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Oligonucleotides Containing a New Type of Acyclic, Achiral Nucleoside Analogue: 1-[3-Hydroxy-2-(hydroxymethyl)prop-1-enyl]thymine

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Abstract—An achiral, acyclic nucleoside analogue has been incorporated once or twice in oligodeoxyribonucleotides by the phosphoramidite method, and conditions found which allow deprotection of the oligonucleotides containing a sensitive modified allylic unit. The binding affinity of the modified oligonucleotides towards complementary DNA and RNA was reduced compared to unmodified DNA (ΔT_m −2 to −6.5 °C). An oligonucleotide with two modifications at the 3′-end showed considerable resistance towards cleavage with a 3′-exonuclease.

Oligonucleotides which are modified to be resistant to degradation by nucleases are promising therapeutic agents (antisense or antigene drugs).[1,2] The attraction of antisense drugs is their high selectivity for a complementary mRNA target based on the fidelity of the Watson–Crick hydrogen binding. Modified oligonucleotides have to fulfil several requirements in order to be useful antisense drugs: they should bind (hybridise) efficiently to RNA, be resistant to degradation, be soluble in water, be able to invade cells, and be able to stop the in vivo processing or translation of RNA. The last requirement may be fulfilled in several ways, for example, by physical blocking of splicing of RNA, physical blocking of initiation of translation of RNA, or by activating RNase H cleavage of RNA hybridised to the oligonucleotide.[3]

Many oligonucleotide modifications have been studied. These include modifications of the phosphate groups (e.g., phosphorothioates, methylphosphonates, and phosphoramidates);[3] of the (deoxy)ribose unit (e.g., 2′-substitution with *O*-alkyl or F,[2] 4′-substitution of *O* with S or CH_2,[4] and introduction of bicyclic derivatives of ribose, as in LNA[5]); and of the bases.[6] More radical modifications include oligonucleotides where the phosphate groups have been replaced (with e.g., amide, hydroxylamine, or formacetal groups),[3] the ribose has been opened to acyclic analogues,[7] or replaced with pyranose, cyclohexene or anhydrohexitol groups.[2] Even the whole sugar-phosphate backbone has been replaced, as in PNA and morpholino-DNA.[3] Oligonucleotides containing acyclic nucleoside analogues usually bind poorly to DNA and RNA due to losses in entropy upon duplex formation, caused by the flexibility of the acyclic units.[7] However, some acyclic analogues, notably PNA, are restricted conformationally by rigid amide linkages and hybridise well to DNA and RNA. The central amide bond in PNA has been replaced with a C=C bond, however with a detrimental effect on the binding properties.[8]

The nucleoside analogue studied in this paper, 1-[3-hydroxy-2-(hydroxymethyl)prop-1-enyl]thymine **1**[9] (Fig. 1) is an acyclic analogue conformationally restricted by a C=C bond. It was designed to mimic the natural nucleosides, and is radically different from PNA (or its C=C-analogue). According to models, **1** can exist in

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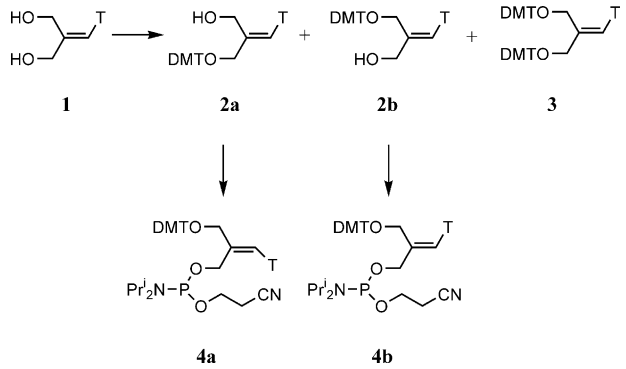


Figure 1.

conformations which positions the two hydroxy groups and the thymine group close to the positions of the 3′-hydroxy group, the 5′-hydroxy group, and thymine in both natural dsDNA (B-type helixes) and dsRNA (A-type helixes). Therefore **1** should be able to replace natural thymidines in both A- and B-type helixes without seriously disturbing the helical structures. The present paper describes our results on the preparation, by phosphoramidite synthesis, of oligodeoxyribonucleotides containing **1**, their ability to hybridise to RNA and DNA, and their resistance to cleavage by a 3′-exonuclease.

Phosphoramidites were prepared from **1**[9] in a standard way. The mono-DMT derivatives **2a** and **2b** were obtained each in 20–25% yield, together with the bis-DMT derivative **3**, from **1** and one equivalent of DMTCl in pyridine (Fig. 1). The three compounds were easily separated on a silica column, eluted with EtOAc–MeOH–Et_3N 98:1:1. The slowest moving compound was identified as the *Z*-isomer **2b** by NOE experiments. Phosphitylation with 2-cyanoethyl *N,N*-diisopropyl-phosphoramidochloridite gave the phosphoramidites **4a** or **4b** in 60–70% yield after purification by column chromatography and precipitation in hexane.[10]

Modified oligonucleotides were synthesised on a T-succinate LCAA CPG support using standard reagents (tetrazole activation, oxidation with Bu^{t}OOH, capping with NMI/Ac_2O/Lu, detritylation with CCl_3COOH).[11] Unwanted phosphitylation at the bases occurred to some degree with the very reactive **4a** and **4b**, but this was remedied by an aqueous wash before oxidation. Attempted oxidation with iodine/water cleaved the allylic C–O bond. This is known to occur for other phosphites with allylic or tertiary substituents.[12] Surprisingly, coupling of the next monomer upon the unit T^β derived from **4b** proceeded with a DMT efficiency of only 80–85%. This low efficiency was independent of the identity of the next phosphoramidite, and was not improved by prolonged or repeated couplings. When the unit was T^α derived from **4a**, the next monomer coupled with a better DMT efficiency (ca. 95%). The reasons for these inefficient couplings are unknown at present.

Deprotection and cleavage of the modified oligonucleotides from the support had to be performed in two steps, because the T^α and T^β units were unstable to aqueous ammonia at the phosphortriester stage. Thus, a tetradecamer, $dT_7T^\beta T_6$, gave, after treatment with concd aq ammonia for 2 h at rt, mainly dT_7p and pdT_6 and no full length product according to polyacrylamide capillary electrophoresis (CE) and electrospray ionization mass spectroscopy (ESI-MS). The T^β unit was unstable to the milder deprotection/cleavage reagent K_2CO_3 in MeOH[13] as well. Allylic groups are known to be removed from allylic phosphortriesters by concd aq ammonia,[14] but their removal in preference to removal of the cyanoethyl group in our system was unexpected. Since the allylic group is removed by an S_N2 or S_N2' process and the cyanoethyl group by a β-elimination (Fig. 2), a hindered weakly nucleophilic base might remove the cyanoethyl group preferentially. Indeed, treatment with neat, dry Pr^i_2NH for 14 h at rt,[15] followed by concd aq ammonia for 2 h at rt, gave full length $dT_7T^\beta T_6$ tetradecamer, although the product was contaminated by shorter sequences according to CE. The tetradecamer was purified by ion exchange HPLC, followed by desalting on a NAP column, to give $dT_7T^\beta T_6$ in ca. 20% yield, 92% pure according to CE.[16] Two other homothymine tetradecamers, containing one T^α in the middle or two T^β unit at the 3′-end, and one nonamer with mixed sequence, $dGCAT^\beta CT^\beta CAC$, were prepared in a similar way. The purified sequences were characterised by CE and ESI-MS.[17]

Oligonucleotides containing the T^α and T^β units were quite stable after the cyanoethyl groups were removed. Thus $dT_7T^\beta T_6$ was not visibly cleaved (CE) for at least 2 months in aq solutions at pH 7. In 32% aq ammonia, 7% was cleaved after 6 h at 55 °C, but less than 1% was cleaved after 24 h at rt. An oligonucleotide with two T^β units at the 3′-end was significantly more stable towards 3′-exonucleolytic cleavage than the unmodified analogue. Thus, $dT_{11}T^\beta T^\beta$ was 65% intact (CE) after cleavage of $dT_{11}T^\beta T^\beta T$ with snake venom phosphordiesterase (Phosphodiesterase I, *Crotalus adamanteus* Venom, Pharmacia) for 2 h, under conditions where $t_{1/2}$ for dT_{14} was 8 min.[18]

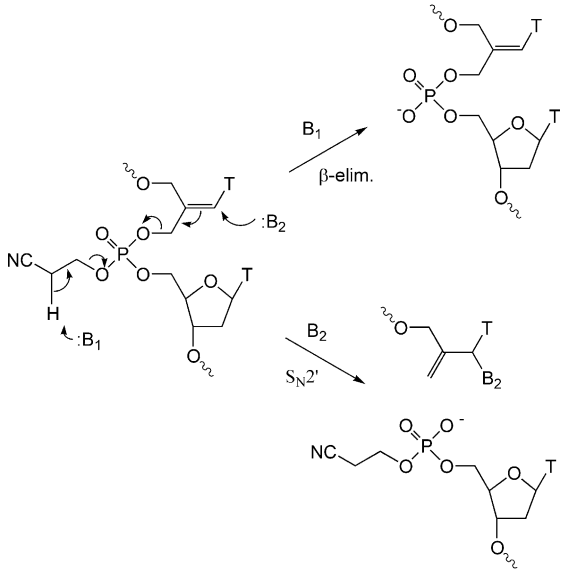


Figure 2.

The hybridisation properties of the modified oligonucleotides towards complementary DNA and RNA single strands were evaluated by thermal denaturation studies (Table 1). These data show that one T^{β} unit in the central region of an oligomer decreased the T_m significantly (ΔT_m −5.0 to −6.5 °C), and that the decrease, as expected, was smaller (ΔT_m −2.0 to −2.5 °C) when T^{β} was situated near the end. No large differences in binding to DNA and RNA complements were observed. A mismatched RNA complement resulted in a depression of T_m which was as large (ΔT_m −15.5 °C) as that found for unmodified dT_{14}. A T^{α} unit resulted in a larger decrease of T_m than a T^{β} unit, as expected since the T^{α} unit is analogous to an α-DNA unit. The hybridisation properties of the T^{β} unit compare favourably with those of other acyclic nucleoside analogues, apart from PNA. Thus the flexible analogue **5** (Fig. 3) built into central regions of oligomers resulted in an average ΔT_m of −13.4 °C, and for several other acyclic analogues ΔT_m values of −6.5 to −10.5 °C have been found, **6** being the best one.[7] Although the saturated analogue of T^{β}, **7**, has not to our knowledge been evaluated for its hybridisation properties, the presence of a double bond in the T^{β} unit seems to have a positive effect on the binding.

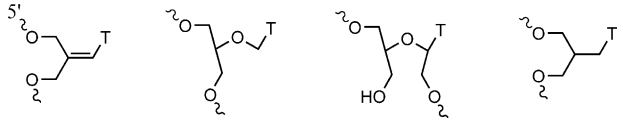
This study shows that a very simple acyclic, achiral monomer like **1** is able to replace a DNA monomer in oligonucleotides without seriously compromising the hybridisation properties. Work is in progress to optimise the coupling and deprotection procedures in order to prepare and study fully modified oligomers, and to examine possible reasons (less than optimal preorganised conformation, reduced solvation, or other factors) for the somewhat unsatisfactory binding properties.

Table 1. Hybridisation data (T_m, °C) for modified and unmodified oligodeoxyribonucleotides with DNA and RNA complements[a]

	dA_{14}	ΔT_m[b]	rA_{14}	ΔT_m[b]	rA_6CA_7	ΔT_m
dT_{14}	36.0		33.5		18.0	−15.5
$dT_7T^{\beta}T_6$	31.0	−5.0	28.0	−5.5	12.5	−15.5
$dT_7T^{\alpha}T_6$	26.0	−10.0	27.0	−6.5		
$dT_{11}T^{\beta}T^{\beta}T$	31.0	−2.5	29.0	−2.0		

	dGTGAGATGC	ΔT_m[b]	rGTGAGATGC	ΔT_m[b]
dGCATCTCAC	39.0		41.0	
$dGCAT^{\beta}CT^{\beta}CAC$	28.0	−5.5	27.5	−6.5

[a] T_m was determined by measuring absorbance at 260 nm against increasing temperature (0.5 °C steps) on equimolar mixtures (3 μM in each strand) of modified oligomer and its complementary DNA or RNA strand in medium salt buffer (10 mM Na_2HPO_4, 100 mM NaCl, 0.1 mM EDTA, pH 7.0). T^{α} and T^{β} are explained in the text.
[b] Change in T_m per modification.



T^{β} unit **5** **6** **7**

Figure 3.

Acknowledgements

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10. Selected data for new compounds. (*E*)-1-[2-(Dimethoxytrityloxymethyl)-3-hydroxyprop-1-enyl]thymine (**2a**): R_f 0.40 (EtOAc–MeOH–Et_3N 98:1:1). NMR (DMSO-d_6): δ_H 11.39 (1H, s, NH), 7.43–6.89 (14H, m, Ar + T-H6), 6.65 (1H, s, NCH=C), 4.90 (1H, t, J = 5.3 Hz, OH), 3.92 (2H, d, J = 5.3 Hz, CH_2OH), 3.74 (6H, s, OCH_3), 3.70 (2H, s, CH_2ODMT), 1.78 (3H, d, J = 1.2 Hz, T-CH_3). FAB^+ MS: 515.2 ($M+H^+$ calcd 515.2). (*Z*)-1-[2-(Dimethoxytrityloxymethyl)-3-hydroxyprop-1-enyl]thymine (**2b**): R_f 0.25 (EtOAc–MeOH–Et_3N 98:1:1). NMR (DMSO-d_6): δ_H 11.30 (1H, s, NH), 7.31–6.86 (14H, m, Ar + T-H6), 6.49 (1H, s, NCH=C), 5.15 (1H, t, J = 5.0, OH), 4.16 (2H, d, J = 5.0, CH_2OH), 3.73 (6H, s, OCH_3), 3.47 (2H, s, CH_2ODMT), 1.60 (3H, d, J = 1.2 Hz, T-CH_3). δ_C 164.1, 158.9, 150.4, 144.5, 140.5, 136.1, 135.5, 130.1, 128.2, 127.3, 124.8, 113.5, 110.6, 87.3, 63.6, 58.9, 55.5, 12.6. FAB^+ MS: 515.2 ($M+H^+$ calcd 515.2). The *Z* configuration of **2b** was determined by ^1H NMR NOE effects from NC*H*=C to CH_2OH. (*Z*)-1-[3-(Dimethoxytrityloxy)-2-[2-cyanoethoxy(diisopropylamino)phosphinoxy - methyl]prop - 1-enyl]thymine (**4a**): NMR ($CDCl_3$): δ_H 7.39–7.14 (10H, m, Ar + NH), 7.12 (1H, q, J 1.2, T-H6), 6.76 (4H, d, J = 8.8 Hz, Ar), 6.70 (1H, s, NCH=C), 4.11–3.96 (2H, m, CH_2OP), 3.80–3.36 (6H, m, CH_2ODMT, CH_2CH_2CN, $CH(CH_3)_2$), 3.72 (6H, s, CH_3O), 2.41 (2H, t, J 6.3, CH_2CH_2CN), 1.87 (3H, d, J = 1.2 HZ, T-CH_3), 1.08–0.95 (12H, m, $CH(CH_3)_2$). δ_P 150.7. (*E*)-1-[3-(Dimethoxytrityloxy)-2-[2-cyanoethoxy(diisopropylamino)phosphinoxy-methyl]prop-1-enyl]thymine (**4b**): NMR ($CDCl_3$): δ_H 7.40–7.18 (10H, m, Ar + NH), 7.14 (1H, s, T-H6), 6.81 (4H, d, J = 8.8 Hz, Ar), 6.70 (1H, s, NCH=C), 4.38 (2H, AB of ABX system, Δ = 27.5 Hz, J_{AB} = 13.4 Hz, J_{AX} = 8.2 Hz, CH_2OP), 3.90–3.53 (6H, m, CH_2ODMT, CH_2CH_2CN, $CH(CH_3)_2$), 3.78 (6H, s, CH_3O), 2.61 (2H, t, J = 6.3 Hz, CH_2CH_2CN), 1.71 (3H, s, T-CH_3), 1.19 (12H, pseudo-triplet, J = 7.0 Hz, $CH(CH_3)_2$). δ_C 164.1, 158.9, 150.2, 144.6, 140.7, 135.6, 133.3, 133.2, 130.2, 128.2, 128.1, 127.3, 125.3, 113.4, 110.4, 87.1, 64.0, 63.7, 58.9,

58.8, 55.5, 43.5, 43.4, 24.9, 24.8, 20.7, 20.6, 12.6. δ_P 149.8. FAB^+ MS: 715.3 ($M+H^+$ calcd 715.3).
11. Oligonucleotide syntheses were performed on a Biosearch 8750 DNA Synthesizer using Pac-protected cyanoethyl phosphoramidites (Millipore) and **4a** or **4b**. The modified phosphoramidites (0.05 M in CH_3CN) were manually coupled for 6 min using tetrazole (0.45 M in CH_3CN) as activator, followed by a washing step (10% H_2O, 0.2% Ac_2O and 0.2% lutidine v/v/v in THF). The capping (standard Ac_2O/lutidine + NMI) was followed by oxidation with *tert*-butyl hydroperoxide (80% in di-*tert*-butyl ether, ca. 0.5 M in CH_2Cl_2–acetone 1:1 v/v) for 3 min, and detritylation (3% TCA in CH_2Cl_2).
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16. Deprotection and cleavage were performed by shaking the support-bound oligonucleotide with neat, dry diisopropylamine (0.5 mL) for 14 h at rt, removal of the diisopropylamine with a syringe, and washing with diethyl ether, followed by shaking with concd aq ammonia (0.5 mL) for 2 h at rt. The supernatant was removed with a syringe and the support washed with water. Speed-Vac evaporation gave the crude product which was purified by HPLC (Pharmacia FPLC) using a Resource-Q 1 mL column and a 0–50% linear gradient over 25 min of buffer A: 20 mM aq NaOH, buffer B: 20 mM aq NaOH, 2.5 M aq NaCl, flow 1 mL/min, detection at 260 nm, 25 °C. Fractions containing the full length product (latest eluted peak) were neutralised with 0.5 M aq HCl, concentrated to ca. 1 mL, and desalted on a NAP 10 column (Pharmacia) by elution with water.
17. $dT_7T^{\beta}T_6$, 92% pure, M 4167.0 (M calcd 4165.7), $dT_7T^{\alpha}T_6$, 81% pure, M 4166.8 (M calcd 4165.7), $dT_{11}T^{\beta}T^{\beta}T$, 85% pure, M 4136.5 (M calcd 4135.7), $dGCAT^{\beta}CT^{\beta}CAC$, 97% pure, M 2598.6 (M calcd 2598.5).
18. To a solution (100 μL) of dT_{14} or $dT_{11}T^bT^bT$ (ca. 1.0 OD), 20 mM Tris, 10 mM $MgCl_2$, pH 8.3 was added snake venom phosphordiesterase (SVP, Phosphodiesterase I (*Crotalus adamanteus* Venom), ca. 0.005 u, Pharmacia) at 37 °C. At 1, 2, 5, 10, 15, 25, 40, 60, and 120 min 10 μL samples were removed and added to stop-solutions (50 μL, 1 M NH_3, 2 mM EDTA). The samples were analysed by CE (30 s electro-injection at 9 kv).