Improved hybridisation potential of oligonucleotides comprising *O*-methylated anhydrohexitol nucleoside congeners

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ABSTRACT

The hybridising potential of anhydrohexitol nucleoside analogues (HNAs) is well documented, but tedious synthesis of the monomers hampers their development. In a search for better analogues, the synthesis of two new methylated anhydrohexitol congeners **1** and **2** was accomplished and the physico-chemical properties of their respective oligomers were evaluated. Generally, oligonucleotides (ONs) containing the 3'-*O*-methyl derivative **1** showed a small increase in thermal stability towards complementary sequences as compared to HNA. Compared to the altritol modification, 3'-*O*-methylation seems to cause a small decrease in thermal stability of duplexes, especially when targeting RNA. These results suggest the possibility of derivatisation of the 3'-hydroxyl group of altritol-containing congeners without significantly affecting the thermal stability of the duplexes. The methyl glycosidic analogues **2** likewise increased the affinity for RNA in comparison with well-known HNA, while at the same time being economically more favorable monomers. However, homopolymers of **2** displayed self-pairing, but not so homopolymers of **1**. Upon incorporation of the hexitols within RNA sequences in an effort to induce a beneficial pre-organised structure, the positive effect of the 3'-*O*-methyl derivative **1** proved larger than that of **2**.

INTRODUCTION

Modulation of gene expression is a continuously growing research area and can be attained by interference with either transcription or translation processes. The use of antisense oligonucleotides to reduce translation is well established, for target validation as well as for different therapeutic indications (1), with the biggest hurdle for the latter being delivery (2). Two different strategies for interaction with the target can be distinguished. The first strategy relies mostly on degradation

of the mRNA target through the assistance of RNase H, which is activated upon recognition of the mixed DNA–RNA duplex. RNase H, however, does not tolerate much modification of the incoming DNA antisense strand, reducing this strategy mainly to the use of phosphorothioate sequences. Alternatively, by modifying either the backbone (3) or the heterocyclic bases (4) of the antisense oligonucleotide (ASO), a strong association with the RNA target can be envisaged to obtain an antisense effect via steric block powerful enough to withstand the read-through power of the ribosomal machinery (5). One way to approach this problem is to synthesise carbohydrate-modified oligonucleotides, exemplified by hexitol nucleic acids (6,7), 2'-*O*-(2-methoxy)ethyl oligonucleotides (8) or bicyclic oligonucleotides (9), with the locked nucleic acid derivatives of the Wengel group (10) showing the strongest affinity for RNA. The strong hybridisation characteristics with complementary RNA are generally attributed (11) to formation of a pre-organised conformation fitting the A-form of double-stranded (ds)RNA, good stacking interactions between the bases interacting in a Watson–Crick type geometry with their complement and efficient hydration of the double-stranded helix.

The strong hybridising potential of anhydrohexitol nucleic acids (HNA) by virtue of their pre-organisation has been well documented (6,7,12) and some interesting biological antisense effects have been reported (13,14), including a steric blockage mechanism as shown in an *in vitro* translation arrest system. However, a further increase in affinity would widen their applicability. The D-altritol nucleic acid (ANA) analogues (15) of HNA with a supplementary hydroxyl at the 3'- α -position have already paved the way and a further increase could be expected upon alkylation of this hydroxyl moiety, in analogy with 2'-*O*-alkylated RNA congeners. In addition, the cost of raw materials needs to be considered, and therefore avoiding reduction of the anomeric position would be advantageous. In view of these arguments, the 3'-*O*-methylated altritol building block **1** and the methyl hexopyranoside congener (1'-*O*-methylated) **2** were evaluated for incorporation into ASOs (within HNA or RNA sequences, respectively) in terms of their hybridising potential with natural oligonucleotides.

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MATERIALS AND METHODS

General

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Reactions were conducted under an atmosphere of nitrogen when anhydrous solvents were used. All reactions were monitored by TLC (Macherey-Nagel Alugram SIL G/UV 254 plates) and column chromatographic purification used silica gel 60 (ICN), 0.040–0.063 mm. NMR spectra were recorded on either a Varian Gemini 200 or a Varian 500 unity spectrometer. Tetramethylsilane (TMS) was used as the internal standard for the ^1H NMR spectra, CDCl_3 ($\delta = 76.9$) or $\text{DMSO}-d_6$ ($\delta = 39.6$) for the ^{13}C NMR spectra. Electrospray ionisation mass spectrometry (ESI-MS) measurements were obtained on a qTof 2 from Micromass. Oligonucleotides were assembled in an ABI 392 synthesiser following standard 1 μmol cycles, except for a 0.11 M amidite concentration for the HNA building blocks, with a coupling time of 3 min. Coupling yields were consistently >95%. Oligos were purified as usual (16) on a Mono Q (Pharmacia) column with a NaCl gradient at pH 12 to disrupt possible secondary structures. MS of the isolated oligos were run following gel filtration and reverse phase HPLC with a 0.025 M TEAB-containing acetonitrile gradient. ESI-MS in negative mode was performed on a quadrupole/orthogonal acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qTof 2; Micromass, Manchester, UK) equipped with a standard ESI interface. Oligonucleotide samples were infused in a 2-propanol:water (1:1) mixture at 3 $\mu\text{l}/\text{min}$. Monoisotopic masses were consistently within 0.5 Da of the calculated masses.

1,5-Anhydro-4,6-*O*-benzylidene-3-*O*-methyl-2-(uracil-1-yl)-2-deoxy-D-*altro*-hexitol (5)

1,5-Anhydro-4,6-*O*-benzylidene-2-(uracil-1-yl)-2-deoxy-D-*altro*-hexitol (**4**) (17) (1.59 g, 4.6 mmol) was co-evaporated with anhydrous acetonitrile (3×10 ml) and dissolved in anhydrous THF (40 ml). NaH (552 mg, 13.8 mmol) was added and the reaction was stirred for 30 min at 0°C , then CH_3I (1.35 ml, 23 mmol) was added. After 5 h stirring at 0°C an additional amount of CH_3I (1 ml, 17 mmol) was added and the reaction was stirred for another 2 h at 0°C . The reaction was quenched with water (20 ml), diluted with EtOAc (200 ml) and washed with NaHCO_3 (2×50 ml). The combined aqueous phase was extracted with dichloromethane (50 ml), then the combined organic phase was dried (Na_2SO_4), filtered and evaporated to dryness. Purification by silica column chromatography (0–2% MeOH/dichloromethane) afforded the methylated nucleoside **5** [829 mg, 2.28 mmol, 50% yield (69% based on recovered starting material)] as a white foam. R_f 0.3 (5% MeOH/dichloromethane).

δ ^1H NMR (CDCl_3): 9.69 (s, 1H, NH), 8.04 (d, $J = 8.1$ Hz, 1H, 6-H), 7.34–7.49 (m, 5H, Ph), 5.80 (d, $J = 8.1$ Hz, 1H, 5-H), 5.30 (s, 1H, PhCH), 4.53 (t, $J = 2.9$ Hz, 1H, 2'-H), 4.37 (dd, $J = 5.5, 9.9$ Hz, 1H, 6'-H), 4.32 (dd, $J = 3.3, 13.2$ Hz, 1H, 1'-H), 4.08 (dt, $J = 5.1, 9.9$ Hz, 1H, H-5'), 4.03 (d, $J = 13.9$ Hz, 1H, 1'-H), 3.86 (br t, 1H, 3'-H), 3.81 (d, $J = 10.3$ Hz, 1H, 6'-H), 3.64 (dd, $J = 2.6, 9.5$ Hz, 1H, 4'-H), 3.63 (s, 3H, OCH_3). δ ^{13}C NMR (CDCl_3): 163.30 (C4), 150.79 (C2), 142.05 (C6), 137.01, 129.03, 128.15, 126.00 (Ph), 102.60 (C5), 102.23 (PhCH), 76.22 (C4'), 74.58 (C3'), 68.70 (C6'), 66.45 (C5'),

64.11 (C1'), 59.41 (OCH_3), 54.64 (C2'). HRMS (thioglycerol) calculated for $\text{C}_{18}\text{H}_{20}\text{N}_2\text{NaO}_6$ $[\text{M}+\text{Na}]^+$: 383.1219, found 383.1229.

1,5-Anhydro-3-*O*-methyl-2-(uracil-1-yl)-2-deoxy-D-*altro*-hexitol (1)

1,5-Anhydro-4,6-*O*-benzylidene-3-*O*-methyl-2-(uracil-1-yl)-2-deoxy-D-*altro*-hexitol (**5**) (390 mg, 1.08 mmol) was dissolved in 90% aqueous trifluoroacetic acid (6 ml) and stirred at room temperature for 1 h. Upon completion, the mixture was evaporated to dryness and co-evaporated with toluene (2×10 ml). Purification by silica column chromatography (5–10% MeOH in dichloromethane) afforded the deprotected nucleoside **1** as a white foam (210 mg, 0.77 mmol, 71% yield). R_f 0.28 (10% MeOH/dichloromethane).

δ ^1H NMR ($\text{DMSO}-d_6$): 11.32 (s, 1H, NH), 7.98 (d, $J = 8.1$ Hz, 1H, 6-H), 5.57 (d, $J = 8.1$ Hz, 1H, 5-H), 4.85 (d, $J = 6.2$ Hz, 1H, 4'-OH), 4.60 (t, $J = 5.9$ Hz, 1H, 6'-OH), 4.46 (d, $J = 3.7$ Hz, 1H, 2'-H), 3.86 (d, $J = 3.7$ Hz, 2H, 1'-H), 3.51–3.68 (m, 5H, 3'-H, 4'-H, 5'-H, 6'-H), 3.39 (s, 3H, OCH_3). δ ^{13}C NMR ($\text{DMSO}-d_6$): 163.42 (C4), 151.31 (C2), 143.27 (C6), 101.35 (C5), 78.01, 77.23 (C3', C5'), 63.60 (C4'), 63.08 (C1'), 60.14 (C6'), 57.62 (OCH_3), 52.77 (C2'). HRMS (thioglycerol) calculated for $\text{C}_{11}\text{H}_{15}\text{N}_2\text{Na}_2\text{O}_6$ $[\text{M}-\text{H}+2\text{Na}]^+$ 317.07255, found 317.07232.

1,5-Anhydro-3-*O*-methyl-5-*O*-monomethoxytrityl-2-(uracil-1-yl)-2-deoxy-D-*altro*-hexitol (6)

1,5-Anhydro-3-*O*-methyl-2-(uracil-1-yl)-2-deoxy-D-*altro*-hexitol (**1**) (460 mg, 1.69 mmol) was co-evaporated with anhydrous pyridine (2×5 ml) and redissolved in anhydrous pyridine (10 ml). Monomethoxytritylchloride (615 mg, 2.0 mmol) was added and the reaction was stirred for 20 h. Following completion, the reaction was quenched with methanol (2 ml), evaporated to dryness and co-evaporated with toluene. Purification by silica gel column chromatography (1–5% MeOH/dichloromethane) afforded the tritylated compound **6** as a white foam (816 mg, 1.50 mmol, 89% yield). R_f 0.79 (5% MeOH/dichloromethane).

δ ^1H NMR ($\text{DMSO}-d_6$): 11.40 (s, 1H, NH), 8.06 (d, $J = 8.1$ Hz, 6-H), 6.88–7.43 (m, 14H, MMTr), 5.58 (d, $J = 8.1$ Hz, 1H, 5-H), 4.80 (d, $J = 6.6$ Hz, 1H, 4'-OH), 4.45 (m, 1H, 2'-H), 3.95 (m, 2H, 1'-H), 3.60–3.86 (m, 5H, MMTr- OCH_3 , 4'-H, 5'-H), 3.54 (t, $J = 3.7$ Hz, 1H, 3'-H), 3.41 (s, 3H, OCH_3), 3.21 (d, $J = 2.6$ Hz, 2H, 6'-H). δ ^{13}C NMR ($\text{DMSO}-d_6$): 163.41 (C4), 151.21 (C2), 143.08 (C6), 158.41, 144.75, 135.28, 127.02–130.36, 113.33 (MMTr), 101.41 (C5), 85.56 (MMTr), 77.37 (C5'), 75.91 (C3'), 63.80 (C4'), 63.25 (C1'), 62.25 (C6'), 58.00 (OCH_3), 55.15 (MMTr), 52.78 (C2'). HRMS (thioglycerol) calculated for $\text{C}_{31}\text{H}_{32}\text{N}_2\text{NaO}_7$ $[\text{M}+\text{Na}]^+$: 567.2107, found 567.1817.

1,5-Anhydro-3-*O*-methyl-4-*O*-(*P*- β -cyanoethyl-*N,N*-diisopropylaminophosphinyl)-6-*O*-monomethoxytrityl-2-(uracil-1-yl)-2-deoxy-D-*altro*-hexitol (7)

The monomethoxytritylated derivative **6** (495 mg, 0.90 mmol) was dissolved in 6 ml of dichloromethane under argon and diisopropylethylamine (470 μl , 2.70 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (305 μl , 1.35 mmol) were added and the solution was stirred for 2 h. An additional amount of 1.35 mmol DIPEA and 0.65 mmol amidite were added and the mixture was stirred for another 2 h, when TLC

indicated completion of the reaction. Water (3 ml) was added, the solution was stirred for 10 min and partitioned between CH_2Cl_2 (50 ml) and aqueous NaHCO_3 (30 ml). The organic phase was washed with aqueous sodium chloride (2×30 ml) and the aqueous phases were back-extracted with CH_2Cl_2 (30 ml). Evaporation of the organics left an oil which was flash purified twice on 40 g silica gel (hexane:acetone:TEA 49:49:2) to afford the product as a foam after co-evaporation with dichloromethane. Dissolution in 2 ml of dichloromethane and precipitation in 60 ml of cold (-70°C) hexane afforded 605 mg (0.81 mmol, 90% yield) of the product **8** as a white powder. R_f (hexane:acetone:TEA 49:49:2) 0.32.

ESI-MS in positive mode (pos.) calculated for $\text{C}_{40}\text{H}_{50}\text{N}_4\text{O}_8\text{P}_1$ 745.33660, found 745.3429 $[\text{M}+\text{H}]^+$; ^{31}P NMR δ (p.p.m., external reference H_3PO_4 capillary) 148.11, 150.40.

Methyl-4,6-*O*-benzylidene-2-(thymine-1-yl)-2-deoxy-D-*altro*-hexopyranoside (**10**)

Thymine (3.78 g, 30 mmol) was suspended in 250 ml of anhydrous DMF to which was added 1.13 g of a 60% oil dispersion of sodium hydride (28 mmol) and the mixture was heated in an oil bath for 1 h at 90°C . The alloside epoxide (18,19) **9** (2.64 g, 10 mmol) was added and the mixture was heated for 4 days at 120°C , after which the reaction was cooled, quenched with sodium bicarbonate and concentrated. The residue was partitioned between 200 ml of ethyl acetate and 200 ml of 5% aqueous sodium bicarbonate and the organics were washed twice with brine. Purification of the organic residue on silica gel (0–2% MeOH/dichloromethane) afforded 2.77 g (7.1 mmol, 71% yield) of **10** as a foam.

UV (MeOH) λ_{max} 269 nm ($\epsilon = 9500$); ^1H NMR (CDCl_3) (500 MHz) δ : 1.96 (s, 3H, 5- CH_3), 3.21 (d, 1H, $J = 3.7$ Hz, OH), 3.47 (s, 3H, 1'- OCH_3), 3.71 (dd, 1H, $J = 3.2, 9.3$ Hz, 4'-H), 3.82 (t, 1H, $J = 11.8$ Hz, 6'- H_a), 4.15 (m, 1H, 3'-H), 4.43 (m, 2H, H5', 6'- H_b), 4.79 (d, 1H, $J = 2.4$ Hz, 2'-H), 4.85 (s, 1H, 1'-H), 5.63 (s, 1H, PhCH), 7.32–7.37 and 7.44–7.49 (m, 5H, arom-H), 7.55 (d, $J = 1.2$ Hz, 6-H), 9.36 (s, 1H, NH); ^{13}C NMR (CDCl_3) δ : 163.45 (C4), 150.65 (C2), 136.85 (C6), 136.81, 129.24, 128.27, 126.18 (Ph), 111.42 (C-5), 102.25 (PhCH), 99.07 (C1'), 75.76 (C4'), 69.06 (C6'), 67.22 (C3'), 58.24 (C5' and C2'), 55.94 (OCH_3), 12.75 (5- CH_3); ESI-MS pos. HRMS calculated for $\text{C}_{19}\text{H}_{23}\text{N}_2\text{O}_7$ $[\text{M}+\text{H}]^+$: 391.1505, found 391.1504.

Methyl-4,6-*O*-benzylidene-2-(thymine-1-yl)-2,3-dideoxy-D-arabino-hexopyranoside (**12**)

Methyl-4,6-*O*-benzylidene-2-(thymine-1-yl)-2-deoxy-D-*altro*-hexopyranoside **10** (390 mg, 1 mmol), obtained in the previous preparation, and 856 mg (7 mmol) dimethylaminopyridine were dissolved in 15 ml of dry dichloromethane. The reaction mixture was cooled to -40°C , and 0.158 ml (2 mmol) of thiophosgene was added with vigorous stirring. The mixture was brought to room temperature and, after stirring for 1 h, 656 mg (4 mmol) of 2,4-dichlorophenol was added and stirring was continued for 2 h more. The mixture was poured into 20 ml of a 1 M solution of KH_2PO_4 and extracted twice with dichloromethane. The organic layers were dried and, following evaporation, the residue was purified by flash chromatography (0–2% MeOH/dichloromethane). The product was immediately used for deoxygenation (FABMS 595 $[\text{M}+\text{H}]^+$).

Then the obtained thiocarbonyl compound was dissolved in 15 ml of anhydrous toluene. After nitrogen gas was bubbled

through the solution for 10 min, 0.41 ml (1.5 mmol) of tributyltin hydride and 20 mg 2,2'-azobis(2-methylpropionitrile) were added and the mixture was heated at 80°C overnight, when TLC indicated completion of the reaction. The mixture was evaporated and purified on silica gel (0–2% MeOH/dichloromethane) affording 320 mg (0.85 mmol, 85% yield) of **12**.

UV (MeOH) λ_{max} 269 nm ($\epsilon = 9700$); ^1H NMR (CDCl_3) δ : 1.98 (d, $J = 1$ Hz, 3H, 5- CH_3), 2.19 (ddd, $J_{3'3'} = 13.7$ Hz, 1H, 3'- H_b), 2.30 (ddd, $J_{3'3'} = 13.2$ Hz, $J_{3'4'} = 12.4$ Hz, $J_{3'2'} = 5.1$ Hz, 1H, 3'- H_a), 3.45 (s, 3H, 1'- OCH_3), 3.72 (ddd, 1H, 4'-H), 3.81 (t, $J = 10.4$ Hz, 1H, 6'- H_a), 3.96 (dt, 1H, $J = 9.9, 4.9$ Hz, 5'-H), 4.36 (dd, $J = 4.8, 10.4$ Hz, 1H, 6'- H_b), 4.77 (s, 1H, 1'-H), 4.81 (dd, 1H, $J = 2.4, 4.9$ Hz, 2'-H), 5.58 (s, 1H, PhCH), 7.32–7.47 (m, 5H, arom-H), 7.72 (s, 1H, 6-H), 9.21 (s, 1H, NH); ^{13}C NMR (CDCl_3) δ : 163.53 (C4), 150.75 (C2), 137.27 (C6), 137.09, 129.18, 128.31, 126.07 (Ph), 110.70 (C5), 102.14 (PhCH), 98.42 (C1'), 73.20 (C4'), 69.11 (C6'), 65.08 (C5'), 55.07 (OCH_3), 53.59 (C2'), 29.60 (C3'), 12.79 (5- CH_3). ESI-MS pos. HRMS calculated for $\text{C}_{19}\text{H}_{23}\text{N}_2\text{O}_6$ $[\text{M}+\text{H}]^+$: 375.1556, found 375.1556.

Methyl-2-(thymine-1-yl)-2,3-dideoxy-D-arabino-hexopyranoside (**2**)

Method A. An aliquot of 500 mg (1.33 mmol) of the benzylidene-protected compound **12** was dissolved in 25 ml of methanol and 2.5 ml of trifluoroacetic acid was added. The solution was stirred for 3 h, evaporated to dryness and co-evaporated twice with dioxane. The residue was dissolved in methanol, adsorbed on silica gel by evaporation and purified by flash chromatography on silica gel (0–15% MeOH/dichloromethane) to afford **2** in 45% yield (172 mg, 0.6 mmol).

Method B. An aliquot of 1.08 g (2.89 mmol) of the benzylidene-protected compound **12** was dissolved in 40 ml of methanol and 0.5 ml of acetic acid was added. The solution was degassed by bubbling nitrogen for 10 min, after which 450 mg 10% Pd on carbon was added and the mixture was hydrogenated overnight on a Parr apparatus at 45 p.s.i. The mixture was filtered, the filter was washed with hot ethanol, the volatiles were removed *in vacuo* and the residue was co-evaporated twice with dioxane. Crystallisation from hexane afforded **2** in 90% yield (743 mg, 2.60 mmol).

Melting point 66 – 67°C (softening) [literature value (23) 49°C]. UV (MeOH) λ_{max} 269 nm ($\epsilon = 9400$) and 209 nm ($\epsilon = 8700$), λ_{min} 235 nm ($\epsilon = 1650$); ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 1.76 (s, 3H, 5- CH_3), 1.73–1.81 (m, 1H, 3'- H_b), 1.97 (dt, 1H, $J = 5.4$ and 13.7 Hz, 3'- H_a), 3.30 (s, 3H, 1'- OCH_3), 3.43 (m, 1H, 5'-H), 3.59–3.67 (dAB, 2H, 6'-H), 3.73 (ddd, 1H, 4'-H), 4.51 (ddd, 1H, 2'-H), 4.75 (t, 1H, 6'-OH), 4.76 (d, $J = 3.4$ Hz, 1H, 1'-H), 4.93 (d, 1H, $J = 3.9$ Hz, 4'-OH), 7.76 (s, 1H, 6-H), 11.24 (s, 1H, NH); ^{13}C NMR ($\text{DMSO}-d_6$) δ : 163.68 (C4), 150.96 (C2), 138.48 (C6), 108.73 (C5), 97.73 (C1'), 75.05 (C5'), 60.52 (C4'), 60.24 (C6'), 54.26 (OCH_3), 52.24 (C2'), 32.26 (C3'), 12.21 (5- CH_3). ESI-MS pos. HRMS calculated for $\text{C}_{12}\text{H}_{18}\text{N}_2\text{O}_6\text{Na}$ $[\text{M}+\text{Na}]^+$: 309.1063, found 309.1063.

6-*O*-dimethoxytrityl-2-(thymine-1-yl)-2,3-dideoxy-D-methylglucopyranoside (**13**)

Following co-evaporation with anhydrous pyridine, an aliquot of 910 mg (3.18 mmol) of the thymine glucopyranoside **2** was

dissolved in 25 ml of pyridine and dimethoxytrityl chloride (1.19 g, 3.5 mmol) was added. The mixture was stirred for 16 h at ambient temperature, quenched with 3 ml of methanol and neutralised with aqueous sodium bicarbonate. The mixture was concentrated and partitioned twice between dichloromethane and aqueous sodium bicarbonate. The organic layer was purified on 40 g silica gel with a methanol step gradient (0–1%) in dichloromethane containing 0.5% pyridine, affording 1.6 g (2.72 mmol, 85%) of **13** as a foam.

^1H NMR 500 MHz (CDCl_3) δ : 1.82 (s, 3H, 5- CH_3), 2.00–2.07 (ddd, 1H, 3'- H_a), 2.12–2.18 (ddd, 1H, 3'- H_b), 2.28 (d, 1H, $J = 3.5$ Hz, 4'-OH), 3.38 (s, 3H, 1'- OCH_3), 3.45 (d, $J = 3.6$ Hz, 2H, 6'-H), 3.69 (dt, 1H, $J = 9$ and 8.5 Hz, 5'-H), 3.78 (s, 6H, 2 $\times$ OCH_3), 3.95 (m, 1H, 4'-H), 4.70 (t, 1H, $J = 6.5$ Hz, 2'-H), 4.75 (s, 1H, 1'-H), 6.84 (2d, 4H, $J = 9$ Hz, arom-H), 7.20–7.47 (m, 9H, arom-H), 7.74 (d, $J = 1.1$ Hz, 6-H), 9.05 (s, 1H, NH); ^{13}C NMR (CDCl_3) δ : 163.59 (C4), 150.88 (C2), 137.98 (C6), 110.51 (C5), 98.14 (C1'), 86.69 (Ph₃C), 72.33 (C5'), 63.54 (C4'), 63.05 (C6'), 55.20 (2 $\times$ CH_3O), 54.91 (1'- OCH_3), 53.50 (C2'), 31.95 (C3'), 12.65 (5- CH_3) + aromatic signals. ESI-MS pos. HRMS calculated for $\text{C}_{33}\text{H}_{36}\text{N}_2\text{O}_8\text{Na}$ 611.2369, found 611.2364 $[\text{M}+\text{Na}]^+$.

6-*O*-dimethoxytrityl-2-(thymine-1-yl)-4-*O*-(*P*- β -cyanoethyl-*N,N*-diisopropylaminophosphinyl)-2,3-dideoxy- β -D-methylglucopyranoside (**14**)

The dimethoxytritylated derivative **13** (800 mg, 1.36 mmol) was dissolved in 10 ml of dichloromethane under argon and diisopropylethylamine (710 μl , 4.08 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (455 μl , 2.05 mmol) were added and the solution was stirred for 15 min, when TLC indicated completion of the reaction. Water (4 ml) was added, the solution was stirred for 10 min and partitioned between CH_2Cl_2 (50 ml) and aqueous NaHCO_3 (30 ml). The organic phase was washed with aqueous sodium chloride (2 $\times$ 30 ml) and the aqueous phases were back-extracted with CH_2Cl_2 (30 ml). Evaporation of the organics left an oil which was flash purified twice on 40 g silica gel (hexane:acetone:TEA 68:30:2) to afford the product as a foam after co-evaporation with dichloromethane. Dissolution in 2 ml of dichloromethane and precipitation in 80 ml of cold (-70°C) hexane afforded 718 mg (0.91 mmol, 67%) of **14** as a white powder. R_f (hexane:acetone:TEA 49:49:2) 0.37.

^{31}P NMR δ (p.p.m., external reference H_3PO_4 capil.) 148.55, 149.01; ^{13}C NMR (CDCl_3) δ : 163.43 (C4), 150.78 (C2), 137.98 (C6), 117.2 (CN), 110.68 (C5), 98.33 and 98.11 (C1'), 86.13 (Ph₃C), 72.57 (C5'), 64.76 and 63.42 (2 $\times$ d, $J = 17.5$ Hz, C4'), 62.68 (C6'), 58.10 and 57.65 (2 $\times$ d, $J = 18.6$ Hz, POCH_2), 55.20 (2 $\times$ CH_3O), 54.88 (1'- OCH_3), 53.60 (C2'), 43.11 (2 $\times$ PNCH_2), 31.97 and 31.80 (C3'), 24.60 and 24.20 (4 $\times$ CHCH_3), 20.20 (CH_2CN), 12.49 (5- CH_3) + aromatic signals (many peaks were broad or doubled, indicating diastereoisomerism). HRMS calculated for $\text{C}_{42}\text{H}_{54}\text{N}_4\text{O}_9\text{P}_1$ $[\text{M}+\text{H}]^+$: 789.36281, found 789.3640.

RESULTS AND DISCUSSION

Synthesis of the new monomers

Synthesis of the 3'-*O*-methylated analogue **1** is depicted in Scheme 1 and followed the pathway previously described for

Scheme 1. Synthesis of the 3'-*O*-methylated altritol congener **1**. (a) 3.2 equiv. uracil, 3 equiv. NaH, DMF, 120°C , 24 h (86%) (**17**); (b) 3 equiv. NaH, 5 equiv. CH_3I , THF, 7 h, 0°C (50%); (c) 90% aqueous TFA (71%); (d) 1.2 equiv. MMTrCl, pyridine (89%); (e) DIEA, CH_2Cl_2 , $(i\text{Pr})_2\text{N}(\text{OCE})\text{PCl}$ (90%).

preparation of altritol monomers (**17**). Ring opening of the 4,6-*O*-benzylidene-protected allitol epoxide **3** with a uracil anion furnished the altritol derivative **4** in 86% yield. Chemoselective methylation without temporary protection of the nucleobase afforded 50% of the methylated nucleoside **5**. Methylation proceeded slowly and the yield was lower than reported for other derivatives (20,21). The slow reaction rate compared to the previously described methylations is probably caused by the axial location of the hydroxyl group. Faster reaction rates are achieved with a primary alcohol (21) and a pseudo-equatorial positioned secondary alcohol (20). The selectivity of the methylation was confirmed by NMR and only a small amount of the $\text{N}^3,3'\text{-O}$ -dimethylated compound was obtained. Following hydrolysis of the benzylidene protecting group, further modification (monomethoxytritylation and phosphitylation) yielded the desired phosphitylated building block **7**, to be used for oligomer assembly.

The 1'-*O*-methylglycosidic analogue (Scheme 2) was obtained starting from the ubiquitous methyl glucopyranoside (**8**). Opening of the epoxide ring of methyl 2,3-anhydro-*allo*-hexopyranoside **9** (18,19) with thymine was sluggish (96 h at 120°C) to afford 71% of the thymidine congener **10**. NMR identification was hampered due to diaxial hindrance of the 1'-methoxy and 3'-hydroxyl groups, causing the C5' signal to resonate at the unusual value of 58 p.p.m. The assignment was fully confirmed, however, by COSY and GHSQC experiments. Barton deoxygenation (22) of the 3'-position (phenoxythiocarbonylation followed by tributyltin hydride reduction) afforded the 3'-deoxy analogue **12** in 85% yield. At this stage, removal of the benzylidene position with acid was possible, but less straightforward because of the glycosidic linkage. Alternatively, this reaction could be accomplished via

Scheme 2. Synthesis of the methylpyranoside building block **2**. (a) $\text{C}_6\text{H}_5\text{CHO}$, ZnCl_2 , 72 h (66%); (b) 6 equiv. $\text{CH}_3\text{C}_6\text{H}_5\text{SO}_2\text{Cl}$, pyridine, 72 h, 60°C (78%); (c) NaOMe , MeOH , CH_2Cl_2 (79%) (19,20); (d) 3 equiv. thymine, 2.8 equiv. NaH , DMF , 96 h, 120°C (71%); (e) 2 equiv. CSCl_2 , 7 equiv. DMAP , CH_2Cl_2 at -40°C followed by 4 equiv. $2,4\text{-Cl}_2\text{C}_6\text{H}_3\text{OH}$ at room temperature for 1 h; (f) 1.5 equiv. Bu_3SnH , AIBN , toluene 80°C (85% over two steps); (g) 10% TFA-MeOH , 3 h (45%); alternatively H_2 , Pd/C in MeOH-HOAc 98:2 for 18 h (90%); (h) DMTrCl , pyridine (85%); (i) DIEA , CH_2Cl_2 , $(\text{iPr})_2\text{N}(\text{OCE})\text{PCl}$ (67%).

hydrogenation in almost quantitative yield, affording the envisaged 1'-O-methylglycoside analogue **2** of 1,5-anhydrohexitol nucleosides. A literature search revealed compounds **10** and **2** to be mentioned in a short communication following the same scheme but without experimental details except for the melting point of **2** (23). Further functionalisation of **2** yielded the desired phosphitylated building block **14**.

Hybridisation potential of the hexitol analogues

HNA oligos were assembled on a propanediol-containing universal support, obviating the need for modified supports. (16) The new analogues were used either for homopolymer synthesis, for incorporation within HNA stretches or for incorporation within stretches of RNA. To evaluate the effect of the alkoxy substituents, the modified analogues were incorporated within pyrimidine hexitol sequences as shown in Table 1. Introduction of the 3'-O-methylated uridine analogue **1** resulted in an increased affinity for a complementary HNA sequence ($\Delta T_m/\text{modification} = +0.6^\circ\text{C}$), and an even more pronounced increase with ANA as the complement ($\Delta T_m/\text{modification} = +1.2^\circ\text{C}$). It should be noted here that two of the modified nucleotides are end-incorporated. Only a slight increase in affinity for RNA was noticed ($\Delta T_m/\text{modification} =$

Table 1. Hybridisation data for hexameric hexitol sequences (6'→4')

Sequence	HNA complement	ANA complement	RNA complement
h(1C1 CC1) (HNA)	52.4 (64)	58.8 (71)	31 (42)
h(UCU CCU) (HNA)	50.7 (61.2)	55	30.5 (40)
a(UCU CCU) (ANA)	54	61.8 (71.2)	38.4 (47.6)
h(TCT CCT) (HNA)	54	60.6	39 (48)
h(2C2 CC2) (HNA)	56.7	62.7	40 (49.5)

T_m values ($^\circ\text{C}$) in 0.1 M (1.0 M) NaCl buffer containing 20 mM phosphate, pH 7.4, with a duplex concentration of 4 μM . **1** denotes a 3'-O-methylated ANA monomer; **2** denotes the 1'-O-methylated HNA monomer.

$+0.2^\circ\text{C}$ and 0.6°C at 1 M NaCl), while none of the hexameric hexitol sequences displayed any interaction with DNA. While the 3'-O-methyl group proved beneficial when introduced into the parent HNA sequence, it was surmounted by the effect of a 3'-hydroxyl moiety as in ANA (15), although a correct comparison is not possible as the ANA sequence was fully modified ($\Delta T_m/\text{modification} = +1.2^\circ\text{C}$).

A more dramatic effect, however, was noted for a methyl substituent introduced at the 5 position of a HNA oligo (thymine bases substituting for uracil), with a $\Delta T_m/\text{modification}$ of 1.1°C when pairing with HNA, 1.8°C with ANA and as high as 2.8°C when pairing with RNA. Within the HNA series the importance of a 5-methyl moiety is clearly greater than the effect of a 3'-O-methyl. While the hydrophobic 5-methyl moiety probably contributes via improved stacking interactions, the effect of the axial 3'-O-methyl group residing in the minor groove is uncertain at this moment, but probably reduces the hydration potential. Methylation of the 3'-hydroxyl group of ANA seems to lower the beneficial influence of a 3'-hydroxyl moiety, as in ANA itself, when pairing with an RNA sequence is envisaged.

Incorporation of the pyranose analogue **2** in comparison with the hexitol T congener endows the oligo with higher affinity for hexitol oligonucleotides ($\Delta T_m/\text{modification} = +0.9^\circ\text{C}$ for HNA and $+0.7^\circ\text{C}$ for ANA) and gives a marginal advantage for pairing with RNA sequences ($\Delta T_m/\text{modification} = +0.3^\circ\text{C}$). Likewise, the effect of an axial 1'-O-methyl group seems additive with the effect of the 5-methyl moiety, making introduction of **2** slightly favourable over addition of a HNA monomer.

Affinity for RNA sequences

In view of their use as ASOs, the pairing potential of hexitol analogues with RNA is most interesting and needed further study. The congeners **1** and **2** were, therefore, incorporated into a mixed sequence HNA octamer and hybridised to their RNA complement. The results (Table 2) confirmed the beneficial influence for both methoxy substituents, with a substantially larger effect for **2** ($\Delta T_m = +1.6^\circ\text{C}$; $\Delta T_m = +3.6^\circ\text{C}$ compared to the hU congener). Care has to be taken when making a comparison with the fully modified ANA sequence, but once more the effect of a 3'-hydroxyl proved larger than the increase obtained with a 3'-O-methyl congener ($\Delta T_m/\text{modification} = +1^\circ\text{C}$ and 0.4°C , respectively), but both modifications proved to be accepted in a mixed sequence environment.

Table 2. Thermal stability of octameric hexitol sequences with single incorporation of methylated analogue **1** or **2** versus RNA

Sequence	T_m (°C) ^a	ΔT_m /modification
h(GCG UA GCG) (full HNA)	52	Reference
h(GCG 1A GCG)	52.4	+0.4
h(GCG TA GCG)	54	+2.0
h(GCG 2A GCG)	55.6	+3.6
a(GCG UA GCG) (full ANA)	59.6	+1.0
r(GCG UA GCG)	47.6	-4.4 ^b

^a T_m values towards complementary RNA, obtained in a buffer consisting of 0.1 M NaCl and 20 mM phosphate, pH 7.4, with a duplex concentration of 4 μ M.

^bData from Hendrix *et al.* (7).

Table 3. Thermal stability of homopolymeric sequences^a

Sequence	HNA complement	RNA complement
(hU) ₁₃ (HNA)	63 (40)	22 (35)
(1) ₁₃ (modified HNA)	78 (>35)	45 (35)
(hT) ₁₃ (HNA)	81.5 (>35)	48 ^b (35)
(2) ₁₃ (modified HNA)	>82 (>35)	57 ^c (33)
(dT) ₁₃ (DNA)	21 (49)	32 (32)

^a T_m values (°C) towards either complementary RNA or HNA, obtained in a buffer consisting of 0.1 M NaCl and 20 mM phosphate, pH 7.4, with a 4 μ M duplex concentration (4 μ M of each strand, 8 μ M for the self-pairing studies). The hypochromicity (%) is given in parentheses.

^bThe T_m of the self-pairing complex **hT**-**hT** was determined as 33°C (26% H).

^c T_m corresponding to the self-pairing complex **2**-**2** at 2 μ Mol.

Effect of homopolymeric sequences

With only **1** and **2** at hand, and although homopolymeric T/A or A/U complexes are known to deviate from the normal A-type RNA duplex, the results obtained with these homopolymeric stretches could be indicative of the interaction potential of fully modified oligonucleotide analogues. Indeed, full modification apparently allowed a cooperative effect of the methyl moieties and a tridecamer of **1** displayed a strong increase in affinity for HNA (ΔT_m /modification = +1.2°C, Table 3) and an even more pronounced increase in affinity for RNA (ΔT_m /modification = +1.8°C), well above the values found for single or multiple isolated incorporation. Likewise, the fully modified tridecamer of **2** showed a strong interaction potential, however, the affinity for RNA could not be determined as it was overshadowed by self-pairing of the homopolymer of **2**. Indeed, within the hexitol series the possibility of T-T self-pairing has been described (24) and substituting **2** for **hT** strongly increased this self-pairing potential (ΔT_m /modification = +2°C). Figure 1 allows a visual inspection of the melting curves for the different homopolymeric hexitol duplexes with the tridecamer of adenosine as the complement. The profile

Figure 1. Stability of duplexes comprising homopolymeric hexitol sequences and their RNA complement (rA)₁₃. Open symbols: circle, (**hU**)₁₃-RN; inverted triangle, (**1**)₁₃-RNA; triangle, (dT)₁₃-RNA. Filled symbols: circle, (**2**)₁₃-RNA; inverted triangle, (**hT**)₁₃-RNA; triangle, (**hT**)₁₃-(**hT**)₁₃.

obtained for the homopolymer of **2** proved identical with that obtained upon omission of the RNA complement. In contrast, absence of the 5-methyl moiety, as in the **hU** congener, abolished (or strongly reduced) self-pairing and likewise self-pairing seemed prohibited by the presence of the 3'-O-methyl group. It is an interesting observation that self-pairing of oligopyrimidines is not only a consequence of sugar modification but, likewise, can be dependent on the substitution pattern of the base moiety.

Incorporation into RNA sequences inducing constraint

Previous studies indicated that hexitol analogues show more resemblance to ribonucleosides. Therefore, incorporation of a single modification of either **1**, **2**, **hU** or **hT** (the respective HNA monomers) into RNA oligonucleotides was studied within different sequence contexts and the modified oligos obtained were evaluated versus RNA complementary sequences (Table 4). It has been documented that a single modified nucleotide when incorporated into natural nucleic acids may induce local geometry changes over several neighbouring base pairs. Therefore, the modified nucleotides were also incorporated within UpXpU, CpXpC, ApXpA and GpXpG motifs. Strong hybridising complexes were obtained, indicative of a pre-organised structure fitting the A-type dsRNA duplex. The thermal stabilities were compared with incorporation of 2'-O-methylated uridine monomers at the same position. As expected from the literature, the 2'-O-methyluridine-containing oligos (25,26) displayed increased affinity for the RNA complement over the non-methylated reference oligos. However, likewise, a systematic increase in affinity was noticed for both the methylated and non-methylated hexitol modification-containing oligos for their respective complementary sequences. The oligos with the plain hexitol modification (**hU**) surpassed the reference RNA oligos in affinity for

Table 4. Thermal stability of RNA sequences (5'→3') containing a single modified building block hybridised to complementary RNA monomers

Entry	Sequence	T_m (°C) ^a	T_m (°C) ^b
A	GCG U U U GCG	51.4 (59.3)	Reference
B	GCG U U _{OMe} U GCG	53.0 (60.8)	+1.6 (1.5)
C	GCG U hU U GCG	54.4 (61.6)	+3.0 (2.3)
D	GCG U 1 U GCG	55.4 (62.4)	+4.0 (3.1)
E	GCG U hT U GCG	55.8	+4.4
F	GCG U 2 U GCG	54.0	+2.6
G	GCU G U G UCG	55.9 (62.8)	Reference
H	GCU G U _{OMe} G UCG	57.3 (64.6)	+1.4 (1.8)
I	GCU G hU G UCG	57.1 (64.7)	+1.2 (1.9)
J	GCU G 1 G UCG	59.3 (66.5)	+3.4 (3.7)
K	GCU G hT G UCG	58.2	+2.3
L	GCU G 2 G UCG	56.3	+0.4
M	GCA C U C ACG	56.9 (63.8)	Reference
N	GCA C U _{OMe} C ACG	58.0 (65.1)	+1.1 (1.3)
O	GCA C hU C ACG	60.0 (66.9)	+3.1 (3.1)
P	GCA C 1 C ACG	60.8 (67.7)	+3.9 (3.9)
Q	GCC A U A CCG	57.1 (64.4)	Reference
R	GCC A U _{OMe} A CCG	58.8 (66.4)	+1.7 (2.0)
S	GCC A hU A CCG	57.2 (64.9)	+0.1 (0.5)
T	GCC A 1 A CCG	58.9 (66.2)	+1.8 (1.8)

U_{OMe}, 2'-*O*-methyluridine; **hU** and **hT**, 1,5-anhydrohexitol monomers.

^a T_m values towards complementary RNA, obtained in a buffer consisting of 0.1 M NaCl (1 M NaCl) and 20 mM phosphate, pH 7.4, with a duplex concentration of 4 μ M.

^b ΔT_m /modification.

their target, while the oligos containing the 3'-*O*-methylated althexitol modification **1** proved to have an even better affinity, surpassing the T_m values for the reference oligos by 3–4°C, except for the ApXpA motif (Table 4, entry T, +1.8°C). However, for this motif incorporation of hexitol U itself did not show any influence on T_m (Table 4, entry S).

On further analysis, one also notices that incorporation of **hU** between two pyrimidine bases (substituting for uridine, Table 4, entries C and O) is much more favourable than incorporation of **hU** in purine motifs (Table 4, entries I and S). A plausible explanation could be the improved pre-organisation of single strands containing purines due to stacking interactions, so that introduction of a pre-organised moiety like **hU** displays larger effects within pyrimidine motifs. Analogous effects of pre-organisation by purine bases through stacking effects can be used to explain the pairing potential of many acyclic or alicyclic oligonucleotide analogues, where adenine homopolymers show interaction with DNA and/or RNA, but the pyrimidine congeners to a much lesser extent (see for example 27–29). Comparing the effects of **1** and **hU**, one also notices that 3'-*O*-methylation of the hexitol ring is advantageous, particularly when the modification is positioned between two purine bases (+1, +0.8, +2.2 and +1.7°C for the U, C, G and A motifs, respectively). The latter effect is more difficult to rationalise.

In contrast, while incorporation of **hT**, as expected, has a larger impact compared to **hU** and while **1** further improves the effect of incorporation of **hU**, the 1'-*O*-methylglycoside **2** diminished the beneficial effect of incorporation of **hT**, but still retained an improved affinity compared to the all RNA oligonucleotide (two examples only). This is in contrast to previous observations (Tables 1 and 2), where incorporation of **2** in HNA proved more beneficial than incorporation of **1**. Clearly, it is possible to incorporate the newly modified monomers into RNA oligonucleotides without compromising the affinity for their respective RNA targets. Indeed, overall a clear increase in affinity is noticed and the 3'-*O*-methyl modification, as in **1**, especially proved superior under these conditions.

CONCLUSIONS

Generally, ONs containing a 3'-*O*-methyl derivative (**1**) showed a small increase in thermal stability towards complementary sequences as compared to HNA, except in the case of a self-complementary octameric HNA sequence for which an increase in thermal stability of 3°C per modification was observed (not shown). Overall and compared to ANA modifications, however, 3'-*O*-methylation seems to cause a small decrease in thermal stability of duplexes between a modified ON and a complementary target, especially when targeting RNA. The introduction of a hydrophobic moiety at the rim of the minor groove does not seem to have a large destabilising effect and the slightly decreased affinity of the methylated analogue as compared to the parent ANA towards complementary sequences is probably due to the reduced ability to form hydrogen bonds, i.e. loss of the ability to act as a proton donor. These results suggest that it is possible to derivatise the 3'-hydroxyl group of ANA sequences without significantly affecting the thermal stability of duplexes with complementary sequences, leaving room for alkylation using different alkyl moieties. In addition, for the glycosidic analogues **2** the potential seems to be there to obtain antisense compounds with higher affinity for RNA in comparison with well-known HNAs, while at the same time being economically more favourable monomers. Introduction of **2** gives improved hybridisation compared to dT as well as in comparison with **hT**. Compound **2** has less stabilising effect compared to **hT** only when a single modification is introduced in the middle of an RNA sequence, but this still surmounts the rU-containing sequence.

Taken together, the present findings eliminate the problem of the supplementary protecting group as necessary in alditol nucleic acids (ANAs) by alkylation of the [S]-hydroxyl group, which is liberated upon opening of the allitol epoxide by introduction of a heterocyclic base moiety (16). Such an alkylation reaction paves the way for a series of new nucleoside analogues, the 3'-*O*-methyl alditol nucleoside analogues (**1**) or, more generally, 3'-*O*-alkyl alditol nucleoside analogues, useful for incorporation into oligonucleotides. In addition, the present report details the use of a ubiquitous methylglucoside as the starting material for synthesis of 3'-deoxy-1'-*O*-methylglycosidic analogues (**2**) of 1,5-anhydrohexitol nucleosides, eliminating the need for reductive deoxygenation of the C1 position. The combination of these structural features and the study of mixed sequences are ongoing. The reason for the beneficial effect of 2'-*O*-methylation of RNA on duplex

stability has been the subject of much speculation, but a clear explanation has never been given. From this and other studies it is obvious that the influence of methylation is sequence-dependent and that many factors may be involved, among which an increase in rigidity of the modified sugar, hydrophobic interactions in the grooves, displacement of ions from the oligos, an enhancement or reduction in base stacking or steric repulsion of pyrimidine bases influencing the base orientation.

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