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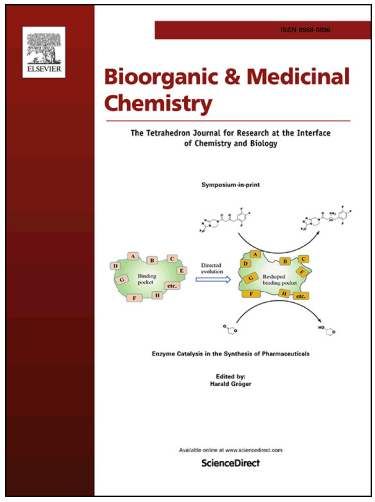

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Syntheses of prodrug-type 2'-*O*-methyldithiomethyl oligonucleotides modified at natural four nucleoside residues and their conversions into natural 2'-hydroxy oligonucleotides under reducing condition

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Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan

†Present address: Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan.

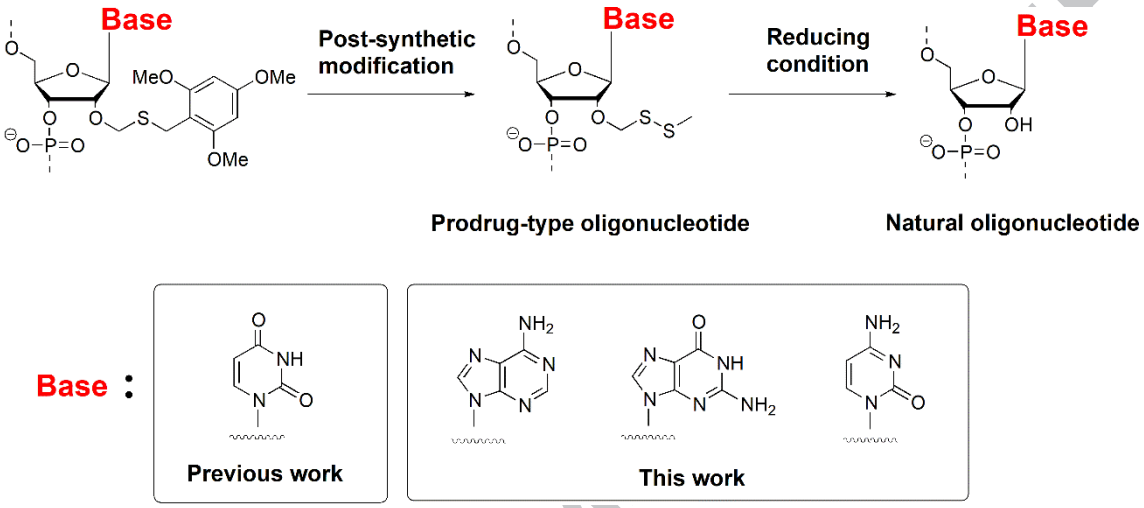
*Corresponding author. TEL & FAX: +81-72-690-1089, E-mail: urata@gly.oups.ac.jp

Keywords: Prodrug-type RNA; reducing environment; 2'-*O*-modified RNA; post-synthetic modification

Abstract

We previously reported that reducing-environment-responsive prodrug-type small interfering RNA (siRNA) bearing 2'-*O*-methyldithiomethyl (2'-*O*-MDTM) uridine exhibits efficient knockdown activity and nuclease resistance. In this report, we describe the preparation of 2'-*O*-MDTM oligonucleotides modified not only at uridine but also at adenosine, guanosine and cytidine residues by post-synthetic modification. Precursor oligonucleotides bearing 2'-*O*-(2,4,6-trimethoxybenzylthiomethyl) (2'-*O*-TMBTM) adenosine, guanosine, and cytidine were reacted with dimethyl(methylthio)sulfonium tetrafluoroborate to form 2'-*O*-MDTM oligonucleotides in the same manner as the oligonucleotide bearing 2'-*O*-TMBTM uridine. Furthermore, the oligonucleotides bearing 2'-*O*-MDTM adenosine, guanosine, and cytidine were efficiently converted into corresponding natural 2'-hydroxy oligonucleotides under the cytosol-mimetic reducing condition.

Graphical abstract



1. Introduction

Selective gene silencing induced by chemically synthesized oligonucleotides, such as antisense oligonucleotide (AON) and small interfering RNA (siRNA), has shown promise for the treatment of several intractable diseases.[1] In the past 30 years, a small number of AON-type drugs were approved[2] and a novel AON-type drug for the treatment of the hereditary transthyretin amyloidosis was approved quite recently.[3] Recently, the clinical use of siRNA has received considerable attention because effective knockdown activity can be induced with lower concentrations of siRNAs than AONs.[4] However, the instability of RNA molecules in serum has hindered the clinical success of RNA-type drugs and only one candidate was approved as the first siRNA-type drug quite recently.[5,6]

2'-*O*-Modified RNAs, including 2'-*O*-alkyl,[7] 2'-fluoro (2'-F),[8,9] 2'-*O*-(2-methoxyethyl) (2'-*O*-MOE),[10] and bridged nucleic acids (BNAs)[11,12] are well used in pharmaceutical research for enhancement of the nuclease resistance. However, the knockdown activities of 2'-*O*-modified siRNAs are often decreased by 2'-*O*-modification because the modification inhibits the formation of an RNA-induced silencing complex (RISC) in the RNA interference (RNAi) pathway.[13,14] In particular, the 5'-end side of the antisense strand of siRNA is essential for the formation of RISC.[15-17] Thus, the 2'-*O*-modifications are mainly introduced at the sense strand or the 3'-end side of the antisense strand of siRNA.

The prodrug concept has been well-received in oligonucleotide chemistry.[18-25] In the prodrug concept, oligonucleotides are protected by bioreversible functional groups to introduce certain properties beneficial for pharmaceutical use, such as enhancement of the nuclease resistance and the cell membrane permeability. These protective groups are cleaved by a trigger reaction to induce the conversion of the prodrugs into natural active oligonucleotides in the target organ. Thus,

prodrug-type oligonucleotides would exhibit both the above-mentioned beneficial properties due to the modification and the gene silencing activity. We originally developed prodrug-type 2'-*O*-modified RNA containing a 2'-*O*-methyldithiomethyl (2'-*O*-MDTM) group, the cleavage of which is triggered by an intracellular reducing environment, and named it "Reducing-Environment-Dependent Uncatalyzed Chemical Transforming (REDUCT)-RNA" (**Figure 1**).[26-28] We reported that the 2'-*O*-MDTM siRNA modified at the uridine residue of anti-luciferase siRNAs exhibits stronger knockdown activity than natural 2'-OH siRNA.[27] Furthermore, the knockdown activity was basically independent of the position and extent of modification.[28] From this basic research, 2'-*O*-MDTM siRNA is expected to exhibit sufficient knockdown activity not only *in vitro* but also *in vivo*. However, the 2'-*O*-MDTM group was introduced only at the uridine residue in our previous reports. In this report, we describe the preparation of 2'-*O*-MDTM oligonucleotides modified at adenosine, guanosine, and cytidine residues by post-synthetic modification and the conversions into natural 2'-OH oligonucleotides under the cytosol-mimetic condition.

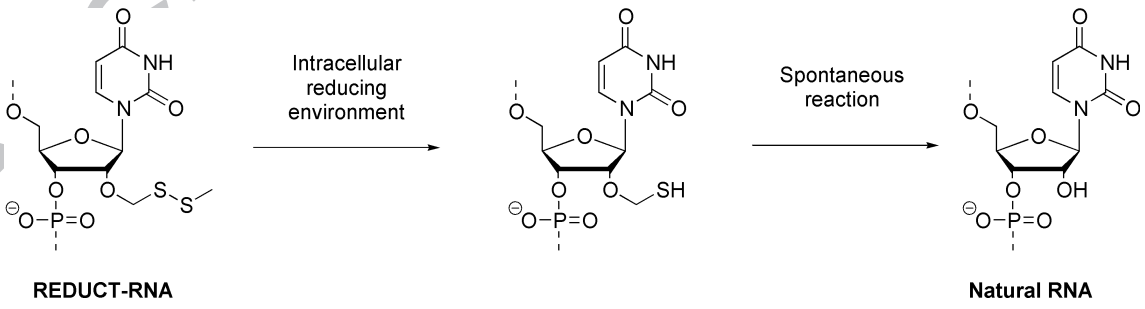


Figure 1. Conversion of Reducing-Environment-Dependent Uncatalyzed Chemical Transforming (REDUCT)-RNA modified at uridine residue.

2. Results and discussion

2.1 Synthesis of 2'-*O*-(2,4,6-trimethoxybenzylthiomethyl)phosphoramidite units

In general, oligonucleotides are synthesized by the phosphoramidite method in which nucleoside phosphoramidites are employed as monomer units. We tried to prepare the phosphoramidite unit bearing a 2'-*O*-MDTM group. However, the 2'-*O*-MDTM phosphoramidite unit was not obtained because an intramolecular reaction occurred between reductive P(III) atom and the disulfide bond. Then, we developed a post-synthetic approach to obtain a 2'-*O*-MDTM oligonucleotide modified at the uridine residue (**Figure 2**).[26] In the post-synthetic approach, the 2,4,6-trimethoxybenzylthiomethyl (TMBTM) group was used as the promoiety, which could be converted into the 2'-*O*-MDTM group by treatment with dimethyl(methylthio)sulfonium tetrafluoroborate (DMTSF). In hopes of applying the method to the preparation of 2'-*O*-MDTM oligonucleotides modified at other nucleoside residues, such as adenosine, guanosine, and cytidine, we synthesized 2'-*O*-TMBTM phosphoramidite units **6a–c** (**Scheme 1**). 2'-*O*-Methylthiomethyl (MTM) derivatives **2a**–**d** were obtained from corresponding 2'-OH derivatives **1a**–**d** in good yields. In the next step, we attempted to synthesize 2'-*O*-TMBTM derivatives **3a**–**c** according to a previously reported synthetic procedure for uridine derivatives.[26] In the synthesis of uridine derivative **3d**, the solvent and excess SO_2Cl_2 were removed completely under reduced pressure after the treatment with SO_2Cl_2 to afford the 2'-*O*-chloromethyl intermediate. However, the 2'-*O*-chloromethyl intermediates of adenosine, guanosine, and cytidine degraded during the removal of the solvent and excess SO_2Cl_2. Therefore, the syntheses of 2'-*O*-TMBTM derivatives **3a**–**c** from corresponding **2a**–**c** were performed in one pot. 2'-*O*-MTM nucleosides **2a**–**c** were treated with SO_2Cl_2 to afford 2'-*O*-chloromethyl intermediate. Subsequently, 2,4,6-trimethoxybenzylmercaptan and excess *N*,*N*-diisopropylethylamine (DIEA) were added to this reaction mixture to afford adenosine, guanosine, and cytidine derivatives **3a**–**c**. In the synthesis of guanosine derivative **3b**,

cyclohexene was added to scavenge chlorine generated from the excess SO_2Cl_2. The 3',5'-*O*-silyl protecting group of 2'-*O*-TMBTM derivatives **3a**–**d** was cleaved off by 3HF-Et_3N. The 5'-hydroxy group was protected with the 4,4'-dimethoxytrityl (DMTr) group to afford **5a**–**d**. Finally, the 3'-hydroxy group of **5a**–**d** was phosphitylated with chlorophosphoramidite reagent under the basic condition to generate the 2'-*O*-TMBTM phosphoramidite units **6a**–**d** in good yields.

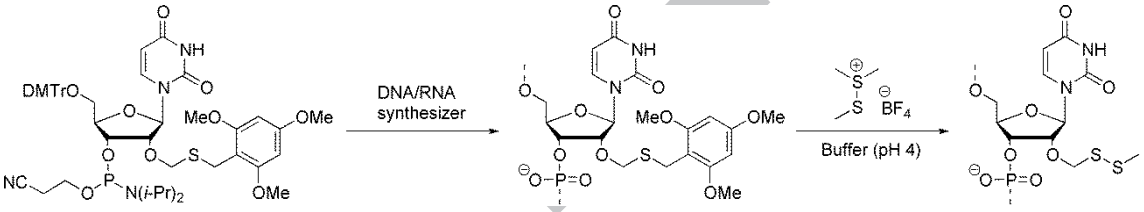
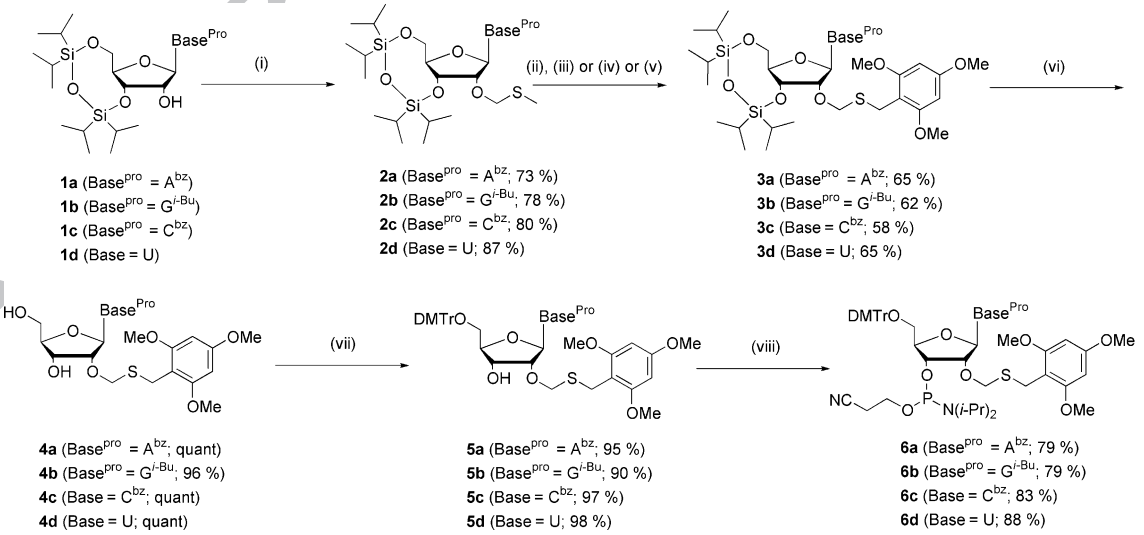


Figure 2. Post-synthetic approach for the synthesis of 2'-*O*-MDTM oligonucleotide modified at uridine residue.



Scheme 1. Syntheses of phosphoramidite units bearing 2'-*O*-TMBTM group. (i) DMSO, Ac_2O,

AcOH; (ii) SO_2Cl_2, CH_2Cl_2; (iii) $(MeO)_3BnSH$, DIEA, CH_2Cl_2 (for **3a** and **c**); (iv) $(MeO)_3BnSH$, DIEA, cyclohexene, CH_2Cl_2 (for **3b**); (v) $(MeO)_3BnSH$, NaH, DMF (for **3d**); (vi) 3HF-Et_3N, THF; (vii) DMTr-Cl, pyridine; (viii) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DIEA, DMAP, CH_2Cl_2.

2.2 Synthesis of 2'-*O*-MDTM oligonucleotides by post-synthetic modification

2'-*O*-TMBTM oligonucleotides **7a**–**d** were synthesized by the solid-phase phosphoramidite method (**Table 1**). Coupling reactions were conducted with 5-ethylthio-1*H*-tetrazole (ETT) as the activator and the coupling time for 2'-*O*-TMBTM phosphoramidites **6a–d** was extended to 600 sec. Furthermore, the oxidation of P(III) to P(V) was conducted with 0.02 M iodine solution. After cleavage from the support and deprotection, 2'-*O*-TMBTM oligonucleotides **7a**–**d** were purified by reversed-phase HPLC (RP-HPLC) and the structures were characterized by MALDI-TOF mass spectrometry (**Table 1**).

Post-synthetic modification for the preparation of 2'-*O*-MDTM oligonucleotides **8a**–**d** from 2'-*O*-TMBTM oligonucleotides **7a**–**d** was conducted by treatment with DMTSF.[26] An aqueous DMTSF suspension was added to 0.1 mM solutions of 2'-*O*-TMBTM oligonucleotides **7a**–**d** in 200 mM sodium acetate buffer (pH 4) at 37 ℃ and the reactions were monitored by RP-HPLC (**Figure 3**). Unexpectedly, the conversion reactions of **7a**–**d** to **8a**–**d** were so fast and almost completed within 1 min without any detectable side reactions. As it is known that DMTSF easily reacts with H_2O,[29] the conversion reactions were conducted by using freshly prepared aqueous DMTSF suspension. After the reactions, the 2'-*O*-MDTM oligonucleotides were purified by RP-HPLC. The disulfide bonds of 2'-*O*-MDTM oligonucleotides might be cleaved in highly concentrated triethylammonium acetate (TEAA) buffer. Then, the solutions of 2'-*O*-MDTM oligonucleotides obtained from the RP-HPLC purification were desalted by a gel filtration column before

concentration. Finally, the structures of the 2'-*O*-MDTM oligonucleotides were confirmed by MALDI-TOF mass spectrometry (**Table 1**). The results indicate that our post-synthetic approach would be applicable to the preparation of oligonucleotides bearing 2'-*O*-MDTM adenosine, guanosine, cytidine as well as uridine.

Table 1. Sequences and characterization of synthesized oligonucleotides (ONs)

ONs	Sequence (5' to 3') [a)]	MALDI-TOF Mass	
		Calcd. $(M-H)^-$	Found
7a	d (GCG TT*A* TTT GCT)	3883.7	3884.2
7b	d (GCG TT*G* TTT GCT)	3899.7	3899.0
7c	d (GCG TT*C* TTT GCT)	3859.7	3859.2
7d	d (GCG TT*U* TTT GCT)	3860.6	3859.1
8a	d (GCG TT**A** TTT GCT)	3749.6	3750.5
8b	d (GCG TT**G** TTT GCT)	3765.6	3765.6
8c	d (GCG TT**C** TTT GCT)	3725.5	3726.1
8d	d (GCG TT**U** TTT GCT)	3726.5	3727.9
9a	d (GCG TT***A*** TTT GCT)	3657.4	3658.0
9b	d (GCG TT***G*** TTT GCT)	3673.4	3672.5
9c	d (GCG TT***C*** TTT GCT)	3633.4	3632.6
9d	d (GCG TT***U*** TTT GCT)	3634.4	3633.7

a) 2'-*O*-modified positions are underlined. Italic (**7a**–**d**), bold (**8a**–**d**), and italic bold (**9a**–**d**) letters indicate 2'-*O*-TMBTM, 2'-*O*-MDTM, and 2'-OH nucleosides, respectively.

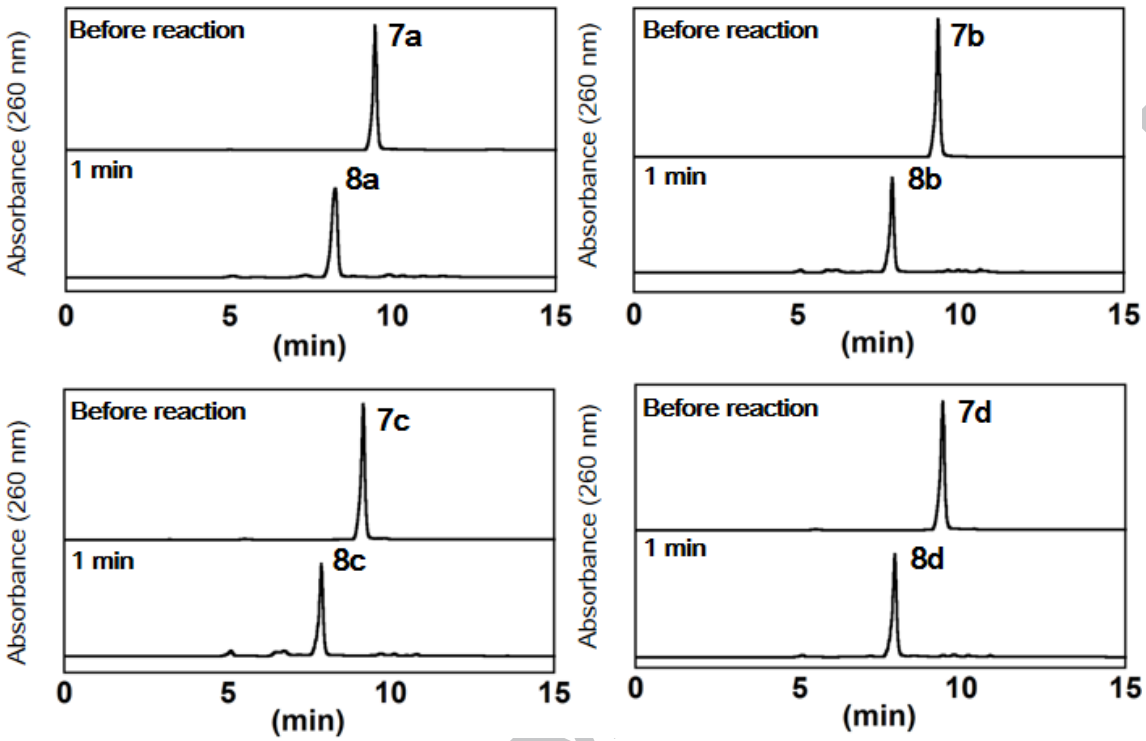


Figure 3. HPLC charts of the conversions of 2'-*O*-TMBTM (**7a**–**d**) into 2'-*O*-MDTM (**8a**–**d**) oligonucleotides by treatment with DMTSF in 200 mM sodium acetate buffer (pH 4) at 37 °C.

2.3 Conversion of 2'-*O*-MDTM oligonucleotides into 2'-OH oligonucleotides under reducing condition.

We already showed that oligonucleotides bearing 2'-*O*-MDTM uridine are efficiently converted into natural oligonucleotides under the cytosol-mimetic reducing condition (10 mM glutathione, GSH). Then, we carried out the conversion of oligonucleotides bearing 2'-*O*-MDTM adenosine, guanosine, cytidine, and uridine residues into corresponding natural 2'-OH oligonucleotides in the reducing condition. 2'-*O*-MDTM oligonucleotides (**8a**–**d**) were treated with 10 mM GSH in 50 mM phosphate buffer (pH 7.0) at 37 °C and the reactions were analyzed by RP-HPLC (**Figure 4**). The 2'-*O*-MDTM groups of oligonucleotides (**8a**–**d)** were cleaved off and the

corresponding 2'-thiohemiacetal intermediates were generated after reaction for 1.0 h. Subsequently, the conversion into 2'-OH oligonucleotides (**9a**–**d**) was almost completed within 6.0 h. The reactions were desalted and the structures were characterized by MALDI-TOF mass spectrometry (**Table 1**). The results indicate that prodrug-type oligonucleotides bearing 2'-*O*-MDTM adenosine, guanosine, and cytidine would be efficiently converted into the corresponding 2'-OH oligonucleotides under the cytosol-mimetic reducing condition in the same manner as prodrug-type oligonucleotides bearing 2'-*O*-MDTM uridine.

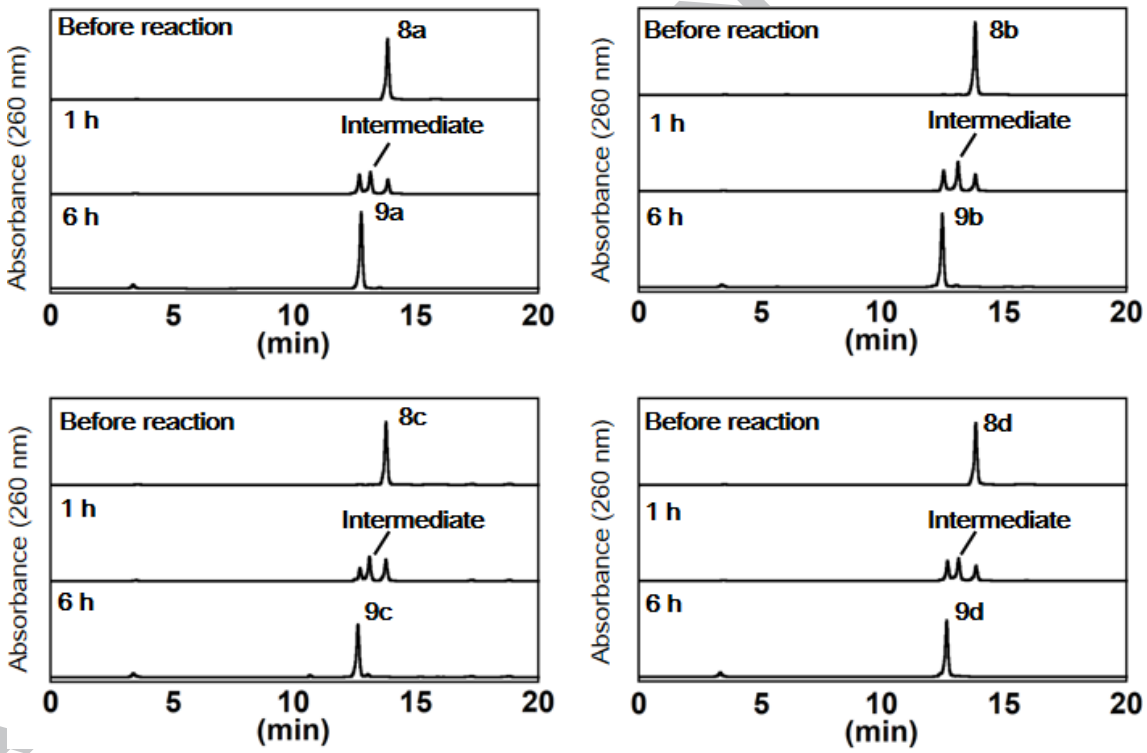


Figure 4. HPLC charts of the conversions of 2'-*O*-MDTM (**8a**–**d**) into 2'-OH (**9a**–**d**) oligonucleotides under cytosol-mimetic reducing condition (10 mM GSH in 50 mM phosphate buffer, pH 7.0 at 37 °C).

3. Conclusions

We showed the preparation method of prodrug-type 2'-*O*-MDTM oligonucleotides modified at four natural nucleoside residues by post-synthetic modification. The 2'-*O*-TMBTM amidites of four natural bases (**6a**–**d**) were obtained in 33–49% yield in 5 steps from protected nucleosides (**1a**–**d**). The 2'-*O*-TMBTM oligonucleotides were synthesized from 2'-*O*-TMBTM amidites (**6a**–**d**) and the oligonucleotides were efficiently reacted with DMTSF to afford 2'-*O*-MDTM oligonucleotides. Furthermore, we confirmed that prodrug-type oligonucleotides bearing 2'-*O*-MDTM adenosine, guanosine, cytidine, and uridine were efficiently converted into the corresponding 2'-OH oligonucleotides under the cytosol-mimetic reducing condition. In our previous report, we successfully obtained the 2'-*O*-MDTM RNA modified at the multiple uridine residues by the post-synthetic modification.[26] Hence, the 2'-*O*-MDTM modifications could be introduced not only at uridine residues but also at multiple natural four nucleoside residues of RNA sequence by using our post-synthetic modification method. The evaluation of the biological properties of siRNAs bearing 2'-*O*-MDTM adenosine, guanosine, cytidine, and uridine residues is ongoing in our laboratory.

4. Experiments

4.1 General methods

All reagents and solvents except 2,4,6-trimethoxybenzylmercaptan were obtained from commercial sources and used without purification. Silica gel chromatography was performed using Wakogel C-400HG or Wakosil C-200 (FUJIFILM Wako Pure Chemical Industries, Japan). TLC was performed on Merck silica gel 60 F_{254} and compounds were visualized under UV light (254 nm). ^{1}H, ^{13}C, and ^{31}P NMR spectra were measured on an Agilent NMR System 600-DD2 NMR spectrometer. Reversed-phase HPLC was performed on Nacalai COSMOSIL 5C18-MS-II ϕ 4.6 × 250 mm

(analytical column) and ϕ 10.0 × 250 mm (purification column) with a linear gradient of acetonitrile in 50 mM triethylammonium acetate (TEAA) (pH 7). Oligonucleotides were synthesized on an Applied Biosystems Model 392 DNA/RNA Synthesizer (Applied Biosystems). The mass spectra of the nucleosides were measured on a JMS-700 mass spectrometer (JEOL, Japan) in the positive-ion mode. The mass spectra of the oligonucleotides were measured on a Voyager-DE STR MALDI-TOF mass spectrometer (AB SCIEX) or a Bruker Microflex MALDI-TOF mass spectrometer (Bruker) in the negative-ion mode. UV quantifications were performed on an Eppendorf BioSpectrometer basic (Eppendorf) by measuring absorbance at 260 nm.

4.2 Syntheses of 2'-*O*-(2,4,6-trimethoxybenzylthiomethyl)phosphoramidites

*4.2.1 Preparation of N-protected-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-O-[(methylthio)methyl]nucleosides (**2a**–**d**)*

To a solution of each of *N*-protected-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-nucleosides (**1a**–**d**) in DMSO (45 eq) were added glacial AcOH (52 eq) and Ac_2O (24 eq). The reaction mixture was stirred at room temperature for 18–24 h. After the reaction, the resulting solution was poured into sat. $NaHCO_3$ aqueous solution and the mixture was extracted with ethyl acetate. The organic layer was washed with distilled water and brine, and then dried over anhydrous sodium sulfate, filtered, and concentrated. The residue was purified by silica gel column chromatography to afford compounds **2a**–**d**.

*4.2.1.1 N-Benzoyl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-O-[(methylthio)methyl]-adenosine (**2a**).*

White solid, 73% yield from **1a**. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 9.12 (1H, br s), 8.78 (1H, s), 8.34 (1H, s), 8.04 (2H, d, *J* = 7.7 Hz), 7.63-7.60 (1H, m), 7.55-7.52 (2H, m), 6.12 (1H, s), 5.07 (1H,

d, *J* = 11.5 Hz), 5.01 (1H, d, *J* = 11.7 Hz), 4.73-4.68 (2H, m), 4.26 (1H, br. d, *J* = 13.2 Hz), 4.20-4.18 (1H, m), 4.04 (1H, dd, *J* = 13.4, 2.4 Hz), 2.21 (3H, s), 1.18–1.02 (28H, m). ^{13}C NMR (151 MHz, $CDCl_3$): 164.6, 152.5, 150.8, 149.4, 140.9, 133.6, 132.8, 128.9, 127.9, 123.3, 88.7, 81.9, 77.6, 74.8, 68.9, 59.6, 17.44, 17.38, 17.33, 17.29, 17.1, 17.03, 17.01, 16.9, 13.45, 13.42, 12.9, 12.8, 12.6. HRMS (FAB): *m/z* calculated for $C_{31}H_{48}N_5O_6SSi_2$ 674.2863 $[M+H]^+$, found 674.2868.

*4.2.1.2 N-Isobutyryl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-O-[(methylthio)methyl]-guanosine (**2b**).*

White to yellow solid, 78% yield from **1b**; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 12.0 (1H, s), 8.51 (1H, s), 8.00 (1H, s), 5.86 (1H, s), 5.02 (1H, d, *J* = 11.4 Hz), 4.96 (1H, d, *J* = 11.4 Hz), 4.50-4.47 (2H, m), 4.24 (1H, br d, *J* = 3.2 Hz), 4.15-4.13 (1H, m), 4.01 (1H, dd, *J* = 14.4, 2.5 Hz), 2.67 (1H, sep, *J* = 6.9 Hz), 2.17 (3H, s), 1.29-1.24 (6H, m), 1.14-0.97 (28H, m). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 178.2, 155.5, 147.4, 146.9, 136.3, 121.9, 87.6, 81.9, 77.8, 74.3, 68.4, 59.6, 36.5, 19.0, 18.9, 17.4, 17.28, 17.23, 17.1, 16.99, 16.97, 16.84, 16.76, 16.71, 13.45, 13.43, 12.9, 12.5. HRMS (FAB): *m/z* calculated for $C_{28}H_{50}N_5O_7SSi_2$ 656.2969 $[M+H]^+$, found 656.2969.

*4.2.1.3 N-Benzoyl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-O-[(methylthio)methyl]-cytidine (**2c**).*

White solid, 80% yield from **1c**; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.80 (1H, br s), 8.37 (1H, br d, *J* = 7.1 Hz), 7.90 (1H, br s), 7.63-7.51 (4H, m), 5.85 (1H, s), 5.14 (1H. d, *J* = 11.4 Hz), 5.01 (1H, d, *J* = 11.4 Hz), 4.40 (1H, br s), 4.31 (1H, d, *J*= 13.2 Hz), 4.22 (2H, dd, *J* = 11.8, 9.9 Hz), 4.01 (1H, d, *J* = 13.5 Hz), 2.21 (3H, s), 1.13–0.92 (28H, m). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 167.7, 162.2, 161.6, 144.8, 133.4, 129.1, 127.6, 98.6, 95.8, 89.9, 82.2, 77.6, 74.4, 67.5, 66.1, 59.3, 17.5, 17.4, 17.3, 17.00, 16.97, 16.9, 16.8, 13.5, 13.3, 13.1, 12.9, 12.5. HRMS (FAB): *m/z* calculated for $C_{30}H_{48}N_3O_7SSi_2$

650.2751 $[M+H]^+$, found 650.2748.

*4.2.1.4 3',5'-O-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2'-O-[(methylthio)methyl]uridine (**2d**).*

White solid, 87% yield from **1d**; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 9.02 (1H, s), 7.90 (1H, d, *J* = 8.2 Hz), 5.73 (1H, s), 5.69 (1H, dd, *J* = 8.2, 2.0 Hz), 4.99 (1H, d, *J* = 11.4 Hz), 4.97 (1H, d, *J* = 11.4 Hz), 4.36 (1H, d, *J* = 4.7 Hz), 4.28-4.23 (1H, m), 4.22 (1H, d, *J* = 4.7 Hz), 4.15-4.13 (1H, m), 3.98 (1H, dd, *J* = 13.5, 2.4 Hz), 2.19 (1H, s), 1.14-0.94 (28H, m). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 163.3, 149.8, 139.3, 101.6. 88.8, 82.0, 77.5, 74.2, 67.8, 59.3, 17.5, 17.4, 17.29, 17.25, 17.24, 17.21, 17.00, 16.97, 16.93, 16.8, 13.4, 13.1, 13.0, 12.8, 12.5. HRMS (FAB): *m/z* calculated for $C_{23}H_{43}N_2O_7SSi_2$ 547.2329 $[M+H]^+$, found 547.2325.

*4.2.2 Preparation of N-protected -3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-O-(2,4,6-trimethoxybenzylthiomethyl)nucleosides (**3a**–**d**)*

To a solution of each of *N*-protected -3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-[(methylthio)methyl]nucleosides (**2a**–**c**) in anhydrous dichloromethane was added dropwise a solution of sulfuryl chloride (1.3 eq) in anhydrous dichloromethane for 1 min, and the reaction mixture was stirred at room temperature for 30 min. A mixture of 2,4,6-trimethoxybenzylmercaptan (2.5 eq) and *N*,*N*-diisopropylethylamine (5.0 eq) in anhydrous dichloromethane was added to the sulfuryl-chloride-treated solution at 0 °C. For the synthesis of guanosine derivative (**3b**), cyclohexene was further added to the solution. The reaction mixture was stirred for 1.5–3.0 h at room temperature and then poured into 0.5 M KH_2PO_4 aqueous solution. The mixture was extracted with ethyl acetate. The organic layer was washed with 0.5 M KH_2PO_4 aqueous solution, distilled water, and brine, and then dried over anhydrous sodium sulfate, filtered, and concentrated. The crude

mixture was purified by silica gel column chromatography to afford compounds **3a**–**c**.

3',5'-*O*-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-(2,4,6-trimethoxybenzylthiomethyl)-uridine (**3d**) was synthesized according to a previously reported procedure.[26]

*4.2.2.1 N-Benzoyl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-O-(2,4,6-trimethoxybenzyl-thiomethyl)adenosine (**3a**).*

White solid, 65% yield from **2a**; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 9.17 (1H, br s), 8.74 (1H, s), 8.17 (1H, s), 8.04 (2H, d, *J* = 7.1 Hz), 7.63-7.60 (1H, m, 1H), 7.53 (2H, t, *J* = 7.9 Hz), 6.10 (2H, s), 6.01 (1H, s), 5.21 (1H, d, *J* = 11.8 Hz), 4.97-4.95 (1H, m), 4.95 (1H, d, *J* = 11.8 Hz), 4.78 (1H, d, *J* = 5.0 Hz), 4.19 (1H, dd, *J* = 13.1, 1.9 Hz), 4.16-4.14 (1H, m), 4.03 (1H, dd, *J* = 13.2, 2.8 Hz), 3.95 (1H, d, *J* = 12.7 Hz), 3.81 (1H, d, *J* = 12.7 Hz), 3.80 (3H, s), 3.77 (6H, s), 1.11–1.05 (28H, m). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 164.5, 160.3, 158.8, 152.3, 150.9, 149.3, 142.0, 133.6, 132.8, 130.1, 128.9, 127.9, 108.1, 90.6, 89.2, 81.8, 74.7, 69.7, 60.0, 55.8, 55.3, 22.9, 17.5, 17.4, 17.33, 17.30, 17.2, 17.1, 17.0, 16.9, 13.4, 13.0, 12.8, 12.7. HRMS (FAB): *m/z* calculated for $C_{40}H_{58}N_5O_9SSi_2$ 840.3493 $[M+H]^+$, found 840.3505.

*4.2.2.2 N-Isobutyryl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-O-(2,4,6-trimethoxy-benzylthiomethyl)guanosine (**3b**).*

White solid, 62 % yield from **2b**, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 11.9 (1H, s), 8.22 (1H, s), 7.95 (1H,s), 6.11 (2H, s), 5.83 (1H, s), 5.15 (1H, d, *J* = 11.7 Hz), 5.01 (1H, d, *J* = 11.5 Hz), 4.59 (1H, d, *J* = 4.7 Hz), 4.51 (1H, dd, *J* = 9.4, 4.7 Hz), 4.21 (1H, br d, *J* = 13.2 Hz), 4.16-4.13 (1H, m), 4.01 (1H, dd, *J* = 13.2, 2.7 Hz), 3.93 (1H, d, *J* = 12.3 Hz), 3.87 (1H, d, *J* = 12.3 Hz), 3.80 (3H, s), 3.78

(6H, s), 2.41 (1H, sep, *J* = 7.0 Hz), 1.19 (3H, d, *J* = 7.0 Hz), 1.15 (3H, d, *J* = 7.0 Hz), 1.12-0.95 (28H, m). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 178.0, 160.5, 158.8, 155.4, 147.2, 147.0, 136.3, 121.9, 107.8, 91.0, 88.1, 81.8, 77.9, 74.1, 69.3, 59.7, 56.0, 55.4, 36.5, 22.9, 18.8, 17.4, 17.29, 17.25, 17.2, 17.05, 16.99, 16.87, 13.4, 12.94, 12.90, 12.6. HRMS (FAB): *m/z* calculated for $C_{37}H_{59}N_5O_{10}SSi_2Na$ 844.3418 $[M+Na]^+$, found 844.3422.

*4.2.2.3 N-Benzoyl-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-O-(2,4,6-trimethoxybenzyl-thiomethyl)cytidine (**3c**).*

White solid, 58% yield from **2c**, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.69 (1H, br s), 8.35 (1H, br d, *J* = 5.0 Hz), 7.89 (2H, br s), 7.62-7.50 (4H, m), 6.13 (2H,s), 5.90 (1H, s), 5.10 (1H, d, *J* = 11.5 Hz), 5.06 (1H, d, *J* = 11.4 Hz), 4.44 (1H, d, *J* = 3.6 Hz), 4.32-4.23 (3H, m), 4.04 (1H, d, *J* = 12.9 Hz), 4.04-4.02 (1H, m), 3.81 (6H, s), 3.80 (3H, s), 3.76 (1H, d, *J* = 12.9 Hz), 1.14-0.94 (28H, m). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 166.1, 162.3, 160.1, 158.9, 144.6, 133.1, 129.0, 127.5, 108.9, 95.7, 92.3, 90.7, 90.0, 82.1, 78.1, 73.3, 70.2, 67.8, 59.5, 55.8, 55.3, 30.3, 22.0, 17.5, 17.4, 17.33, 17.30, 17.1, 16.99, 16.98, 16.87, 13.5, 13.3, 13.1, 12.9, 12.8, 12.6. HRMS (FAB): *m/z* calculated for $C_{39}H_{57}N_3O_{10}SSi_2Na$ 838.3200 $[M+Na]^+$, found 838.3199.

*4.2.2.4 3',5'-O-(1,1,3,3-Tetraisopropyldisiloxane-1,3-diyl)-2'-O-(2,4,6-trimethoxybenzylthiomethyl)-uridine (**3d**).*

White solid, 65% yield from **2d**, ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.31 (1H, s), 7.85 (1H, d), 6.12 (2H, s), 5.76 (1H,s), 5.66 (1H, d, *J* = 8.2 Hz), 4.99 (2H, s), 4.44 (1H, d, *J* = 4.6 Hz), 4.29-4.24 (2H, m), 4.15 (1H, dd, *J* = 9.7, 1.8 Hz), 3.99 (1H, dd, *J* = 13.5, 2.4 Hz), 3.95 (1H, d, *J* = 13.2 Hz), 3.81 (6H, s), 3.80, (3H, s), 3.77 (1H, d, *J* = 12.9 Hz), 1.11-0.96 (28H, m). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 163.0, 160.2, 158.9, 149.5, 139.7, 108.4, 101.4, 90.6, 89.2, 82.0, 77.6, 73.3, 68.3, 59.5,

55.8, 55.3, 22.0, 17.5, 17.4, 17.3, 17.2, 17.1, 16.99, 16.95, 16.8, 13.5, 13.0, 12.9, 12.6. HRMS (FAB): *m/z* calculated for $C_{39}H_{57}N_3O_{10}SSi_2Na$ 735.2778 $[M+Na]^+$, found 735.2772.

*4.2.3 Preparation of N-protected-2'-O-(2,4,6-trimethoxybenzylthiomethyl)-5'-O-(4,4'-dimethoxy-trityl)nucleosides (**5a–d**)*

To a solution of each of *N*-protected-3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-(2,4,6-trimethoxybenzylthiomethyl)nucleosides (**3a**–**d**) in THF were added $Et_3N{\cdot}3HF$ (3 eq) and Et_3N (3 eq). The reaction mixture was stirred at room temperature for 1.5–3.0 h. The mixture was added to sat. $NaHCO_3$ aqueous solution and extracted with $CHCl_3$. The organic layer was washed with distilled water and brine, and then dried over anhydrous sodium sulfate, filtered, and concentrated. The crude mixture was purified by silica gel column chromatography to afford *N*-protected-2'-*O*-(2,4,6-trimethoxybenzylthiomethyl)nucleosides **4a**–**d**. Each of compounds **4a**–**d** was dissolved in anhydrous pyridine and 4,4'-dimethoxytrityl chloride (1.5 eq) was added to the solution. The reaction mixture was stirred at room temperature for 1.5–3.0 h. The mixture was added to sat. $NaHCO_3$ aqueous solution and extracted with ethyl acetate. The organic layer was washed with distilled water and brine, and then dried over anhydrous sodium sulfate, filtered, and concentrated. The crude mixture was purified by silica gel column chromatography to afford compounds **5a**–**d**.

*4.2.3.1 N-Benzoyl-2'-O-(2,4,6-trimethoxybenzylthiomethyl)-5'-O-(4,4'-dimethoxytrityl)adenosine (**5a**).*

White solid, 95% yield from **3a**; ^1H NMR (600 MHz, $CDCl_3$) δ: 9.00 (1H, br s), 8.71 (1H,

s), 8.18 (1H, s), 8.02 (2H, d, *J* = 8.2 Hz), 7.63-7.60 (1H, m), 7.55-7.51 (2H, m), 7.44-7.16 (9H, m), 6.84-6.79 (4H, m), 6.21 (1H, d, *J* = 5.3 Hz), 6.12 (2H, s), 4.94 (1H, d, *J* = 12.0 Hz), 4.91 (1H, t, *J* = 5.2 Hz), 4.73 (1H, d, *J* = 12.0 Hz), 4.57 (1H, dd, *J* = 9.1, 4.4 Hz), 4.27 (1H, dd, *J* = 7.3, 3.8 Hz), 3.85 (2H, d, *J* = 4.1 Hz), 3.81 (6H, s), 3.80 (3H, s), 3.78 (6H, s), 3.50 (1H, dd, *J* = 10.6, 3.2 Hz), 3.41 (1H, dd, *J* = 9.6, 4.1 Hz), 3.02 (1H, d, *J* = 4.7 Hz). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 160.8, 158.8, 158.6, 152.1, 150.6, 150.2, 147.3, 143.3, 139.4, 133.4, 133.0, 130.1, 129.1, 129.0, 128.2, 127.9, 127.8, 127.6, 127.1, 124.6, 113.2, 106.2, 90.7, 89.6, 88.0, 82.3, 81.4, 75.7, 71.0, 63.3, 55.8, 55.4, 55.2, 24.1. HRMS (FAB): *m/z* calculated for $C_{49}H_{50}N_5O_{10}S$ 900.3277 $[M+H]^+$, found 900.3282.

*4.2.3.2 N-Isobutyryl-2'-O-(2,4,6-trimethoxybenzylthiomethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine (**5b**).*

White solid, 86% yield from **3b**; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 11.9 (1H, s), 7.78 (1H, s), 7.56-7.53 (3H, m), 7.41-7.16 (7H, m), 6.84-6.77 (4H, m), 6.11 (2H, s), 5.83 (1H, d, *J* = 7.1 Hz), 5.20 (1H, dd, *J* = 7.0, 5.0 Hz), 4.95 (1H, d, *J* = 12.0 Hz), 4.63 (1H, d, *J* = 12.0 Hz), 4.52-4.50 (1H, m), 4.19 (1H, dd, *J* = 5.3, 2.9 Hz), 3.86 (1H, d, *J* = 12.3 Hz), 3.84 (1H, d, *J* = 12.4 Hz), 3.80 (3H,s), 3.78 (6H, s), 3.77 (3H, s), 3.76 (3H, s), 3.51 (1H, dd, *J* = 10.7, 1.9 Hz), 3.12 (1H, dd, *J* = 10.7, 3.4 Hz), 2.95 (1H, d, *J* = 2.9 Hz), 1.59 (1H, sep, *J* = 6.8 Hz), 0.92 (3H, d, *J* = 6.8 Hz), 0.70 (3H, d, *J* = 6.7 Hz). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 178.1, 160.7, 158.8, 158.7, 155.4, 148.1, 147.0, 144.9, 139.4, 139.1, 136.1, 135.7, 130.04, 130.02, 129.1, 128.1, 128.0, 127.8, 127.7, 127.1, 127.0, 122.6, 113.24, 113.22, 113.1, 106.6, 90.6, 86.4, 86.2, 84.4, 78.7, 74.9, 69.9, 63.7, 55.8, 55.4, 55.2, 36.0, 23.7, 18.5, 18.5. HRMS (FAB): *m/z* calculated for $C_{46}H_{52}N_5O_{11}S$ 882.3383 $[M+H]^+$, found 882.3389.

*4.2.3.3 N-Benzoyl-2'-O-(2,4,6-trimethoxybenzylthiomethyl)-5'-O-(4,4'-dimethoxytrityl)cytidine (**5c**).*

White solid, 97% yield from **3c**; ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.59 (1H, br s), 8.51 (1H,

br s), 7.87 (2H, br s), 7.61 (1H, t, *J* = 7.6 Hz), 7.52 (2H, t, *J* = 7.6 Hz), 7.45-7.43 (2H, m), 7.35-7.26 (7H, m), 6.90-6.87 (4H, m), 6.12 (2H, s), 5.97 (1H, s), 5.09-5.02(2H, m), 4.47-4.43 (1H, m), 4.29 (1H, d, *J* = 5.3 Hz), 4.05-4.02 (1H, m), 3.91 (1H, d, *J* = 12.7 Hz), 3.832 (3H, s), 3.830 (3H, s), 3.82 (6H, s), 3.81 (1H, d, *J* = 12.9 Hz), 3.79 (3H, s), 3.59-3.54 (2H, m), 2.99 (1H, d, *J* = 8.8 Hz). ^{13}C NMR (151 MHz, $CDCl_3$) δ: 160.6, 160.5, 158.85, 158.82, 158.7, 158.6, 147.3, 144.1, 139.4, 135.7, 135.3, 133.4, 133.2, 130.2, 130.1, 129.1, 128.3, 128.0, 127.84, 127.75, 127.5, 127.2, 127.1, 113.3, 113.2, 106.9, 106.8, 90.7, 89.2, 87.0, 85.6, 83.6, 81.4, 80.2, 80.0, 74.6, 73.8, 68.4, 67.7, 61.4, 61.2, 55.9, 55.38, 55.37, 55.2, 23.5, 22.9. HRMS (FAB): *m/z* calculated for $C_{48}H_{50}N_3O_{11}S$ 876.3165 $[M+H]^+$, found 876.3160.

*4.2.3.4 2'-O-(2,4,6-Trimethoxybenzylthio-methyl)-5'-O-(4,4'-dimethoxytrityl)uridine (**5d**)*

White solid, 98% yield from **3d**; spectral data are available in a previous report.[26]

*4.2.4 Preparation of N-protected-2'-O-(2,4,6-trimethoxybenzylthiomethyl)-3'-O-[(2-cyanoethyl)-(N,N-diisopropylamino)phosphoramidyl]-5'-O-(4,4'-dimethoxytrityl)nucleosides (**6a–d**)*

To a solution of each of *N*-protected-2'-*O*-(2,4,6-trimethoxybenzylthiomethyl)-5'-*O*–(4,4'-dimethoxytrityl)nucleosides (**5 a – d**) in anhydrous dichloromethane were added 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (1.5 eq), *N*,*N*-diisopropylethylamine (2.0 eq), and 4-dimethylaminopyridine (0.5 eq) at 0°C. After the reaction mixture was stirred for 0.5–1.5 h at room temperature, sat. $NaHCO_3$ aqueous solution was added and the mixture was extracted with ethyl acetate. The organic layer was washed with distilled water and brine and then dried over

anhydrous sodium sulfate, filtered, and concentrated. The crude mixture was purified by silica gel column chromatography to afford compounds **6a**–**d**.

*4.2.4.1 N-Benzoyl-2'-O-(2,4,6-trimethoxybenzylthio-methyl)-3'-O-[(2-cyanoethyl)-(N,N-diisopropylamino)phosphoramidyl]-5'-O-(4,4'-dimethoxytrityl)adenosine (**6a**).*

White solid, 79% yield from **5a**; ^{31}P NMR (243 MHz, $CDCl_3$) δ: 151.4, 150.7. HRMS (FAB): *m/z* calculated for $C_{58}H_{67}N_7O_{11}PS$ 1100.4356 $[M+H]^+$, found 1100.4360.

*4.2.4.2 N-Isobutyryl-2'-O-(2,4,6-trimethoxybenzylthio-methyl)-3'-O-[(2-cyanoethyl)-(N,N-diisopropylamino)phosphoramidyl]-5'-O-(4,4'-dimethoxytrityl)guanosine (**6b**).*

White solid, 79% yield from **5b**; ^{31}P NMR (243 MHz, $CDCl_3$) δ: 150.8, 150.4. HRMS (FAB): *m/z* calculated for $C_{55}H_{69}N_7O_{12}PS$ 1082.4461 $[M+H]^+$, found 1082.4459.

*4.2.4.3 N-Benzoyl-2'-O-(2,4,6-trimethoxybenzylthio-methyl)-3'-O-[(2-cyanoethyl)-(N,N-diisopropylamino)phosphoramidyl]-5'-O–(4,4'-dimethoxytrityl)cytidine (**6c**)*

White solid, 83% yield from **5c**; ^{31}P NMR (243 MHz, $CDCl_3$) δ: 150.9, 150.7. HRMS (FAB):*m/z* calculated for $C_{57}H_{67}N_5O_{12}PS$ 1076.4244 $[M+H]^+$, found 1076.4241.

*4.2.4.4 2'-O-(2,4,6-Trimethoxybenzylthio-methyl)-3'-O-[(2-cyanoethyl)-(N,N-diisopropylamino)-phosphoramidyl]-5'-O-(4,4'-dimethoxytrityl)uridine (**6d**)*

White solid, 88% yield from **5d**; spectral data are available in a previous report.[26]

388 **4.3 Oligonucleotide synthesis**

389 Oligonucleotides were synthesized on a 1 μmol scale on an ABI Model 392 DNA/RNA
390 synthesizer in the trityl-on mode. The standard ABI CE DNA synthesis protocol was employed
391 except for the following points. The coupling wait time was extended to 600 sec for the coupling of
392 compounds **6a**–**d** with 5-ethylthio-1*H*-tetrazole (ETT) as the activator. 0.02 M iodine solution was
393 used for phosphite triester oxidation. Synthesized oligonucleotides were treated with concentrated
394 aqueous ammonia at 55 ℃ for 8.0 h. After the removal of ammonia in the suspensions, controlled
395 pore glasses (CPGs) were removed by filtration with Millex-LG 0.20 μm (Millipore). The crude
396 oligonucleotides were analyzed and purified by RP-HPLC. The obtained oligonucleotide solutions
397 were concentrated in a centrifugal evaporator (EYELA, Japan). After the addition of 2.0 M TEAA,
398 the oligonucleotides were applied to a Sep-Pak C18 plus (Waters) and then washed with 100 mM
399 TEAA. Subsequently, the 5'-DMTr group of the oligonucleotides was removed by adding 2% TFA
400 aqueous solution and desalted with water. Finally, the oligonucleotides were eluted with 50%
401 acetonitrile/water and concentrated in a centrifugal evaporator. The oligonucleotide solutions were
402 quantified on a UV spectrometer and lyophilized. The structures were characterized by MALDI-TOF
403 mass spectrometry.
404
405 **4.4 Conversion of 2'-*O*-TMBTM oligonucleotides into 2'-*O*-MDTM oligonucleotides**
406 To a solution of oligonucleotides (**7a**–**d**) (0.1 mM each) in 200 mM sodium acetate buffer
407 (pH 4) was added freshly prepared DMTSF aqueous suspension (ca. 300 eq) and the reaction was
408 left to stand at 37 ℃. After the reaction was monitored by RP-HPLC, excess DMTSF and buffer
409 were removed with a gel filtration column (GE Healthcare NAP-25). Oligonucleotides **8a**–**d** were
410 purified by RP-HPLC and the solutions were desalted by a gel filtration column (GE Healthcare,

NAP-25). The oligonucleotide solutions were concentrated and quantified on a UV spectrometer. The structures were characterized by MALDI-TOF mass spectrometry.

4.5 Conversion of 2'-*O*-MDTM oligonucleotides into 2'-OH oligonucleotides under glutathione-based reducing condition

Each of oligonucleotides **8a**–**d** (2 nmol) was dissolved in 36 µL of 50 mM sodium phosphate buffer (pH 7). To the solution was added 4 µL of 100 mM GSH aqueous solution to adjust the final concentrations of each oligonucleotide and GSH to 50 µM and 10 mM, respectively. The solution was incubated at 37 °C and the reaction was monitored by RP-HPLC. The samples were desalted by ZipTip C_{18} (Millipore) and the structures were characterized by MALDI-TOF mass spectrometry.

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