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Synthesis and Hybridization Property of Sugar and Phosphate Linkage Modified Oligonucleotides

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Abstract—1-[3-Deoxy-5-*O*-(4,4′-dimethoxytriphenylmethyl)-3-*C*-hydroxymethyl-2-*O*-(2-methoxyethoxymethyl)-β-D-*erythro*-pentofuranosyl]thymine (**13**) was synthesized from 1,2-isopropylidene-D-xylose (**1**) as a building block of modified oligonucleotides. Three types of novel oligonucleotides were synthesized from **13** and their T_ms were compared with those of the corresponding natural oligonucleotides. It was found that our synthesized oligomers had lower affinity to DNA and RNA than the natural oligomers.

Introduction

Since oligonucleotides complementary to messenger RNA or viral RNA were proved to inhibit the production of proteins and viral replications in the 1970s,[1,2] and the natural gene regulation via antisense RNA was discovered in the 1980s,[3,4] antisense research for the development of therapeutics has been explored extensively. Many oligonucleotides also have been used as important tools to study specific genes, proteins and their functions in cells.

Antisense oligonucleotides bind to complementary messenger RNA following Watson–Crick base pairing rules, which result in the inhibition of protein synthesis.[5–7] Natural oligonucleotides which are added from outside of cells do not show enough antisense effects because of rapid degradation by cell nucleases. In order to overcome the degradation by nucleases and to improve affinity to DNA and RNA, base and sugar moieties of nucleoside structures have been modified extensively.

Various conjugation approaches to improve delivery to cells have also been reported, among which liposomes are being used for many in vitro and in vivo experiments for antisense screenings.[8–10] The progress of the Human Genome Project and other sequencing projects has led to many gene sequences now being available, and the opportunity for the antisense agents in various diseases and biological experiments is growing rapidly.

Vitravene, the first antisense drug, was approved by the Food and Drug Administration (FDA) for the treatment of AIDS patients infected with cytomegalovirus retinitis.[11] The phosphorothioate oligomers, however, have shown side effects, causing several immune responses probably due to protein bindings.[12–14] Since phosphorothioate linkage is believed to cause these undesired effects, several approaches which use non-phosphorothioates or less phosphorothioate linkages have been attempted. In 1997, the second generation oligonucleotides consisted of mixed backbone of phosphorothioate and 2′-*O*-alkylated oligonucleotides entered the human clinical trials.[15]

Recently, many sugar backbone modified oligonuleotides have been reported to increase resistance to nucleases and to give better binding affinities to mRNA.[6,16,17] When we recently started the antisense research program and focused on the structure modification of nucleosides, oligonucleotide analogues with electronegative moiety at the C2′ position appeared to have certain superior characteristics such as the preference of A-type geometry for duplex formation.[18] Incorporation of methoxyethoxymethyl (MEM) group into the 2′ hydroxyl position of the sugar backbone was chosen, expecting more 3′-endo sugar conformation (A type duplex) due to the *gauche* effect[19] between the furanose ring oxygen and the oxygen of 2′-OMEM. In view of giving more flexibility of phosphate linkage which may induce A conformation with

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RNA, the 3′-hydroxymethyl group was also designed with 2′-*O*-MEM substituent. Although several syntheses of 2′,3′-dideoxy-3′-*C*-hydroxymethyl nucleosides have been published,[20,21] 3′-deoxy-2′-*O*-substituted-3′-*C*-hydroxymethyl nucleoside has not been reported. Thus, we report the synthesis of 3′-deoxy-2′-*O*-substituted-3′-*C*-hydroxymethyl nucleoside and its oligomers and hybridization study.

Results and Discussion

Nucleoside synthesis

To synthesize the desired oligonucleotides, the parent nucleoside was first synthesized from the condensation of the silylated thymine with the appropriate 3-deoxy-3-*C*-hydroxymethyl sugar derivative whose synthesis is shown in Scheme 1.

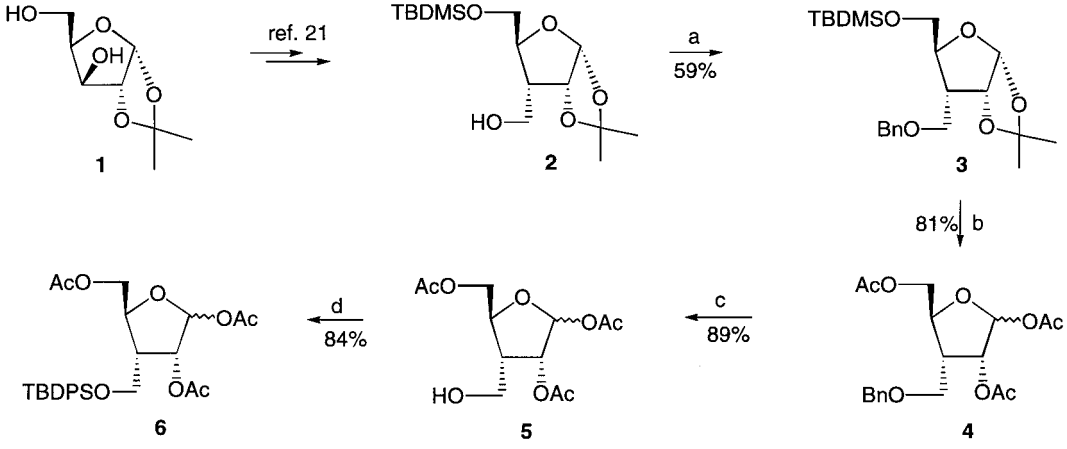
1,2-*O*-Isopropylidene-α-D-xylofuranose (**1**) was converted to **2** by the known method reported by Lin and his co-workers.[21] Treatment of **2** with benzyl bromide in DMF at 0 °C gave the benzylate **3**. Deprotection of the 1,2-*O*-isopropylidene and 5-*O*-TBDMS groups of **3** with 50% acetic acid at 100 °C for 3 h followed by acetylation with acetic anhydride in pyridine afforded the acetate **4** as an anomeric mixture. We first tried to synthesize the desired target nucleoside from compound **4**, but in the last step, the 4,4′-dimethoxytrityl (DMT) group of the 5′-position was too unstable under the reaction conditions that remove the benzyl protecting group to obtain the desired product. Therefore, benzyl protecting group had to be changed to the *tert*-butyldiphenylsilyl group which could be easily deprotected under the mild conditions.

Thus, benzyl group of the compound **4** was removed by the catalytic hydrogen transfer reaction (palladium hydroxide on carbon and cyclohexene in ethanol) to yield the 3-*C*-hydroxymethyl derivative **5**, which was treated with *tert*-butyldiphenylsilyl chloride and imidazole in DMF to give 1,2,5-tri-*O*-acetyl-3-deoxy-3-*C*-[(*tert*-butyldiphenyl)silyloxymethyl]-D-ribofuranose (**6**) which serves as a good glycosyl donor for the synthesis of the desired nucleoside.

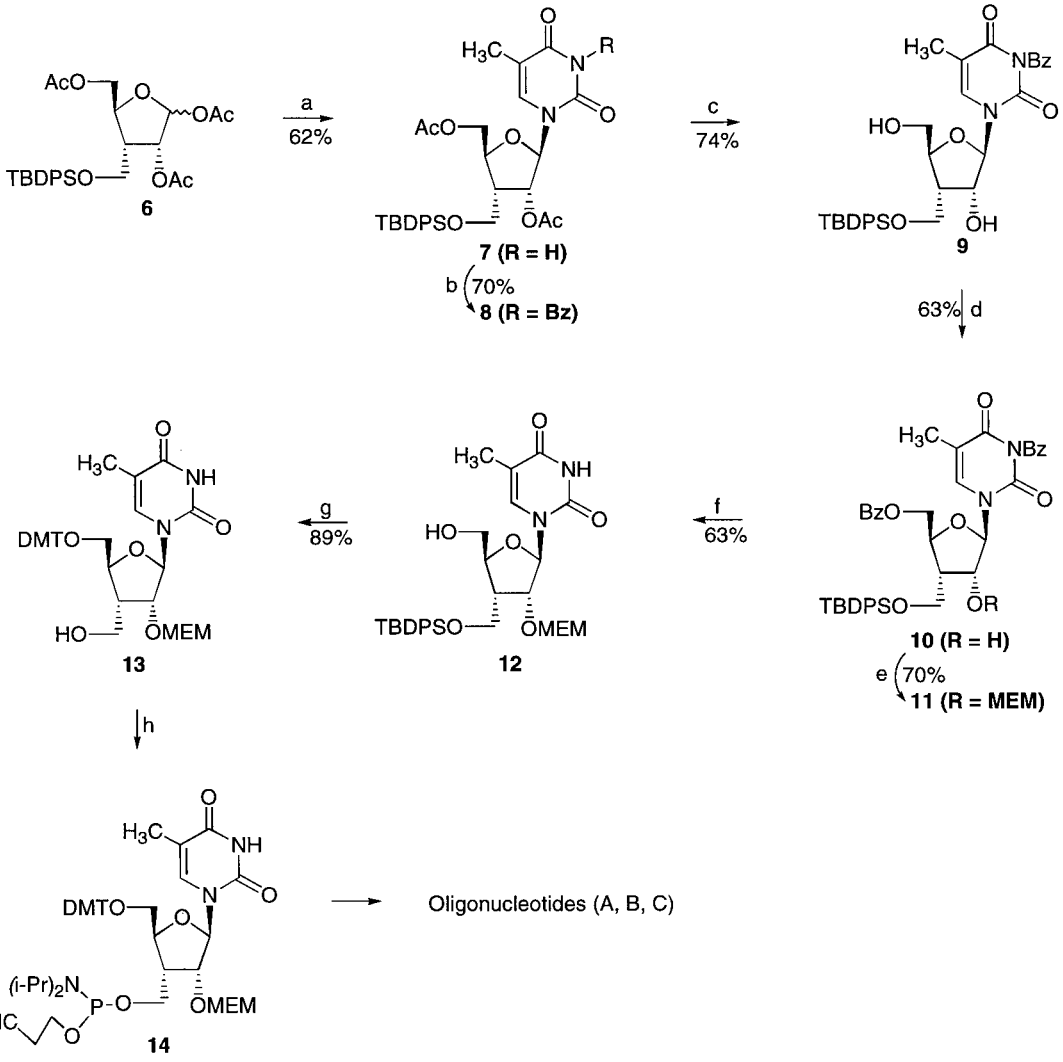
Synthesis of 1-[3-deoxy-3-*C*-hydroxymethyl-2-*O*-(2-methoxyethoxymethyl)-5-*O*-(4,4′-dimethoxytrityl)-β-D-*erythro*-pentofuranosyl]thymine (**13**) from the acetate **6** is illustrated in Scheme 2. Condensation of the acetate **6** with silylated thymine in the presence of TMSOTf (trimethylsilyl trifluoromethanesulfonate) gave **7** (62%) which was treated with benzoyl chloride in pyridine at 80 °C to produce the N^3-benzoylated derivative **8** in 70% yield. Selective deacetylation of **8** in the presence of benzoyl group was achieved with K_2CO_3 in MeOH at −5 °C to afford the diol **9**. It has to be mentioned that 2′-*O*-acetyl group of **8** could be selectively removed over 5′-*O*-acetyl or N^3-benzoyl group in small scale (30 mg) reaction, but in large scale (1.61 g) reaction, both acetyl groups were cleaved under the same reaction conditions. Thus, 5′-hydroxyl group of **9** had to be protected to the benzoate **10** by treating with benzoyl chloride in pyridine at −5 °C. The remaining 2′-hydroxyl group of **10** was reacted with 2-methoxyethoxymethyl chloride (MEM-Cl) and *N,N*-diisopropylethylamine in methylene chloride at 50 °C for two days to give **11**, which was treated with methanolic ammonia to afford 5′-*O*- and N^3-deprotected nucleoside **12**. Compound **12** was treated with 4,4′-dimethoxytrityl chloride in pyridine at room temperature for 24 h followed by the treatment with tetra-*n*-butylammonium fluoride in THF to yield the desired nucleoside **13**. The nucleoside **13** was converted to the phosphoramidite **14**, which was used for the synthesis of the desired oligonucleotides A–C.

Oligonucleotide synthesis and T_m studies

Assembly of oligonucleotides A–C was accomplished by using ABI 392 DNA/RNA synthesizer on a 1 μmol scale from **14** and commercial 2′-deoxynucleoside phosphoramidites. Standard protocols were used except 10 min coupling time for the incorporation of modified nucleotide. All oligomers were characterized by digestion to constituent bases followed by HPLC analysis and mass spectrometric analysis. Their duplex melting temperatures with complementary DNA or RNA sequences



Scheme 1. Reagents: (a) BnBr, NaH, *n*-Bu_4NI, DMF, 0°C. (b) i. 50% AcOH, 100°C; ii. Ac_2O, pyridine. (c) $Pd(OH)_2$, cyclohexene, EtOH, 80 °C. (d) TBDPSCl, imidazole, DMF, rt.



Scheme 2. Reagents: (a) Silylated thymine, TMSOTf, CH_2Cl_2, rt to 50 °C. (b) BzCl, pyridine, 80°C. (c) K_2CO_3, MeOH, −5 °C. (d) BzCl, pyridine, −5 °C. (e) MEMCl, CH_2Cl_2, *i*-Pr_2NEt. (f) NH_3, MeOH, rt. (g) i. DMTCl, pyridine, rt; ii. *n*-Bu_4NF, THF, rt. (h) (*i*-$Pr)_2NPCl(O)CH_2CH_2CN$, *i*-Pr_2NEt, rt.

(5′-AGGGAGAGAAAG-3′) under low (100 mM NaCl) and high (1 M NaCl) ionic strength condition are summarized in Table 1. The result of T_m studies with oligomers A–C containing one to four modified nucleosides showed that introduction of 2′-methoxyethoxymethyl group to extended phosphate linkage decreased melting temperature significantly, indicating destabilization with DNA (−4∼−8 °C/mod.).

It was observed that the ionic strength of buffer for T_m measurement did not affect T_m changes since the same degree of T_m drop was observed in 100 mM NaCl and 1 M NaCl buffer solution. On the other hand, T_m was less decreased with RNA complementary sequence (−1∼−2.5 °C/mod.). The better affinity of the modified oligonucleotides A–C for RNA target than DNA target might be caused from the preference of 3′-endo form (A type duplex) due to the *gauche* effect[19] between the furanose ring oxygen and the electronegative 2′-OMEM group.

Recently, oligonucleotides (18-mer) containing a 3′-α-*C*-methylene phosphodiester linkage have been synthesized and reported to have lower affinity to DNA and RNA.[22] When A–C dodecamers were compared to these oligomers in hybridization property, the presence of 2′-OMEM substituent in 3′-extended oligonucleotide appeared to destabilize DNA duplex more. However, the duplex with RNA showed the same degree of T_m decrease. Therefore, it can be concluded that the presence of the 2′-OMEM group interrupts the hybridization with DNA because of the steric hindrance of the 3′-extended linkage. However, it has the positive effect with RNA probably due to the electronegative effect of substituents giving same degree of T_m decrease in comparison to oligonucleotides of 3′-α-*C*-methylene phosphodiester linkage.

Conclusions

The introduction of the electronegative OMEM group at the 2′-position of the 3′-extended oligonucleotide did not give better hybridization property with DNA as well as RNA than the corresponding natural oligomers. Even though application of these oligomers to antisense

Table 1. T_m, ΔT_m, and ΔT_m/mod. values of synthesized oligomers and natural oligomers

Condition	Oligomer	Sequence	T_m	ΔT_m	ΔT_m/mod.
DNA 1M NaCl	Natural	5′-CTTTCTCTCCCT-3′	54.8	—	—
	A	5′-CTTTCTC**T**CCCT-3′	48.8	−6	−6
	B	5′-CTT**T**CTC**T**CCCT-3′	38.9	−15.9	−7.95
	C	5′-C**T**T**T**C**T**C**T**CCCT-3′	30.0	−24.8	−6.2
DNA 100 mM NaCl	Natural	5′-CTTTCTCTCCCT-3′	42.9	—	—
	A	5′-CTTTCTC**T**CCCT-3′	38.8	−4.1	−4.1
	B	5′-CTT**T**CTC**T**CCCT-3′	27.9	−15	−7.5
	C	5′-C**T**T**T**C**T**C**T**CCCT-3′	18.1	−24.8	−6.2
RNA 100 mM NaCl	Natural	5′-CTTTCTCTCCCT-3′	53.8	—	—
	A	5′-CTTTCTC**T**CCCT-3′	52.8	−1	−1
	B	5′-CTT**T**CTC**T**CCCT-3′	49.8	−4	−2
	C	5′-C**T**T**T**C**T**C**T**CCCT-3′	43.8	−10	−2.5

T indicates 3′-deoxy-3′-*C*-hydroxymethyl-2′-*O*-methoxyethoxymethyl-5-methyluridine.

technology does not seem to be promising, they may still find their uses in other areas like protein studies.

Experimental

Ultra violet (UV) spectra were recorded on a Beckman DU-68 spectrophotometer and ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker DPX (250 or 300 MHz) spectrometer using $CDCl_3$ or DMSO-d_6 and chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane as internal standard for ^1H and ^{13}C NMR and from H_3PO_4 as external standard for ^{31}P NMR; coupling constants are in hertz. FAB mass spectra were recorded on Jeol HX 110 spectrometer. Laser desorption mass spectra for modified oligonucleotides were obtained using Voyage MALDI Workstation at Korea Basic Science Center (Taejon, Korea). Elemental analyses were performed by the general instrument laboratory of Ewha Womans University, Korea. TLC was performed on Merck precoated $60F_{254}$ plates. Column chromatography was performed using silica gel 60 (230–400 mesh, Merck). All the anhydrous solvents were distilled over CaH_2 or P_2O_5 or Na/benzophenone prior to the reaction.

3-*C*-(Benzyloxymethyl)-3-deoxy-5-*O*-[(*tert*-butyldimethyl)-silyl]-1,2-*O*-isopropylidene-α-D-ribofuranose (3). To a solution of **2**[21] (10.26 g, 0.03 mol) in DMF (40 mL) was added sodium hydride (60% dispersion in mineral oil, 1.93 g, 0.05 mol) at 0 °C and benzyl bromide (5.75 mL, 0.05 mol) and tetra-*n*-butylammonium iodide (1.18 g, 3.00 mmol) were successively added to the mixture. After being stirred at room temperature for 3 h, the reaction mixture was neutralized with acetic acid and extracted with EtOAc (250 mL). The organic layer was dried over anhydrous $MgSO_4$ and evaporated to give a syrup, which was purified by silica gel column chromatography (hexane:EtOAc = 20:1) to afford **3** (7.77 g, 59%) as a syrup: ^1H NMR (DMSO-d_6) δ 0.13 (s, 6H, 2×CH_3), 0.97 (s, 9H, *t*-butyl), 1.35 (s, 3H, CH_3), 1.48 (s, 3H, CH_3), 2.12–2.19 (m, 1H, 3-H), 3.52–3.97 (m, 5H, 3-CH_2, 3-H, and 5-H), 4.46 (d, 1H, J = 11.8 Hz, CH_a-Ph), 4.56 (d, 1H, J = 11.8 Hz, CH_b-Ph), 4.76 (t, 1H, J = 4.1 Hz, 2-H), 5.83 (d, 1H, J = 4.1 Hz, 1-H), 7.32–7.39 (m, 5H, Ph). Anal. calcd for $C_{22}H_{36}O_5Si$: C, 64.67; H, 8.88. Found: C, 64.88; H, 8.90.

1,2,5-Tri-*O*-acetyl-3-*C*-(benzyloxymethyl)-3-deoxy-D-ribofuranose (4). A solution of **3** (7.77 g, 0.02 mol) in 50% AcOH (155 mL) was heated at 100 °C for 1 h. The reaction mixture was allowed to cool and evaporated to give a syrup, which was treated with pyridine (28 mL) and acetic anhydride (28 mL) and the solution was stirred at room temperature for 48 h. The solvent was removed under vacuum and the residue was dissolved in EtOAc (200 mL). The organic layer was washed with H_2O (50 mL) and brine (50 mL), dried over anhydrous $MgSO_4$ and filtered. The filtrate was evaporated and the residue was purified by silica gel column chromatography (hexane:EtOAc = 4:1) to give **4** (5.83 g, 81%, α/β = 1/9) as a syrup: ^1H NMR ($CDCl_3$) (β-anomer) δ 2.01 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.07 (s, 3H, CH_3), 2.65–2.72 (m, 1H, 3-H), 3.51 (dd, 1H, J = 6.8, 9.1 Hz, 3-CH_a), 3.65 (dd, 1H, J = 7.6, 9.1 Hz, 3-CH_b), 4.10 (dd, 1H, J = 6.3, 11.4 Hz, 5-CH_a), 4.23–4.29 (m, 1H, 4-H), 4.34 (dd, 1H, J = 2.9, 11.4 Hz, 5-CH_b), 4.45 (d, 1H, J = 11.5 Hz, CH_a-Ph), 4.54 (d, 1H, J = 11.5 Hz, CH_b-Ph), 5.30 (d, 1H, J = 4.6 Hz, 2-H), 6.09 (s, 1H, 1-H), 7.26–7.39 (m, 5H, Ph). Anal. calcd for $C_{19}H_{24}O_8$: C, 59.99; H, 6.36. Found: C, 59.59; H, 6.71.

1,2,5-Tri-*O*-acetyl-3-deoxy-3-*C*-hydroxymethyl-D-ribofuranose (5). To a solution of **4** (4.42 g, 12 mmol) in ethanol was added cyclohexene (50 mL) and palladium hydroxide on carbon (1.52 g) and the reaction mixture was heated at 80 °C for 5 h. The solution was filtered through a Celite and the filtrate was evaporated to give a syrup, which was purified by silica gel column chromatography (hexane:EtOAc = 1:1) to yield **5** (3.09 g, 89%) as a syrup: ^1H NMR ($CDCl_3$) (β-anomer) δ 2.14 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 2.43–2.48 (m, 1H, 3-H), 3.64–3.86 (m, 2H, 3-CH_2), 4.11–4.32 (m, 4H, OH, 4-H and 5-H), 5.35 (d, 1H, J = 2.5 Hz, 2-H), 6.08 (s, 1H, 1-H). Anal. calcd for $C_{12}H_{18}O_8$: C, 49.65; H, 6.25. Found: C, 49.99; H, 6.70.

1,2,5-Tri-*O*-acetyl-3-*C*-[(*tert*-butyldiphenyl)silyloxymethyl]-3-deoxy-D-ribofuranose (6). To a solution of **5** (3.09 g, 10.65 mmol) and imidazole (2.18 g, 32.01 mmol) in DMF (30 mL) was added *tert*-butyldiphenylsilylchloride (4.16 mL, 16 mmol). After being stirred at room temperature for 2 h, the reaction mixture was evaporated and extracted with EtOAc (150 mL). The

organic layer was washed with brine (50 mL), dried ($MgSO_4$) and evaporated. The residue was purified by silica gel column chromatography (hexane:EtOAc = 1.5:1) to give **6** (4.73 g, 84%). Major anomer: ^{1}H NMR ($CDCl_3$) δ 1.04 (s, 9H, *t*-butyl), 1.98 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 2.09 (s, 3H, CH_3), 2.56–2.67 (m, 1H, 3-H), 3.64–3.86 (m, 3H, 3-CH_2 and 4-H), 4.07–4.27 (m, 2H, 5-H), 5.30 (d, 1H, J = 4.9 Hz, 2-H), 6.08 (s, 1H, 1-H), 7.40–7.68 (m, 10H, 2×Ph). Anal. calcd for $C_{28}H_{36}O_8Si$: C, 63.61; H, 6.86. Found: C, 63.97; H, 7.26.

1-[2,5-Di-*O*-acetyl-3-*C*-(*tert*-butyldiphenyl)silyloxymethyl-3-deoxy-β-D-*erythro*-pentofuranosyl]thymine (7). A solution of thymine (0.83 g, 6.61 mmol) in 1,1,1,3,3,3-hexamethyldisilazane (HMDS, 10 mL) and catalytic amount of ammonium sulfate was refluxed overnight and concentrated to dryness under nitrogen. To this residue was added a solution of **6** (2.69 g, 5.09 mmol) in CH_2Cl_2 followed by TMSOTf (1.38 mL, 6.61 mmol) and the mixture was stirred overnight at room temperature and heated at 50 °C for 24 h. The reaction mixture was diluted with saturated $NaHCO_3$ solution (20 mL) and extracted with CH_2Cl_2 (70 mL) and the organic phase was washed with H_2O (20 mL) and brine (20 mL), dried ($MgSO_4$) and evaporated. The residue was purified by silica gel column chromatography ($CHCl_3$:MeOH = 50:1) to give **7** (1.87 g, 62%) as a white foam: ^{1}H NMR ($CDCl_3$) δ 1.06 (s, 9H, *t*-butyl), 1.76 (s, 3H, 5-CH_3), 2.01 (s, 3H, CH_3), 2.10 (s, 3H, CH_3), 2.52–3.01 (m, 1H, 3′-H), 3.71 (dd, 1H, J = 6.0, 11.7 Hz, 3′-CH_a), 3.76 (dd, 1H, J = 5.6, 11.7 Hz, 3′-CH_b), 4.25–4.42 (m, 3H, 4′-H and 5′-H), 5.36 (dd, 1H, J = 3.8, 7.3 Hz, 2′-H), 5.91 (d, 1H, J = 3.8 Hz, 1′-H), 7.38–7.67 (m, 11H, 2×Ph and H-6). Anal. calcd for $C_{31}H_{37}N_2O_8Si$: C, 62.71; H, 6.28; N, 4.72. Found: C, 62.96; H, 6.26; N, 4.98.

1-[2,5-Di-*O*-acetyl-3-*C*-(*tert*-butyldiphenyl)silyloxymethyl-3-deoxy-β-D-*erythro*-pentofuranosyl]-N^3-benzoylthymine (8). To a solution of **7** (1.87 g, 3.14 mmol) in pyridine was added benzoyl chloride (1.46 mL, 12.57 mmol) and the reaction mixture was stirred at 80 °C overnight and diluted with CH_2Cl_2 (40 mL). MeOH (5 mL) was added to the mixture and stirred for 30 min. The solvent was evaporated and the residue was extracted with EtOAc (150 mL) and the organic layer was washed with H_2O (30 mL) and brine (30 mL), dried ($MgSO_4$) and evaporated. The residue was purified by silica gel column chromatography (hexane:EtOAc = 2.5:1) to give **8** (1.54 g, 70%): ^{1}H NMR (CDCl3) δ 1.06 (s, 9H, *t*-butyl), 1.95 (s, 3H, 5-CH_3), 2.00 (s, 3H, CH_3), 2.12 (s, 3H, CH_3), 2.53–2.64 (m, 1H, 3′-H), 3.68–3.78 (m, 2H, 3′-CH_2,), 4.24–4.43 (m, 3H, 4′-H and 5′-H), 5.36 (dd, 1H, J = 3.9, 7.3 Hz, 2′-H), 5.94 (d, 1H, J = 3.9 Hz, 1′-H), 7.38–7.67 (m, 15H, 3×Ph), 8.04 (s, 1H, H-6). Anal. calcd for $C_{38}H_{42}N_2O_9Si$: C, 65.31; H, 6.06; N, 4.01. Found: C, 65.71; H, 5.91; N, 4.28.

1-[3-*C*-(*tert*-Butyldiphenyl)silyloxymethyl-3-deoxy-β-D-*erythro*-pentofuranosyl]-N^3-benzoylthymine (9). To a solution of **8** (1.61 g 2.30 mmol) in MeOH (50 mL) was added K_2CO_3 (1.27 g, 9.19 mmol) at −5 °C and the mixture was stirred at −5 °C for 30 min. The mixture was neutralized with acetic acid, filtered through a short silica gel pad and then evaporated. The residue was purified by silica gel column chromatography (hexane: EtOAc = 2:1) to afford **9** (1.05 g, 74%): ^{1}H NMR ($CDCl_3$) δ 1.12 (s, 9H, *t*-butyl), 2.00 (s, 3H, 5-CH_3), 2.12 (t, 1H, J = 5.3 Hz, 5′-OH, D_2O exchangeable), 2.43–2.49 (m, 1H, 3′-H), 3.17 (d, 1H, J = 4.0 Hz, 2′-OH, D_2O exchangeable), 3.68–3.76 (m, 1H, 5′-H_a), 3.96 (d, 2H, J = 4.0 Hz, 3′-CH_2), 3.97–4.04 (m, 1H, 5′-H_b), 4.32 (td, 1H, J = 2.8, 9.3 Hz, 4′-H), 4.48–4.52 (m, 1H, 2′-H), 5.68 (d, 1H, J = 1.9 Hz, 1′-H), 7.44–7.96 (m, 16H, 3×Ph and H-6). Anal. calcd for $C_{34}H_{38}N_2O_7Si$: C, 66.43; H, 6.23; N, 4.56. Found: C, 66.77; H, 6.01; N, 4.43.

1-[5-*O*-Benzoyl-3-*C*-(*tert*-butyldiphenyl)silyloxymethyl-3-deoxy-β-D-*erythro*-pentofuranosyl]-N^3-benzoylthymine (10). To a solution of **9** (0.839 g, 1.37 mmol) in pyridine (20 mL) was added benzoyl chloride (0.24 mL, 2.05 mmol) at −5 °C and the mixture was stirred for 40 min. After MeOH (5 mL) was added to the mixture, the whole mixture was stirred for 20 min. The solvent was removed under vacuum. The residue was dissolved in EtOAc (100 mL) and the organic layer was washed with H_2O (20 mL) and brine (20 mL), dried ($MgSO_4$) and filtered. The filtrate was evaporated and the residue was purified by silica gel column chromatography (hexane:EtOAc = 3:1) to give **10** (0.62 g, 63%) as a white foam: ^{1}H NMR ($CDCl_3$) δ 1.09 (s, 9H, *t*-butyl), 1.72 (s, 3H, 5-CH_3), 2.51–2.58 (m, 1H, 3′-H), 3.17 (br s, 1H, 2′-OH), 4.01 (s, 1H, 3′-CH_a), 4.04 (s, 1H, 3′-CH_b), 4.46 (dd, 1H, J = 4.1, 12.5 Hz, 5′-H_a), 4.56 (dd, 1H, J = 1.9, 6.1 Hz, 2′-H), 4.59–4.65 (m, 1H, 4′-H), 4.77 (dd, 1H, J = 2.1, 12.5 Hz, 5′-H_b), 5.72 (d, 1H, J = 1.9 Hz, 1′-H), 7.40–8.04 (m, 21H, 4×Ph and H-6). Anal. calcd for $C_{41}H_{42}N_2O_8Si$: C, 68.50; H, 5.89; N, 3.90. Found: C, 68.91; H, 6.03; N, 3.54.

1-[5-*O*-Benzoyl-3-*C*-(*tert*-butyldiphenyl)silyloxymethyl-3-deoxy-2-*O*-(2-methoxyethoxymethyl)-β-D-*erythro*-pentofuranosyl]-N^3-benzoylthymine (11). To a solution of **10** (0.70 g, 0.97 mmol) in CH_2Cl_2 (10 mL) added *N,N*-diisopropylethylamine (0.51 mL, 2.92 mmol) and 2-methoxyethoxymethyl chloride (0.33 mL, 2.92 mmol) and the reaction mixture was stirred at 50 °C for two days. The solvent was removed under vacuum and the residue was purified by silica gel column chromatography (hexane: EtOAc = 2.5:1) to give **11** (0.586 g, 75%) as a white foam: ^{1}H NMR ($CDCl_3$) δ 1.06 (s, 9H, *t*-butyl), 1.67 (s, 3H, 5-CH_3), 2.54–2.69 (m, 1H, 3′-H), 3.28 (s, 3H, OCH_3), 3.37 (t, 2H, J = 4.3 Hz, CH_2OCH_3), 3.45–3.61 (m, 2H, $CH_2CH_2OCH_3$), 3.89 (dd, 1H, J = 7.9, 10.7 Hz, 3′-Ha), 4.03 (dd, 1H, J = 5.3, 10.7 Hz, 3′-Hb), 4.44 (dd, 1H, J = 1.3, 5.9 Hz, 2′-H), 4.52–4.61 (m, 2H, 4′-H and 5′-Ha), 4.72 (d, 1H, J = 6.9 Hz, OCHaO), 4.88–4.93 (m, 2H, 5′-H_b and OCH_bO), 5.86 (d, 1H, J = 1.3 Hz, 1′-H), 7.39–8.08 (m, 21H, 4×Ph and H-6). Anal. calcd for $C_{45}H_{50}N_2O_{10}Si$: C, 66.98; H, 6.25; N, 3.47. Found: C, 67.04; H, 6.35; N, 3.41.

1-[3-*C*-(*tert*-Butyldiphenyl)silyloxymethyl-3-deoxy-2-*O*-(2-methoxyethoxymethyl)-β-D-*erythro*-pentofuranosyl]-thymine (12). A solution of **11** (0.58 g, 0.72 mmol) in methanolic ammonia (50 mL) was stirred at room temperature for two days. The solvent was evaporated

and the residue was purified by silica gel column chromatography (hexane:EtOAc = 1:1) to afford **12** (0.49 g, 100%): ^{1}H NMR ($CDCl_3$) δ 1.11 (s, 9H, *t*-butyl), 1.96 (s, 3H, 5-CH_3), 2.32 (t, 1H, J = 5.8 Hz, 5′-OH, D_2O exchangeable), 2.44–2.53 (m, 1H, 3′-H), 3.31 (s, 3H, OCH_3), 3.41 (t, 2H, J = 4.5 Hz, CH_2OCH_3), 3.50–3.65 (m, 2H, $CH_2CH_2OCH_3$), 3.79–4.09 (m, 4H, 3′-CH_2 and 5′-H), 4.25 (td, 1H, J = 2.8, 9.1 Hz, 4′-H), 4.39 (dd, 1H, J = 2.1, 6.2 Hz, 2′-H), 4.72 (d, 1H, J = 6.9 Hz, OCH_aO), 4.90 (d, 1H, J = 6.9 Hz, OCH_bO), 5.81 (d, 1H, J = 2.1 Hz, 1′-H), 7.43–7.87 (m, 11H, 2×Ph and H-6), 8.13 (br s, 1H, NH, D_2O exchangeable). Anal. calcd for $C_{31}H_{42}N_2O_8Si$: C, 62.18; H, 7.07; N, 4.68. Found: C, 62.47; H, 7.09; N, 4.65.

1-[3-Deoxy-3-*C*-hydroxymethyl-2-*O*-(2-methoxyethoxymethyl)-5-*O*-(4,4′-dimethoxytriphenylmethyl)-β-D-*erythro*-pentofuranosyl]thymine (13). A solution of **12** (0.485 g, 0.81 mmol) and 4,4′-dimethoxytrityl chloride (0.550 g, 1.22 mmol) in pyridine (15 mL) was stirred at room temperature for 24 h. MeOH (1 mL) was added to the mixture and stirred for 30 min. The solvent was removed under reduced pressure. The residue was dissolved in EtOAc (100 mL). The organic layer was washed with H_2O (15 mL) and brine (15 mL), dried ($MgSO_4$) and filtered. The filtrate was evaporated to give the residue, which was treated with tetra-*n*-butylammonium fluoride (1.0 M solution, 0.97 mL, 0.97 mmol) in THF (10 mL) at 0 °C. After being stirred at 0 °C for 1 h, the reaction mixture was stirred at room temperature for 3 h. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (hexane:EtOAc = 1:1 to $CHCl_3$:MeOH = 30:1) to give **13** (0.48 g, 89%) as a pale yellow foam: ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 3H, 5-CH_3), 2.68–2.79 (m, 1H, 3′-H), 3.25 (dd, 1H, J = 3.0, 11.1 Hz, 5′-H_a), 3.42–3.46 (m, 4H, OCH_3 and OH), 3.49–3.91 (m, 13H, 3′-CH_2, 2×OCH_3, CH_2CH_2 and 5′-H_b), 4.21–4.26 (m, 1H, 4′-H), 4.52 (d, 1H, J = 5.3 Hz, 2′-H), 4.85 (d, 1H, J = 6.7 Hz, OCH_aO), 5.23 (d, 1H, J = 6.7 Hz, OCH_bO), 5.86 (s, 1H, 1′-H), 6.85–7.83 (m, 14H, C_6H_5, 2×C_6H_4 and H-6), 8.41 (br s, 1H, 3-NH, D_2O exchangeable). Anal. calcd for $C_{36}H_{42}N_2O_{10}$: C, 65.24; H, 6.39; N, 4.23. Found: C, 65.64; H, 6.64; N, 4.63.

1-[3-Deoxy-5-*O*-dimethoxytrityl-3-*C*-hydroxymethyl-2-*O*-(2-methoxyethoxymethyl)-β-D-*erythro*-pentofuranosyl]-thymine 2-cyanoethyl *N*,*N*-diisopropylphosphor amidite (14). A solution of **13** (0.23 g, 0.35 mmol) in freshly distilled dichloromethane (5 mL) containing *N*,*N*-diisopropylchlorophosphoramidite (160 μL, 0.7 mmol) and *N*,*N*-diisopropylethylamine (183 μL, 1.05 mmol) was stirred at room temperature for 2 h under nitrogen. Saturated $NaHCO_3$ solution (20 mL) was added to the reaction mixture. The organic layer was separated and washed with brine (10 mL) and water (10 mL), dried over anhydrous Na_2SO_4 and evaporated. The residue was purified by silica gel column chromatography (Et_3N(0.5%)/EtOAc (25%)/CH_2Cl_2) to give **14** (0.18 g, 61%) as a diastereomeric mixture: ^{1}H NMR ($CDCl_3$) δ 1.11 (d, 3H, NHCHCH_3), 1.13 (d, 3H, NHCHCH_3), 1.28 (d, 3H, NHCHCH_3), 1.30 (d, 3H, NHCHCH_3),), 1.40 (s, 3H, 5-CH_3), 1.42 (s, 3H, 5-CH_3), 2.77 (m, 2 H), 3.30 (m, 2 H), 3.37 (s, 3H, OCH_3), 3.38 (s, OCH_3), 3.76–3.50 (m, 2 H), 3.81 (s, 6H, OCH_3), 4.24 (m, 2 H), 4.49 (m, 2 H), 4.87 (m, 2 H), 4.97 (m, 2 H), 6.00 (dd, 1H, 1′-H), 6.86–6.83 (m, 8 H), 7.45–7.23 (m, 18 H), 7.62 (s, 1H, H-6), 7.69 (s, 1H, H-6), 8.19 (s, 1H, NH); ^{31}P NMR ($CDCl_3$) δ 147.1, 149.6; FAB-MS (*m*/*e*) 863 $(M+H)^+$.

Oligonucleotide synthesis and purification

All oligonucleotides were synthesized using ABI 392 DNA/RNA synthesizer on a 1 μmol scale. For the modified phosphoramidite the coupling was extended to 10 min. The solid supports and protecting groups were cleaved by the treatment of concentrated NH_4OH at 55 °C for 17 h. The solution was lyophilized with addition of triethylamine every hour to prevent detritylation. Triethylammonium acetate solution (TEAA 100 mM, 0.5 mL∼1 mL, pH 7) was added and the residue was purified by reverse phase HPLC (Hamilton PRP-1, 300 mm×7 mm, 18∼28% CH_3CN/100 mM TEAA in 20 min, pH 7, monitored 260 nM). The desired fractions were lyophilized to dryness and water (2×1 mL) was added and lyophilized again to remove TEAA salt. The treatment of remaining residue with 0.3 mL of AcOH for 20 min cleaved trityl groups. After lyophilization with ethanol (0.3 mL), the residue was taken up in 1 mL of H_2O, extracted with diethyl ether (3×1 mL), and then lyophilized to dryness. After the addition of 1 mL of water to the dry DNA pellet the solution was quantified by UV absorbance at 260 nM at 70 °C. The extinction coefficients of the natural nucleoside used for calculation were as follows: dAMP, 15200: dCMP, 7700: TMP, 8830: dGMP, 11500. The extinction coefficients of 2′-methoxyethoxymethylthymidine at 260 nM was presumed same as TMP. The composition of oligonucleotides was confirmed by enzyme hydrolysis followed by HPLC analysis (Hewlett–Packard, ODS hypersil, C-18; 20 mM K_2HPO_4, pH 5.6 (A), MeOH (B), 100% A to 40% B, 20 min) and mass spectrometric data. The purity of the oligomers A–C was found to be more than 99%. RNA (5′-AGGGAGAGAAAG-3′) was purchased from the Midland Certified Reagent Company (Midland, TX, USA) on 1 μmol scale.

Laser desorption mass spectral data for oligonucleotides A–C

5′-d(CTTTCTCTCCCT)-3′ (**A**): calculated mass, 3615; observed mass, 3619: 5′-d(CTTTCTCTCCCT)-3′ (**B**): calculated mass, 3734; observed mass, 3737: 5′-d(CTTTCTCTCCCT)-3′ (**C**): calculated mass, 3972; observed mass, 3974.

T_m measurement

Melting temperatures were determined using Beckmann DU 650 spectrometer equipped with Beckmann High Performance Temperature Controller. Nitrogen gas was passed over the cell at lower temperature than room temperature to prevent the condensation of moisture. The temperature was increased from 5 °C to 90 °C in 1 °C steps (1 °C/min). The modified oligonucleotides were hybridized with complementary DNA or RNA

sequences in a medium salt buffer containing 100 mM NaCl or 1 M NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0 and 2.5 mM of each oligonucleotide. Melting temperatures were evaluated by taking the first derivative of the absorbance versus temperature curve.

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