ODN3	5′-GCTTCTC**1**AGAATG	35.3	−7.3	37.7	−6.1	4281.03	4281.28
ODN4	5′-GCTTC**1**C**1**AGAATG	27.6	−7.5	25.3	−9.3	4310.06	4309.10
ODN5	5′-GCT**1**CTCTAGAATG	34.4	−8.2	37.6	−6.2	4281.03	4279.91
ODN6	5′-GC**11**CTCTAGAATG	25.2	−8.7	29.5	−7.2	4310.06	4311.91
ODN7	5′-GCTTCTCTAGAA**1**G	39.1	−3.5	42.0	−1.8	4281.03	4280.07
ODN8	5′-GCTTC**2**CTAGAATG	29.1	−13.5	28.6	−15.2	4281.03	4280.38
ODN9	5′-GCTTCTC**2**AGAATG	31.5	−11.1	29.6	−14.2	4281.03	4282.38
ODN10	dT14	31.8[d]		28.3[e]			
ODN11	αdT14	32.0		42.5			
ODN12	5′-TTTT**1**T**1**TTTTTTT	<10		10.6	−8.9	4253.06	4253.55
ODN13	5′-TTTT**2**T**2**TTTTTT	<10		10.3	−9.0	4253.06	4252.11
ODN14	α 5′-(TTTT**1**T**1**TTTTTT)	13.7	−9.2	22.5	−10.0	4253.06	4253.31
ODN15	α 5′-(TTTT**2**T**2**TTTTTT)	<10		<10		4253.06	4253.65

		T_m (°C) (ODN/ 3′−CGAAGAG***C***TCTTAC)	ΔT_m (C)	
ODN16	5′-GCTTCTCTAGAATG	32.2	−10.4	**ODN1** as references
ODN17	5′-GCTTCTC**1**AGAATG	30.3	−5.0	**ODN3** as references
ODN18	5′-GCTTCTC**2**AGAATG	28.2	−3.3	**ODN9** as references

[a] T_m was determined by measuring absorbance at 260 nm against increasing temperature (1.0 °C) on equimolar mixtures (1.5 μM in each strand) of modified oligomer and its complementary DNA/RNA and single mismatched DNA in medium salt buffer (10 mM NaH_2PO_4, 100 mM NaCl, 0.1 mM EDTA, pH 7.00); **1** and **2** are explained in the text; ΔT_m = change in T_m per modification.
[b] Target: 3′-CGAAGAGATCTTAC.
[c] Target: 3′-CGAAGAGAUCUUAC.
[d] Target: dA_{14}.
[e] Target: rA_{14}.

temperature per modification (ΔT_m) are listed in Table 1.

The thermal melting data showed that insertion of **1** in the central region of a mixed oligomer sequence decreased the T_m significantly (ΔT_m −7.0 to −9.0 °C), and that the decrease was smaller (ΔT_m −2.0 to −3.5 °C) when **1** was situated near the end. This is in agreement with previous studies for acyclonucleotide systems.[8–11] The insertion of **2** led to a more dramatic decrease in T_m (ΔT_m −11.0 to −15.0 °C) when compared with the nucleotide **1**. The fully modified oligomer gave no melting neither towards DNA nor RNA. The difference in ΔT_m between matched and a C/T mismatched duplexes was larger for insertion of **1** than for **2** (**ODN16**–**ODN18**). The destabilisation effect observed in the case of **2** is in the same order of magnitude as previously observed for the ethyleneglycol linker.[12] The consequence is that in case of incorporation **2** we have very little base pairing and it could as well be considered as a mismatch. However, insertions of the α-nucleoside analogue **1** gave ODNs, where base pairing is more likely as evaluated from mismatched meltings. Only small differences in binding abilities to DNA and RNA complements were observed. To evaluate further the possible rotation around C-4′–C-5′ bond we synthesised ODNs possessing incorporation as next-nearest neighbours of **1** and **2** either in d(β T)$_{14}$ (**ODN12**, **ODN13**, Table 1) or in d(α T)$_{14}$ (**ODN14**, **ODN15**). In both cases decreases in T_m were observed. In the case of hybridisation with RNA, melting data could be determined for the nucleoside **1** which could allow comparison of incorporation into an α T sequence with incorporation into a β T sequence. Per modification, the drop in melting temperature was nearly the same whatever incorporated in an α T sequence or in a β T sequence. This may indicate that the new nucleoside modification behave both as α and β nucleoside.

The proposed system **1** having relatively short ethyleneglycol backbone and thymine situated in the α-position of the furanose ring was not diminished the hybridisation properties larger than other acyclic nucleotide analogues, apart from PNA.[8] Thus an average ΔT_m of −6.0 to −10.5 °C has been found for incorporation into central regions of oligomers summarised by Nielsen et al.[8] and acyclo DNA (T) (Fig. 1) being the worst one with ΔT_m of −13.4 °C.[9] The conformational restriction by C=C bond decreased the binding towards DNA and RNA from ΔT_m of −2.0 °C in the end of the duplex to ΔT_m of −5.5 °C in the middle of the duplex.[10] ODNs with (*N*-thymin-1-ylacetyl)-1-arylserinol backbone has been found quite efficient in their binding capacity towards DNA duplex and triplex structures, but the dropping ΔT_m of −9.0 °C has also been observed when the monomer has been inserted in the middle of the sequence.[11] The optimum flexibility and rigidity of the sugar ring and phosphodiester backbone in conjunction with the proper position of the base moieties is a key factor in the modeling of new DNA/RNA analogues. When this is combined by the above findings of incorporation of **1** into α and β T sequences we still think it is possible to develop new nucleotides which can give interesting hybridisation properties for incorporation in both α and β nucleotide sequences. The perspective for the fully modified sequence is the

hybridisation both in a parallel and in an antiparallel fashion, but the design of a proper phosphodiester backbone linker is still needed to be found.

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6. Selected data for new compounds. 1-(2,3-Dideoxy-6-*O*-(4,4′-dimethoxytrityl)-3-phthalimido-α-D-*arabino*-hexofuranosyl)thymine (**5**): R_f 0.40 (5% MeOH/$CHCl_3$). NMR ($CDCl_3$): δ_H 1.80 (3H, s, CH_3), 2.60 (1H, br.s., OH), 2.75 (1H, m, H-2′α), 2.95 (1H, m, H-2′β), 3.32 (2H, m, H-6′), 3.50 (1H, m, H-5′), 3.80 (6H, s, OCH_3), 4.72 (1H, dd, $J=5.8$ and 3.6, H-3′), 5.28 (1H, t, $J=6.6$, H-4′), 6.34 (1H, t, $J=6.2$ Hz, H-1′), 6.70–6.80 and 7.00–7.50 (14H, m, DMT), 7.70–7.90 (5H, m, H-6, Phth), 8.80 (1H, s, NH). δ_C 12.3, 37.2, 51.3, 55.1, 55.2, 64.3, 69.5, 82.6, 86.2, 90.3, 110.3, 112.9, 113.1, 123.5, 123.7, 126.8, 127.7, 127.9, 128.1, 129.1, 129.9, 131.5, 134.2, 135.6, 135.9, 136.8, 139.5, 144.6, 149.7, 150.0, 158.5, 163.9, 168.3. UV-MALDI MS: 726.245 ($[M+Na]^+$ calcd 726.242). 1-{2,3-Dideoxy-6-*O*-[4,4′-dimethoxytrityl]-5-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-3-phthalimido-α-D-*arabino*-hexofuranosyl}thymine (**6**): R_f 0.62 (5% MeOH/$CHCl_3$). NMR ($CDCl_3$): δ_H 0.90 (6H, m, CH_3 [Pr^i]), 1.05 (6H, m, CH_3 [Pr^i]), 1.80 (3H, s, CH_3), 2.19 (2H, m, CH_2CN), 2.60–2.90 (4H, m, CH_2CH_2CN, H-2′), 3.20–3.60 (5H, m, H-6′, H-5′, CH [Pr^i]), 3.78 (6H, s, OCH_3), 5.00 (1H, m, H-3′), 5.23 (1H, m, H-4′), 6.40 (1H, m, H-1′), 6.70–6.80 and 7.00–7.50 (14H, m, DMT), 7.70–7.90 (5H, m, H-6, Phth), 8.60 (1H, br.s, NH). δ_P 150.6 (s) and 151.1 (s) in the ratio 3:4. UV-MALDI MS: 926.348 ($[M+Na]^+$ calcd 926.350). 1-[2,3-Dideoxy-6-*O*-(*tert*-butyltrimethylsilyl)-5-*O*-(4,4′-dimethoxytrityl)-3-phthalimido-α-D-*arabino*-hexofuranosyl]thymine (**7**): R_f 0.45 (5% MeOH/$CHCl_3$). NMR ($CDCl_3$): δ_H 0.06 (6H, s, $2\times CH_3$ [TBDMSi]), 0.98 (9H, s, t-C_4H_9), 2.09 (3H, s, CH_3), 2.79 (2H, m, H-2′), 3.50 (1H, dd, $J=2.6$, 11.0 Hz, H-6′), 3.75 (1H, dd, $J=3.6$, 11.0 Hz, H-6′), 3.90 (7H, m, OCH_3, H-5′), 4.98 (1H, t, $J=5.7$ Hz, H-4′), 5.40 (1H, m, H-3′), 6.62 (1H, t, $J=6.7$ Hz, H-1′), 6.70–6.80 and 7.00–7.50 (14H, m, DMT), 7.75–7.95 (5H, m, H-6, Phth), 8.54 (1H, s, NH). δ_C −5.4, −5.6, 12.5, 18.2, 25.9, 39.1, 51.2, 55.1, 55.2, 61.2, 73.2, 82.2, 86.0, 87.9, 110.5, 112.6, 113.1, 123.3, 123.7, 126.6, 127.3, 127.8, 128.6, 129.1, 130.6, 131.9, 134.0, 135.9, 136.0, 136.8, 139.5, 144.3, 149.9, 150.0, 158.3, 163.6, 167.7. UV-MALDI MS: 840.331 ($[M+Na]^+$ calcd 840.329). 1-{2,3-Dideoxy-6-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-5-*O*-[4,4′-dimethoxytrityl]-3-phthalimido-α-D-*arabino*-hexofuranosyl}thymine (**9**): R_f 0.35 (5% MeOH/$CHCl_3$). NMR ($CDCl_3$): δ_H 0.95 (6H, m, CH_3 [Pr^i]), 1.05 (6H, m, CH_3 [Pr^i]), 1.90 (3H, s, CH_3), 2.50–2.75 (6H, m, CH_2CH_2CN, H-2′), 3.25–3.60 (5H, m, H-6′, H-5′, CH [Pr^i]), 3.75 (6H, s, OCH_3), 4.70 (1H, m, H-3′), 5.20 (1H, m, H-4′), 6.40 (1H, m, H-1′), 6.50–6.70 and 7.00–7.50 (14H, m, DMT), 7.70–7.90 (5H, m, H-6, Phth), 8.05 (1H, br.s, NH). δ_P 146.4 (s) and 148.5 (s) in the ratio 1:1. UV-MALDI MS: 926.345 ($[M+Na]^+$ calcd 926.350).
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