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A post-synthetic approach for the synthesis of 2′-*O*-methyldithiomethyl-modified oligonucleotides responsive to a reducing environment†

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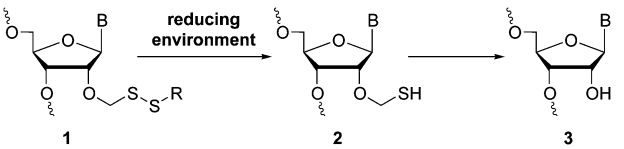
Based on a novel concept, a Reducing-Environment-Dependent Uncatalyzed Chemical Transforming RNA, "REDUCT RNA", we established a post-synthetic approach for the synthesis of 2′-*O*-methyldithiomethyl-modified oligonucleotides from 2′-*O*-(2,4,6-trimethoxybenzylthiomethyl)-oligonucleotides by treatment with dimethyl(methylthio)sulfonium tetrafluoroborate. 2′-*O*-methyldithiomethyl oligonucleotides were easily converted into 2′-hydroxy oligonucleotides under reducing conditions, such as those found in the intracellular environment.

Technologies based on ribozymes[1] and RNA interference[2] (RNAi) provide attractive gene silencing strategies different from small molecule approaches. However, such gene silencing technologies need to overcome some hurdles before they can be used in practical applications. Exogenous unmodified RNAs are rapidly degraded by nucleases and exhibit poor cell permeation. To circumvent these problems, numerous chemical modifications of RNA have been reported. Among them, the 2′-*O*-modifications conferred efficient resistance to nucleases on oligonucleotides[3] but usually decreased the activities of small interfering RNAs (siRNAs) and ribozymes.[4,5] Indeed, siRNA activity virtually disappeared with the full 2′-*O*-methyl modification of oligoribonucleotides.[4,6] These drawbacks of the modifications would be circumvented with prodrug-type RNA bearing transient modifications as biolabile groups. Based on this idea, Debart *et al.* reported oligonucleotides bearing 2′-*O*-acyloxymethyl groups that were designed to be cleaved by intracellular esterases to liberate native RNA.[7,8] In mammals, however, high carboxyesterase activities in the serum are found[9] and this may lead to cleavage of the promoieties to liberate native RNA before delivery of the modified RNA into target cells.

It is known that the intracellular concentration of glutathione is very high (1–10 mM) compared to the plasma concentration (≈2 μM), creating a reducing environment inside cells.[10–13] Therefore, we designed 2′-*O*-alkyldithiomethyl RNA **1**, whose promoiety's disulfide bond was expected to be cleaved in the reducing environment inside cells to liberate native RNA **3** *via* unstable thiohemiacetal derivative **2** (Scheme 1). As far as we know, there is only one report of the synthesis of 2′-*O*-alkyldithiomethyl RNA, in which the *t*-butyldithiomethyl group is employed as a novel protecting group for the 2′-hydroxy groups in the chemical synthesis of RNA.[14] However, the instability of the phosphoramidite units bearing the alkyldithiomethyl group would limit their application, because the trivalent phosphorus atom on such phosphoramidites is capable of allowing intramolecular attack on the disulfide bond.[14,15] Thus, the development of a novel approach for the synthesis of 2′-*O*-alkyldithiomethyl-modified RNA is required for its practical use.



Scheme 1 Conversion of 2′-*O*-alkyldithiomethyl-modified nucleotides into native nucleotides in a reducing environment.

Here, we report a post-synthetic approach for the synthesis of 2′-*O*-methyldithiomethyluridine-containing oligonucleotides. We also show the functional basis of the 2′-*O*-methyldithiomethyl-modified oligonucleotides, which are efficiently converted into the 2′-hydroxy oligonucleotides under reducing conditions, namely Reducing-Environment-Dependent Uncatalyzed Chemical Transforming RNA (REDUCT RNA).

Originally, we focused on the 4-methoxybenzylthio group, which was reported to be easily converted into the methyldithio group by treatment with dimethyl(methylthio)sulfonium tetrafluoroborate (DMTSF).[16] Thus, we synthesized 2′-*O*-(4-methoxybenzyl)thiomethyl uridine derivative **4a** and attempted to convert it into 2′-*O*-methyldithiomethyl derivative **6** (Scheme 2). However, the reaction gave 2′-*O*-fluoromethyl uridine derivative **8** as the major product. This result should be due to the stronger electron-donating ability of the 2′-oxygen atom to the methylthiosulfonium moiety of **5a** than the 4-methoxybenzyl group. Thus, we designed a novel 2′-*O*-promoiety, the 2,4,6-trimethoxybenzylthiomethyl (TMBTM) group, which has a stronger electron-donating characteristic than the 4-methoxybenzylthiomethyl group. Indeed, TMBTM-containing

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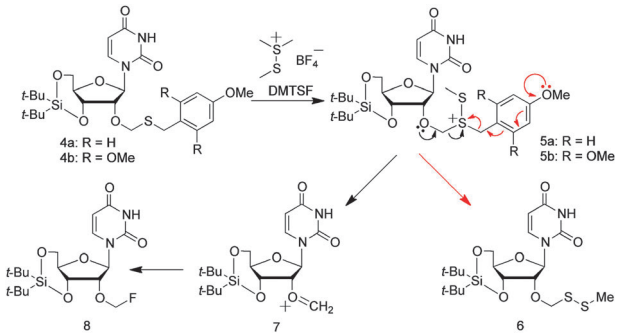
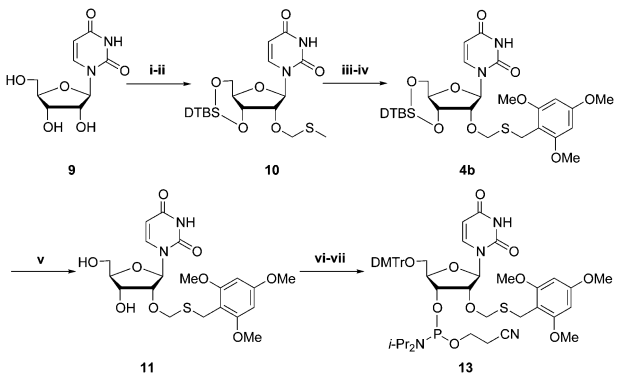


Scheme 2 Conversion of 2′-*O*-benzylthiomethyluridine derivatives into a 2′-*O*-methyldithiomethyl or a 2′-*O*-fluoromethyl derivative.



Scheme 3 Synthesis of a 2′-*O*-TMBTM uridine amidite unit. Conditions: (i) DTBS-$(OTf)_2$, DMF, 0 °C, 1 h; (ii) DMSO, Ac_2O, AcOH, r.t., 24 h, 74% (from **9**); (iii) SO_2Cl_2, CH_2Cl_2, r.t., 0.75 h; (iv) 2,4,6-$(MeO)_3$BnSH, NaH, DMF, 0 °C, 2 h, 72% (from **10**); (v) $Et_3N{\cdot}3HF$, THF, r.t., 0.3 h, 89%; (vi) DMTr-Cl, pyridine, r.t., 1.5 h, 95%; (vii) $[(^iPr)_2N]_2POCE$, diisopropylammonium tetrazolide, CH_2Cl_2, r.t., 15 h, 92%.

uridine derivative **4b** was converted into methyldithiomethyl-containing uridine derivative **6** in excellent yield.

To evaluate the conversion of the 2′-*O*-TMBTM group into the methyldithiomethyl group in oligonucleotides, we synthesized amidite building block **13** bearing the 2′-*O*-TMBTM groups (Scheme 3). We initially protected the 3′,5′-hydroxy groups of uridine with the di-*tert*-butylsilanediyl (DTBS) group and subsequently introduced the methylthiomethyl (MTM) group into the 2′-hydroxyl group in 74% yield. The resulting 2′-*O*-MTM derivative **10** was treated with sulfuryl chloride to afford the 2′-*O*-chloromethyl derivative,[17] which was treated with 2,4,6-trimethoxybenzylmercaptan[18] in the presence of sodium hydride to give 2′-*O*-TMBTM-derivative **4b** in 72% yield. Removal of the DTBS group with $Et_3N{\cdot}3HF$ afforded 2′-*O*-TMBTM-uridine **11** in 89% yield. The 5′-hydroxy group of **11** was protected by the 4,4′-dimethoxytrityl (DMTr) group, and finally the 3′-hydroxy group was phosphitylated to afford phosphoramidite unit **13** in 87% yield. The overall yield of **13** obtained from uridine **9** in seven steps was 42%. Amidite **13** was completely stable for several months at −20 °C and also showed excellent stability even in solution. Dissolving **13** in acetonitrile resulted in less than 2% degradation within one week (Fig. S1, ESI†). This is in marked contrast to 2′-*O*-*t*-butyldithiomethyl-modified amidites.[14]

By using amidite unit **13**, oligonucleotides bearing one to three 2′-*O*-TMBTM groups shown in Table 1 were synthesized.

Table 1 Synthesized oligonucleotides bearing 2′-*O*-TMBTM groups[a]

		MALDI-TOF mass	
ODN	Sequence (5′–3′)	Calcd $[M-H]^-$	Found
14a	d(GCGTT**X**TTTGCT)[a]	3860.6	3859.1
15a	d(GCGTT**X**T**X**TGCT)[a]	4088.9	4090.0
16a	d(GCG**X**T**X**TXTGCT)[a]	4317.2	4316.4
17a	d(GCGTT**XXX**TGCT)[a]	4317.2	4318.4

[a] **X**: 2′-*O*-TMBTM-uridine.

5-Ethylthio-1*H*-tetrazole (ETT) was employed as an activator for the coupling reaction and the coupling time was extended to 900 s. Oxidation of the trivalent phosphorus was conducted by using 0.02 M iodine solution. The synthesized oligonucleotides were deprotected and cleaved from the support with concentrated aqueous ammonia at 55 °C for 10 h. A nearly single peak for the crude oligonucleotides bearing the 5′-*O*-DMTr group was observed using HPLC. A typical chromatogram of the crude 5′-*O*-dimethoxytritylated oligonucleotide is shown in Fig. S2 (ESI†). After HPLC purification followed by detritylation, the structures were characterized using MALDI-TOF mass spectrometry (Table 1).

Post-synthetic modifications of oligonucleotides **14a–17a** into the corresponding 2′-*O*-methyldithiomethyl oligonucleotides **14b–17b** were conducted by treatment with DMTSF. A 0.1 mM solution of the oligonucleotide in 200 mM sodium acetate buffer (pH 4) was treated with DMTSF (final concentration: 30 mM). Fig. 1 shows the HPLC analysis of the conversion of **15a** into **15b**. The reaction efficiently proceeded without the generation of much by-product within 3 h. Without the buffer, depurination took place due to the strong acidity of DMTSF (data not shown). Depurination was well prevented by appropriate pH control to pH 4 with sodium acetate buffer, although the reaction rate was somewhat low compared with the non-buffered system. Other oligonucleotides bearing 2′-*O*-TMBTM (**14a**, **16a**, and **17a**) were similarly converted into 2′-*O*-methyldithiomethyl oligonucleotides **14b**, **16b**, and **17b** by DMTSF treatment. The structures of **14b–17b** were confirmed using MALDI-TOF mass spectrometry (Table S1, ESI†). Thus, our post-synthetic approach successfully provided 2′-*O*-methyldithiomethyl oligonucleotides in an easy and practical manner.

To evaluate the resistance of 2′-*O*-methyldithiomethyl oligonucleotides to 3′-exonuclease, we synthesized oligonucleotide **18** 5′-d(TTTTTTTTTU_{MDTM}T)-3′ (U_{MDTM}: 2′-*O*-methyldithiomethyluridine) as well as thymidine 10-mer **19** as control. Each 10-mer was incubated with snake venom phosphodiesterase (SVPDE) and

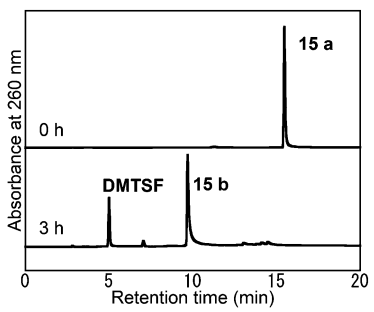


Fig. 1 HPLC analysis of the conversion of **15a** into **15b** by treatment with DMTSF.

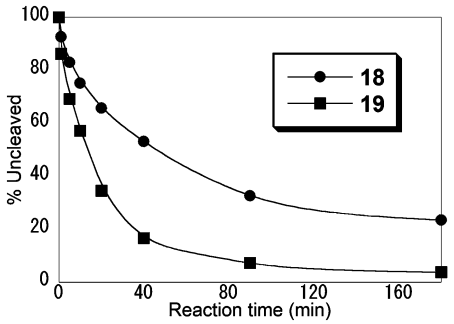


Fig. 2 Kinetics of degradation of oligonucleotides 5′-d(TTTTTTTTU$_{MDTM}$T)-3′ **18** and 5′-d(TTTTTTTTTT)-3′ **19** with SVPDE.

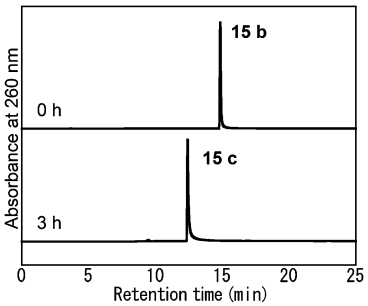


Fig. 3 HPLC analysis of the reductive conversion of **15b** into **15c** by treatment with 10 mM DTT.

aliquots were removed and analyzed using HPLC. The half-life ($t_{1/2}$) of **19** was less than 20 min, whereas that of **18** was extended to approximately 40 min (Fig. 2). The result suggests that oligonucleotides bearing the 2′-*O*-methyldithiomethyl group are significantly more resistant to SVPDE than native DNA. Similar results were also obtained with 5% fetal bovine serum (Fig. S5, ESI†).

To be activated in the intracellular environment, 2′-*O*-methyldithiomethyl oligonucleotides need to be converted into 2′-hydroxy oligonucleotides under reducing conditions. To assess the reductive conversion of the 2′-*O*-methyldithiomethyl group into a 2′-hydroxy group, 2′-*O*-methyldithiomethyl oligonucleotide **15b** was treated with 10 mM 1,4-dithiothreitol (DTT) as the reductant in 100 mM Tris–HCl buffer (pH 8). The redox reaction proceeded cleanly and efficiently. Deprotection of the promoieties of **15b** was completed within 3 h to afford the corresponding 2′-hydroxy oligonucleotide **15c** (Fig. 3). The structure of **15c** was confirmed to be 2′-hydroxy oligonucleotide by MALDI-TOF mass analysis (Table S1, ESI†). Similarly, the other 2′-*O*-methyldithiomethyl oligonucleotides (**14b**, **16b**, and **17b**) were reductively converted into 2′-hydroxy oligonucleotides **14c**, **16c**, and **17c**, respectively. Furthermore, the reductive conversion under more physiological conditions (10 mM glutathione, pH 7.0) was conducted. 2′-*O*-methyldithiomethyl oligonucleotides **14b** and **16b** were gradually converted into the corresponding 2′-hydroxy oligonucleotides **14c** and **16c** (Fig. S3 and S4, ESI†).

Here, we proposed two novel concepts. One is the utilization of an alkyldithiomethyl group, which is designed to be cleaved under reducing conditions similar to that in the cytoplasm, as the promoiety of nuclease resistant prodrug-type RNA (REDUCT RNA). Indeed, oligonucleotides containing the 2′-*O*-methyldithiomethyl group were rapidly and efficiently converted into the corresponding 2′-hydroxy oligonucleotides. The results demonstrated that the REDUCT RNA would be highly beneficial for *in vivo* stabilization and activation in cells for the widespread application of functional RNAs. The second concept is a novel post-synthetic approach for the synthesis of oligonucleotides containing the 2′-*O*-methyldithiomethyl group. To avoid utilization of unstable amidite units containing the 2′-*O*-alkyldithiomethyl group, the novel post-synthetic approach was developed. This efficient synthetic method would make practical applications of oligonucleotides containing the alkyldithiomethyl group possible. The synthesis of TMBTM-modified amidite units other than the uridine derivative and the conversion of TMBTM-modified oligonucleotides consisting of four natural bases into the 2′-*O*-methyldithiomethyl oligonucleotides are ongoing.

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