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Synthesis and triplex-forming properties of oligonucleotides capable of recognizing corresponding DNA duplexes containing four base pairs

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ABSTRACT

A triplex-forming oligonucleotide (TFO) could be a useful molecular tool for gene therapy and specific gene modification. However, unmodified TFOs have two serious drawbacks: low binding affinities and high sequence-dependencies. In this paper, we propose a new strategy that uses a new set of modified nucleobases for four-base recognition of TFOs, and thereby overcome these two drawbacks. TFOs containing a 2'-deoxy-4N-(2-guanidoethyl)-5-methylcytidine ($d^{g}C$) residue for a C-G base pair have higher binding and base recognition abilities than those containing 2'-OMe-4N-(2-guanidoethyl)-5-methylcytidine ($_{2'\text{-OMe}}{}^{g}C$), 2'-OMe-4N-(2-guanidoethyl)-5-methyl-2-thiocytidine ($_{2'\text{-OMe}}{}^{g}C_{s}$), $d^{g}C$ and 4S-(2-guanidoethyl)-4-thiothymidine (^{gs}T). Further, we observed that N-acetyl-2,7-diamino-1,8-naphtyridine ($^{DA}N_{ac}$) has a higher binding and base recognition abilities for a T-A base pair compared with that of dG and the other DNA derivatives. On the basis of this knowledge, we successfully synthesized a fully modified TFO containing $^{DA}N_{ac}$, $d^{g}C$, 2'-OMe-2-thiothymidine ($_{2'\text{-OMe}}{}^{s}T$) and 2'-OMe-8-thioxoadenosine ($_{2'\text{-OMe}}{}^{s}A$) with high binding and base recognition abilities. To the best of our knowledge, this is the first report in which a fully modified TFO accurately recognizes a complementary DNA duplex having a mixed sequence under neutral conditions.

INTRODUCTION

Triplex-forming oligonucleotides (TFOs) can recognize the major groove of the corresponding DNA duplex *via* Hoogsteen base pairs. (1) A TFO could be a useful molecular tool for gene regulation (2–7) and specific gene modification. (8,9) However, unmodified TFOs have two serious drawbacks: low binding affinities and high sequence-dependencies. A large number of chemists have introduced various chemical modifications into the sugar moieties (10–14) and nucleobases of TFOs (15–18) to increase their binding affinities under neutral conditions. For example, TFOs containing LNAs/BNAs (13) and 5-propynylpyrimidines (17) more strongly bind to targeted DNA duplexes than unmodified TFOs. A recent study conducted by us yielded the interesting result that the strong stacking effect of thiocarbonyl groups of 2'-OMe-2-thiothymidine ($_{2'\text{-OMe}}{}^{s}T$; Figure 1a) (19) and 2'-OMe-8-thioadenosine ($_{2'\text{-OMe}}{}^{s}A$; Figure 1b) (19,20) can increase the binding affinities of TFOs.

Unmodified TFOs can bind only to the sites of homopurine-homopyrimidine in DNA duplexes because unmodified nucleobases cannot form selective and strong base pairs with C or T residues in the central DNA strands of the triplexes. Seidman et al. reported that 2'-OMe-4*N*-(2-guanidoethyl)-5-methylcytidine ($_{2'\text{-OMe}}{}^{g}C$; Figure 1c) formed a stable base pair with the CG site in the DNA duplex. (21) Recently, Hari and Obika also developed a new nucleoside, 2',4'-BNA, which has 4-[(3*S*)-3-guanidinopyrrolidino]-5-methylpyrimidin-2-one nucleobase to target a CG base pair. (22) On the other hand, Fox et al. for the first time tried four-base recognition by TFO containing 5-(3-aminoprop-1-ynyl)uracil for AT, 3-methyl-2 aminopyridine for GC, 6-(3-aminopropyl)-7-methyl-3H-pyrrolo[2,3-d]pyrimidin-2(7H)-one) for CG and N-(4-(3-acetamidophenyl)thiazol-2-yl-acetamide) (S; Figure 1d) for TA at physiological pH. (23) However, the

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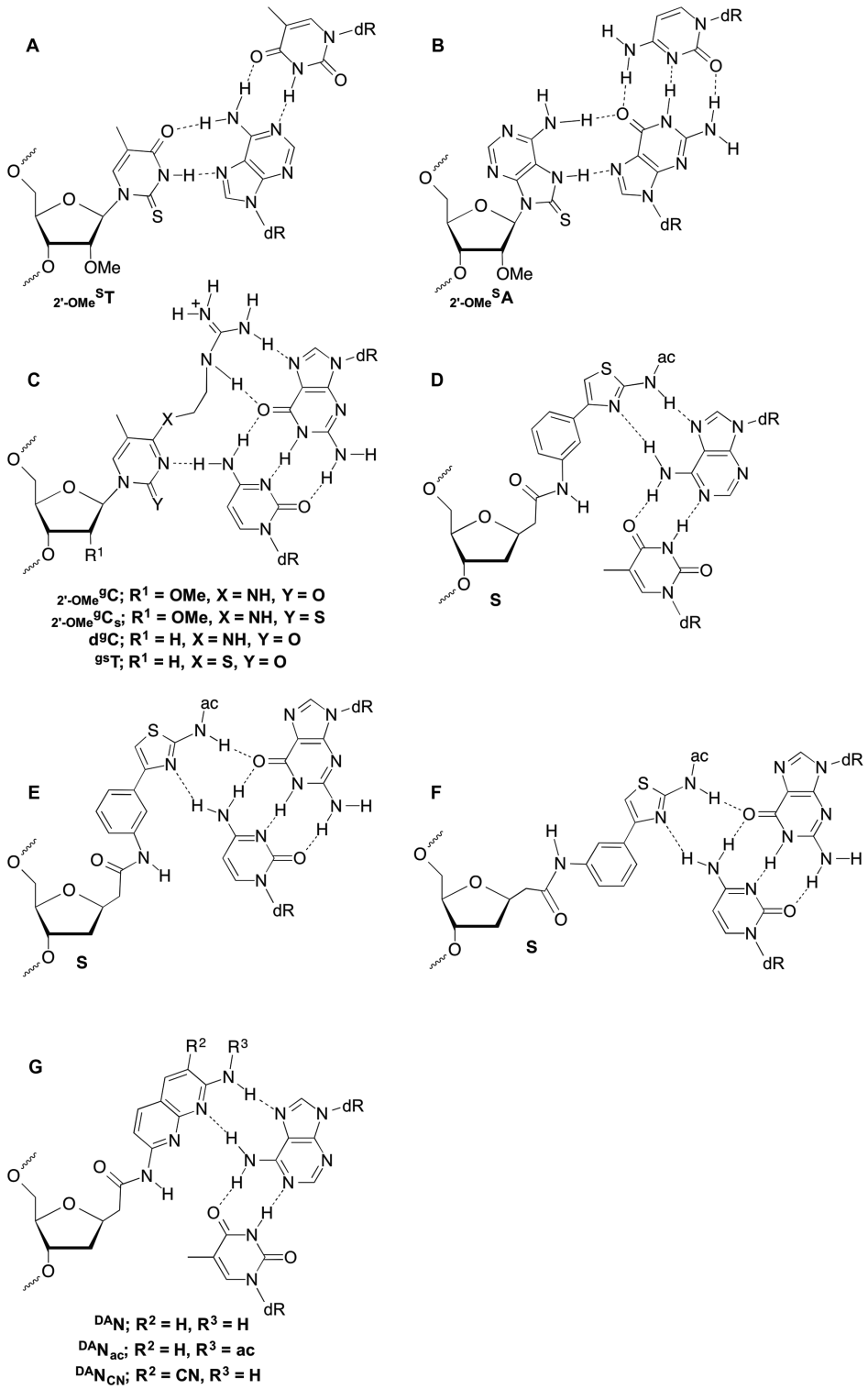


Figure 1. Chemical structures of modified nucleosides incorporated into triplex-forming oligonucleotides.

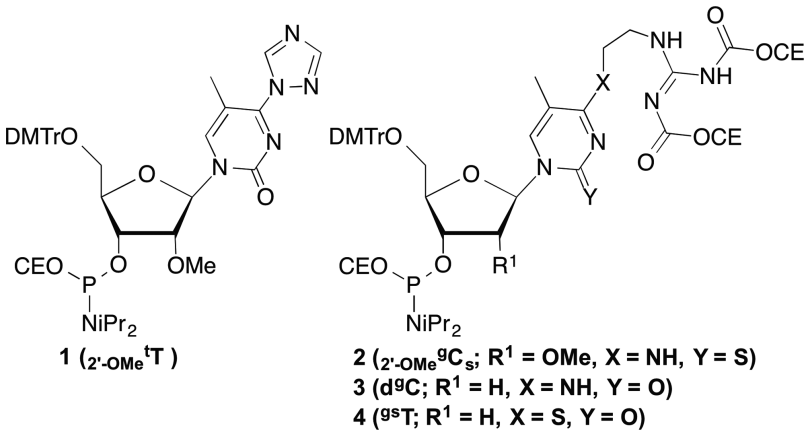
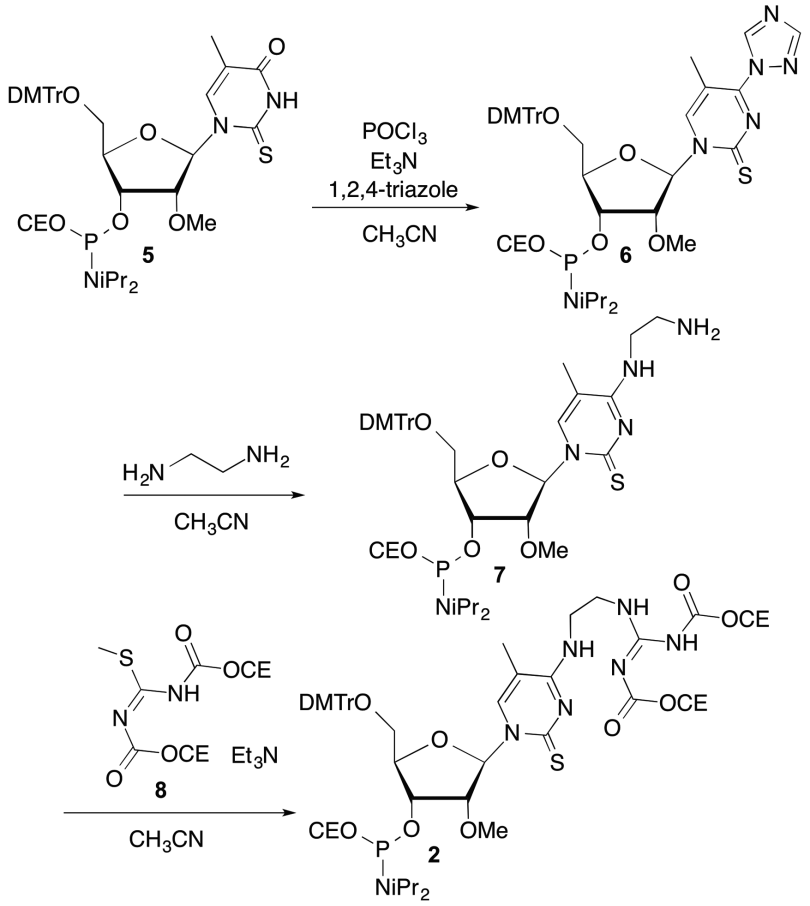


Figure 2. Chemical structures of modified nucleoside incorporated into triplex-forming oligonucleotides.



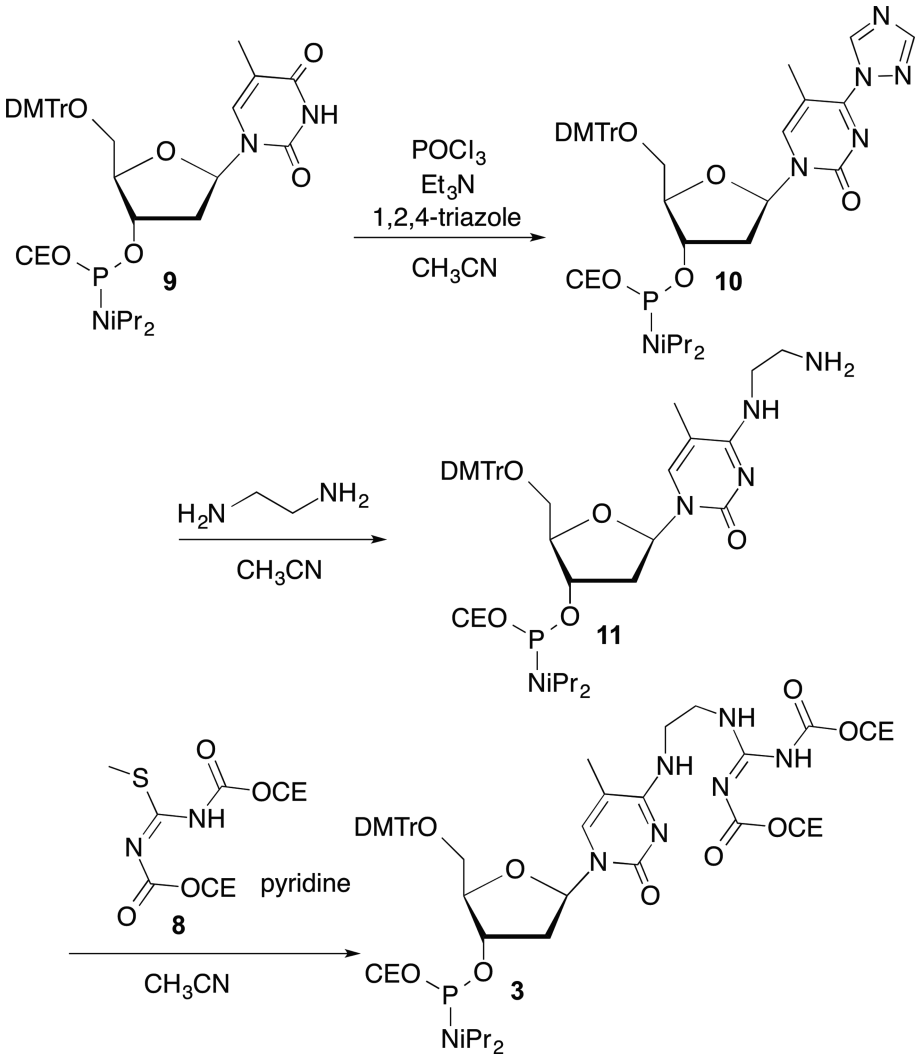
Scheme 1. Synthesis of $_{2'\text{-OMe}}{}^{g}C_s$ phosphoramidite unit **2**.

binding ability of S to TA was slightly lower than that to CG under neutral conditions. Consequently, in this paper we propose a new strategy that uses a new set of modified nucleobases for four-base recognition of TFO and thereby overcomes the high sequence-dependencies.

MATERIALS AND METHODS

General Remarks

^{1}H, ^{13}C and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded at 500, 120 and 203 MHz, respectively. The chemical shifts were measured from tetramethylsilane for the ^{1}H NMR spectra and $CDCl_3$ (77 ppm) for the ^{13}C NMR spectra, with 85% phosphoric acid (0 ppm) for the ^{31}P NMR spectra. Column chromatography was performed



Scheme 2. Synthesis of $d^{g}C$ phosphoramidite unit **3**.

with silica gel C-200 purchased from Wako Co. Ltd., and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. Anion-exchange HPLC was conducted on a Waters Alliance system with a Waters 3D UV detector and a GenPak^{TM} FAX column (Waters, 4.6 × 100 mm). A linear gradient (10–60%) of Solvent I (1 M NaCl in 25 mM phosphate buffer (pH 6.0)) in solvent II (25 mM phosphate buffer (pH 6.0)) was used at 50°C at a flow rate of 1.0 ml/min for 45 min. ESI mass was performed using Mariner^{TM} (PerSeptive Biosystems Inc.). MALDI-TOF mass was performed using Bruker Daltonics [Matrix: 3-hydroxypicolinic acid (100 mg/ml) in H_2O-diammoniumhydrogen citrate (100 mg/ml) in H_2O (10:1, v/v)]. Highly cross-linked polystyrene was purchased from ABI.

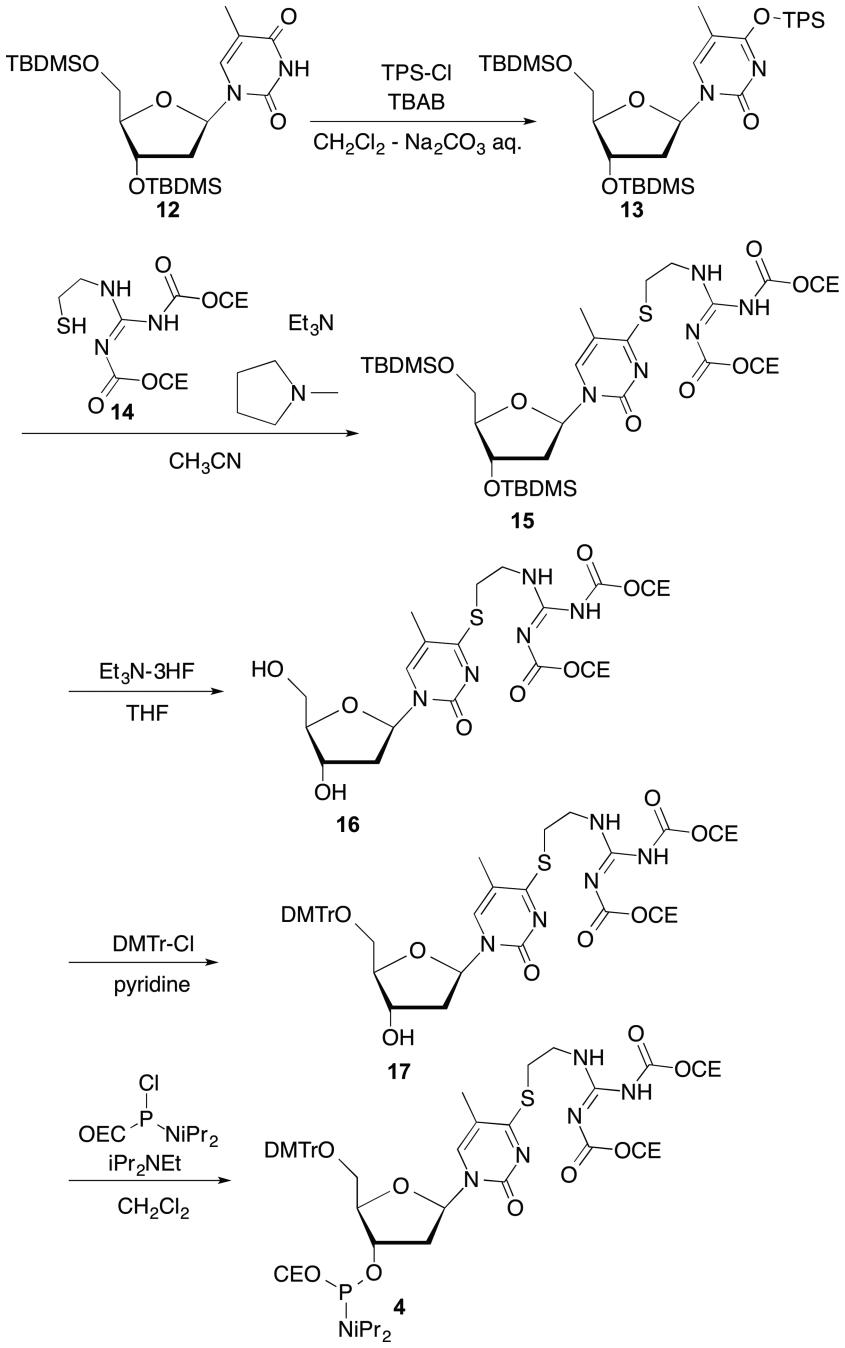
Synthesis of TFO 1

Chain elongation of TFO was carried out on a $_{2'\text{-OMe}}$U-loaded CPG resin (1 μmol) in an RNA synthesizer using the standard procedure for RNA synthesis. The resin was treated with a mixture of ethylenediamine-diisopropylethylamine, CH_3CN (500 μl, 1:1:8, v/v/v), at room temperature for 3 h. Following introduction of a guanidine group by using a solution of DMF solution (500 μl) of 1-pyrazolecarboxamidine (1 M) and diisopropylethylamine (1 M) at 55°C for 15 h, the resin was treated with 28% NH_4OH at room temperature for 2 h. The resin was then removed by filtration and washed with 0.1 M ammonium acetate buffer (1 ml x 3). The filtrate was then purified by anion-exchange HPLC to give TFO 1.

TFO 1: MALDI-TOF Mass (M+H) calcd for $[C_{184}H_{250}N_{44}O_{137}P_{17}+ H]^+$ 5796.7, found 5794.2.

Synthesis of TFOs 2–4

Chain elongation of TFO was carried out on a $_{2'\text{-OMe}}$U-loaded CPG resin (1 μmol) in an RNA synthesizer using the standard procedure for RNA synthesis. Following removal of 2-cyanoethyl groups using 10% DBU (50 μl) in CH_3CN-*N*,*O*-bis(trimethylsilyl)acetamide (450 μl, 1:1, v/v) for 4 h, the resin was treated with 28% NH_4OH (500 μl) at room temperature for 2 h. The resin was then removed by filtration and washed with 0.1 M ammonium acetate buffer (1



Scheme 3. Synthesis of ^{gs}T phosphoramidite unit **4**.

ml x 3). The filtrate was purified by anion-exchange HPLC to give the TFO.

TFO 2: MALDI-TOF Mass (M+H) calcd for $[C_{184}H_{249}N_{44}O_{136}P_{17}S + H]^+$5812.7, found 5812.5.

TFO 3: MALDI-TOF Mass (M+H) calcd for $[C_{183}H_{247}N_{44}O_{136}P_{17} + H]^+$5766.6, found 5766.3.

TFO 4: MALDI-TOF Mass (M+H) calcd for $[C_{183}H_{246}N_{43}O_{136}P_{17} + H]^+$5783.7, found 5781.4.

Synthesis of TFOs 6–8

Chain elongation of TFO was carried out on a $_{2'\text{-OMe}}$U-loaded CPG resin (1 μmol) in an RNA synthesizer using the standard procedure for RNA synthesis. The resin was treated with 28% NH_4OH (500 μl) at room temperature for 2 h. It was subsequently removed by filtration and washed with 0.1 M ammonium acetate buffer (1 ml x 3). The filtrate was then purified by anion-exchange HPLC to give the TFO.

TFO 6: MALDI-TOF Mass (M+H) calcd for $[C_{185}H_{243}N_{42}O_{136}P_{17} + H]^+$5755.9, found 5757.0.

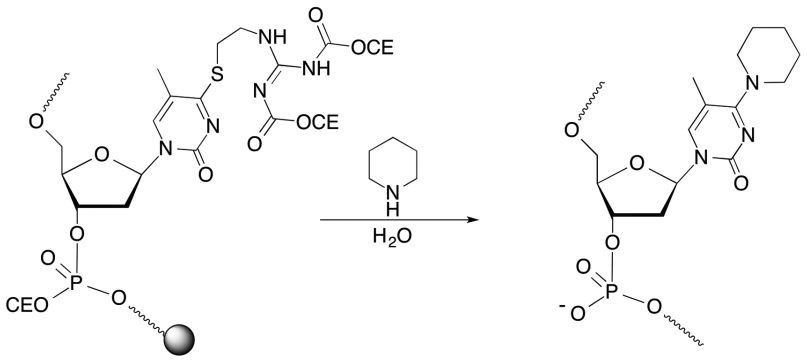


Figure 3. Decomposition of ^{gs}T by treatment with piperidine.

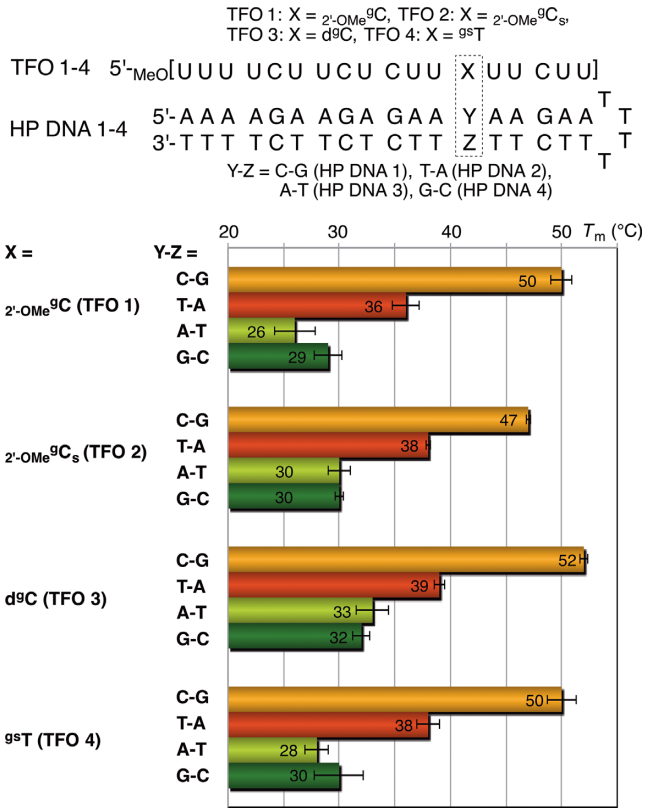


Figure 4. Comparison of the stabilities of the triplexes formed between HP DNAs 1–4 and TFOs 1–4. The T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate (pH 7.0), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM spermine and 1.5 μM triplex. The values represent the mean ± SD for three separate experiments.

TFO 7: MALDI-TOF Mass (M+H) calcd for $[C_{187}H_{245}N_{42}O_{137}P_{17} + H]^+$5797.9, found 5797.7.

TFO 8: MALDI-TOF Mass (M+H) calcd for $[C_{186}H_{242}N_{43}O_{136}P_{17} + H]^+$5780.9, found 5781.6.

Synthesis of TFO 10

Chain elongation of TFO was carried out on a $_{2'\text{-OMe}}$U-loaded CPG resin (1 μmol) in an RNA synthesizer using the standard procedure for RNA synthesis. Following removal of 2-cyanoethyl groups using 10% DBU (50 μl) in CH_3CN-*N*,*O*-bis(trimethylsilyl)acetamide (450 μl, 1:1, v/v) for 4 h, the resin was treated with 28% NH_4OH (500 μl) at room temperature for 2 h. It was subsequently removed by filtration and washed with 0.1 M ammonium acetate buffer (1 ml x 3). The filtrate was then evaporated under reduced pressure and the residue rendered anhydrous by repeated coevaporation with dry CH_3CN (1 ml x 5), and then dissolved in 1 M TBAF-THF (500 μl). Following stirring at room temperature for 30 min, the crude mixture was purified by Sep-Pak C18 cartridge and anion-exchange HPLC.

TFO 6: MALDI-TOF Mass (M+H) calcd for 6905.0, found 6909.7.

T_m measurement

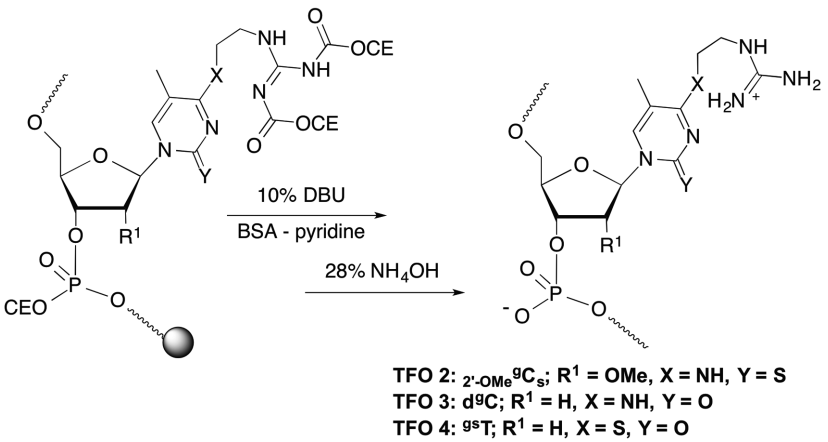
An appropriate TFO (1.5 μM) and its complementary 1.5 μM HP DNA were dissolved in a buffer (pH 7.0) containing 10 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM spermine. The solution was kept at 90°C for 5 min for complete dissociation of the duplex to single strands, then cooled at the rate of 0.5°C/min, and kept at 5°C for 5 min. Then, the melting temperatures (T_m) were determined at 260 nm or 290 nm (TFO 10) using a UV spectrometer by increasing the temperature at a rate of 0.5°C/min.

Fluorescence measurement

An appropriate TFO (0.1 μM) and its complementary 0.1 μM HP DNA were dissolved in a buffer (pH 7.0) containing 10 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM spermine. The solution was kept at 90°C for 5 min for complete dissociation of the duplex to single strands, then gradually cooled to 20°C. The fluorescence profiles were subsequently determined using a fluorescence spectrometer at 20°C. The excitation wavelength was 340 nm.

Computational methods

Molecular dynamic simulations were carried out using the AMBER 12 program package. The sequences of triplexes were 5′-TTCTTATTCTT-3′/5′-AAGAATAAGAA-3′/5′-(2′-*O*-methyl)-UUC*UUXUUC*UU-3′, where C* denotes protonated 2′-*O*-methyl cytidine and X denotes modified nucleosides. The charges of non-canonical modified residues were determined by RESP charge fitting (HF/6–31G(d), iop(6/33 = 2)). The leaprc.ff12SB were used for



Scheme 4. Synthesis of **TFO 2–4** on polymer supports.

nucleic acids and the additional force field parameters were taken from GAFF. The initial triplexes were solvated in a periodic box with 10 Å of water molecules, explicitly described by the TIP3P water model. Sodium and chloride ions were added to make 0.1 M NaCl solution. Minimization and equilibration (10.3 ns) were executed in accordance with a protocol used in previous work. Unrestrained extended simulations (30 ns) were performed with the Berendsen algorithm to maintain the temperature (300 K). During the MD simulations, hydrogen vibrations were removed using SHAKE bond constraints, allowing a longer time step of 2 fs. Long-range electrostatic interactions were treated using the Particle Mesh Ewald approach and a 10 Å cutoff. Postprocessing analysis of the trajectories was carried out using the cpptraj module (v13.17) embedded in AmberTools and R (3.0.1). All of the figures were created with Pymol.

RESULTS AND DISCUSSION

Synthesis and triplex formation of TFOs containing $_{2'\text{-OMe}}{}^{g}C$, $_{2'\text{-OMe}}{}^{g}C_s$, $d^{g}C$ and ^{gs}T

First, we examined four modified pyrimidines, $_{2'\text{-OMe}}{}^{g}C$, $_{2'\text{-OMe}}{}^{g}C_s$, $d^{g}C$ and ^{gs}T, as shown in Figure 1c, for binding to a C-G base pair of the targeted duplex. These modified nucleosides were designed to evaluate the stabilizing effects of the triplexes by introduction of a thiocarbonyl group at 2-position $_{2'}$- and a sulfur atom at 4-position into $_{2'\text{-OMe}}{}^{g}C$, as reported by Seidman, or removal of the 2'-O-methyl group of the nucleoside. Introduction of these pyrimidine derivatives into the TFOs was carried out by using the corresponding phosphoramidite units **1–4** in Figure 2. Phosphoramidite unit **1** was synthesized by treating $_{2'\text{-OMe}}T$ phosphoramidite compound with phosphoryl chloride and 1, 2, 4-triazole. Scheme 1 shows the synthesis of $_{2'\text{-OMe}}{}^{g}C_s$ phosphoramidite unit **2**. $_{2'\text{-OMe}}{}^{s}T$ phosphoramidite unit was converted to compound **6** by using phosphoryl chloride and then 1, 2, 4-triazole and introduction of a 2-aminoethylamino group into the 4-position of the pyrimidine (19,24) was carried out to obtain compound **7**. $_{2'\text{-OMe}}{}^{g}C_s$ phosphoramidite unit **2** was synthesized by treating compound **7** with *S*-methylthiourea derivative **8** in 68% isolated yield from compound **5** (3 steps, Scheme 1). $d^{g}C$ phosphoramidite unit **3** was also synthesized in the same manner as $_{2'\text{-OMe}}{}^{g}C_s$ phosphoramidite unit **2**, as shown in Scheme 2. Scheme 3 shows the synthesis of ^{gs}T phosphoramidite unit **4**. Introduction of a guanidoethylthio group into the 4-position of the pyrimidine was carried out by using 2,4,6-triisopropylbenzenesulfonyl (TPS) group as a leaving group. The isolated yield of compound **15** was 52% from compound **12**. Subsequently, the TBDMS groups of compound **15** were removed by treatment with Et_3N-3HF at room temperature. 5'-Tritylation and 3'-phosphitylation of the nucleoside was carried out to obtain the ^{gs}T phosphoramidite unit **4** by general procedures.

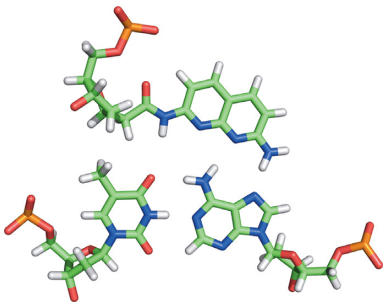
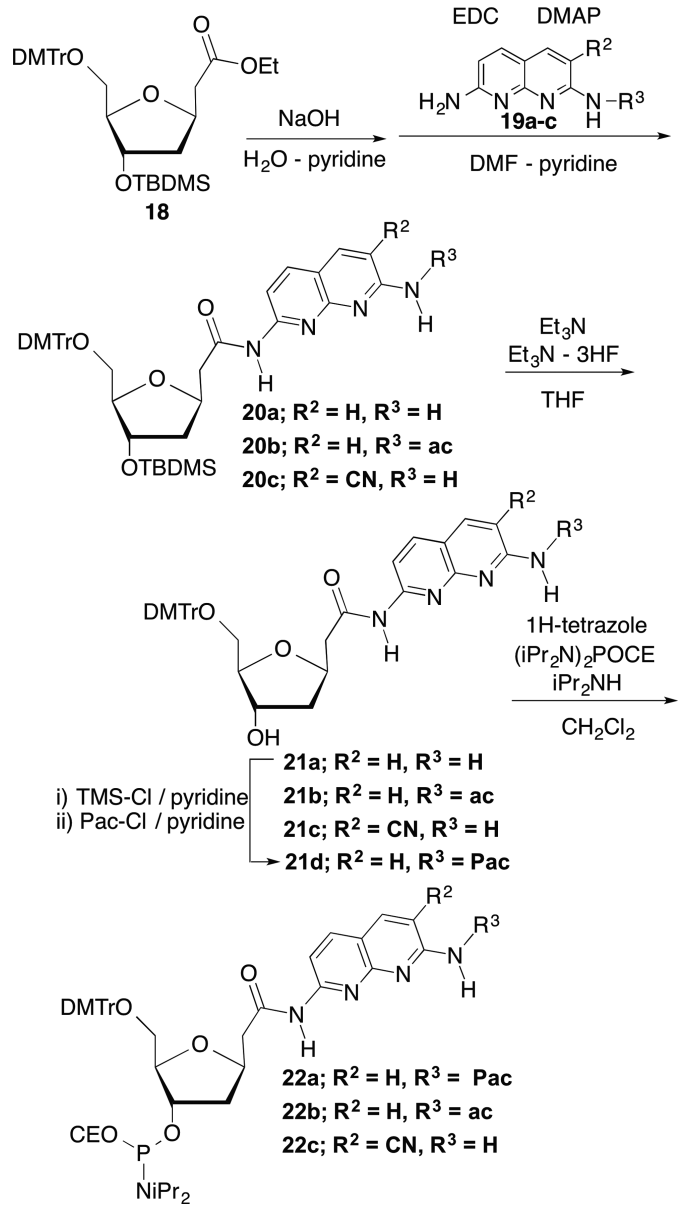


Figure 5. Final snapshot of a $^{DA}N_{ac}$-TA base pair after molecular dynamic (MD) simulation of the triplex.

In the synthesis of TFO 1–4 containing $_{2'\text{-OMe}}{}^{g}C$, $_{2'\text{-OMe}}{}^{g}C_s$, $d^{g}C$ and ^{gs}T (Figure 4), phosphoramidite units **1–4**, $_{2'\text{-OMe}}U$ and $_{2'\text{-OMe}}C$ phosphoramidite units, and $_{2'\text{-OMe}}U$-loaded CPG resin having a succinate linker were used in the phosphoramidite approach. After chain elongation using phosphoramidite unit **1**, a guanidoethyl group was introduced into TFO 1 by treating the oligonucleotide containing a 4-triazolylthymine residue with ethylenediamine and 1-pyrazolylcarboxamidine hydrochloride. When the cyanoethyl groups were incorporated into the phosphate and guanidino groups and the succinate linker were cleaved by treatment with piperidine, nucleophilic substitution at the 4-position of thymine was observed, as shown in Figure 3. Therefore, we released oligonucleotides from the resins us-



Scheme 5. Synthesis of ^{DA}N, $^{DA}N_{ac}$ and $^{DA}N_{CN}$ phosphoramidite units **20a-c**.

ing ammonium hydroxide in the synthesis of TFOs 2–4 after deprotection of the cyanoethyl groups using DBU in the presence of bis(trimethylsilyl)acetamide (BSA) (Scheme 4). These TFOs were purified by anion-exchanged HPLC and identified by MALDI-TOF mass spectrometry.

The binding and base recognition abilities of TFO 1–4 were observed by measuring the T_m values of triplexes formed between TFO 1–4 and HP DNA 1–4, as shown in Figure 4. The T_m value of the triplex containing a d^gC:C-G base pair was higher than that of the other triplexes. These results indicate that introduction of a methoxy group into the 2'-position and a sulfur atom into the 4- or 2-position of the pyrimidine bases marginally reduces the binding abilities of the TFOs. Additionally, it was found that the base recognition ability of TFO 3 containing a d^gC residue, which corresponds to the difference (ΔT_m) in the T_m value between the matched and the other mismatched triplexes, was high enough to distinguish the one-mismatch base pairs because differences in T_m values between the matched triplex and the mismatched triplexes were more than 13°C. The base recognition ability of TFO 3 was similar to that of TFO 1 and TFO 4. Therefore, we decided to use d^gC at the opposite site to a C-G base pair to synthesize our new fully modified TFO (TFO 10 in Figure 8).

Synthesis and triplex formation of TFOs containing ^{DA}N, $^{DA}N_{ac}$ and $^{DA}N_{CN}$

S has two aromatic rings and the bond between phenyl and thiazole can be free to rotate. Therefore, S may form mismatched base pairs with CG in two binding manners, as shown in Figure 1e and f. If the rotation of the bond between two aromatic rings was fixed, one of the binding man-

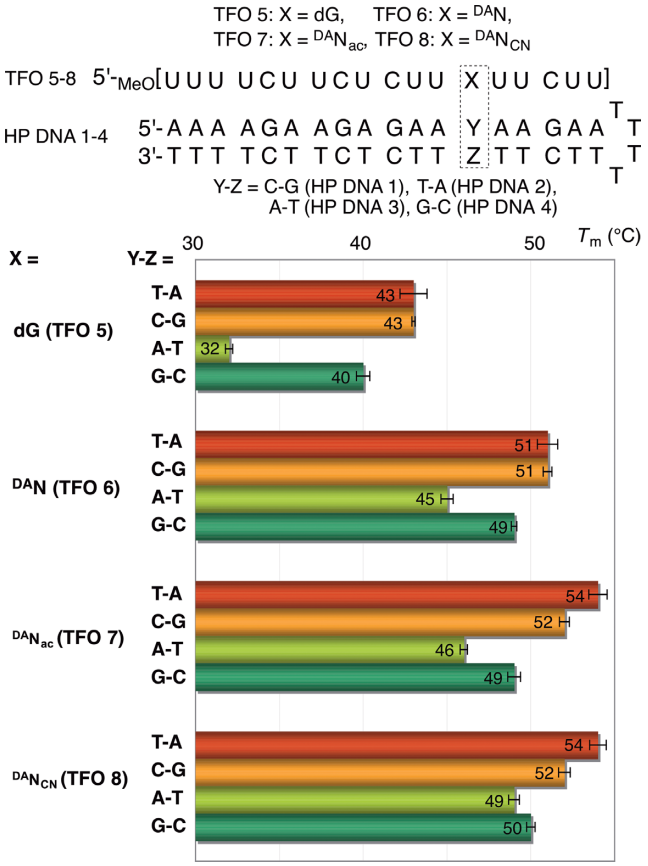


Figure 6. Comparison of the stabilities of the triplexes formed between HP DNAs 1–4 and TFOs 5–8. The T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate (pH 7.0), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM spermine and 1.5 μM triplex. The values represent the mean ± SD for three separate experiments.

ners (Figure 1f) might be inhibited. In this study, the three 2,4-diamino-1,8-naphtyridine derivatives (^{DA}N, ^{DA}Nac and $^{DA}N_{CN}$ in Figure 1g) (25,26) were designed for binding to a TA base pair of the targeted duplex to increase the base recognition abilities of TFOs. Figure 5 shows the stable structure of a $^{DA}N_{ac}$-TA base pair during the molecular dynamic (MD) simulation of the triplex. This result indicates that ^{DA}N derivatives can form two or three hydrogen bonds to a TA base pair in the triplex.

Synthesis of ^{DA}N, $^{DA}N_{ac}$, $^{DA}N_{CN}$ phosphoramidite units **22a-c** is shown in Scheme 5. Following hydrolysis of compound **18** (27) using sodium hydroxide, the resulting compound was condensed with ^{DA}N, $^{DA}N_{ac}$, or $^{DA}N_{CN}$ **19a-c** in the presence of EDC. The TBDMS groups of 3'-hydroxyl groups of **20a-c** were removed by treatment with Et_3N-3HF. The amino group of ^{DA}N can react with phosphoramidite compounds because of the high nucleophilicity, although the reactivity of the amino group of $^{DA}N_{CN}$ is low in the phosphoramidite approach. Therefore, a phenoxyacetyl group was introduced into the amino group of ^{DA}N to avoid side reactions during chain elongation. Finally, 3'-phosphitylation was carried out to afford the ^{DA}N, $^{DA}N_{ac}$, $^{DA}N_{CN}$ phosphoramidite units **22a-c**.

In the synthesis of TFO 6–8 containing ^{DA}N, $^{DA}N_{ac}$ and $^{DA}N_{CN}$, after chain elongation using the phosphoramidite units **22a-c**, deprotection and release of the oligonucleotides from the resins were carried out by treatment with 28% ammonium hydroxide for 2 h at room temperature. These TFOs were purified by anion-exchanged HPLC and identified by MALDI-TOF mass spectrometry.

Figure 6 shows the T_m values of the triplexes formed between TFO 5–8 and HP DNA 1–4. The T_m values of the triplexes containing ^{DA}N (TFO 6) were higher than those of the triplexes containing dG (TFO 5). It was also found that introduction of acetyl (TFO 7) and cyano (TFO 8) groups into ^{DA}N increased their ability to bind to HP DNA 1. The base recognition ability of $^{DA}N_{ac}$ was higher than that of dG and the other ^{DA}N derivatives. Interestingly, the ability of $^{DA}N_{ac}$ to bind to the T-A base pair was higher than its ability to bind to the C-G base pair under neutral conditions. To the best of our knowledge, this is the first report of a new modified nucleobase that can distinguish between T-A and C-G base pairs in DNA duplexes under neutral conditions.

Figure 7 shows the fluorescence profiles of TFO 7 and its complexes with equimolar HP DNAs 1–4. The fluorescent strength of $^{DA}N_{ac}$ incorporated into TFO 7 was interestingly decreased by addition of HP DNAs 1–4. The reduction in the fluorescence may have resulted from the increase in the hydrophobicity around $^{DA}N_{ac}$ by formation of the triplexes and the fluorescent quenching effect of the guanine base in the case of HP DNAs 1 and 4. (28,29) It was also observed that the fluorescence strength of $^{DA}N_{ac}$ in the triplex formed between TFO 7 and HP DNA 2 was similar to that in the triplex formed between TFO 7 and HP DNA 3. These results show that the hydrophobicity around $^{DA}N_{ac}$ in the triplex formed between TFO 7 and HP DNA 2 was similar to that in the triplex formed between TFO 7 and HP DNA 3. However, the ability of TFO 7 to bind to HP DNA 2 was much higher than its ability to bind to HP DNA 3, as shown in Figure 6. These results indicate that the $^{DA}N_{ac}$ of TFO 7 formed the hydrogen bonds to the T-A base pair in the triplex though the $^{DA}N_{ac}$ intercalated into the hydrophobic site of the triplex without a hydrogen bond.

Synthesis and triplex formation of fully modified TFO 10 containing $^{DA}N_{ac}$, $d^{g}C$, $_{2'\text{-OMe}}{}^{s}T$ and $_{2'\text{-OMe}}{}^{s}A$

Finally, synthesis of the fully modified TFO 10 containing $^{DA}N_{ac}$, $d^{g}C$, $_{2'\text{-OMe}}{}^{s}T$ and $_{2'\text{-OMe}}{}^{s}A$ was carried out to measure the binding and base recognition of the fully modified TFO to HP DNAs 5–13 with the mixed sequences. Although we previously reported synthesis of the $_{2'\text{-OMe}}{}^{s}A$ containing a benzoyl group as a protecting group, it was observed that the $^{DA}N_{ac}$ moieties were decomposed under the conditions for deprotection of the N-benzoyl group (treatment with 28% NH_4OH for 8 h). Therefore, a phenoxyacetyl group, which can be easily cleaved under mild conditions compared with a benzoyl group, was introduced into the amino group of 2'-*O*-methyl-8-(2-trimethylsilylethylthio)adenosine **23** in the presence of trimethylsilyl chloride, as shown in Scheme 6. After tritylation of the 5'-hydroxy group, the 3'-phosphitylation was carried out to give the $_{2'\text{-OMe}}{}^{s}A$ phosphoramidite unit **26**.

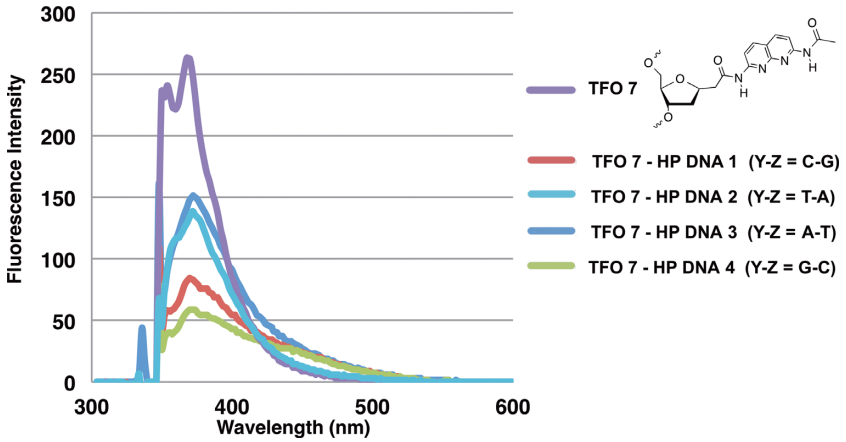
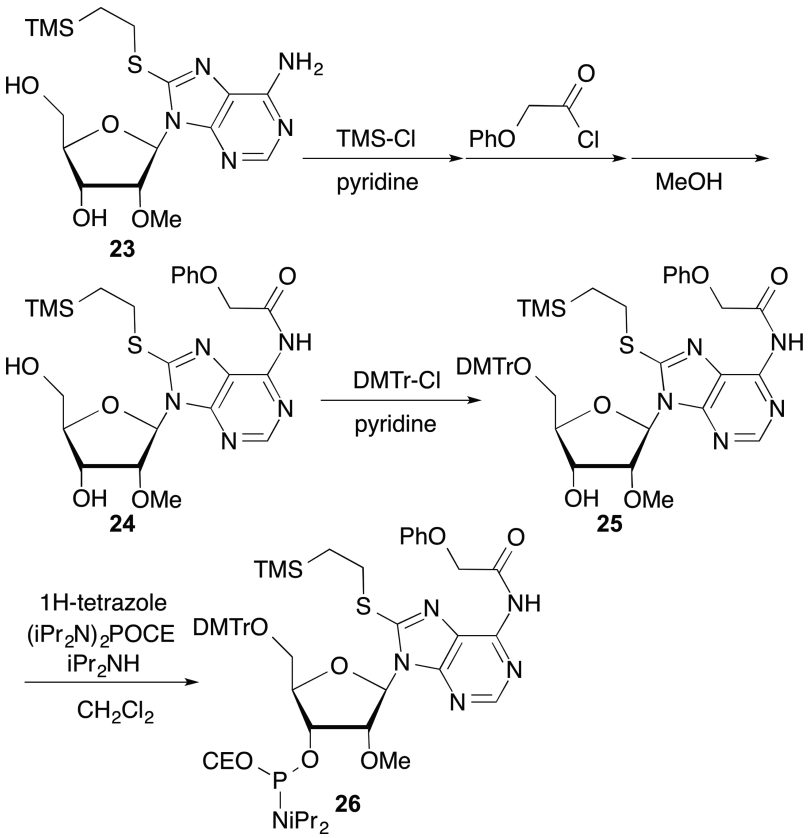


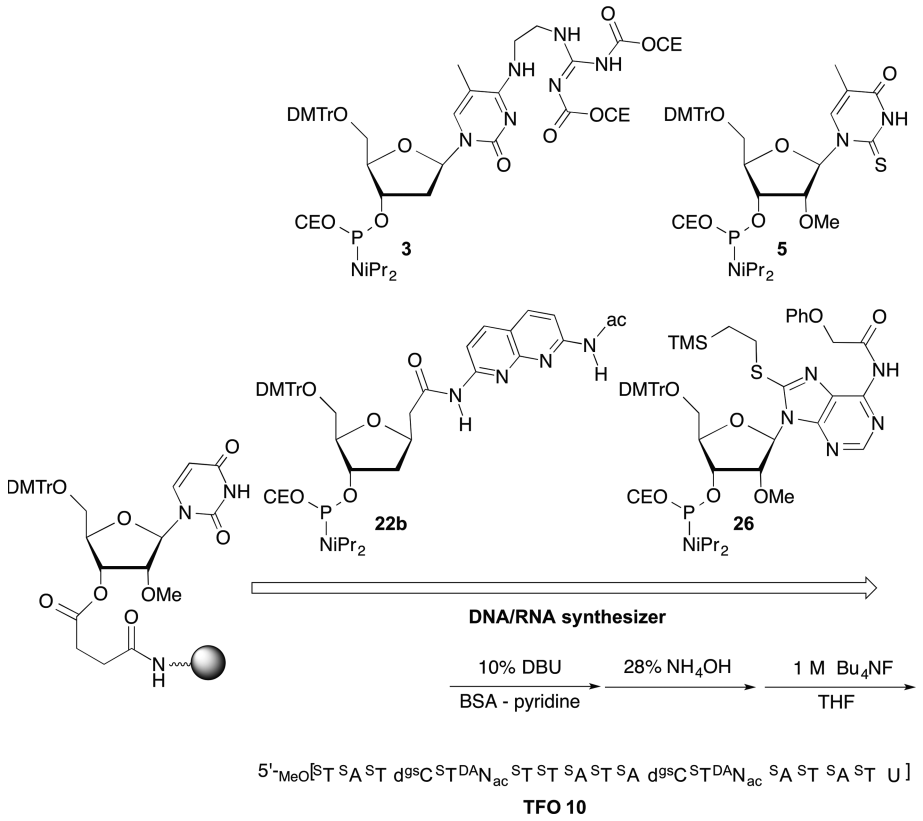
Figure 7. Fluorescence profiles of 0.1 μM TFO 7 and its complexes with equimolar HP DNAs 1–4 measured in a buffer containing 10 mM sodium cacodylate (pH 7.0), 100 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM spermine at 20°C. The excitation wavelength was 340 nm.



Scheme 6. Synthesis of $_{2'\text{-OMe}}{}^{S}$A phosphoramidite units **26**.

In the synthesis of fully modified TFO 10, after chain elongation using phosphoramidite units **3**, **5**, **22b** and **26**, deprotection and release of the oligonucleotides from the resins were carried out by treatment with 28% ammonium hydroxide for 2 h at room temperature, as shown in Scheme 7. These TFOs were purified by anion-exchanged HPLC in 3% yield and identified by MALDI-TOF mass spectrometry.

Figure 8 shows the T_m values of the triplexes formed between TFO 9–10 and HP DNA 5–13. The T_m value of the triplex formed between the fully modified TFO 10 and the complementary HP DNA 5 was 50°C, whereas that formed between the unmodified TFO 9 and HP DNA 5 was not detected. The ΔT_m between the $^{DA}N_{ac}$:T-A matched base pair and the $^{DA}N_{ac}$:C-G mismatched base pair in the triplexes formed between TFO 10 and HP DNA 6, and TFO 10 and HP DNA 9 was 3°C and 2°C, respectively. On the other hand, the T_m values of the triplexes containing other mismatched base pairs were much lower than those of the triplexes formed between TFO 10 and HP DNA 5. It was



Scheme 7. Synthesis of TFO **10,** containing $_{2'\text{-OMe}}{}^{s}$T, $_{2'\text{-OMe}}{}^{s}$A, d^{g}C and $^{DA}N_{ac}$.

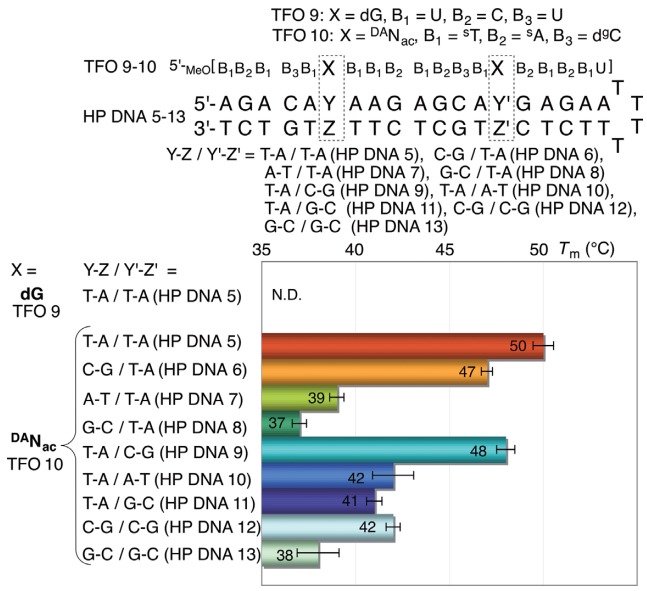


Figure 8. Comparison of the stabilities of the triplexes formed between HP DNAs 5–13 and TFOs 9–10. The T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate (pH 7.0), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM spermine and 1.5 μM triplex. The values represent the mean ± SD for three separate experiments.

also observed that the T_m value of the triplex containing two $^{DA}N_{ac}$:C-G mismatched base pairs (the triplex formed between TFO 10 and HP DNA 12) decreased by 8°C compared with that of the triplex containing fully matched base pairs. These results indicate that $^{DA}N_{ac}$ in fully modified TFO can bind to a T-A base pair strongly and selectively. To the best of our knowledge, this is the first report in which a fully modified TFO accurately recognized a complementary DNA duplex with mixed sequence under neutral conditions.

The T_m values of the triplexes formed between TFO 10 and HP DNA 5, 6 and 9 were measured under the previous Fox's conditions (50 mM sodium phosphate buffer (pH 7.0), 200 mM NaCl, 3.0 uM each oligonucleotide) to compare with the T_m values of the triplexes containing Fox's modified TFO (23). The T_m values of the triplexes formed between TFO 10 and HP DNA 5, 6 and 9 were 39°C, 36°C and 37°C, respectively. These results show that the base recognition ability of our modified TFO 10 (ΔT_m = 2–3°C) is higher than that of the previous TFO (ΔT_m = -1°C) under the neutral conditions without $MgCl_2$ and spermine, although the binding ability of TFO 10 (T_m = 39°C) is lower than that of the previous TFO (T_m = 49°C). Introduction of the cationic functional groups into the 5-position of d^{g}C and $_{2'\text{-OMe}}{}^{s}$T and the 2' position of $_{2'\text{-OMe}}{}^{s}$A and $_{2'\text{-OMe}}{}^{s}$T might increase the binding ability of the modified TFO.

CONCLUSIONS

In summary, we found that the TFO containing a d^{g}C residue for a C-G base pair has higher binding and base recognition abilities than those containing $_{2'\text{-OMe}}{}^{g}$C, $_{2'\text{-OMe}}{}^{g}$Cs and ^{gs}T. We also observed that $^{DA}N_{ac}$ forms hydro-

gen bonds with the T-A base pair in a triplex and stabilizes the structures of triplexes. Consequently, we successfully synthesized a fully modified TFO containing $^{DA}N_{ac}$, $d^{g}C$, $_{2'\text{-OMe}}{}^{s}T$ and $_{2'\text{-OMe}}{}^{s}A$ with high binding and base recognition abilities. These results should be very useful for nanotechnology and gene therapy using TFOs. Further studies are now underway in those directions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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