Investigation of the binding behaviors of isonucleoside-incorporated oligonucleotides with complementary sequences

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ABSTRACT: Oligonucleotides consisting of isonucleoside 2',5'-anhydro-3'-nucleobase-*D*-mannitol incorporated in 1'→4' linkage mode were synthesized. Their binding behaviors with complementary sequences were investigated via thermal denaturation and CD spectra. 6'-*O*-methyl-2',5'-anhydro-3'-(thymin-1-yl)-*D*-mannitol incorporated oligonucleotide was also synthesized to investigate the effect of hydroxy groups of isonucleosides on duplex formation. The results showed that the 6'-OH free isonucleoside-modified oligonucleotide was able to form a B-like duplex with 3'→5' complementary native oligodeoxynucleotide in the 1'→4' direction. The free hydroxy group in the isonucleoside made a significant contribution to the affinity of the modified oligonucleotide to the complementary sequence, which was confirmed by molecular dynamics simulation.

Key Words: Isonucleoside, nucleoside, oligonucleotide, chemical modification

Introduction

Antisense oligonucleotides manipulate the expression of specific genes by selective hybridization to target mRNA or DNA and can be used as an effective and specific therapeutic agent (*1-5*). They have several disadvantages, though, including instability with respect to cellular nuclease, unsatisfactory binding affinity, insufficient membrane penetration and low bioavailability, and restriction of the application of natural oligonucleotides. Various types of structure-modified oligonucleotides have been developed to overcome these problems (*6*). Modification of the phosphate linkage by phosphorothioate (PS) has been the first successful strategy for antisense drug development, and the first antisense drug (Fomivirsen) was approved by the FDA in 1998 for the treatment of cytomegalovirus-induced retinitis in patients with AIDS. 2'-*O*-substituted oligonucleotide is another type of well investigated modification that has been proven to be stable with respect to DNA or RNA cleaving enzymes (*7*). The mixed backbone containing PS and 2'-*O*-modified oligonucleotide retains RNase H activation properties that are present in PS antisense oligonucleotide and generally absent in 2'-*O*-modified antisense oligonucleotide. Furthermore, this mixed antisense oligonucleotide reduces the side effects caused by PS antisense oligonucleotide and improves the character of pharmacology, pharmacokinetics, and pharmacodynamics (*8*). Locked nucleic acid (LNA), which consists of a conformational restriction with a 2'-*O*-4'-C-methylene bridge, has been reported to exhibit unprecedented affinity to complementary DNA or RNA and shows promise as a therapy (*9-11*).

Isonucleoside is a type of modified nucleoside in which the nucleobase is transferred from C1'- to other positions of ribose, displaying both chemical and enzymatic stabilities (*12,13*). Though the incorporation of isonucleoside in the native structure of oligodeoxynucleotide would slightly decrease the stability of the duplex, the excellent antagonizing ability of the isonucleoside-incorporated oligonucleotide with respect to the hydrolysis of nuclease provides a promising way to design a stable antisense oligonucleotide (*14*). More recent investigation has shown that siRNA with isonucleoside modification on the sense strand still retains the gene silencing effect (*15*). Various types of isonucleoside-modified oligonucleotides (**1**-**5**) are reported to display different hybrid properties than complementary sequences. The homo-oligomer of modified mode **1**, **4**, or **5** was able to bind to the complementary sequence but the modified mode **2** or **3** did not show obvious hybridization ability (*13*). Molecular modeling showed that in the case of modified form **4** or **5** the 6'-OH group of each unit was located in the groove area when hybridized to $d(A)_{14}$, where it was able to form hydrogen bonds with water in

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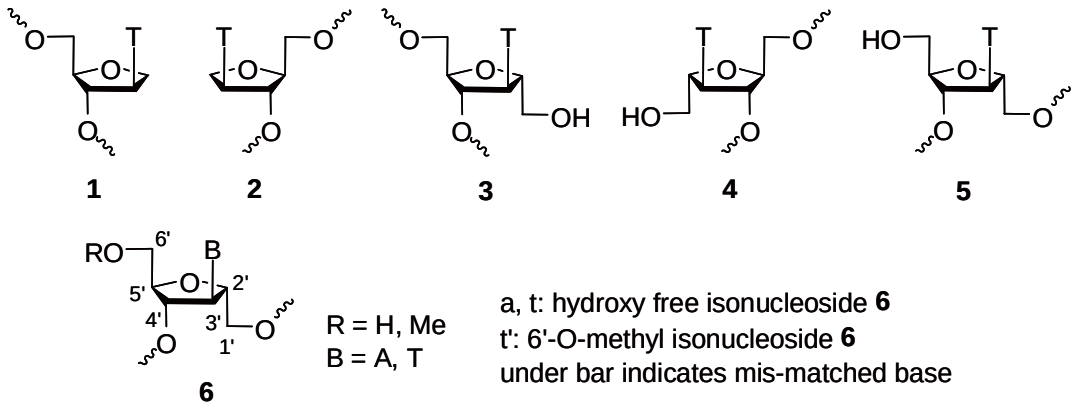


I: 5'-TTT TTT TTT TTTT-3'
II: 1'-ttt ttt ttt ttt-4'-5'-T-3'
III: 1'-t't't' t't't' t't't' t't't' -4'-5'-T-3'
IV: 5'-AAA AAA AAA AAAA-3'
V: 1'-ttt taa att aat aat aa-4'-5'-A-3'
VI: 5'-TTT TAA ATT AAT AAT AAA-3'
VII: 5'-AAA ATT TAA TTA TTA TTT-3'
VIII: 5'-TTT ATT ATT AAT TTA AAA-3'
IX: 5'-TCT AAC CCT AACT-3'
X: 5'-AGT TAG GGT TAGA-3'
XI: 5'-AGT TAG GGT TAG-3'-1'-a-4'
XII: 5'-aGT TAG GGT TAG-3'-1'-a-4'
XIII: 5'-AGT TAG GGT TAGT-3'
XIV: 5'-AGT TAG GGT TAGT-3'

Figure 1. Structures of isonucleosides and the sequences of isonucleoside-incorporated oligonucleotides.

the medium and may have contributed to the stability of duplex formation, while in the case of modified mode **3** most of the 6'-OH groups were directed to the inside of the duplex. These results indicate that the hydoxy group played a crucial role in duplex formation. In this work, an oligonucleotide constructed with isonucleoside **6** was designed and synthesized and the role of 6'-OH was validated by methyl protection. The stability and binding mode of the duplexes (Figure 1) formed by the modified oligonucleotide and its native counterpart were investigated by melting behavior and CD spectra. The results may provide some insight into the design of chemically modified antisense nucleotides.

Materials and Methods

General methods

All solvents were dried and distilled prior to use. Thin layer chromatography (TLC) was performed using silica gel GF-254 (Qing-Dao Chemical Co., China) plates with detection by UV. Column chromatography was performed on silica gel (200-300 mesh or flash, Qing-Dao Chemical Co.). Evaporations were carried out under reduced pressure with a bath temperature below 45°C.

^{1}H-NMR spectra were recorded on Varian VXR-300 or Varian Inova 500 with TMS as an internal standard. ESI-TOF MS were recorded on ABI QSTAR. ^{31}P-NMR spectra were recorded on Bruker AC 2007 or Varian VXR-300. MALDI-TOF MS were recorded on Bruker BIFLEX III or Kratos PC Axima-plus. UV spectra were recorded with Varian Cary 300.

Molecular optimization was performed using the Gaussian 98 program. Unrestrained molecular dynamics simulations were performed for the oligonucleotide duplexes studied by using the Amber 8 suite of programs (*16*). The Amber99 force field was used to describe the DNA. An 8Å truncated octahedron of TIP3P water was added to solvate the structures and counterions of Na^{+} were placed next to each phosphate group. The simulations were conducted for 1.5 ns at a constant pressure (1 atm) and temperature (300K). Stable trajectories of geometry and energy terms were acquired after 500 ps of simulations and the averaged structures during the last 800 ps period of simulations were generated.

Synthesis

1'-O-Benzoyl-3'-deoxy-3'-(adenin-9-yl)-2',5'-anhydro-D-mannitol (10) and 6'-O-benzyol-3'-deoxy-3'-(adenin-9-yl)-2',5'-anhydro-D-mannitol (11)

Saturated ammonia/anhydrous methanol (5 mL) was added to a solution of compound **9** (0.8 g, 1.6 mmol) in anhydrous methanol (10 mL). The reaction mixture was stirred at room temperature for about 1 hour and monitored by TLC. Once the fully deprotected product was detected, the reaction was stopped by removing the solvent under reduced pressure. The mixture was partially separated by silica gel column chromatography (CH_2Cl_2/CH_3OH: 80/1-10/1) to provide the mono-benzoyl protected mixture of products (**10**, **11**) (150 mg, 48.7%) in a ratio of about 1:1, and starting material **9** was collected. Compounds **10** and **11** were separated carefully by silica gel column chromatography for structure characterization.

Compound 10: ^{1}H-NMR (300MHz, DMSO-d_6) δ: 3.65 (m, 2H, 6'-H), 3.91 (dd, 1H, 1'-H), 4.13 (dd, 1H, 6'-H), 4.39 (m, 1H, 5'-H), 4.71 (m, 1H, 4'-H), 4.97 (m, 1H, 2'-H), 5.20 (m, 1H, 3'-H), 7.26 (s, 2H, D_2O exchangeable, 6-NH_2), 7.43 (m, 2H, Ar-H), 7.62 (m, 3H, Ar-H), 8.09 (s, 1H, 2-H), 8.19 (s, 1H, 8-H).

Compound 11: ^{1}H-NMR (300MHz, DMSO-d_6) δ: 3.64 (dd, 2H, 1'-H), 3.90 (m, 1H, 5'-H), 4.37 (m, 2H, 2'-H, 4'-H), 4.73 (m, 2H, 6'-H), 4.93 (m, 1H, 3'-H), 7.30 (s, 2H, D_2O exchangeable, 6-NH_2), 7.48 (m, 2H, Ar-H), 7.65 (m, 1H, Ar-H), 7.79 (d, 2H, Ar-H), 8.11 (s, 1H, 2-H), 8.33 (s, 1H, 8-H).

Mixture of compounds **10** and **11**: ESI-TOF$^+$: 386 $[M + H]^+$.

1'-O-Benzoyl-3'-deoxy-3'-(6-N-benzoyl-adenin-9-yl)-2',5'-anhydro-D-mannitol (12) and 6'-O-benzoyl-3'-deoxy-3'-(6-N-benzoyl-adenin-9-yl)-2',5'-anhydro-D-mannitol (13)

The mixture of compounds **10** and **11** (0.33 g, 0.86 mmol) was dissolved in anhydrous pyridine (10 mL), TMSCl (1.7 mL, 13.4 mmol) was added in an ice bath. The solution was stirred at room temperature for about 1 h till no starting material was detected by TLC, and then benzoyl chloride (0.8 mL, 6.83 mmol) was added. After 2 h of stirring, ammonia was added in drops to adjust the solution to pH 9 and stirred at room temperature for about 3 h. After evaporation, the residue was separated by silica gel chromatography (CH_2Cl_2/CH_3OH: 80/1-30/1) to provide a mixture of compounds **12** and **13** (0.407 g, 96%). Compound **12** was partially obtained as white crystals by recrystallization from ethanol.

Compound **12**: ^{1}H-NMR (300MHz, DMSO-d_6) δ: 3.47 (m, 2H, 6'-H), 4.19 (m, 1H, 5'-H), 4.48 (m, 1H, 4'-H), 4.60 (m, 2H, 1'-H), 5.00 (m, 2H, 2'-H, 3'-H), 7.64 (m, 6H, Ar-H), 8.05 (m, 2H, Ar-H), 8.13 (m, 2H, Ar-H), 8.61 (s, 1H, 2-H), 8.69 (s, 1H, 8-H), 11.19 (s, 1H, D_2O exchangeable, NH).

Mixture of compounds **12** and **13**: ESI-TOF$^+$: 490 $[M]^+$.

6'-O-(4,4-Dimethoxytriphenylmethyl)-1'-O-benzoyl-3'-deoxy-3'-(6-N-benzoyl-adenin-9-yl)-2',5'-anhydro-D-mannitol (14) and 1'-O-(4,4-dimethoxytriphenyl methyl)-6'-O-benzyol-3'-deoxy-3'-(6-N-benzoyl-adenin-9-yl)-2',5'-anhydro-D-mannitol (15)

The mixture of compounds **12** and **13** (0.2 g, 0.41 mmol) was dissolved in anhydrous pyridine (10 mL), and dimethoxytrityl chloride (0.15 g, 0.43 mmol) was added. The solution was stirred at room temperature for 24 h. After evaporation, the residue was purified by silica gel chromatography (CH_2Cl_2/CH_3OH: 100/1-10/1) to provide compound **14** (101 mg, 35%) and compound **15** (111 mg, 34%) as white foam, respectively.

Compound **14**: ^{1}H-NMR (500MHz, DMSO-d_6) δ: 3.24 (m, 2H, 6'-H), 3.73 (2s, 6H, DMT-OCH_3), 4.16 (m, 1H, 5'-H), 4.48 (m, 2H, 1'-H), 4.89 (m, 1H, 4'-H), 4.93 (m, 1H, 2'-H), 5.08 (m, 1H, 3'-H), 5.83 (d, J = 6Hz, 1H, D_2O exchangeable, 4'-OH), 6.86 (m, 4H, Ar-H), 7.23 (m, 1H, Ar-H), 7.30-7.35 (m, 7H, Ar-H), 7.48-7.58 (m, 7H, Ar-H), 7.66 (m, 2H, Ar-H), 7.81 (d, 2H, Ar-H), 8.67 (s, 1H, 2-H), 8.71 (s, 1H, 8-H), 11.20 (s, 1H, D_2O exchangeable, 6-NH). ESI-TOF$^+$: 792 $[M + H]^+$.

Compound **15**: ^{1}H-NMR (500MHz, DMSO-d_6) δ: 3.09 (m, 1H, 1'-H), 3.20 (m, 1H, 1'-H), 4.27 (m, 1H, 5'-H), 4.51 (m, 1H, 6'-H), 4.62 (m, 1H, 6'-H), 4.65 (m, 1H, 2'-H), 4.93 (m, 1H, 4'-H), 5.11 (m, 1H, 3'-H), 5.97 (d, J = 6Hz, 1H, D_2O exchangeable, 4'-OH), 6.77 (m, 4H, Ar-H), 7.08-7.10 (m, 4H, Ar-H), 7.15-7.21 (m, 5H, Ar-H), 7.52-7.72 (m, 6H, Ar-H), 8.06 (d, 2H, Ar-H), 8.11 (m, 2H, Ar-H), 8.65 (s, 1H, 2-H), 8.64 (s, 1H, 8-H), 11.20 (s, 1H, 6-NH, D_2O exchangeable). ESI-TOF$^+$: 792 $[M + H]^+$.

1'-O-(4,4-Dimethoxytriphenylmethyl)-4'-O-(2-cyanoethyl-N,N-diisopropyl) phosphoramidite-1'-O-benzoyl-3'-deoxy-3'-(6-N-benzoyl-adenin-9-yl)-2',5'-anhydro-D-mannitol (16)

Compound **15** (110 mg, 0.14 mmol) was dried in a vacuum and dissolved in anhydrous THF (2 mL) in an argon atmosphere. Diisopropylethylamine (DIPEA, 97 μL, 0.56 mmol) and 2-cyanoethyl-*N,N*-diisopropyl-chlorophosphoramidite (62 mL, 0.28 mmol) were added to the solution. The mixture was stirred at 0°C for 10 min and continued to be stirred at room temperature for 40 min. Then, the reaction mixture was quenched by addition of MeOH (1 mL). After stirring for 10 min, EtOAc (15 mL) was added and the organic layer was washed with 5% aqueous $NaHCO_3$ (2 × 5 mL), followed by H_2O (5 mL) and then drying over anhydrous Na_2SO_4. After evaporation, the oily residue was purified by silica gel column chromatography (petroleum ether/AcOEt/CH_2Cl_2: 10/1/1-4/1/1, 0.3% Et_3N) to provide compound **16** as a white foam (120 mg, 86%). ^{31}P-NMR ($CDCl_3$, ppm) δ: 150.7, 151.6.

6-O-Benzoyl-2,5:3,4-dianhydro-D-talitol (17) and 1-O-benzoyl-2,5:3,4-di-anhydro-D-talitol (18)

Saturated ammonia/anhydrous methanol (20 mL) was added to the solution of compound **7** (7.0 g, 20.0 mmol) in anhydrous methanol (60 mL). The solution was stirred at room temperature till the fully deprotected product was detected by TLC (identified by phosphatomolybdic acid in ethanol). After evaporation, the residue was separated by silica gel column chromatography (petroleum ether/EtOAc: 10/1-2/1) to provide a mixture of compounds **17** and **18** (in total: 1.9 g, 80%), and starting material (3.7 g) was collected.

Compound **17**: ^{1}H-NMR (300MHz, $CDCl_3$) δ: 3.43 (m, 2H, H-1), 3.85 (m, 1H, 2-H), 4.01 (m, 2H, 3-H, 4-H), 4.34 (m, 3H, 5-H, 6-H), 4.88 (m, 1H, 1-OH), 7.55 (m, 2H, Ar-H), 7.67 (m, 1H, Ar-H), 8.01 (m, 2H, Ar-H).

Compound **18**: ^{1}H-NMR (300MHz, $CDCl_3$) δ: 3.43 (m, 2H, 6-H), 4.00 (m, 3H, 3-H, 4-H, 5-H), 4.32 (m, 1H, 2-H), 4.38 (d, 2H, 1-H), 4.90 (m, 1H, 6-OH), 7.56 (m, 2H, Ar-H), 7.68 (m, 1H, Ar-H), 7.97 (m, 2H, Ar-H).

6-O-Benzoyl-1-O-methyl-2,5:3,4-dianhydro-D-talitol (19) and 1-O-benzoyl-6-O-methyl-2,5:3,4-dianhydro-D-talitol (20)

The mixture of compounds **17** and **18** (3.4 g, 13.6 mmol) was dissolved in an ether solution of CH_2N_2 (150 mL), silica gel (5 g, 200-300 mesh) was added, and the solution was stirred at room temperature for 2 h. After evaporation under reduced pressure, the residue was purified on a silica gel column (petroleum ether/EtOAc: 10/1-5/1) to provide a mixture of compounds **19** and **20** (in total: 2.5 g, 70%) as a white syrup. ^{1}H-NMR (300MHz, $CDCl_3$) δ: 3.37 (s, 3H, OCH_3), 3.42 (s, 3H, OCH_3). Anal. Calcd. for $C_{14}H_{16}O_5$: C, 63.63; H, 6.10. Found: C, 63.44; H, 5.98.

6'-O-Benzyl-1'-O-methyl-3'-deoxy-3'-(thymin-l-yl)-2',5'-anhydro-D- mannitol (21) and 1'-O-benzyl-6'-O-methyl-3'-deoxy-3'-(thymin-1-yl)-2',5'-anhydro-D-mannitol (22)

Thymine (2.8 g, 22.6 mmol) in anhydrous DMF, DBU (7 mL, 46 mmol) was added to the solution of compounds **19** and **20** (4 g, 15.1 mmol) in drops at room temperature. The solution was stirred at room temperature for 30 min and then at 90-100°C for 48 h. After evaporation, the residue was applied to a silica gel column (petroleum ether/EtOAc: 5/1-2/1) to provide a mixture of **21** and **22** (in total: 2.0 g, 52%) as a yellow syrup. Unreacted starting materials **19** and **20** were collected. The mixture was characterized by ^{1}H-NMR, and two sets of signals were observed.

1'-O-Methyl-3'-deoxy-3'-(thymin-l-yl)-2',5'-anhydro-D-mannitol (23) and 6'-O-methyl-3'-deoxy-3'-(thymin-l-yl)-2',5'-anhydro-D-mannitol (24)

Saturated ammonia/anhydrous methanol (15 mL) was added to the solution of compounds **21** and **22** (0.29 g, 0.74 mmol) in methanol (15 mL). The solution was stirred at room temperature overnight. After evaporation, the residue was applied to a silica gel column (CH_2Cl_2/CH_3OH: 50/1-10/1) to provide a mixture of **23** and **24** (in total: 0.18 g, 83%) as a yellow syrup. The mixture was characterized by ^{1}H-NMR, and two sets of signals were observed.

Compound **23**: ^{1}H-NMR (500MHz, DMSO-d_6) δ: 1.76 (s, 3H, 5-CH_3), 3.24 (s, 3H, O-CH_3), 3.34 (m, 2H, 1'-H), 3.54 (m, 2H, 6'-H), 3.68 (m, 1H, 5'-H), 4.05 (m, 1H, 2'-H), 4.20 (m, 1H, 4'-H), 4.68 (m, 1H, 3'-H), 7.58 (s, 1H, 6-H).

Compound **24**: ^{1}H-NMR (500MHz, DMSO-d_6) δ: 1.76 (s, 3H, 5-CH_3), 3.24 (s, 3H, O-CH_3), 3.25-3.50 (m, 4H, 1'-H, 6'-H), 3.80 (m, 1H, 5'-H), 3.88 (m, 1H, 2'-H), 4.16 (m, 1H, 4'-H), 4.63 (m, 1H, 3'-H).

6'-O-Dimethoxyltrityl-1'-O-methyl-3'-deoxy-3'-(thymin-l-yl)-2',5'-anhydro-D-mannitol (25) and 1'-O-dimethoxyltrityl-6'-O-methyl-3'-deoxy-3'-(thymin-l-yl)-2',5'-anhydro-D-mannitol (26)

Similar to the preparation of **14** and **15** from **12** and **13**, compounds **25** and **26** were obtained from **23** and **24** as a white foam at a 74% yield.

Compound **25**: ^{1}H-NMR (500MHz, DMSO-d_6) δ: 1.79 (s, 3H, 5-CH_3), 3.09 (m, 2H, 1'-H), 3.32 (s, 3H, 1'-OCH_3), 3.49 (m, 2H, 6'-H), 3.73 (s, 6H, DMT-OCH_3), 3.84 (m, 1H, 5'-H), 4.01 (m, 1H, 2'-H), 4.15 (m, 1H, 4'-H), 4.74 (m, 1H, 3'-H), 5.60 (d, J = 6Hz, 2H, D_2O exchangeable, 4'-OH), 6.84 (m, 4H, Ar-H), 7.20-7.32 (m, 8H, Ar-H), 7.53 (s, 1H, 6-H), 11.28 (s, 1H, D_2O exchangeable, NH). ESI-TOF$^+$ MS: 611 $[M + Na]^+$.

Compound **26**: ^{1}H-NMR (500MHz, DMSO-d_6) δ: 1.79 (s, 3H, 5-CH_3), 3.13 (m, 2H, 6'-H), 3.32 (s, 3H, 6'-OCH_3), 3.45 (m, 2H, 1'-H), 3.73 (s, 6H, DMT-OCH_3), 3.85 (m, 1H, 5'-H), 4.01 (m, 1H, 2'-H), 4.19 (m, 1H, 4'-H), 4.65 (m, 1H, H-3'), 5.61 (d, J = 6Hz, 1H, D_2O exchangeable, 4'-OH), 6.84 (m, 4H, Ar-H), 7.20-7.32 (m, 8H, Ar-H), 7.56 (s, 1H, 6-H), 11.28 (s, 1H, D_2O exchangeable, NH). ESI-TOF$^+$ MS: 611 $[M + Na]^+$.

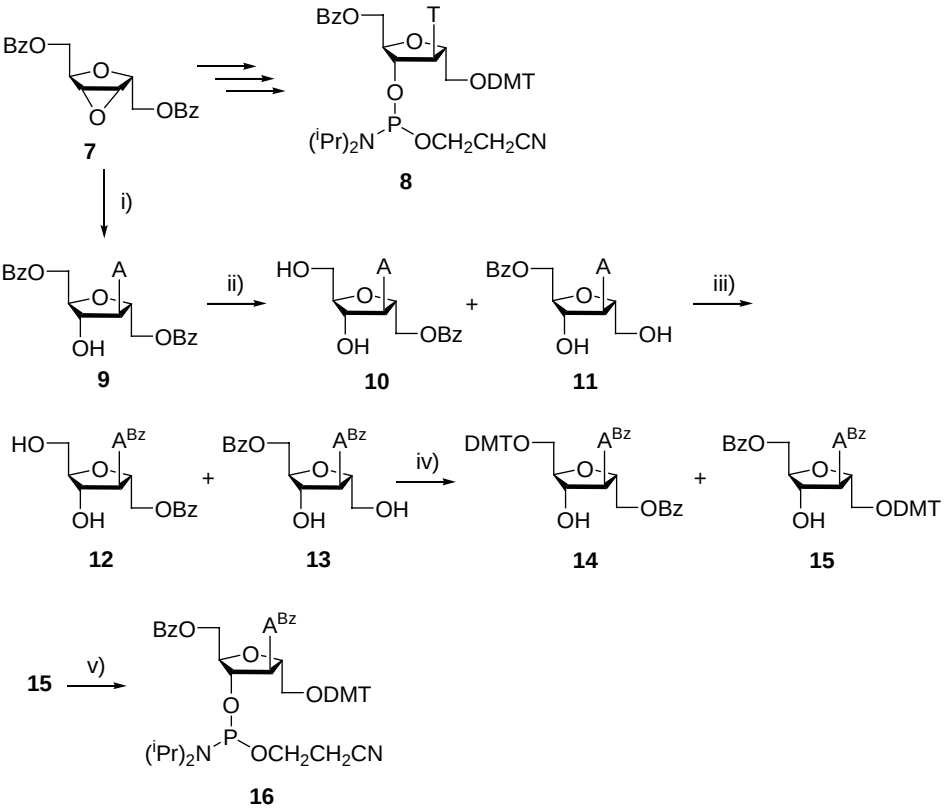
1'-O-(4,4-Dimethoxytriphenylmethyl)-4'-O-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidite-6'-O-methyl-3'-deoxy-3'-(thymin-l-yl)-2',5'-anhydro-D-mannitol (27)

Similar to the preparation of **16** from **15**, compound **27** was obtained from **26** as a white foam at a 74% yield. ^{31}P-NMR ($CDCl_3$, ppm) δ: 157.2.

Solid-phase synthesis of oligonucleotides

Oligonucleotide synthesis was carried out on a 1-μM scale with a DNA synthesizer (model 392A, Applied Biosystems) applying regular phosphoramidite chemistry. Cleavage and deprotection of the oligonucleotides were performed in a concentrated aqueous ammonia solution at 80°C for 2 h. The oligonucleotides were purified by PAGE (DMT-off) or HPLC (C18, DMT-on) and desalted by OPC (ABI). The pure oligonucleotides were lyophilized and stored at -20°C.

Thermal denaturation and CD spectra



Scheme 1. Reagents and conditions: i) adenine, DBU, DMF, 90-100°C; ii) NH_3/CH_3OH; iii) a. TMSCl, b. BzCl, pyridine, c. NH_3/H_2O; iv) DMTCl, pyridine; v) $ClP(OCH_2CH_2CN)N(^iPr)_2$, $EtN(^iPr)_2$, THF.

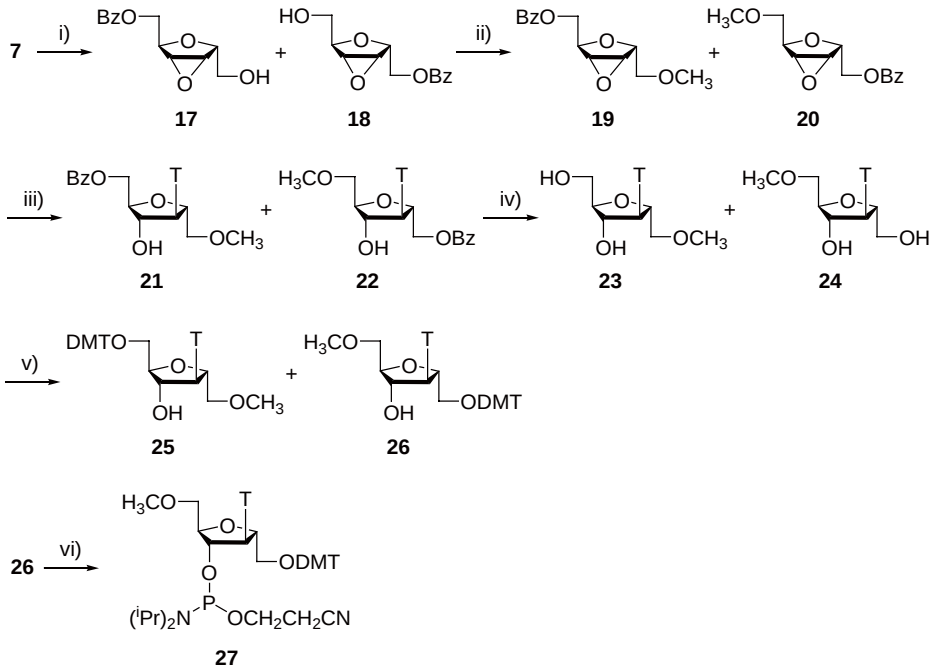
The oligomers were dissolved in a buffer containing 0.14 M NaCl, 0.01 M Na_2HPO_4, and 1.0 mM EDTA at pH 7.2. The solution containing oligonucleotide at a concentration of 4 μM was mixed with an equimolar amount of its complementary sequence. Sample were incubated at 80°C for 5 min, then gradually cooled to 4°C, and kept at this temperature for 12 h. Then, the sample was used for the investigation of thermal denaturation and CD spectra. Thermal denaturations were recorded on a Varian Cary 300 spectrophotometer at 260 nm. Sample temperature was increased at 0.5°C/min intervals between 20 and 80°C. CD spectra were measured at 5°C with a J720 polarized spectrophotometer in thermostatically controlled 1 cm cuvettes.

Results and Discussions

Synthesis of oligonucleotides

Isonucleoside phosphoramidite **8**, suitable for solid-phase oligonucleotide synthesis, was obtained by starting with 2',5'-anhydro-1',6'-*O*-dibenzoyl-3'-(thymin-1-yl)-*D*-mannitol, which was prepared from *D*-glucosamine in five steps based on a previous strategy (*13*). The isonucleoside phosphoramidite **16** was also obtained by a similar method (Scheme 1). The amino group of compound **10** or **11** was protected selectively by the "one-pot" method (*17*), in which compound **10** or **11** was first treated with trimethylchlorosilane to protect the hydroxyl groups, and then benzoyl chloride was added to provide *N*-acylation products. Finally, the trimethyl group was hydrolyzed in a basic environment (pH 9) by adding ammonia water (25%) to the reaction solution. Under these pH conditions, the benzoyl group remained. Compounds **10** and **11** or **12** and **13** possessed very similar polarity and were difficult to separate, so the mixture was directly used for the subsequent reaction. The mixture of compounds **12** and **13** reacted with dimethoxytrityl chloride and provided compounds **14** and **15**, which were then separated by silica gel chromatography.

Isonucleoside phosphoramidite **27** was provided by the key intermediate epoxide **7** via six steps (Scheme 2). Compound **7** was debenzoylated with 50% NH_3/CH_3OH at room temperature to provide compounds **17** and **18** in a ratio of about 4:1. Compounds **17** and **18** were difficult to separate, so the mixture was used in the following reaction till the dimethoxytrityl group was used for the protection of the 1'-OH group. Compounds **17** and **18** were methylated in CH_2N_2/ether in which silica gel was used as a catalyst to provide compounds **19** and **20**, which was then reacted with thymine under basic conditions to provide isonucleosides **21** and **22**. After debenzoylation and protection with dimethoxytrityl groups, compounds **25** and **26** were provided, which were then separated



Scheme 2. Reagents and conditions: i) NH_3/CH_3OH; ii) CH_2N_2, ether; iii) Thymine, DBU, DMF, 90-100°C; iv) NH_3/CH_3OH; v) $ClP(OCH_2CH_2CN)N(^iPr)_2$, $EtN(^iPr)_2$, THF.

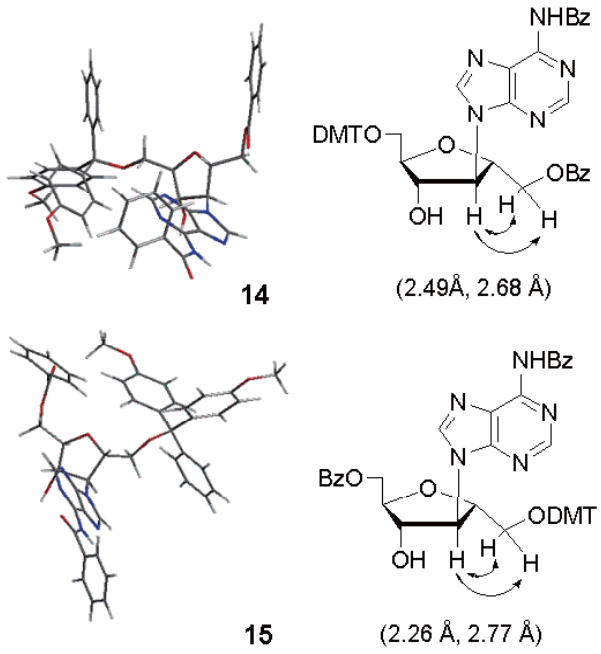


Figure 2. Characterization of compounds **14** and **15** by NOE and molecular modeling.

by silica gel chromatography. The phosphoramidite **27** was obtained by the phosphination of compound **26**. The structures of **14**, **15**, **25** and **26** were identified by ^{1}H-NMR, H-H COSY, and NOESY spectra. The proton signals of the CH_2 connected to OBz appeared to be lower than those of the CH_2 connected to ODMT. Molecular modeling implied that there was a relatively strong NOE between the protons of 1'-H and 3' -H (Figure 2), which was in accordance with NMR characterization.

All isonucleoside-incorporated oligonucleotides were assembled on an automated DNA synthesizer on a 1 μ-mol scale. Synthesis was followed by the standard phosphoramidite protocol except for three injections and a prolonged coupling time of 80 sec every time to ensure adequate coupling yields. For convenience, commercially available nucleotide-attached CPG was used in the synthesis of isonucleoside-incorporated oligonucleotide and one native nucleotide was retained at the 3'-end of the sequence. Universal CPG (Proligo) was used for the synthesis of oligonucleotide with a single isonucleoside modified at the 3'-end. The coupling efficiency was obtained by measuring the release of DMT (95-97%). The crude DMT-protected oligomers were purified by HPLC (C18 column) and then detritylated in 80% acetic acid for 30 min and desalted by OPC (oligonucleotide purification cartridge, ABI) to provide the pure oligomer products. The purities of oligomers were confirmed by capillary electrophoresis or by HPLC analysis. The molecular weights were confirmed by MALDI-TOF mass spectrometry.

The base paring properties of isonucleoside-incorporated oligonucleotides with respect to their complementary sequences were investigated by thermal denaturation and circular dichroism (CD) spectra. Table 1 summarizes the melting temperatures (Tm values) determined by ultra-violet (UV) spectroscopy. The results indicate that oligomer **II** was able to bind with its complementary sequence **IV** though the Tm value was a little lower than that of the native control (**I**/**IV**) (Table 1). When the 6'-OH of isonucleoside was

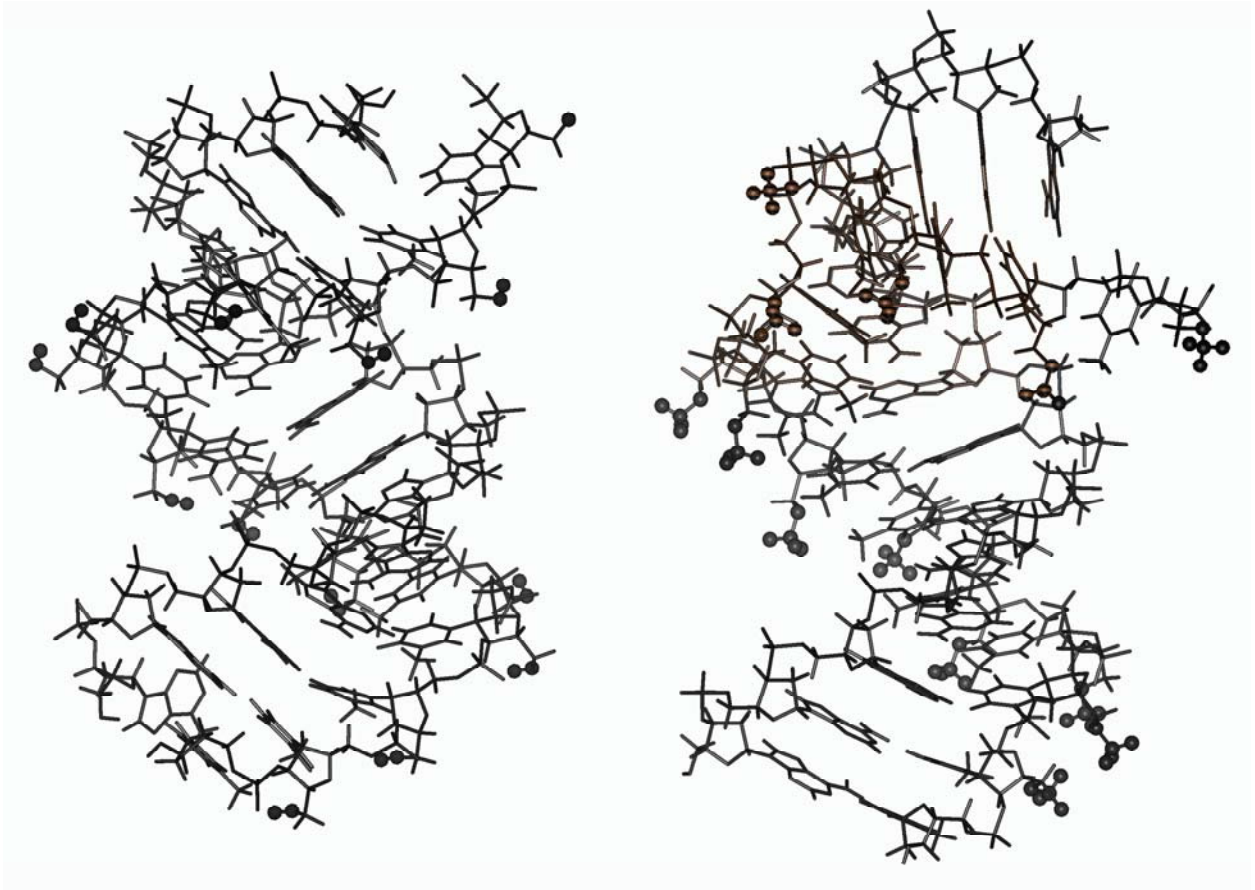


Figure 3. Average structures of duplexes formed by isonucleoside-incorporated oligonucleotides and their native complementary sequences. 6'-OH and 6'-OMe are highlighted with filled-in circles. (Left) duplex **II/IV**; (Right) duplex **III/IV**.

Table 1. Melting temperature of duplexes formed by isonucleoside-incorporated oligonucleotides and their complementary sequences

Duplex	Tm (ΔT) (°C)
I/IV	34.9
II/IV	33.1 (-1.8)
III/IV	-
V/VII	-
V/VIII	29.0 (-8.0)
VI/VII	-
VI/VIII	37.0
X/IX	42.0
XI/IX	40.0 (-2.0)
XII/IX	41.1 (-1.1)
XIII/IX	39.0 (-3.0)
XIV/IX	40.2 (-1.8)

ΔT is the difference in melting temperature between the modified duplex and native oligodeoxynucleotide duplex.

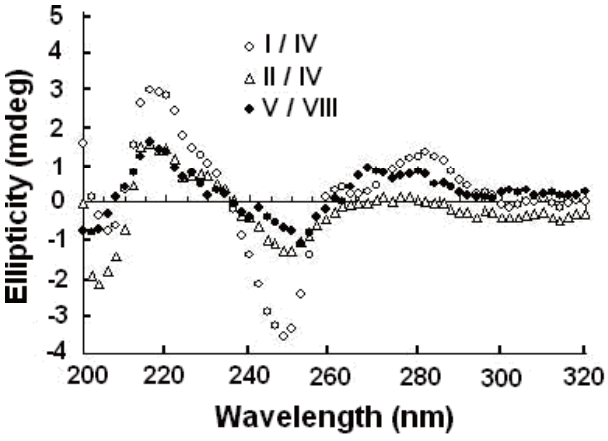


Figure 4. CD spectra of duplexes formed by isonucleoside-incorporated sequences and native complementary sequences.

protected by a methyl group, as shown by oligomer III, no obvious binding to the native complementary sequence was observed. This fact undoubtedly demonstrates that 6'-OH contributed to the stability of the isonucleoside-incorporated duplex. Molecular dynamics simulation also supported this conclusion (Figure 3). Simulation results showed that Watson-Crick base paring and base stacking were retained in the case of free 6'-OH, whereas in the case of 6'-OMe base paring and stacking were disturbed.

Theoretically, isonucleoside-incorporated oligonucleotide has two possible ways to bind with its counterpart, *i.e.*, binding to the 5'→3' native complementary sequence in the 1'→4' or 4'→1' direction. A mixed purine-pyrimidine sequence **V** has been designed to clarify the mode of binding. Thermal denaturation studies showed that a relatively stable duplex was formed between sequences **V** and **VIII**, but no obvious binding was observed between sequence **V** and **VII**. The results indicated that isonucleoside-

incorporated oligonucleotide bound with 5'→3' native complementary oligodeoxynucleotide in the 4' →1' direction. Table 1 also shows that the effect of isonucleoside on the decrease in melting temperature in the mixed purine-pyrimidine sequence (**II/IV**) was much more obvious than in the homo-pyrimidine sequence (**V/VIII**). Single nucleoside modification at one or two ends of the sequence led to the decrease in melting temperature for about 1-2°C, but the effect was a little weaker than the corresponding mis-matched sequence. Considering the increased enzymatic stability of the modified oligonucleotide, the amount of decrease in the binding ability was acceptable. This implied that a stable chemically modified antisense oligonucleotide could be designed by attaching an isonucleoside at the ends of the native sequence.

CD spectra were used to study the conformation of the above duplexes (Figure 4). The spectrum of the duplex dT_{14}/dA_{14} (**I/IV**) showed a positive Cotton peak at 217 nm and a negative Cotton peak at 248 nm, which is the typical character of a B-form DNA conformation. Duplex **II/IV** and **V/VIII** also displayed very similar spectra, which suggested that the modified oligonucleotide duplexes adopted similar B-form conformations. The low intensities of the Cotton peaks of modified duplexes resulted from the poor base stacking of duplexes in which Watson-Crick base paring was disturbed by the torsion of the backbone originating from the introduction of isonucleoside.

In summary, oligonucleotides consisting of isonucleoside 2',5'-anhydro-3'-nucleobase-*D*-mannitol or 6'-*O*-methyl-2',5'-anhydro-3'-(thymin-1-yl)-*D*-mannitol incorporated in 1'→4' linkage mode were synthesized. Their binding behaviors with complementary sequences were investigated via thermal denaturation and CD spectra. A mixed purine-pyrimidine sequence showed that the modified oligonucleotide was able to form a duplex with 3'→5' complementary native oligodeoxynucleotide in the 1'→4' direction. All duplexes were able to adopt B-like DNA conformations. The free hydroxy group in isonucleoside contributed to the modified oligonucleotide's affinity to the complementary sequence, which was in accordance with the results of molecular modeling. The results provided some insight into the design of chemically stable antisense oligonucleotides, *e.g.* by incorporating an isonucleoside at one or two ends of the sequence.

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