Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Stable Triplex Formation Using the Strong Stacking Effect of Consecutive Thionucleoside Moieties Incorporated into Triplex-Forming Oligonucleotides

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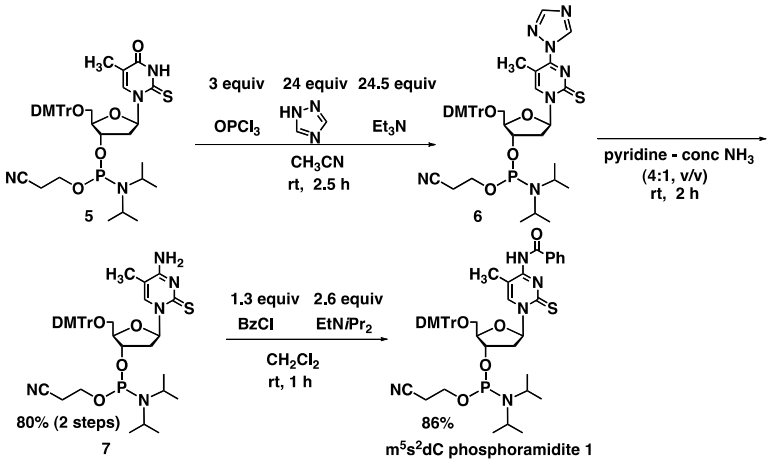
Content

General Remarks ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded at 270, 68 and 109 MHz, respectively. The chemical shifts were measured from tetramethylsilane for ^{1}H NMR spectra and $CDCl_3$ (77 ppm) for ^{13}C NMR spectra and 85% phosphoric acid (0 ppm) for ^{31}P NMR spectra. Column chromatography was performed 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 done on a Waters Aliance system with a Waters 3D UV detector and a Gen-PakTM FAX column (Waters, 4.6 x 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 by use of Mariner^{TM} (PerSeptive Biosystems Inc.). MALDI-TOF mass was performed by use of Bruker Daltonics [Matrix: 3-hydoroxypicolinic acid (100mg/ml) in H_2O-diammoniumhydrogen citrate (100 mg/ml) in H_2O (10:1, v/v)]. Highly cross-linked polystyrene was purchased from ABI.



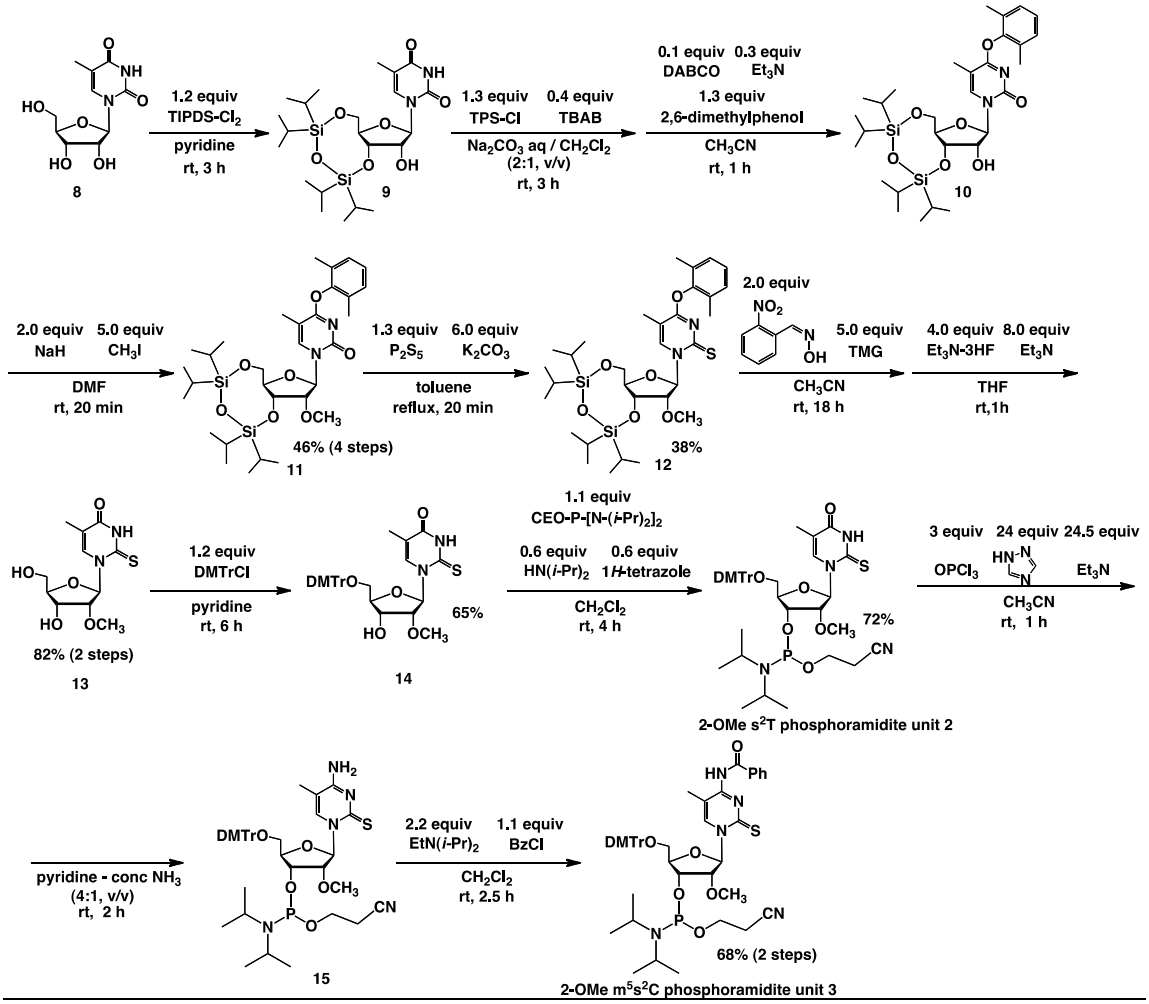
***Scheme S1*. Synthesis of phosporamidite unit 1.**

Synthesis of compound 7. 1,3,4-Triazole (1.09 g, 15.8 mmol) was suspended in dry CH_3CN (5 mL). To the solution was added slowly phosphoryl chloride (301 mg, 1.97 mmol) at 0 ˚C. After the mixture was stirred at 0 ˚C for 10 min, Et_3N (1.59 g, 15.8 mmol) was added to the mixture. After being stirred at 0 ˚C for 30 min, the dry CH_3CN

(5 mL) solution of s^2T phosphoramidite unit **5** (500 mg, 0.66 mmol) was added to the mixture. After the mixture was stirred at room temperature for 3 h, the mixture was partitioned between CH_2Cl_2 (100 mL) and saturated brine (100 mL). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was treated with pyridine – 28% conc NH_3 aq (4:1, v/v, 125 mL) at room temperature for 2 h. The mixture was evaporated under reduced pressure. The residue was chromatographed on a column of NH-silica gel (20 g) with hexane- $CHCl_3$ (30:70–0:100, v/v) to give **2.** (462 mg, 93%)^{1}H NMR ($CDCl_3$) δ 1.02-1.16 (m, 12H), 1.39, 1.44 (2s, 3H), 2.25-2.37 (m, 1H), 2.40, 2.63 (2t, 2H, *J* = 6.0 Hz), 2.77-2.93 (m, 1H), 3.31-3.36 (m, 1H), 3.50-3.60 (m, 3H), 3.63-3.65 (m, 1H), 3.79 (s, 6H), 4.15 (brs, 1H), 4.58-4.70 (m, 1H), 5.27 (brs, 1H), 6.80-6.85 (m, 4H), 7.03, 7.09 (2t, 1H, , *J* = 6.0 Hz), 7.22-7.29 (m, 7H), 7.40 (t, 2H, *J* = 7.3 Hz), 8.02, 8.08 (2s, 1H), 9.16 (brs, 1H). ^{13}C NMR ($CDCl_3$) δ 12.6, 16.2, 24.5, 40.6, 40.9, 55.2, 58.3, 61.9, 62.5, 71.6, 72.6, 85.4, 86.8, 90.1, 106.5, 113.1, 117.5, 127.1, 127.9, 128.2, 130.2, 135.3, 138.9, 144.2, 158.7, 160.5, 178.4. ^{31}P NMR ($CDCl_3$) δ 149.6, 150.5 HRMS (ESI) calcd for $[C_{40}H_{50}N_5O_6PS+H]^+$ 760.3298, found 760.3285.

Synthesis of phosphoramidite unit 1. To the dry THF solution (5mL) of the 2'-deoxy-5-methyl-2-thiocytine 3'-*O*-phosphoramidite derivative **7** (385 mg, 0.52 mmol) was added *N*-ethyldiisopropylamine (378 μL, 1.24 mmol) and benzoylcloride (145 μL, 0.83 mmol). After being stirred at room temperature for 1.5 h, the mixture was diluted with $CHCl_3$ (50 mL), and the mixture was washed three times with saturated $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (5 g) with 1% hexane-$CHCl_3$ (80:20–60:40, v/v) containing 1% triethylamine. The fractions were collected, evaporated under reduced pressure, and finally evaporated by repeated coevaporation three times each with toluene and $CHCl_3$ to remove the last traces of

triethylamine to give **1** (210 mg, 47%) (462 mg, 93%)^{1}H NMR ($CDCl_3$) δ 1.06-1.19 (m, 12H), 1.61, 1.63 (2s, 3H), 2.35-2.40 (m, 1H), 2.43, 2.63 (2t, 2H, *J* = 6.5 Hz), 2.74-2.92 (m, 1H), 3.37-3.41 (m, 1H), 3.56-3.67 (m, 4H), 3.77-3.83 (m, 7H), 4.20, 4.24 (2s, 1H), 4.63-4.67 (m, 1H), 6.85-6.95 (m, 5H), 7.15-7.18 (m, 1H), 7.24-7.34 (m, 8H), 7.41-7.44 (m, 4H), 7.52 (t, 1H, *J* = 7.3 Hz), 8.07, 8.11 (2s, 1H), 7.29 (d, 2H, , *J* = 7.5 Hz). ^{13}C NMR ($CDCl_3$) δ 13.0, 20.1, 20.2, 23.4, 24.5, 40.1, 43.1, 43.2, 43.3, 55.2, 55.25, 55.28, 58.0, 58.2, 62.1, 62.4, 77.2, 86.0, 86.9, 90.1, 90.2, 113.2, 113.3, 117.2, 117.3, 117.5, 127.2, 128.0, 128.1, 128.2, 128.8, 129.1, 129.8, 130.07, 130.12, 130.2, 132.5, 135.2, 136.9, 144.1, 156.1, 158.7. ^{31}P NMR ($CDCl_3$) δ 149.6, 150.2 HRMS (ESI) calcd for $[C_{47}H_{54}N_5O_7PS+H]^+$ 864.3554, found 864.3550.



***Scheme S2*. Synthesis of phosporamidite units 2 and 3.**

Synthesis of compound 11.

Compound **8** (25.8 g, 100 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine and finally dissolved in dry pyridine (500 mL). 1,3-Dichloro-1,1,3,3-tetraisopropyl-1,3-disiloxane (34.4 mL, 120 mmol) was added, and the mixture was stirred at room temperature for 3 h. The mixture was quenched by addition of MeOH. The mixture was partitioned between $CHCl_3$ and saturated brine. The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by chromatography with $CHCl_3$-MeOH to afford compound **9**. Subsequently, the compound **9** was dissolved in CH_2Cl_2 (500 mL). Tetrabutylammonium bromide (12.9 g, 40 mmol), 2,4,6,-

triisopropylbenzenesulfonylchloride (39.4 g, 130 mmol), and 0.2 M Na_2CO_3 aq. (1 L) were added. After being stirred at room temperature for 3 h, the mixture was partitioned between CH_2Cl_2 and Na_2CO_3 aq. The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was rendered anhydrous by repeated coevaporation with dry pyridine. The residue was further coevaporated with dry toluene and finally dissolved in dry CH_3CN (600 mL). 2,6-dimethylphenol (12.2 g, 100 mmol), triethylamine (41.9 mL, 300 mmol), and 1,4-diazabicyclo[2.2.2]octane (39.4 g, 130 mmol) were added. After being stirred at room temperature for 1 h, the mixture was was partitioned between $CHCl_3$ and saturated brine. The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by chromatography with hexane- $CHCl_3$ and $CHCl_3$–MeOH to afford the deprotected compound **10** (40.2 g, 66.4 mmol).

Compound **10** (40.2 g, 66.4 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine. The residue was further coevaporated with dry toluene and finally dissolved in dry DMF (600 mL). CH_3I (22 mL, 332 mmol) and NaH (3.3 g, 133 mmol) were added, and the mixture was stirred at room temperature for 20 min. The mixture was quenched by addition of acetic acid and diluted with ethyl acetate. The ethyl acetate solution was washed twice with saturated brine. The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by chromatography with hexane-ethyl acetate to afford **6**. (28.3 g, 46%):^{1}H NMR ($CDCl_3$) δ 0.99-1.13 (m, 28H), 2.10 (s, 6H), 2.15 (s, 3H), 3.68 (s, 3H), 3.82 (d, 1H, *J* = 3.5 Hz), 3.98 (d, 1H, *J* = 14 Hz), 4.15-4.21 (m, 2H), 4.28 (d, 1H, *J* = 14 Hz), 5.75 (s, 1H), 7.03 (s, 3H),

3.96-4.00 (m, 2H), 4.12-4.27 (m, 3H), 6.19 (s, 1H), 6.98 (s, 3H), 8.21 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 12.6, 12.9, 14.1, 13.7, 16.7, 17.1, 17.2, 17.3, 17.6, 17.7, 59.4, 59.7, 68.2, 77.6, 81.9, 83.5, 89.9, 103.6, 125.9, 128.8, 128.9, 130.0, 130.3, 140.9, 149.7, 155.6, 169.9. HRMS (ESI) calcd for $[C_{31}H_{50}N_2O_7Si_2+H]^+$ 619.3229, found 619.3213.

Synthesis of compound 12. Compound **11** (11 g, 17.7 mmol) was rendered anhydrous by

repeated coevaporation with dry pyridine. The residue was further coevaporated with dry toluene and finally dissolved in dry toluene (200 mL). Phosphorus pentasulfide (10.3 g, 23 mmol) and K_2CO_3 (14.8 g, 106 mmol) were added. The resulting mixture

was refluxed for 20 min and then cooled to room temperature. The mixture was partitioned between $CHCl_3$ and saturated brine. The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by chromatography with hexanes-ethyl acetate to afford compound **6** (24.3 g, 38% yield):
^{1}H NMR ($CDCl_3$) δ 0.87-1.07 (m, 28H), 2.04 (s, 6H), 2.14 (s, 3H), 3.68 (s, 3H), 3.96-4.00 (m, 2H), 4.12-4.27 (m, 3H), 6.19 (s, 1H), 6.98 (s, 3H), 8.21 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 12.4, 12.6, 12.7, 12.8, 13.0, 13.3, 13.5, 16.3, 16.5, 16.8, 16.9, 17.0, 17.1, 17.2, 17.4, 59.3, 60.3, 68.3, 68.3, 77.2, 82.2, 82.7, 94.1, 108.9, 125.5, 125.8, 129.3, 128.7, 129.9, 130.3, 142.4, 149.2, 164.1, 179.6. HRMS (ESI) calcd for $[C_{31}H_{50}N_2O_6SSi_2+H]^+$ 635.3001, found 635.3008.

Synthesis of compound 13. Compound **12** (4.3 g, 6.78 mmol) was rendered anhydrous by repeated coevaporation with dry pyridine. The residue was further coevaporated with dry toluene and finally dissolved in dry CH_3CN (30 mL). A solution of 1,1,3,3-tetramethylguanidine (2.5 mL, 20.3 mmol) and *syn*-*o*-nitrobenzaldoxime (2.4 g, 20.3 mmol) in CH_3CN (30 mL) was added. After being stirred at room temperature for 18 h, the mixture was was partitioned between $CHCl_3$ and saturated brine. The combined organic extracts were dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by chromatography with hexane- $CHCl_3$ to afford the deprotected compound. The deprotected compound was rendered anhydrous by repeated coevaporation with dry pyridine. The residue was further coevaporated with dry toluene and finally dissolved in dry THF (100 mL). To the solution were added Et_3N-3HF (4.4 mL, 27 mmol) and Et_3N(7.5 mL, 54 mmol). After being stirred at room temperature for 1 h, the mixture was concentrated in vacuo. The residue was purified by chromatography with $CHCl_3$-MeOH to give compound **13**.(1.6 g, 82%):
^{1}H NMR (DMSO) δ 1.80 (s, 3H), 3.44 (s, 3H), 3.60 (d, 1H, *J* = 12 Hz), 3.74 (d, 1H, *J* = 12.5 Hz), 3.78 (s, 1H), 3.87 (d, 1H, *J* = 5.0 Hz), 4.12 (s, 1H), 5.16 (brs, 1H), 5.35 (brs, 1H), 6.58 (s, 1H), 8.15 (s, 1H), 12.59 (brs, 1H); ^{13}C NMR (DMSO) δ 12.6, 58.5, 59.5, 67.8, 83.5, 84.6, 90.9, 115.0, 137.0, 160.6, 174.6. HRMS (ESI) calcd for $[C_{11}H_{16}N_2O_5S+H]^+$ 289.0853, found 289.0894.

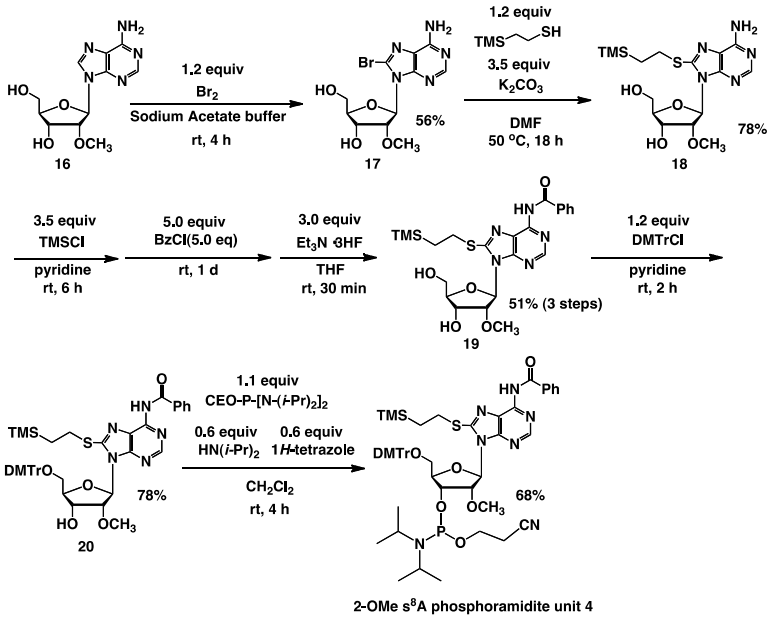
Synthesis of compound 14. Compound **13** (1.6 g, 7.0 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry pyridine (70 mL). To the solution was added DMTr-Cl (2.9 g, 8.4 mmol). After being stirred at room temperature for 6 h, the mixture was partitioned between $CHCl_3$ (200 mL) and saturated brine (200 mL). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with $CHCl_3$-MeOH (100:0–97:3, v/v) containing 1% Et_3N to give compound **14.** (2.7g, 65%) ^{1}H NMR ($CDCl_3$) δ 1.30 (s, 3H), 3.49 (d, 1H, *J* = 11 Hz), 3.63 (d, 1H, *J* = 11 Hz), 3.79 (s, 3H) 3.81 (s, 6H), 3.99 (d, 1H, *J* = 4.5 Hz), 4.09 (d, 1H, *J* = 7.5 Hz), 4.55 (t, 1H, *J* = 6.3 Hz), 6.68 (s, 1H), 6.86 (d, 4H, *J* = 8.0 Hz), 7.28-7.44 (m, 9H), 7.93 (s, 1H), 10.21 (brs, 1H). ^{13}C NMR ($CDCl_3$) δ 11.8, 55.1, 55.2, 60.0, 60.1, 61.4, 68.6, 83.2, 83.3, 83.9, 84.0, 86.8, 91.6, 91.7, 113.1, 113.2, 116.6, 123.7, 127.1, 128.0, 128.2, 130.0, 130.1, 135.1, 135.2, 136.1, 136.2, 136.3, 144.1, 149.5, 158.6, 158.7, 160.8, 174.0. HRMS (ESI) calcd for $[C_{32}H_{34}N_2O_7S+Na]^+$ 613.1979, found 613.1985.

Synthesis of phosphoramidite unit 2. Compound **14** (2.7 g, 4.6 mmol) was rendered anhydrous by repeated coevaporation with dry CH_3CN (3ml x 3) and dissolved in dry CH_2Cl_2 (20 ml). To the mixture was added diisopropylamine (387 μL, 2.74 mmol), 2-cyanoethoxy[bis(*N*,*N*-diisopropylamino)]phosphine (2.18 mL, 6.7 mmol) and 1-*H*-tetrazole (192 mg, 2.74 mmol). After the mixture was stirred at room temperature for 4 h, water (5 ml) was added to the mixture. After being stirred at room temperature for 10 min, the mixture was partitioned between $CHCl_3$ (200 ml) and brine (200 ml). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with hexane-$CHCl_3$ (50:50–0:100, v/v) containing 1% Et_3N and then $CHCl_3$-MeOH (100:0–97:3, v/v) containing 1% Et_3N to give the fractions containing phosphoramidite unit **2**.

The fractions were collected and evaporated under reduced pressure. The residue was finally evaporated by repeated coevaporation three times each with toluene and $CHCl_3$ to remove the last traces of Et_3N to give phosphoramidite unit **2**. (3.6 g, 72%) ^{1}H NMR ($CDCl_3$) δ 1.00–1.26 (m, 15H) 2.38, 2.66 (2t, 2H, *J* = 6.0 Hz), 3.37-3.45 (m, 2H), 3.59-3.62 (m, 2H), 3.65, 3.68 (2s, 3H), 3.73-3.95 (m, 8H), 4.04-4.08 (m, 1H), 4.23-4.31 (m, 1H), 4.45-4.62 (m, 1H), 6.59-6.68 (m, 1H), 6.82-6.86 (m, 4H), 7.29-7.42 (m, 9H), 7.92, 7.98 (2s, 1H), 9.21 (brs, 1H); ^{13}C NMR ($CDCl_3$) δ 11.6, 24.5, 24.6, 43.2, 43.3, 55.2, 55.3, 92.4, 113.1, 113.2, 116.5, 116.6, 127.3, 128.0, 128.3, 128.5, 130.3, 130.3, 130.4, 135.1, 136.4, 144.0, 158.8; ^{31}P NMR ($CDCl_3$) 151.5, 151.6. HRMS (ESI) calcd for $[C_{41}H_{51}N_4O_8PS+Na]^+$ 813.3057, found 813.3035.

Synthesis of phosphoramidite unit 3. 1,3,4-Triazole (2.1 g, 30.4 mmol) was suspended in dry CH_3CN (10 mL). To the solution was added slowly phosphoryl chloride (343 μL, 1.97 mmol) at 0 ˚C. After the mixture was stirred at 0 ˚C for 10 min, Et_3N (4.2 mL, 30.4 mmol) was added to the mixture. After being stirred at 0 ˚C for 30 min, the dry CH_3CN (10 mL) solution of phosphoramidite unit **2** (1.0 g, 1.26 mmol) was added to the mixture. After the mixture was stirred at room temperature for 1 h, the mixture was partitioned between CH_2Cl_2 (100 mL) and saturated brine (100 mL). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was treated with pyridine – 28% conc NH_3 aq (4:1, v/v, 20 mL) at room temperature for 30 min. The mixture was evaporated under reduced pressure. The residue was chromatographed on a column of NH-silica gel with hexane- $CHCl_3$ (30:70–0:100, v/v) to give compound **15** (880 mg). To the dry CH_2Cl_2 solution (15mL) of the compound **15** (880 mg, 1.1 mmol) were added *N*-ethyldiisopropylamine (423 μL, 2.42 mmol) and benzoylcloride (140 μL, 1.21 mmol). After being stirred at room temperature for 2.5 h, the mixture was diluted with $CHCl_3$ (50 mL), and the mixture was washed three times with saturated $NaHCO_3$. The organic layer was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with 1% hexane-$CHCl_3$ (80:20–60:40, v/v) containing 1% triethylamine. The fractions were collected, evaporated under reduced pressure, and finally evaporated by repeated coevaporation three times each with

toluene and $CHCl_3$ to remove the last traces of triethylamine to give phosphoramidite unit **3** (770 mg, 78%). ^{1}H NMR ($CDCl_3$) δ 0.98–1.31 (m, 15H) 2.38, 2.64 (2t, 2H, *J* = 6.3 Hz), 3.37-3.44 (m, 2H), 3.57-3.90 (m, 10H), 4.06, 4.15 (2d, 1H, *J* = 4.0 Hz), 4.31, 4.37 (2d, 1H, *J* = 9.5 Hz), 4.53-4.58 (m, 1H), 6.41 (s, 1H, *J* = 4.0 Hz), 6.81-6.86 (m, 4H), 7.22-7.51 (m, 10H), 7.71 (d, 4H, *J* = 7.5 Hz), 8.66, 8.67 (2s, 1H); ^{13}C NMR ($CDCl_3$) δ 1.9, 13.5, 20.0, 20.1, 20.3, 20.4, 24.4, 24.5, 24.6, 24.7, 43.1, 43.2, 43.3, 55.2, 57.8, 58.0, 58.2, 60.1, 60.3, 60.4, 68.7, 68.9, 69.7, 69.8, 81.9, 83.1, 86.9, 94.6, 95.1, 113.2, 115.9, 116.4, 117.3, 117.4, 127.2, 127.3, 128.0, 128.5, 128.6, 128.8, 129.1, 130.4, 133.1, 133.8, 135.0, 144.0, 144.1, 145.4, 153.6, 158.8, 161.0, 161.1, 171.7, 171.8, 178.5; ^{31}P NMR ($CDCl_3$) 151.5, 151.6. HRMS (ESI) calcd for $[C_{41}H_{51}N_4O_8PS+Na]^+$ 916.3479, found 916.3462.



***Scheme S3*. Synthesis of phosporamidite unit 4.**

Synthesis of compound 17. Bromine (6.8 g, 42 mmol) was suspended in 2 M sodium acetate buffer (pH 4.3, 50 mL). After being stirred at room temperature for 1 h, the solution was slowly added to 2 M sodium acetate buffer (pH 4.3, 140 mL) of 2'-*O*-

methyladenosine **16** (10 g, 35.5 mmol). After being stirred at room temperature for 6 h, 5% sodium thiosulfate (100 mL) and 2 M sodium hydroxide (200 mL) was added to the mixture. The resulting precipitates were collected by filtration with water to give the compound **17** (7.1 g, 56%). ^{1}H NMR (DMSO) δ 3.31 (s, 3H, 2'-*O*-Me), 3.51-3.55 (m, 1H), 3.67-3.69 (m, 1H), 4.00 (d, 1H, *J* = 2.5 Hz), 4.42 (d, 1H, *J* = 2.5 Hz), 4.86 (dd, 1H, *J* = 5.3 Hz, 6.8 Hz), 5.34 (d, 1H, *J* = 5.0 Hz), 5.47 (dd, 1H, *J* = 3.8 Hz, 8.3 Hz), 5.92 (d, 1H, *J* = 6.5 Hz), 7.58 (bs, 2H, 6-NH_2), 8.14(s, 1H),. ^{13}C NMR (DMSO) δ 22.9, 58.2, 62.6, 69.6, 80.7, 88.0, 89.2, 120.3, 124.6, 127.7, 136.9, 150.3, 150.5, 153.3, 155.9. HRMS (ESI) calcd for $[C_{11}H_{14}BrN_5O_4+H]^+$ 360.0307, found 360.0283.

Synthesis of compound 18. Compound **17** (5 g, 13.9 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in DMF (140 mL). To the solution were added K_2CO_3 (6.7 g, 48.7 mmol) and 2-(trimethylsilyl)ethanthiol (2.7 ml, 16.7 mmol). After being stirred at 50 °C for 18 h, the mixture was partitioned between AcOEt (500 mL) and saturated brine (300 mL). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with $CHCl_3$-MeOH (100:0–95:5, v/v) to give **18.** (4.48 g, 78%) ^{1}H NMR (DMSO) δ 0.05 (s, 9H) 0.86-0.90 (m, 2H), 0.99-1.02 (m, 2H), 3.34(s, 3H, OMe) 3.51-3.56 (m, 1H), 3.66-3.68 (m, 1H), 3.99 (bs, 1H), 4.38 (bs, 1H), 4.77 (dd, 1H, *J* = 5.3 Hz, 6.8 Hz), 5.32 (brs, 1H), 5.67 (dd, 1H, *J* = 3.3 Hz, 8.3 Hz), 5.87 (d. 1H, *J* = 6.5 H), 7.30 (s, 2H), 8.07 (s, 1H). ^{13}C NMR (DMSO) δ -1.0, 17.3, 17.5, 30.1, 34.5, 36.4, 58.1, 62.8, 69.7, 75.7, 80.9, 87.6, 87.9, 120.4, 149.2, 150.9, 152.2, 155.3, 163.0. HRMS (ESI) calcd for $[C_{16}H_{27}N_5O_4SSi+H]^+$ 414.1631, found 414.1603.

Synthesis of compound 19. Compound **18** (1.5 g, 3.6 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry pyridine (36 mL). To the solution was added tirimethylsilyl chloride (1.6 mL, 12.6

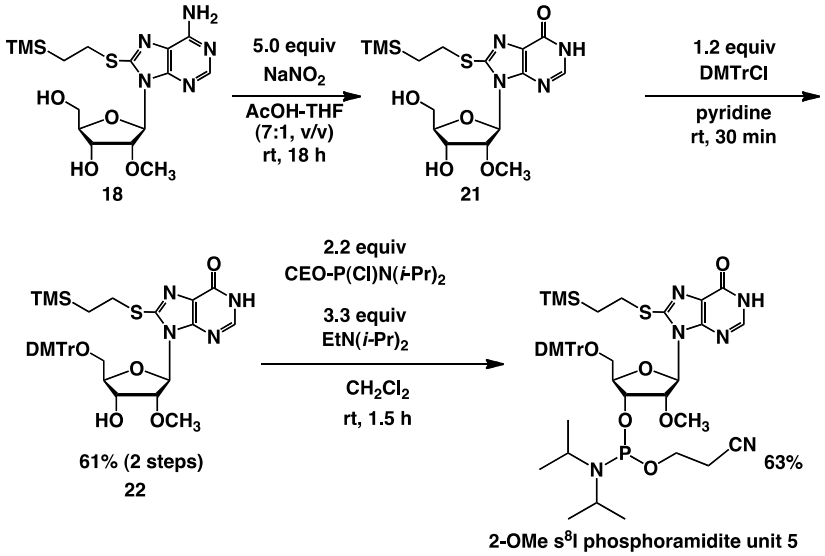
mmol). After being stirred at room temperature for 6 h, benzoyl chloride was added to the mixture. After being stirred at room temperature for 1 day, to the solution was added 28% ammonium hydroxide. After being stirred at room temperature for 10 min, the mixture was evaporated under reduced pressure. The mixture was partitioned between $CHCl_3$ (200 mL) and saturated brine (200 mL). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was dissolved in dry THF (20 mL) and treated with Et_3N-3HF (1.4 mL, 8.7 mmol) for 30 min. The mixture was partitioned between $CHCl_3$ (500 mL) and saturated brine (300 mL). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with $CHCl_3$-MeOH (100:0–95:5, v/v) to give **19.** (950 mg, 51%) ^{1}H NMR (DMSO) δ -0.08 (s, 9H) 1.13-1.16 (m, 2H), 3.30-3.27 (m, 5H), 3.55-3.57 (m, 1H), 3.69-3.71 (m, 1H), 3.98 (brs, 1H), 4.43 (brs, 1H), 4.89 (t, 1H, *J* = 5.8 Hz), 5.10 (brs, 1H), 5.37 (d, 1H, *J* = 6.0 Hz), 5.85 (d, 1H, *J* = 6.5 Hz), 7.54 (t, 1H, *J* = 7.8 Hz), 7.63 (t, 1H, *J* = 7.3 Hz), 8.04 (d, 1H, *J* = 7.5 Hz), 8.65 (s, 1H), 11.11 (brs, 1H). ^{13}C NMR ($CDCl_3$) δ -1.6, 17.7, 29.7, 58.9, 63.4, 70.7, 88.2, 88.4, 124.5, 128.0, 129.1, 133.0, 134.0, 147.6, 150.7, 152.7, 155.4, 164.6. HRMS (ESI) calcd for $[C_{23}H_{31}N_5O_5SSi+H]^+$ 518.1893, found 518.1918.

Synthesis of compound 20. Compound **19** (950 mg, 1.8 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry pyridine (18 mL). To the solution was added DMTr-Cl (740 mg, 2.2 mmol). After being stirred at room temperature for 2 h, the mixture was partitioned between $CHCl_3$ (200 mL) and saturated brine (200 mL). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with $CHCl_3$-MeOH (100:0–97:3, v/v) containing 1% Et_3N to give **20.** (1.4 g, 78%) ^{1}H NMR ($CDCl_3$) δ 0.09 (s, 9H), 1.15 (t, 2H, *J* = 9.0 Hz), 3.31-3.49 (m, 5H) 3.75 (s, 6H), 4.23 (d, 1H, *J* = 5.0 Hz), 4.76 (d, 1H, *J* = 4.5 Hz), 5.04 (t, 1H, *J* =

4.8 Hz), 6.04 (d, 1H, *J* = 4.5 Hz), 6.77 (dd, 4H, *J* = 6.3 Hz, 8.3 Hz), 7.17-7.56 (m, 13H), 8.01 (d, 2H, *J* = 7.5 Hz), 8.55 (s, 1H), 9.26 (s, 1H). ^{13}C NMR ($CDCl_3$) δ -1.5, 17.8, 29.6, 55.4, 58.9, 63.6, 70.3, 80.8, 84.4, 86.4, 87.4, 113.3, 123.9, 127.0, 127.9, 128.0, 128.5, 129.1, 130.4, 132.7, 134.2, 136.2, 136.3, 145.1, 147.1, 151.3, 153.7, 155.4, 158.6, 158.7, 164.8. HRMS (ESI) calcd for $[C_{44}H_{49}N_5O_7SSi+H]^+$ 820.3200, found 820.3191.

Synthesis of phosphoramidite unit 4. Compound **20** (1.0 g, 1.2 mmol) was rendered anhydrous by repeated coevaporation with dry CH_3CN (3ml x 3) and dissolved in dry CH_2Cl_2 (20 ml). To the mixture was added diisopropylamine (100 µl, 0.73 mmol), 2-cyanoethoxy[bis(*N*,*N*-diisopropylamino)]phosphine (470 µl, 1.5 mmol) and 1-*H*-tetrazole (50 mg, 0.73 mmol). After the mixture was stirred at room temperature for 4 h, water (5 ml) was added to the mixture. After being stirred at room temperature for 10 min, the mixture was partitioned between $CHCl_3$ (100 ml) and brine (100 ml). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with hexane-$CHCl_3$ (50:50–0:100, v/v) containing 1% Et_3N and then $CHCl_3$-MeOH (100:0–97:3, v/v) containing 1% Et_3N to give the fractions containing phosphoramidite unit **4**. The fractions were collected and evaporated under reduced pressure. The residue was finally evaporated by repeated coevaporation three times each with toluene and $CHCl_3$ to remove the last traces of Et_3N to give phosphoramidite unit **4**. (831 mg, 68%) ^{1}H NMR ($CDCl_3$) δ 0.02, 0.11 (2s, 9H), 1.12-1.26 (m, 14H), 2.42-2.71 (m, 2H), 3.31-3.68 (m, 10H), 3.78-79 (m, 6H), 3.92-3.98 (m, 1H), 4.34-4.39 (m, 1H), 4.77-4.93 (m, 1H), 5.17-5.27 (m, 1H), 5.97-5.98 (m, 1H), 6.75-6.80 (m, 4H), 7.20-7.23 (m, 3H), 7.27-7.31 (m, 6H), 7.31-7.42 (m, 2H), 7.54-7.56 (m, 2H), 7.62-7.62 (m, 1H), 8.01 (d, 2H, *J* = 7.0 Hz), 8.51, 8.55 (2s, 1H), 8.86 (s, 1H); ^{13}C NMR ($CDCl_3$) δ -1.5, 17.8, 20.3, 20.4, 20.6, 24.8, 29.5, 43.3, 43.4, 43.5, 43.6, 55.4, 58.4, 58.6, 58.8, 59.2, 59.4, 62.9, 63.3, 70.6, 70.8,

71.5, 71.6, 79.7, 79.9, 83.8, 86.4, 87.2, 87.3, 113.2, 117.7, 118.0, 123.7, 126.9, 127.9, 128.4, 128.5, 129.1, 130.3, 130.3, 132.8, 134.3, 136.0, 136.2, 144.9, 145.0, 146.9, 151.1, 151.2, 153.5, 155.7, 155.8, 158.6, 164.5; ^{31}P NMR ($CDCl_3$) 150.9, 152.1. HRMS (ESI) calcd for $[C_{53}H_{66}N_7O_8PSSi+H]^+$ 1020.4279, found 1020.4197.



***Scheme S4*. Synthesis of phosporamidite unit 5.**

Synthesis of compound 22. Compound **18** (1.2 g, 2.9 mmol) was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in AcOH-H_2O (7:1, v/v, 29 mL). To the solution were added $NaNO_s$ (1 g, 14.5 mmol) and 2-(trimethylsilyl)ethanthiol (2.7 ml, 16.7 mmol). After being stirred at room temperature for 18 h, the mixture was partitioned between $CHCl_3$ (200 mL) and saturated brine (200 mL). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The resulting residue was rendered anhydrous by coevaporation three times with dry pyridine and finally dissolved in dry pyridine (29 mL). To the solution was added DMTr-Cl (1.2 g, 3.5 mmol). After being stirred at room temperature for 2 h, the mixture was partitioned between $CHCl_3$ (200 mL) and saturated brine (200 mL). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column

of silica gel with $CHCl_3$-MeOH (100:0–97:3, v/v) containing 1% Et_3N to give **20.** (1.3 g, 62%) ^1H NMR ($CDCl_3$) δ 0.09 (s, 9H, TMS), 1.00-1.03 (m, 2H), 3.40-3.47 (m, 7H), 3.74-3.77 (m, 6H) .415 (m, 1H), 4.65 (dd, 1H, *J* = 5.5 Hz, J = 11.5 Hz), 4.79 (t, 1'H, *J* = 5.0 Hz), 6.00 (d, 1H, *J* = 4.5 Hz), 6.77 (d, 4H, *J* = 8.0 Hz), 7.17-7.45 (m, 9H), 7.73 (s, 1H), 12.6 (s, 1H). ^{13}C NMR ($CDCl_3$) δ -1.5, 16.9, 29.6, 55.4, 58.9, 63.8, 70.4, 81.2, 84.3, 86.5, 87.6, 113.2, 125.6, 127.0, 127.9, 128.5, 130.4, 136.2, 143.3, 145.1, 150.6, 151.1, 158.6, 158.7. HRMS (ESI) calcd for $[C_{37}H_{44}N_4O_7SSi+H]^+$ 717.2778, found 717.2774.

Synthesis of phosphoramidite unit 5. Compound **22** (1.0 g, 1.4 mmol) was rendered anhydrous by repeated coevaporation with dry CH_3CN (3ml x 3) and dissolved in dry CH_2Cl_2 (14 ml). To the mixture was added diisopropylethylamine (803 µl, 4.6 mmol) and 2-cyanoethoxy(*N*,*N*-diisopropylamino)chlorophosphine (686 µl, 3.1 mmol). After the mixture was stirred at room temperature for 1.5 h, water (5 ml) was added to the mixture. After being stirred at room temperature for 10 min, the mixture was partitioned between $CHCl_3$ (100 ml) and brine (100 ml). The organic phase was collected, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel with hexane-$CHCl_3$ (50:50–0:100, v/v) containing 1% Et_3N and then $CHCl_3$-MeOH (100:0–97:3, v/v) containing 1% Et_3N to give the fractions containing **21**. The fractions were collected and evaporated under reduced pressure. The residue was finally evaporated by repeated coevaporation three times each with toluene and $CHCl_3$ to remove the last traces of Et_3N to give phosphoramidite unit **5**. (808 mg, 63%) ^1H NMR ($CDCl_3$) δ 0.06, 0.07 (2s, 9H), 0.99-1.24 (m, 14H), 2.40-2.70 (m, 2H), 3.33-3.67 (m, 10H), 3.74, 3.75 (2s, 6H), 3.92-3.98 (m, 1H), 4.30-4.36 (m, 1H), 4.66-4.84 (m, 1H), 4.87-5.04 (m, 1H), 5.99 (d, 1H, *J* = 6.0 Hz), 6.74-6.80 (m, 4H), 7.17-7.44 (m, 9H), 7.77, 7.80 (2s, 1H), 8.86 (s, 1H), 13.50 (brs, 1H); ^{13}C NMR ($CDCl_3$) δ -1.4, 16.8, 20.5, 20.6, 24.8, 24.9, 29.7, 43.4, 43.5,

43.6, 43.7, 55.3, 58.4, 58.8, 59.3, 59.4, 86.5, 86.6, 87.4, 113.2, 118.0, 125.6, 127.0, 127.9, 128.5, 128.6, 130.3, 130.4, 136.1, 145.0, 150.7, 151.4, 151.5, 158.7; ^{31}P NMR ($CDCl_3$) 150.7, 152.0. HRMS (ESI) calcd for $[C_{46}H_{61}N_6O_8PSSi+H]^+$ 917.3857, found 917.3872.

Synthesis of TFOs 2-12 and 15-17.

(For TFO containing s^8A and s^8I) The synthesis of TFOs was carried out on a T-loaded CPG resin (1 μmol scale) in ABI 392 DNA synthesizer. (Activator: 0.25 M solution of 5-benzylthio-1H-tetrazole, Oxidizer: 0.02 M solution of I_2) The fully protected oligomer after chain elongation was deprotected and released from the resin by treatment with a 28% ammonia solution at 50 ^{o}C for 8 h. The mixture was evaporated and the resulting residue was rendered anhydrous by repeated coevaporation with dry CH_3CN. After the coevaporation, the oligomer was dissolved in 1 M Tetrabutylammonium fluoride/THF and the mixture was stirred at room temperature for 2 h. The crude mixture was purified by Sep-Pak C18 cartridge and anion-exchange HPLC to give TFO.

(For TFO without s^8A and s^8I) The synthesis of TFOs was carried out on a T-loaded CPG resin (1 μmol scale) in ABI 392 DNA synthesizer. (Activator: 0.25 M solution of 5-benzylthio-1H-tetrazole, Oxidizer: 0.02 M solution of I_2) The fully protected oligomer after chain elongation was deprotected and released from the resin by treatment with a 28% ammonia solution at 50 ^{o}C for 8 h. The crude mixture was purified by Sep-Pak C18 cartridge and anion-exchange HPLC.

TFO 2: TTTCTTm^{5}s^{2}CTTCTT, Yield 78%, MALDI-TOF Mass (M+H) calcd for $[C_{118}H_{156}N_{27}O_{76}P_{11}S + H]^+$ 3572.6, found 3569.4

TFO 3: TTTCs^{2}Ts^{2}TCs^{2}Ts^{2}TCTT, Yield 68%, MALDI-TOF Mass (M+H) calcd for $[C_{117}H_{154}N_{27}O_{75}P_{11}S_4 + H]^+$ 3606.5, found 3609.7

TFO 4: TTTCs^{2}Ts^{2}Tm^{5}s^{2}Cs^{2}Ts^{2}TCTT, Yield 70%, MALDI-TOF Mass (M+H) calcd for $[C_{118}H_{156}N_{27}O_{74}P_{11}S_5 + H]^+$ 3636.5, found 3638.5

TFO 5: TTTm^{5}s^{2}Cs^{2}Ts^{2}Tm^{5}s^{2}Cs^{2}Ts^{2}Tm^{5}s^{2}CTT, Yield 21%, MALDI-TOF Mass (M+H) calcd for $[C_{120}H_{160}N_{27}O_{72}P_{11}S_7 + H]^+$ 3696.5, found 3695.2

TFO 6: TTTs^{2}Ts^{2}TCCCCs^{2}Ts^{2}TTT, Yield 61%, MALDI-TOF Mass (M+H) calcd for $[C_{126}H_{165}N_{30}O_{81}P_{12}S_4 + H]^+$ 3895.6, found 3898.2

TFO 7: TTTs^{2}Ts^{2}Tm^{5}s^{2}Cm^{5}s^{2}Cm^{5}s^{2}Cm^{5}s^{2}Cs^{2}Ts^{2}TTT, Yield 54%, MALDI-TOF Mass (M+H) calcd for $[C_{130}H_{173}N_{30}O_{77}P_{12}S_8 + H]^+$ 4015.5, found 4013.7

TFO 8: TTTs^{2}Ts^{2}Ts^{8}A s^{8}A s^{8}A s^{8}A s^{2}Ts^{2}TTT, Yield 34%, MALDI-TOF Mass (M+H) calcd for $[C_{130}H_{166}N_{38}O_{77}P_{12}S_8 + H]^+$ 4119.5, found 4122.1.

TFO 9: s^{2}Ts^{2}Ts^{2}Ts^{2}Ts^{2}T m^{5}s^{2}C s^{2}T m^{5}s^{2}C s^{2}T m^{5}s^{2}C s^{2}T m^{5}s^{2}C s^{2}T m^{5}s^{2}C T, Yield 24%, MALDI-TOF Mass (M+H) calcd for $[C_{150}H_{301}N_{35}O_{84}P_{14}S_{14} + H]^+$ 4718.5, found 4719.2.

TFO 10: 2-OMe[s^{2}Ts^{2}Ts^{2}Ts^{2}Ts^{2}T m^{5}s^{2}C s^{2}T m^{5}s^{2}C s^{2}T m^{5}s^{2}C s^{2}T m^{5}s^{2}C s^{2}T m^{5}s^{2}C]T, Yield 5%, MALDI-TOF Mass (M+H) calcd for $[C_{164}H_{229}N_{35}O_{98}P_{14}S_{14} + H]^+$ 5138.7, found 5139.9.

TFO 11: 2-OMe[s^{2}Ts^{2}Ts^{2}Ts^{2}Ts^{2}T s^{8}A s^{2}T s^{8}A s^{2}T s^{8}A s^{2}T s^{8}A s^{2}T s^{8}A]T, Yield 14%, MALDI-TOF Mass (M+H) calcd for $[C_{164}H_{229}N_{35}O_{98}P_{14}S_{14} + H]^+$ 5138.7, found 5264.3.

TFO 12: 2-OMe[$s^2Ts^2Ts^2Ts^2Ts^2T$ s^8I s^2T s^8I s^2T s^8I s^2T s^8I s^2T s^8I]T, Yield 18%, MALDI-TOF Mass (M+H) calcd for $[C_{164}H_{201}N_{63}O_{98}P_{14}S_{14} + H]^+$ 5502.5, found 5499.4.

TFO 15: 2-OMe[s^8A s^8A s^8A s^2T s^2T s^2T s^2T s^2T s^8A s^2T]T, Yield 16%, MALDI-TOF Mass (M+H) calcd for $[C_{120}H_{160}N_{34}O_{71}P_{10}S_{10} + H]^+$ 3843.5, found 38.4.

TFO 16: TTT$s^2Ts^2Ts^2Tm^5s^2Cs^2Ts^2Ts^2$TTT, Yield 29%, MALDI-TOF Mass (M+H) calcd for $[C_{120}H_{158}N_{25}O_{74}P_{11}S_7 + H]^+$ 3698.5, found 3699.2

TFO 17: TTT$s^2Ts^2Ts^2TCs^2Ts^2Ts^2$TTT, Yield 58%, MALDI-TOF Mass (M+H) calcd for $[C_{119}H_{156}N_{25}O_{75}P_{11}S_6 + H]^+$ 3668.5, found 3668.4

Table S1. Selectivity of TFO 3-4 containing C and m^5s^2C at pH 7.0.

TFO **3-4** 5'-T T T C s^2T s^2T X s^2T s^2T C T T
X = C (TFO **3**), m^5s^2C (TFO **4**)

HP **1, 5-7** 5'-G A A A G A A Y A A G A A A C $^{T}_{T}$
3'-C T T T C T T Y' T T C T T T G $_{T}^{T}$
Y-Y' = G-C (HP **1**), C-G (HP **5**), A-T (HP **6**), T-A (HP **7**)

entry	oligonucleotides	T_m (°C) [a]	ΔT_m (°C)
1	TFO 3 – HP 1	37	-
2	TFO 3 – HP 5	15	22[b]
3	TFO 3 – HP 6	9	28[b]
4	TFO 3 – HP 7	10	27[b]
5	TFO 4 – HP 1	44	-
6	TFO 4 – HP 5	16	28[c]
7	TFO 4 – HP 6	12	32[c]
8	TFO 4 – HP 7	11	33[c]

[a] The T_m values are accurate within ±0.5 °C. The T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate buffer (pH 7.0), 500 mM NaCl, 10 mM $MgCl_2$, and 2 μM triplex. [b] ΔT_m is the difference in the T_m value between the matched triplex (entry 1) and the mismatched triplexes (entries 2–4). [c] ΔT_m is the difference in the T_m value between the matched triplex (entry 5) and the mismatched triplexes (entries 6–8).

TFO**16** 5'-T T T s^2T s^2T s^2T m^5s^2C s^2T s^2T s^2TT T

HP **8** 5'-G A A A A A A G A A A T T C^{T}T
3'-C T T T T T T C T T T A A G$_{T}$T

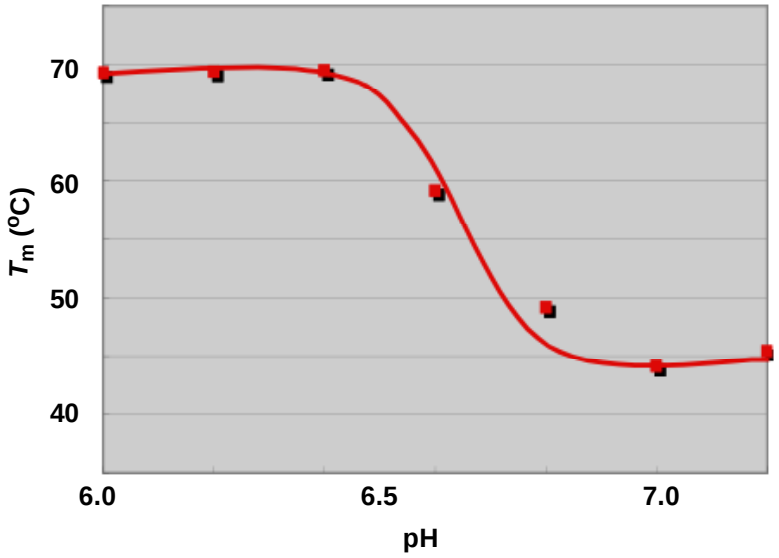


Figure S1. T_m values for DNA triplexes formed between TFO **16** and HP **8** at varied pH conditions (pH 6.0–7.2) The T_m values are accurate within ±0.5 °C. The T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate buffer (pH 6.0–7.2), 500 mM NaCl, 10 mM $MgCl_2$, and 2 μM triplex.

TFO **17** 5'-T T T s^2T s^2T s^2T C s^2T s^2T s^2TT T

HP **8** 5'-G A A A A A A G A A A T T C^{T}T

3'-C T T T T T T C T T T A A G$_{T}$T

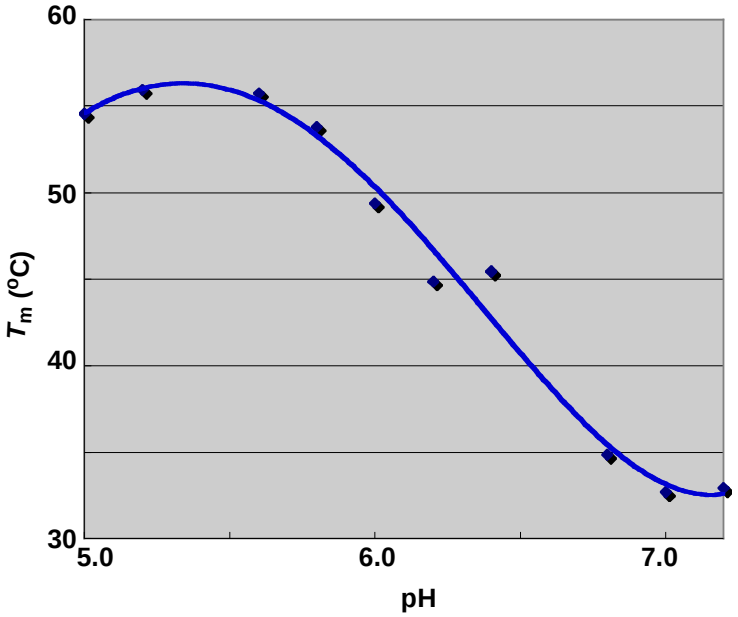


Figure S2. T_m values for DNA triplexes formed between TFO **17** and HP **8** at varied pH conditions (pH 5.0–7.2) The T_m values are accurate within ±0.5 °C. The T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate buffer (pH 5.0–7.2), 500 mM NaCl, 10 mM $MgCl_2$, and 2 μM triplex.

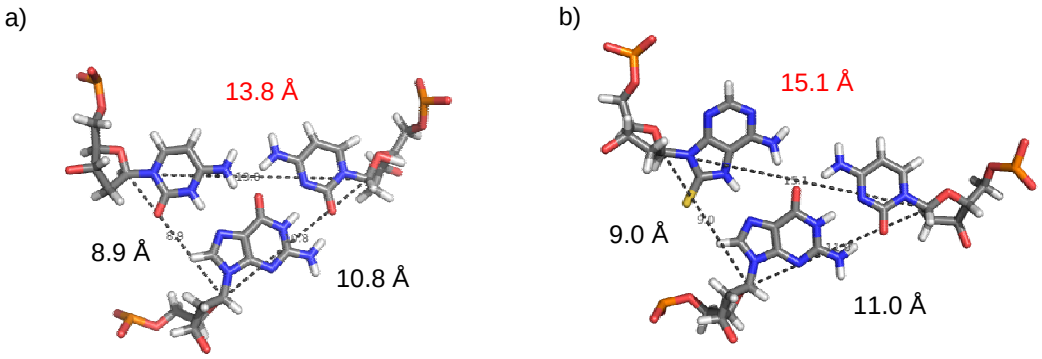


Figure S3. Computer modelling of a DNA triplexes for distance between the C1' atoms in the neighboring mononucleotide units a) C^{+}-G-C, b) s^{8}A-G-C.

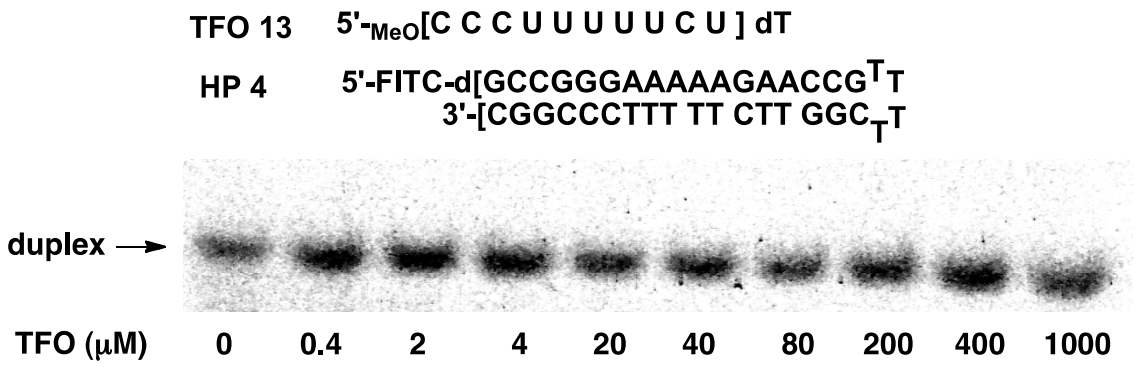


Figure S4. Electrophoretic mobility shift assay of the triplex formed between TFO 13 and HP 4 (40 nM) on 10% nondenaturing polyacrylamide gel at pH 7.0. Indicated concentrations of TFO 13 were incubated with HP 4 for 2 h at 37 °C.

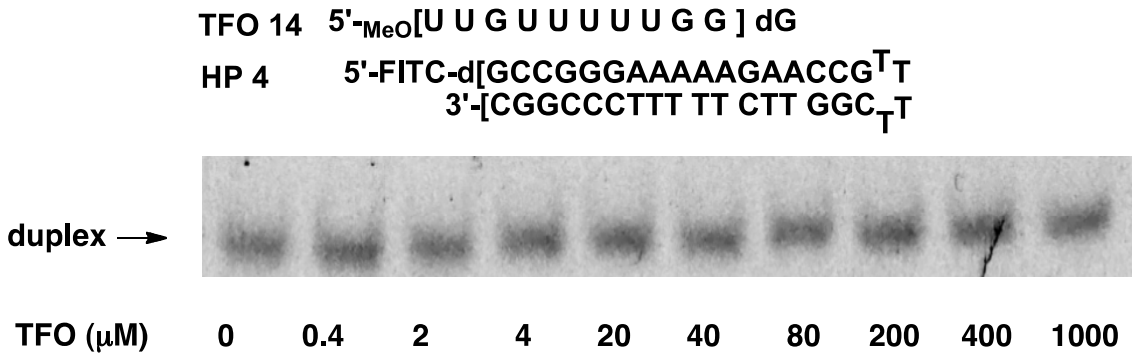


Figure S5. Electrophoretic mobility shift assay of the triplex formed between TFO 14 and HP 4 (40 nM) on 10% nondenaturing polyacrylamide gel at pH 7.0. Indicated concentrations of TFO 14 were incubated with HP 4 for 2 h at 37 °C.

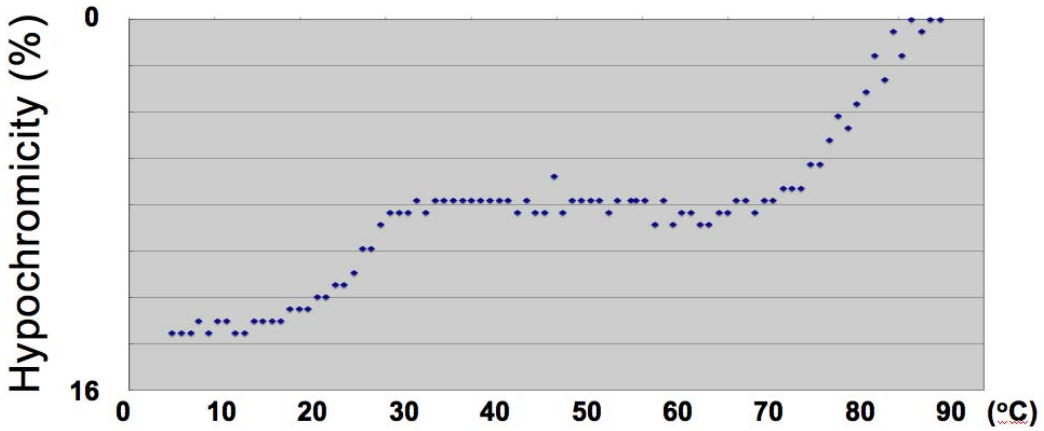


Figure S6. Melting curve of the triplex formed between .TFO 9 and HP 3 by UV analysis at 260 nm.

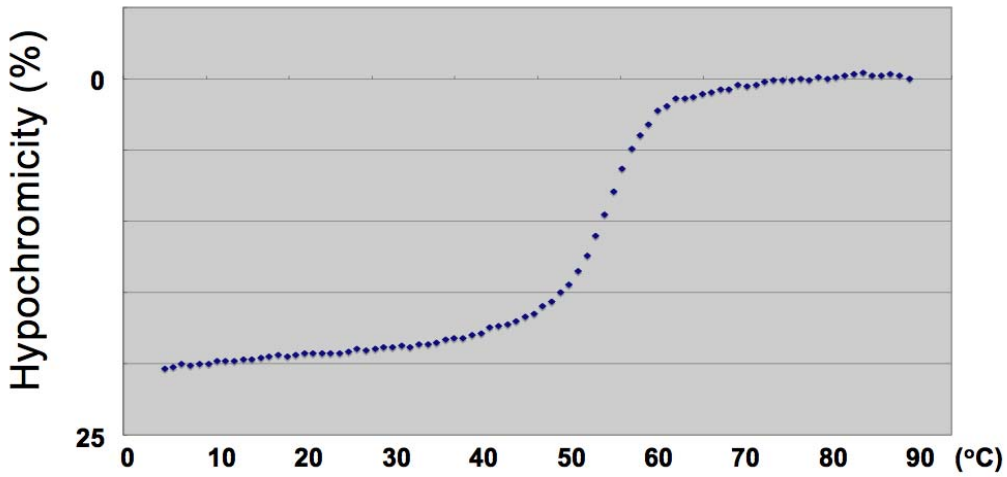


Figure S7. Melting curve of the triplex formed between .TFO 10 and HP 3 by UV analysis at 290 nm.

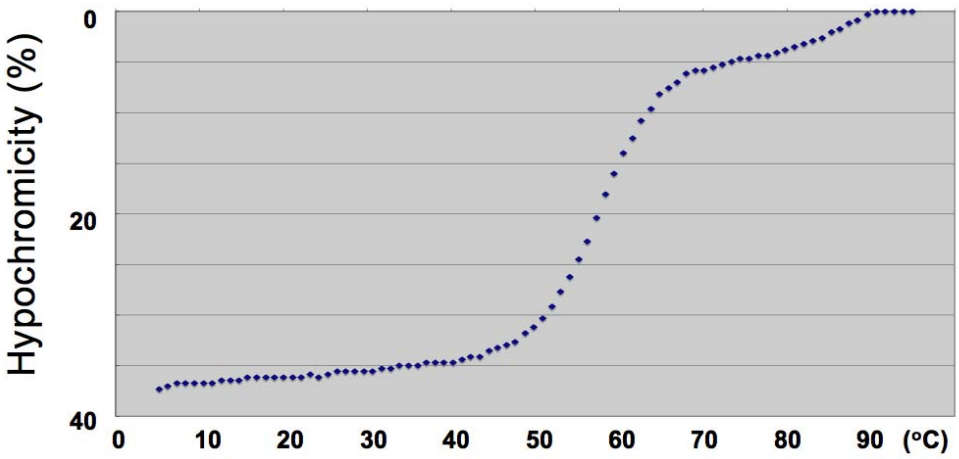


Figure S8. Melting curve of the triplex formed between .TFO 11 and HP 3 by UV analysis at 290 nm.

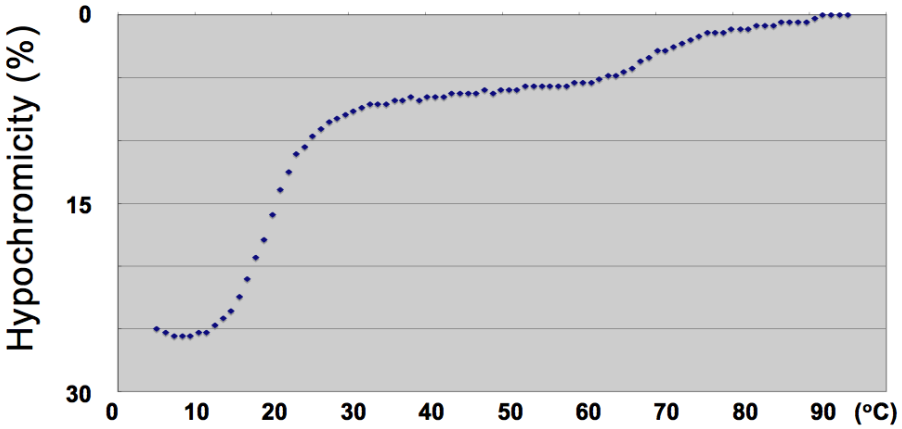


Figure S9. Melting curve of the triplex formed between .TFO 12 and HP 3 by UV analysis at 290 nm.