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A NEW ODORLESS PROCEDURE FOR THE SYNTHESIS OF 2′-DEOXY-6-THIOGUANOSINE AND ITS INCORPORATION INTO OLIGODEOXYNUCLEOTIDES

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□ *6-S-{2-[(2-ethylhexyl)oxycarbonyl]ethyl)}-3′,5′-O-bis(*tert*-butyldimethylsilyl)-2′-deoxy-6-thioguanosine (**2**) was synthesized in high yield from the corresponding 6-O-mesitylenesulfonyl derivative by the reaction with 2-ethylhexyl 3-mercapto-propionate. The phosphoramidite precursor derived from **2** was successfully applied to an automated DNA synthesizer to produce 2′-deoxy-6-thioguanosine containing ODN. The results showed that 2-ethylhexyl 3-mercaptopropionate is useful as an odor less reagent and also as an S-protecting group of 2′-deoxy-6-thioguanosine.*

Keywords 2′-Deoxy-6-thioguanosine; odorless synthesis; 2-Ethylhexyl 3-mercaptopropionate; phosphoroamidite; DNA synthesis

INTRODUCTION

Oligonucleotides containing a thio-substituted base, such as 6-thioguanine (6-thio-dG, 6-thio-G) and 4-thiouracil (4-thio-U), have been widely used for various purposes in biology.[1–7] Their photo-induced cross-linking ability has been used to study the three-dimensional interaction of RNA-RNA or RNA-proteins at the atomic level.[1–4] The high nucleophilicity of the sulfur atom of 6-thioguanosine was used as a modification site of oligode-oxynucleotides (ODN).[5–7] Recently, we developed the functionality-transfer reaction for the site-specific modification of DNA and RNA by utilizing the ODN containing 2′-deoxy-6-thioguanosine. The site-specific NO modification of the target cytosine was demonstrated by

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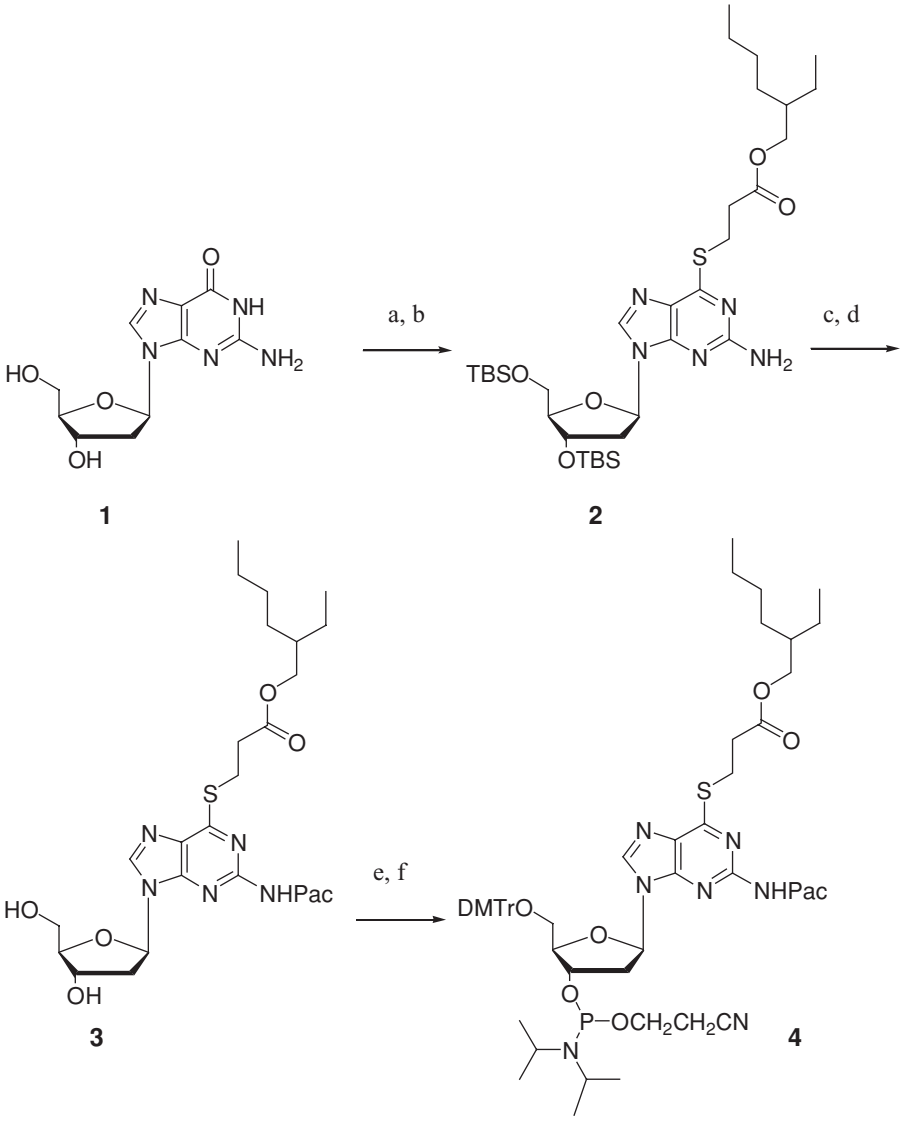
the NO transfer reaction from *S*-nitroso-6-thioguanosine in the ODN.[8,9] The nitrosylated amino group of cytosine caused deamination to form uracil, and its potential application as an artificial tool for RNA editing is now under investigation. This strategy was expanded to the functionality-transfer reaction of the relatively large organic entity to the amino group of cytosine base, and site-specific covalent modification of RNA has been achieved.[10,11]

The excellent synthesis of 2′-deoxy-6-thioguanosine, the phosphoramidite derivative and its incorporation into the ODN have already been reported.[12–18] One approach utilized the preformed 2′-deoxy-6-thioguanosine to protect the 6-thiocarbonyl group with the cyanoethyl group[12][13] or the 2,4-dinitorophenyl group.[17,18] These protecting groups are useful, because they can be removed under mild alkaline conditions. For the other approach, the 6-carbonyl group was transformed into a labile leaving group such as a halide or arylsulfonyloxy group for displacement with 2-cyanoethanethiol.[14–16] In both approaches, odorous agents, such as 2-cyanoethanethiol or hydrogen sulfide, are used. We now describe a new practical synthesis of the 3′-*O*-phosphoramidite derivative of 6-*S*-protected 2′-deoxy-6-thioguanosine by using 2-ethylhexyl 3-mercaptopropionate[19,20] as an odorless and inexpensive reagent and its incorporation into the oligodeoxynucleotides.

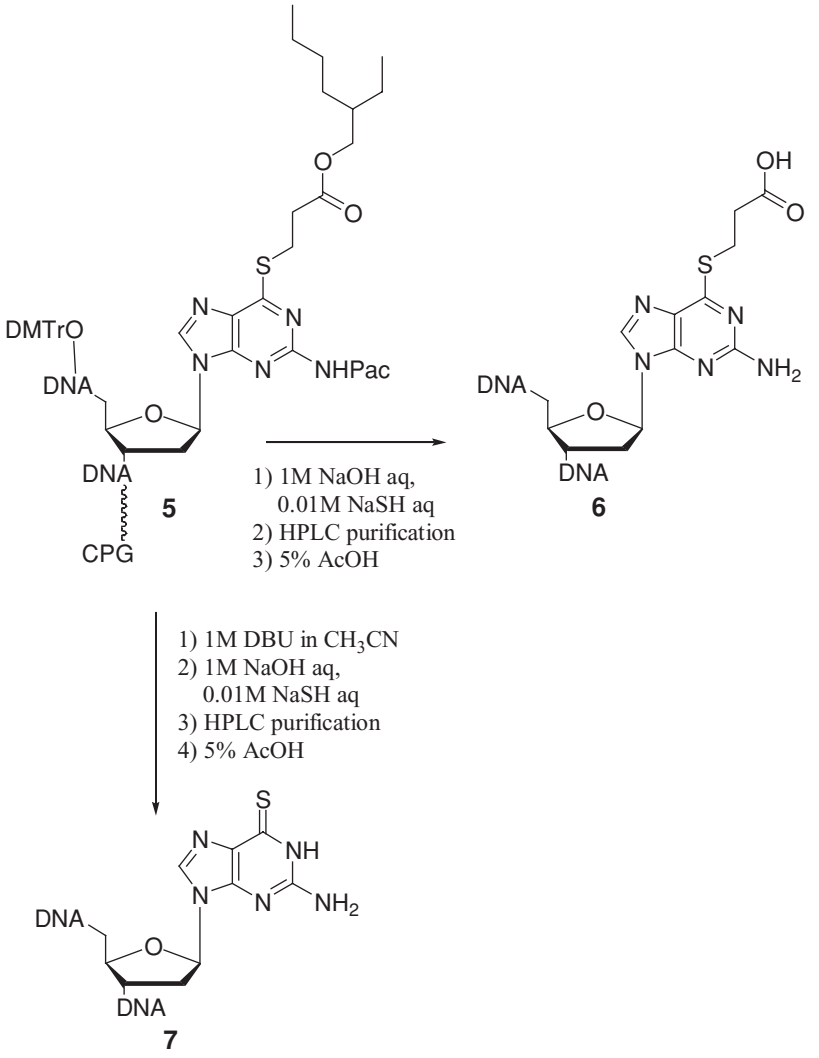
RESULTS AND DISCUSSION

The synthetic route for 2′-deoxy-6-thioguanosine phosphoramidite is shown in Scheme 1. 2′-Deoxyguanosine **1** protected with *tert*-butyldimethylsilyl (TBDMS) groups was reacted with 2-mesitylenesulfonyl chloride to activate the 6-position of guanine, and the formed intermediate was treated with 2-ethylhexyl 3-mercaptopropionate in the presence of *N*-methylpyrrolidine. This one-flask, two-step procedure gave **2** in a good yield (95%) after purification using a silica gel column. As the reaction and workup can be easily performed without any smell, this procedure can be applied to a large scale synthesis. Although racemic 2-ethylhexyl 3-mercaptopropionate was used, two diasteroisomers of **2** did not produce any trouble either in purification by column chromatography or in structure determination by NMR. The 2-amino group of **2** was protected with phenoxyacetyl chloride, and the TBDMS groups were deprotected with TBAF to give the corresponding diol product **3** in 71% yield for two steps. The 5′-hydroxyl group of **3** was selectively protected with 4,4′-dimethoxytrityl (DMTr) chloride in pyridine (94%), and the phoshporamidite precursor **4** was synthesized by a standard procedure with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in 77% yield after silica gel column chromatography followed by precipitation in hexane at −78°C. The amidite precursor **4** was applied to an automated DNA synthesizer for incorporation into the ODN 5′-d(CTTTGsTTCTCCTTTCT).



SCHEME 1 Synthesis of 6-*S*-protected 2′-deoxy-6-thioguanosine by using 2-ethylhexyl 3-mercaptopropionate. (a) TBDMSCl, imidazole, DMF, room temperature, 94%; (b) 2-mesitylenesulfonyl chloride, Et_3N, DMAP, CH_2Cl_2, 0°C to room temperature, then *N*-methylpyrrolidine, 2-ethylhexyl 3-mercaptopropionate, 0°C to room temperature, 95%; (c) phenoxyacethyl chloride, 1-HOBt, pyridine, CH_3CN, room temperature, 84%; (d) TBAF, THF, room temperature 85%; (e) DMTrCl, pyridine, room temperature, 94%; (f) amidite reagent, DIPEA, CH_2Cl_2, 0°C, 77%.

When the synthesized ODN**5** was cleaved from the CPG resin by the treatment with a 1M NaOH and 0.01M NaSH solution, the 2-ethylhexylpropionate group was not deprotected but was hydrolyzed to give ODN**6** after HPLC purification and subsequent DMTr deprotection with 5% AcOH (Scheme 2). On the other hand, the prior treatment of ODN**5**



SCHEME 2 Conditions for deprotection of 2-ethylhexylpropionate and cleavage from the CPG resin.

with 1M DBU in CH_3CN was effective for the β-elimination to remove the 2-ethylhexylpropionate group from ODN**5** on the CPG resin. Subsequent cleavage of the ODN from the CPG resin by the treatment with a 1M NaOH and 0.01M NaSH solution produced the DMTr-protected ODN**7**. An alkaline 0.01M NaSH solution was used to protect against air oxidation and sulfur loss. The HPLC analysis of the crude products indicated that both the DNA synthesis and the deprotection of the 2-ethylhexylpropionate group efficiently proceeded (Figure 1A). Finally, the terminal 5′-DMTr group was deprotected with 5% AcOH to give the desired ODN**7** containing 2′-deoxy-6-thioguanosine as a single peak in ca 60% isolated yield (Figure 1B). There

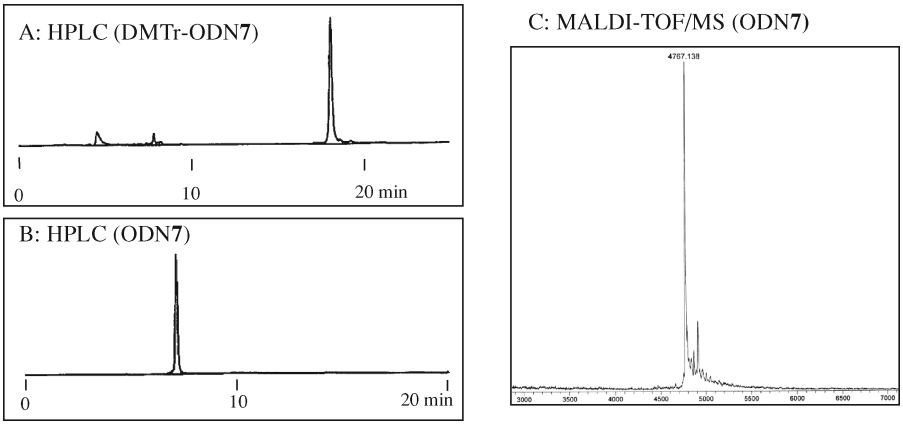


FIGURE 1 HPLC Chart and MALD-TOF/MS of the synthesized DNA. A) HPLC chart of the crude sample of DMTr-protected ODN7. B) HPLC of ODN7 purified by HPLC after the treatment of 5% AcOH. C) MALDI-TOF/MS of ODN7 calcd. 4767.79 ([M-H]−), found 4767.12.

was no peak with a lower molecular weight than the calculated one in the MALDI-TOF/MS spectra of the ODN**7** (Figure 1C), confirming that desulfurization did not take place during the ODN synthesis and isolation procedure.

CONCLUSION

In conclusion, we have established the efficient and odorless synthesis of 6-*S*-{2-[(2-ethylhexyl)oxycarbonyl]ethyl)}-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxy-6-thioguanosine (**2**) from the corresponding 6-*O*-mesitylenesulfonyl derivative by the reaction with 2-ethylhexyl-3-mercaptopropionate; an odorless and inexpensive reagent. The phosphoramidite precursor was also derived from **2** by the conventional method, and was successfully applied to an automated DNA synthesizer to produce 2′-deoxy-6-thioguanosine containing ODN. The described procedure is expected to be useful as a standard protocol for the synthesis of ODN incorporating 2′-deoxy-6-thioguanosine.

EXPERIMENTAL

6-*S*-{2-[(2-ethylhexyl)oxycarbonyl]ethyl)}-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxy-6-thioguanosine(2)

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2′-deoxy-6-guanosine (1 g, 2.0 mmol) was dried by azeotropic evaporation with dry CH_3CN, and dissolved in dry CH_2Cl_2 (60 mL). To the suspension were added dry Et_3N (1.1 ml, 7.9 mmol), 2-mesitylenesulfonyl chloride (530 mg, 2.4 mmol), and DMAP (12.3 mg, 0.010 mmol) at 0°C under an argon atmosphere. After being stirred for 12 hours at room temperature, the reaction mixture was

cooled to 0°C. *N*-methylpyrrolidine (2.1 mL, 20 mmol) and 2-ethylhexyl 3-mercaptopropionate (4.6 mL, 20 mmol) were added to the mixture, and then the mixture was stirred at room temperature for 5.5 hours. The reaction mixture was diluted with CH_2Cl_2 (40 mL) and washed with 1M KH_2PO_4 (70 mL × 3). The combined organic layers were dried over Na_2SO_4, then evaporated. The crude product was purified by silica gel chromatography (hexane/ethyl acetate = 4:1) to give **2** (1.34 g, 95%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ: 7.93 (1H, s), 6.28 (1H, t, J = 6.7 Hz), 5.13 (2H, brs), 4.57–4.54 (1H, m), 4.05–3.98 (2H, m), 3.97–3.95 (1H, m), 3.79 (1H, dd, J = 11.2, 4.2 Hz), 3.73 (1H, dd, J = 11.2, 3.4 Hz), 3.56 (2H, t, J = 7.0 Hz), 2.81 (2H, t, J = 7.0 Hz), 2.62 (1H, ddd, J = 13.1, 6.6, 6.1 Hz), 2.23 (1H, ddd, J = 13.1, 6.1, 3.7 Hz), 1.57–1.52 (1H, m), 1.33 (2H, quint, J = 7.3 Hz), 1.30–1.20 (6H, m), 0.89 (9H, s), 0.88 (9H, s), 0.86 (6H, t, J = 7.3 Hz), 0.09 (6H, s), 0.03 (6H, s); IR (neat): 3321, 3193, 1734, 1591, 1564, 1255, 836 cm^{-1}; HR-ESIMS calcd for $C_{33}H_{61}N_5O_5SSi_2$ $(M+H)^+$: m/z 696.4005, found: 696.4006.

6-*S*-{2-[(2-ethylhexyl)oxycarbonyl]ethyl}-2-*N*-phenylacetyl-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-2′-deoxy-6-thioguanosine

Compound **2** (1.18 g, 1.7 mmol) was dried by azeotropic evaporation with dry CH_3CN, and dissolved in dry CH_3CN (5 mL) and dry pyridine (5 mL). To the solution was added 1-hydroxybenzotriazol (573 mg, 4.2 mmol) in the presence of molecular sieves 4A at room temperature. After being stirred for 1.5 hours, phenoxyacetyl chloride (1.2 mL, 8.7 mmol) was added to the reaction mixture. The reaction mixture was stirred for an additional 8.5 hours, diluted with ethyl acetate (50 mL), and filtered through Celite. The filtrate was washed with brine (50 mL × 2), dried over Na_2SO_4, then evaporated. The crude product was purified by silica gel chromatography (hexane/ethyl acetate = 5:1) to give the title compound (1.18 g, 84%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ: 8.84 (1H, brs), 8.16 (1H, s), 7.33 (2H, t, J = 7.6 Hz), 7.04 (1H, t, J = 7.6 Hz), 7.01 (2H, d, J = 7.9 Hz), 6.40 (1H, t, J = 6.4 Hz), 4.73 (2H, s), 4.61–4.58 (1H, m), 4.03–3.98 (3H, m), 3.84 (1H, dd, J = 11.3, 4.3 Hz), 3.75 (1H, dd, J = 11.3, 3.4 Hz), 3.60 (2H, t, J = 7.0 Hz), 2.88 (2H, t, J = 7.0 Hz), 2.63 (1H, ddd, J = 12.9, 6.6, 6.1 Hz), 2.41 (1H, ddd, J = 12.9, 6.1, 3.7 Hz), 1.57–1.54 (1H, m), 1.35–1.25 (8H, m), 0.89 (9H, s), 0.88 (9H, s), 0.85 (6H, t, J = 7.3 Hz), 0.09 (6H, s), 0.06 (6H, s); IR (neat): 3408, 1730, 1576, 1495, 1380, 1216, 837; ESI-MS: m/z 830.5 $(M+H)^+$

6-*S*-{2-[(2-ethylhexyl)oxycarbonyl]ethyl}-2-*N*-phenylacetyl-2′-deoxy-6-thioguanosine(3)

To a solution of the above compound (993 mg, 1.2 mmol) in dry THF (10 mL) was added TBAF (946 mg, 3.0 mmol) at room temperature.

After being stirred for 1.5 hours, the reaction mixture was evaporated. The residue was purified by silica gel chromatography ($CHCl_3$/MeOH = 99:1) to give **3** (613 mg, 85%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ: 9.03 (1H, s), 8.15 (1H, s), 7.33 (2H, t, J = 7.9 Hz), 7.04 (1H, t, J = 7.9 Hz), 7.02 (2H, d, J = 7.9 Hz), 6.40 (1H, t, J = 6.7 Hz), 4.95–4.94 (1H, m), 4.67 (2H, s), 4.14–4.13 (1H, m), 4.05–3.98 (2H, m), 3.94 (1H, dd, J = 10.1, 2.4 Hz), 3.85 (1H, dd, J = 10.1, 2.4 Hz), 3.59 (2H, t, J = 7.0 Hz), 3.02–2.92 (1H, m), 2.86 (2H, t, J = 7.0 Hz), 2.83 (2H, brs), 1.56–1.53 (1H, m), 1.57–1.54 (1H, m), 1.33–1.24 (8H, m), 0.85 (6H, t, J = 7.3 Hz); IR (neat): 3398(br), 1729, 1578, 1495, 1380, 1214; HR-ESIMS calcd for $C_{29}H_{39}N_5O_7S$ $(M+H)^+$: m/z 602.2643, found: 602.2678.

6-*S*-{2-[(2-ethylhexyl)oxycarbonyl]ethyl}-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-phenylacetyl-2′-deoxy-6-thioguanosine

Compound **3** (370 mg, 0.62 mmol) was dried by azeotropic evaporation each with dry CH_3CN and dry pyridine, and dissolved in dry pyridine (6 mL). To the solution was added 4,4′-dimethoxytrityl chloride (521 mg, 1.54 mmol) at room temperature, and then the mixture was stirred for 1 hour. The reaction mixture was diluted with ethyl acetate (40 mL), washed water and brine (each 40 mL). The organic phase was dried over Na_2SO_4, then evaporated. The residue was purified by silica gel chromatography ($CHCl_3$/MeOH = 49:1, 0.5% pyridine) to give the title compound (520 mg, 94%) as a pale yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ: 8.92 (1H, s), 8.06 (1H, s), 7.39 (2H, d, J = 7.3 Hz), 7.34 (2H, t, J = 7.9 Hz), 7.28 (4H, d, J = 8.9 Hz), 7.22 (2H, t, J = 7.3 Hz), 7.16 (1H, t, J = 7.3 Hz), 7.05 (1H, t, J = 7.9 Hz), 7.00 (2H, d, J = 7.9 Hz), 6.77 (4H, d, J = 8.9 Hz), 6.64 (1H, t, J = 6.7 Hz), 4.81–4.79 (1H, m), 4.62 (2H, s), 4.24–4.21 (1H, m), 4.07–3.99 (2H, m), 3.74 (6H, s), 3.60 (2H, t, J = 7.0 Hz), 3.43 (1H, dd, J = 10.1, 4.9 Hz), 3.42 (1H, brs), 3.34 (1H, dd, J = 10.1, 4.3 Hz), 2.87 (2H, t, J = 7.0 Hz), 2.72 (1H, ddd, J = 13.4, 6.7, 6.7 Hz), 2.64 (1H, ddd, J = 13.4, 6.1, 3.7 Hz), 1.59–1.54 (1H, m), 1.38–1.26 (8H, m), 0.87 (6H, t, J = 7.3 Hz); IR (neat): 3405, 1727, 1576, 1508, 1380, 1249, 729; HR-ESIMS calcd for $C_{50}H_{57}N_5O_9S$ $(M+H)^+$: m/z 904.3950, found: 904.3933.

6-*S*-{2-[(2-ethylhexyl)oxycarbonyl]ethyl}-5′-*O*-(4,4′-dimethoxytrityl)-2-*N*-phenylacetyl-2′-deoxy-6-thioguanosine-3′-*O*-(2-cyanoethyl)(*N,N*-diisopropyl)phosphoramidite(4)

The above compound (308 mg, 0.34 mmol) was dried by azeotropic evaporation each with dry CH_3CN, and dissolved in dry CH_2Cl_2 (3.5 mL). To the solution was added diisopropylethylamine (360 μL, 2.1 mmol) at 0°C. After being stirred for 25 minutes, *N*,*N*-diisopropylchlorophosphoramidite (190 μL, 0.85 mmol) was added, and then the mixture was stirred for an additional 1 hour at the same temperature. The reaction mixture was quenched with saturated aqueous $NaHCO_3$ (15 mL), extracted with

ethyl acetate (15 ml × 3). The organic phases were dried over Na_2SO_4, then evaporated. The residue was purified by silica gel chromatography (hexane/ethyl acetate = 2:1) to give the purified material, which was precipitated in hexane at −78°C. The hexane was removed by decantation, and the solid material was dried in a vacuum for several hours to give **4** (288 mg, 77%) as a pale yellow foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ: 8.78 (1H, brs), 8.06 (0.5H, s), 8.05 (0.5H, s), 7.39–7.33 (4H, m), 7.29–7.16 (7H, m), 7.05 (1H, t, J = 7.9 Hz), 7.02 (2H, d, J = 7.9 Hz), 6.78–6.74 (4H, m), 6.43 (1H, t, J = 6.1 Hz), 4.79–4.74 (3H, m), 4.30–4.26 (1H, m), 4.08–4.00 (2H, m), 3.87–3.54 (6H, m), 3.77 (3H, s), 3.76 (3H, s), 3.40–3.31 (2H, m), 2.93–2.89 (2H, m), 2.86–2.68 (2H, m), 2.61 (1H, t, J = 6.4 Hz), 2.45 (1H, t, J = 6.4 Hz), 1.59–1.55 (1H, m), 1.39–1.26 (8H, m), 1.19–1.10 (12H, m), 0.87 (6H, t, J = 7.3 Hz); ^{31}P NMR (161 MHz, $CDCl_3$): δ: 149.6; IR (neat): 3406, 2252, 1728, 1575, 1508, 1379, 1249, 1178, 1035, 728; HR-ESIMS calcd for $C_{50}H_{57}N_5O_9S$ $(M+H)^+$: m/z 1104.5028, found: 1104.5050.

Synthesis of Oligodeoxynucleotides (ODN7)

ODN **7** was synthesized at a 1 μmol scale on a Model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA) with standard β-cyanoethyl phosphoramidite chemistry. The 5′-terminal dimethoxytrityl-bearing ODNs were deprotected with 1.0 M DBU in dry CH_3CN at room temperature for 5 hours to remove the 2-[(2-ethylhexyl)oxycarbonyl]ethyl group, and removed from the solid support by treatment with 1 M NaOH and 0.01 M NaSH (1 mL) for 4 hours. The crude product was purified by reverse-phase HPLC (Column: nacalai tesque: COSMOSIL 5C18-AR-II, 10 × 250 mm; Solvent: A: 0.1M TEAA Buffer, B: CH_3CN, B: 10–40% per 20 minutes, linear gradient; flow rate: 3.0 ml/min; monitored by UV detector at 254 nm). The dimethoxytrityl group of the purified ODN was cleaved with 5% AcOH and the mixture was additionally purified by HPLC (solvent: A: 0.1M TEAA Buffer, B: CH_3CN, B: 10–30% per 20 minutes). The isolated ODN**7** was quantified by UV measurement to be 60% isolated yield. MALDI-TOFMS (m/z) **7**: calcd 4767.79, found 4767.12.

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