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Synthesis of 2′-*O*-[2-(*N*-Methylcarbamoyl)ethyl]ribonucleosides Using Oxa-Michael Reaction and Chemical and Biological Properties of Oligonucleotide Derivatives Incorporating These Modified Ribonucleosides

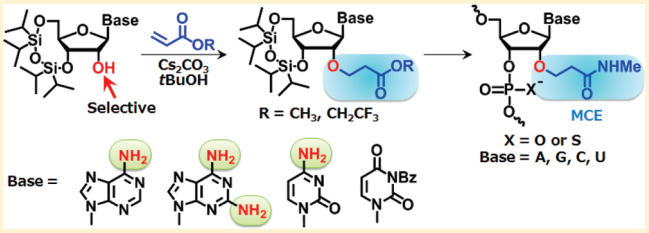
Takeshi Yamada,[†] Natsuki Okaniwa,[†] Hisao Saneyoshi,[†] Akihiro Ohkubo,[†] Kohji Seio,[†] Tetsuya Nagata,[‡] Yoshitsugu Aoki,[‡] Shin'ichi Takeda,[‡] and Mitsuo Sekine*[,†]

[†]Department of Life Science, Tokyo Institute of Technology, J2-12, 4259 Nagatsuta, Midoriku, Yokohama 226-8501, Japan

[‡]Department of Molecular Therapy, Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan

Supporting Information

ABSTRACT: To develop oligonucleotides containing new 2′-*O*-modified ribonucleosides as nucleic acid drugs, we synthesized three types of ribonucleoside derivatives modified at the 2′-hydroxyl group with 2-(methoxycarbonyl)ethyl (MOCE), 2-(*N*-methylcarbamoyl)ethyl (MCE), and 2-(*N*,*N*-dimethylcarbamoyl)ethyl (DMCE) groups, as key intermediates, via the oxa-Michael reaction of the appropriately protected ribonucleoside (U, C, A, and G) derivatives. Among them, the 2′-*O*-MCE ribonucleosides were found to be the most stable under basic conditions. To study the effects of the 2′-*O*-modification on the nuclease resistance of oligonucleotides incorporating the 2′-*O*-modified ribonucleosides and their hybridization affinities for the complementary RNA and DNA strands, 2′-*O*-MCE-ribonucleoside phosphoramidite derivatives were successfully synthesized and subjected to the synthesis of 2′-*O*-MCE-oligonucleotides and 2′-*O*-methyl-oligonucleotides incorporating 2′-*O*-MCE-ribonucleosides. The 2′-*O*-MCE-oligonucleotides and chimeric oligomers with 2′-*O*-MCE and 2′-*O*-methyl groups thus obtained demonstrated complementary RNA strands and much higher nuclease resistances than the corresponding 2′-*O*-methylated species. Finally, we incorporated the 2′-*O*-MCE-ribonucleosides into antisense 2′-*O*-methyl-oligoribonucleotides to examine their exon-skipping activities in splicing reactions related to pre-mRNA of mouse dystrophin. The exon-skipping assay of these 2′-*O*-methyl-oligonucleotide incorporating 2′-*O*-MCE-uridines showed better efficacies than the corresponding 2′-*O*-methylated oligoribonucleotide phosphorothioate derivatives.



INTRODUCTION

A number of 2′-*O*-modified ribonucleosides have been designed and synthesized for oligonucleotide-based therapeutics, represented by the silencing of mRNAs using siRNAs,[1] antisense RNAs,[2] and shRNAs[3] and the suppression of specific proteins using RNA aptamers.[4] Various modifications with substituent groups, such as 2′-*O*-methyl,[5] 2′-*O*-methoxyethyl,[6] 2′-*O*-aminopropyl,[7] and 2′-*O*-[2-(methylamino)-2-oxoethyl],[8] have been reported to date. The incorporation of these 2′-*O*-modified nucleosides into oligoribonucleotides has proven to be useful for improving their stability against hydrolysis by nucleases in serum,[9] controlling their biodistribution in cells and organs,[10] and enhancing their hybridization affinities for the complementary RNAs.[11] 2′-*O*-Modification of ribonucleosides was also used for the introduction of tethers with other small molecules such as lipids,[12] dyes,[13] and peptides.[14] Several 2′-*O*-modified ribonucleoside derivatives have been synthesized via the ring-opening reaction of 2,2′-anhydrouridine[15] and the alkylation of appropriately protected ribonucleosides with alkyl halides under strong basic conditions.[16] Meanwhile, we independently reported the synthesis and properties of 2′-*O*-cyanoethyl-modified (2′-*O*-CE) oligonucleotides[17] (Figure 1). 2′-*O*-CE oligoribonucleotides demonstrated higher hybridization affinities for the cDNAs and RNAs than the corresponding unmodified species; they also demonstrated favorable nuclease resistances. These results indicated that 2′-*O*-CE oligonucleotides would be available for oligonucleotide therapy; however, they were somewhat unstable under basic conditions. The 2′-*O*-CE group was gradually removed via β-elimination under basic conditions such as aqueous ammonia. We also found that the CE group could be used as a protecting group of 2′-OH in RNA synthesis, since this group could be removed by treatment with 1 M Bu_4NF in THF.[18]

On the basis of the previous results, we designed three types of ribonucleosides modified at the 2′-hydroxyl group with 2-(methoxycarbonyl)ethyl (MOCE) (**1**), 2-(*N*-methylcarbamoyl)ethyl (MCE) (**2**), and 2-[(*N*,*N*-dimethylcarbamoyl)ethyl

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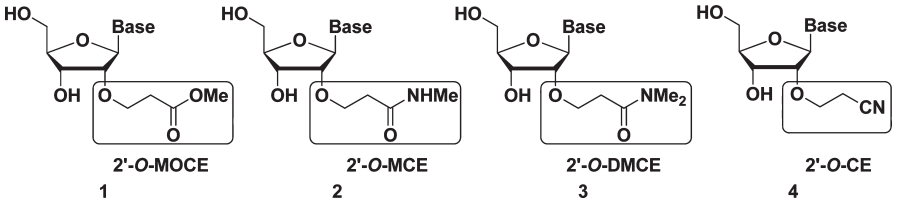


Figure 1. The structures of 2′-*O*-MOCE, 2′-*O*-MCE, 2′-*O*-DMCE, and 2′-*O*-CE ribonucleosides.

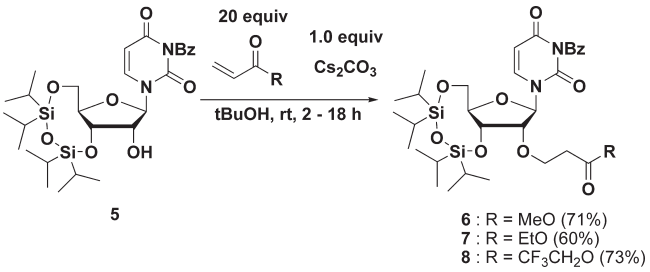
(DMCE) (**3**) groups as novel monomer components of 2′-*O*-modified oligoribonucleotides, as shown in Figure 1. We expected that these modifiers could be introduced via Michael additions similar to the $CsCO_3$-mediated 2′-*O*-cyanoethylation used for the synthesis of 2′-*O*-CE ribonucleosides (**4**) followed by amidation. It seemed that the transformation of the cyano group in the CE group to weaker electron-withdrawing groups, such as methoxycarbonyl, *N*-methylcarbamoyl, and *N*,*N*-dimethylcarbamoyl, could suppress the loss of these 2′-*O*-modifiers due to β-elimination during the entire synthetic process involving ammonia treatment required for removal of base-protecting groups. The MCE and DMCE groups have amide functions in their structures. Prakash et al. previously reported that oligoribonucleotides incorporating 2′-*O*-[2-(methylamino)-2-oxoethyl]ribonucleosides with an amide group similar to that of 2′-*O*-MCE ribonucleosides showed favorable nuclease resistance.[9]

In this paper, we report the details of the synthesis of new 2′-*O*-modified ribonucleosides, which involved highly selective oxa-Michael additions for purine nucleotides without alkylation of the base residues, such as cytosine, adenine, and 2-aminoadenine, and several unique properties of oligonucleotide derivatives incorporating these 2′-*O*-modified ribonucleosides. In addition to these synthetic studies, promising biological properties of 2′-*O*-MCE/2′-*O*-Me chimeric RNA oligomers, involving an effective exon skipping of the pre-mRNA of mouse dystrophin, are also described.

RESULTS AND DISCUSSION

Oxa-Michael Addition of the Secondary 2′-*O*-Hydroxyl Group of Uridine to Acrylates. We tested various α,β-unsaturated carbonyl compounds, such as alkyl acrylates (CH_2═$CHC(O)OR$: R = CH_3, C_2H_5, *n*-C_4H_9, *t*-C_4H_9, and CF_3CH_2), acrylamide, *N*,*N*-dimethylacrylamide, acrolein, and methyl vinyl ketone, as the acceptors of the oxa-Michael addition of N^3-benzoyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine (**5**)[19] in *t*-BuOH in the presence of cesium carbonate at room temperature for 2−18 h, as shown in Scheme 1. Before compound **5** was selected for the Michael addition donor, 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine[20] was tested. However, the alkylation reaction on the uridine moiety was faster than Michael addition to the 2′-hydroxyl group. When methyl, ethyl, and 2,2,2-trifluoroethyl esters of acrylic acid were used, the oxa-Michael reactions proceeded smoothly to give 2′-*O*-alkylated compounds **6**, **7**, and **8**, respectively. Other acceptors, such as *n*-butyl acrylates, *tert*-butyl alkylate, acrylamide, *N*,*N*-dimethylacrylamide, acrolein, and methyl vinyl ketone, were ineffective under these conditions. The reaction rate increased by increasing the substrate concentration from 0.1 to 0.2 M. The polymerization of the Michael acceptors did not occur in most cases, except for the reaction of **5** with acrolein.

Scheme 1. Oxa-Michael Reaction to Compound 5[a]



[a] Conditions: acrylate ester derivatives, Cs_2CO_3, *t*-BuOH, rt, 2−18 h; yield: **6** (71%), **7** (60%), **8** (73%).

Functional Group Transformation of 2′-*O*-[2-(Alkoxycarbonyl)ethyl]uridine. Scheme 2 shows the synthesis of 5′-*O*-(4,4′-dimethoxytrityl)uridine derivatives modified at the 2′-position with MOCE, MCE, and DMCE groups. The benzoyl group of the methyl ester compound **6** was selectively removed by treatment with 5 equiv of *n*-$PrNH_2$ in THF to give the deprotetected compound **9**. The methyl ester group was inert under these conditions. When compound **6** was treated with 40% $MeNH_2$ in MeOH, the monoalkyl amide **10** was obtained in a good yield. However, treatment of compound **6** with 2 M Me_2NH in THF did not give the desired dialkyl amide **11** but instead gave the debenzoylated compound **9** quantitatively. The use of 50% Me_2NH in H_2O resulted in the formation of a hydrolyzed compound as the main product (data not shown). Therefore, we chose the more reactive ester **8** for the synthesis of **11**. As a result, treatment of compound **8** with 2 M Me_2NH in THF easily gave compound **11**. Treatment of compounds **9**−**11** with $Et_3N{\cdot}3HF$ gave the resulting 3′,5′-unprotected intermediates **12**−**14**, which were allowed to react with DMTrCl to give compounds **15**−**17**, respectively.

Chemical Properties of the MOCE, MCE, and DMCE Groups of 15−17 under Basic Conditions. We investigated the stability of the MOCE, MCE, and DMCE groups of **15**−**17** under basic conditions; Table 1 summarizes these results. We treated 10 mg of each compound with 1 mL of 1 M NaOH, 28% NH_4OH, and 1 M TBAF in THF, and checked the time course of the reaction by silica gel thin layer chromatography analysis.

Treatment with 1 M NaOH easily hydrolyzed the methyl ester **15** to give the carboxylic acid **18** in 65% yield. The ammonia treatment of compound **15** also resulted in rapid ammonolysis, giving rise to the amide compound **19**. These results showed that hydrolysis and ammonolysis were much faster than β-elimination, and once the ester was transformed to the amide or the carboxylic acid, further β-elimination was well suppressed. We isolated and identified the hydrolyzed product **18** and the amidate derivative **19** by ^1H NMR and HRMS. Rapid removal of the MOCE group took place when compound **15** was treated

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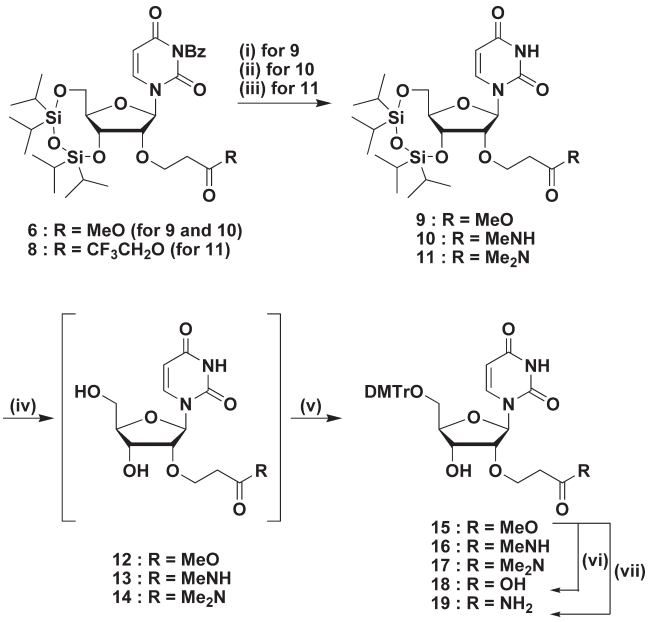
Table 1. Chemical Properties of Compound 15−17

conditions[a]	compd **15**	compd **16**	compd **17**
1 M NaOH	hydrolysis within 5 min	stable for 24 h	hydrolysis in 18 h
28% NH_4OH	amidation within 5 min	stable for 24 h	stable for 24 h
1 M TBAF/THF	elimination within 5 min	stable for 24 h	elimination[b]

[a] All reactions were done at room temperature. [b] $t_{1/2}$ = 2 h. The reaction was completed at 40 °C for 2 h.

Scheme 2. Preparation of Compound 15−19[a]



[a] Conditions: (i) *n*-$PrNH_2$, THF, rt, 4 h, yield: **9** (71%); (ii) 40% $MeNH_2$ in MeOH, rt, 4 h, yield: **10** (66%); (iii) 2 M Me_2NH in THF, rt, 2 h, yield: **11** (81%); (iv) $Et_3N \cdot 3HF$, THF, rt; (v) DMTrCl, pyridine, rt, yield: **15** (90%, 2 steps), **16** (51%, 2 steps), **17** (70%, 2 steps); (vi) 0.1 M NaOHaq, rt, 30 min, yield: **18** (65%); (vii) 28% NH_4OH, rt, 30 min, yield: **19** (99%).

with 1 M TBAF in THF. In our previous study, we reported that the cyanoethyl group was removed from 2′-*O*-cyanoethyluridine by using this reagent.[18]

The monoalkyl amide **16** was very stable under all tested conditions. In addition, it was also stable under the conditions of 28% NH_4OH at 55 °C for 5 h, which are the conditions for the oligonucleotide synthesis in this study. The amide proton of the MCE group was supposed to be dissociated faster than the α-proton under basic conditions, so that the dissociated species suppressed further deprotonation at the α-position.

The *N*,*N*-dialkyl amide **17** demonstrated the most unique properties. The DMCE group was stable under ammonia treatment but could be removed by treatment with 1 M TBAF.

Table 1 shows that the MCE group is the most stable among the three modified groups. Therefore, it is tolerable in the standard RNA synthesis. This result led us to synthesize the 2′-*O*-MCE-ribonucleoside 3′-phosphoramidite derivatives **20**−**23**, as shown in Figure 2.

Synthesis of 2′-*O*-MCE-Ribonucleoside 3′-*O*-Phosphoramidite Derivatives 20−23. For the synthesis of 2′-*O*-MCE-ribonucleoside 3′-phosphoramidite derivatives, we applied the Michael addition to the *N*-protected cytidine, adenosine,

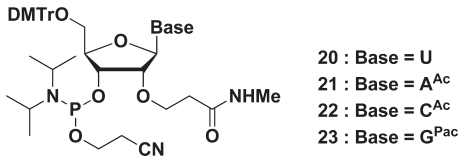
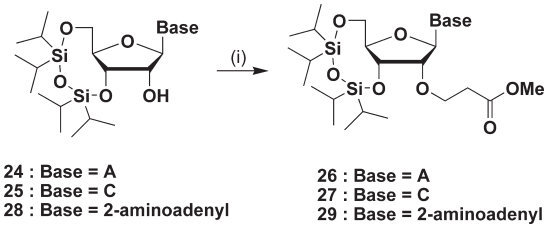


Figure 2. The structures of 2′-*O*-MCE-RNA-phosphoramidites **20**−**23**.

Scheme 3. Selective Oxa-Michael Reactions to Compounds 26, 27, and 29[a]



[a] Conditions: (i) Cs_2CO_3, methyl acrylate, *t*-BuOH, rt, 6−18 h, yield: **26** (72%), **27** (75%), **29** (79%).

and guanosine derivatives such as 6-*N*-benzoyl-3′,5′-(1,1,3,3-tetraisopropyldisilane-1,3-diyl)adenosine,[20] 4-*N*-benzoyl-3′,5′-(1,1,3,3-tetraisopropyldisilane-1,3-diyl)cytidine,[20] and 2-*N*-phenoxyacetyl-3′,5′-(1,1,3,3-tetraisopropyldisilane-1,3-diyl)guanosine.[21] However, these reactions gave complex mixtures in all cases. These results indicated that there were side reactions on the *N*-protected base moieties that had amido protons.

Several research groups have reported the selective alkylation of the 2′-hydroxyl group of ribonucleosides, such as adenosine and 2-aminoadenosine, with alkylating reagents under basic conditions.[22] On the basis of these results, we studied whether a Michael reaction would be available in the presence of *N*-free nucleobases.

The results are shown in Scheme 3. We found that the *N*-unmasked adenosine derivative **24** underwent smooth regioselective Michael additions with methyl acrylate to give the desired 2′-*O*-MOCE-adenosine derivative **26** in 72% yield. Similar conditions were also tested on *N*-unmasked cytidine derivative **25**. Alkylation on the nucleobase moiety was observed as time passed at room temperature, but Michael addition to the 2′-hydroxyl group was much faster than the side reaction so that the desired 2′-*O*-MOCE-cytidine derivative **27** was selectively obtained in 75% yield.

The reaction of 3′,5′-*O*-TIPDS-guanosine[20] was also tested under Cs_2CO_3 conditions, but the reaction resulted in formation of a complex mixture. The fully base-protected species, i.e., 6-*O*-diphenylcarbamoyl-2-*N*-dimethylaminomethylene-3′,5′-*O*-TIPDS-guanosine,[23] was also examined, but the rate of Michael reaction was very slow. The dimethylaminomethylene (dmf) and diphenylcarbamoyl (dpc) group were gradually degraded so that the reaction

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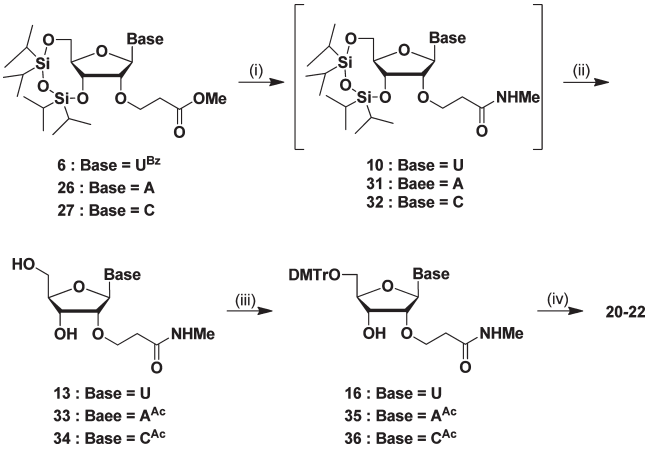
Scheme 4. Synthesis of Phosphoramidites 20−22[a]



[a] Conditions: (i) 40% $MeNH_2$ in MeOH, EtOH, rt; (ii) AcCl, pyridine, rt (for **31** and **32**); (c) $Et_3N\cdot 3HF$, Et_3N, THF, rt, yield: **13** (73%, 2 steps), **33** (60%, 3 steps), **34** (53%, 3 steps); (iii) DMTrCl, pyridine, rt, yield: **35** (73%) and **36** (52%); (iv) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, diisopropylammonium 1*H*-tetrazolide, MeCN, rt, yield: **20** (60%), **21** (76%), **22** (48%).

gave a complex mixture. Finally, based on the successful results using the *N*-free adenosine and cytidine derivatives **24** and **25**, we chose 3′,5′-*O*-TIPDS-2-aminoadenosine **28**[24] as a precursor for the synthesis of 2′-*O*-MCE-guanosine derivative **30** (see Scheme 5) since the 2-aminoadenine moiety can be converted to the guanosine one.[24] Consequently, it was found the reaction of *N*-unmasked 2-aminoadenosine derivative **28** with methyl acrylate in *t*-BuOH gave the desired 2′-*O*-MOCE-2-aminoadenosine product **29** in 78% yield without base modification.

The imino proton of uridine ($pK_a = 9.25$)[25] is apparently more acidic than the amino protons of adenosine or cytidine (probably more than that of aniline ($pK_a = 30.6$)[26]) and the 2′-OH proton ($pK_a = 12.14-12.86$).[27] Therefore, it was considered that the imino proton of uridine would be easily dissociated by cesium carbonate as a base so that, as compared to the nondissociated species, the dissociated one became more reactive toward the Michael acceptors. In contrast to the dissociation property of the uracil base, the amino protons of adenosine and cytidine could not be dissociated by $CsCO_3$. Therefore, it was expected that this property was dependent on the inherent nucleophilicity of the nucleobases when a Michael reaction competitively occurs on the unprotected nucleobase sites toward a $CsCO_3$-mediated dissociated 2′-hydroxyl group.

Scheme 4 shows the synthesis of phosphoramidite compounds **20**−**22**. We converted the 2′-*O*-MOCE derivatives **6**, **26**, and **27** to the *N*-free 2′-*O*-MCE-uridine derivative **10**, adenosine derivative **31**, and cytidine derivative **32** via treatment with methylamine. Under these conditions, the N^3-benzoyl group of the uridine derivative **6** was simultaneously removed to give the 2′-*O*-MCE disilylated derivative **10**, which in turn was allowed to react with $Et_3N\cdot 3HF$ in THF to give 2′-*O*-MCE-urdine (**13**) in 73% yield. In the case of cytidine derivative **27**, a trace amount of the β-elimination product **25** was formed by methylamine treatment. The *N*-free intermediates **31** and **32** thus obtained from **26** and **27** were treated in situ with acetyl chloride in pyridine followed by $Et_3N\cdot 3HF$ in THF to give the 2′-*O*-MCE *N*-acetyl adenosine (**33**) and cytidine (**34**) in 51% and 53% yields, respectively. 2′-*O*-MCE *N*-acetyl derivatives **33** and cytidine derivative **34** were treated with DMTrCl to give the 5′-*O*-protected adenosine derivative **35** and cytidine derivative **36** in 73% and 52% yields, respectively. The 3′-phosphitylation of 5′-*O*-DMTr derivatives **16**, **35**, and **36** with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite[28] in the presence of diisopropylammonium 1*H*-tetrazolide gave the phosphoramidite uridine derivatives **20**, adenosine derivative **21**, and cytidine derivative **22** in 66%, 76%, and 48% yields, respectively.

For the guanosine 3′-phosphoramidite building block **23**, we succeeded in synthesizing this compound via a six-step reaction from the 2-aminoadenosine derivative **29**, as shown in Scheme 5. The treatment of 2′-MOCE derivative **29** with methylamine yielded the 2′-*O*-MCE derivative compound **37** in good yield (77%). In this reaction, the selective conversion of **29** to **37** occurred without the β-elimination of the MCE group. We attempted the selective acylation of the 2-amino group of compound **37** by using isobutyryl chloride and phenoxyacetyl chloride. The latter gave a better result, and the 2-*N*-phenoxyacetyl derivative **38** could be easily obtained by reprecipitation. The deamination of compound **38** with $NaNO_2$ in the presence of AcOH gave the desired guanosine derivative **39**. Since we observed the partial elimination of the TIPDS group during this reaction, we treated the resulting product **39** with $Et_3N\cdot 3HF$ in THF in situ to give the 3′,5′-unprotected product **30** in an overall yield of 71%. The usual dimethoxytritylation of compound **30** followed by the 3′-phosphitylation of the resulting 5′-protected compound **40** gave the guanosine 3′-*O*-phosphoramidite derivative **23** in a good yield (80%).

Synthesis of 2′-*O*-MCE/2′-*O*-Me-Chimeric Oligonucleotides. 2′-*O*-MCE-oligonucleotides and chimeric oligonucleotides partially modified with 2′-*O*-MCE-ribonucleosides were synthesized by using the ribonucleoside 3′-phosphoramidite building blocks **20**−**23**, according to the standard phosphoramidite[40,41] procedure. We carried out the synthesis of these modified oligonucleotide using an ABI-392 DNA/RNA synthesizer and CPG resins. Either 5-benzylthio-1*H*-tetrazole (BTT)[29] or 5-[bis(3,5-trifluoromethyl)phenyl]-1*H*-tetrazole[30] was used as an activator. We used BTT for the synthesis of ORNs 1−5 (Table 2). BTT was also used for the synthesis of ORN 6; however, the coupling reaction with compound **23** did not proceed well. Compound **23** was less reactive than the other phosphoramidites **20**−**22**. Therefore, we tested several activators to obtain an optimized coupling reaction using compound **23** at room temperature for 10 min. When 1*H*-tetrazole or BTT was used as the activator, the coupling efficiency was lower than 10%. On the other hand, the use of 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole gave a much better result with more than 90% coupling efficiency, and thereby, 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole was the best choice for our reagent. The release of oligonucleotides from the resin and simultaneous deprotection of the protecting groups were carried out by using 28% NH_4OH at 55 °C for 5 h. In all cases, we obtained the desired products as the main peaks. The side products derived from β-elimination and transamidation were not observed. 3-[(Dimethylaminomethylidene)amino]-3*H*-1,2,4-dithiazole-5-thione (DDTT)[31] was used as a sulfurization agent for the synthesis of ORNs 10 and 12. We characterized the synthesized oligonucleotides by MALDI-TOF.

Hybridization Properties of 2′-*O*-MCE-Oligonucleotides. We measured the T_m values of the duplexes of 2′-*O*-MCE-oligonucleotides with the cDNA and RNA strands in 10 mM sodium phosphate buffer (pH 7.0) in the presence of 100 mM

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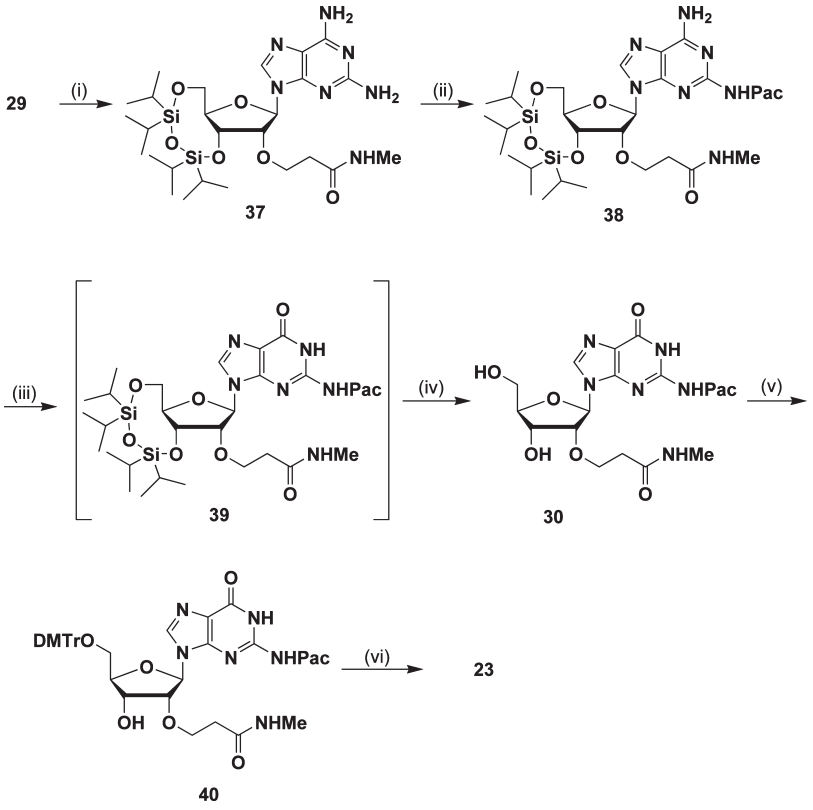
Scheme 5. Synthesis of Phosphoramidite 23[a]



[a] Conditions: (i) $MeNH_2$ in MeOH, EtOH, rt, yield: **37** (77%); (ii) phenoxyacetyl chloride, pyridine, −10 °C, yield: **38** (73%); (iii) $NaNO_2$, AcOH, H_2O, rt; (iv) $Et_3N \cdot 3HF$, Et_3N, THF, rt; yield: **30** (71%); (v) DMTrCl, pyridine, rt, yield: **40** (75%); (vi) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite, diisopropylammonium 1*H*-tetrazolide, MeCN, rt, yield: **23** (80%).

Table 2. Synthesized Oligonucleotides Used in This Study and Mass Data

entry	sequence[a]	backbone[b]	calcd mass	obsd mass
ORN 1	5′-**UUU UUU UUU UUU**-3′	PO	4630.0	4628.8
ORN 2	5′-(2′-*O*-CE)-**UUU UUU UUU UUU**-3′	PO	4236.4	4238.4
ORN 3	5′-(2′-*O*-Me)-UUU UUU UUU UUU-3′	PO	3779.5	3779.5
ORN 4	5′-**AAA AAA AAA AAA**-3′	PO	4908.8	4908.3
ORN 5	5′-(2′-*O*-Me)-CGU AGA CUA UCU C-3′	PO	4241.7	4240.3
ORN 6	5′-(2′-*O*-Me)-CGU A**G**A CUA UCU C-3′	PO	4315.8	4314.8
ORN 7	5′-(2′-*O*-Me)-CGU AG**A** CUA UCU C-3′	PO	4315.8	4315.2
ORN 8	5′-(2′-*O*-Me)-CGU AGA **C**UA UCU C-3′	PO	4315.8	4315.3
ORN 9	5′-(2′-*O*-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3′	PO	10019.8	10025.9
ORN 10	5′-(2′-*O*-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3′	PS	10483.2	10492.2
ORN 11	5′-(2′-*O*-Me)-C**U**C CAA CAG CAA AGA AGA **U**GG CA**U** **UU**C **U**AG-3′	PO	10446.0	10453.6
ORN 12	5′-(2′-*O*-Me)-C**U**C CAA CAG CAA AGA AGA **U**GG CA**U** **UU**C **U**AG-3′	PS	10909.4	10915.1

[a] **B**: 2′-*O*-MCE-RNA. [b] PO, PS means phosphate backbone, and phosphorothioate backbone, respectively.

NaCl and 0.1 mM EDTA. These results are summarized in Table 3. The data were compared with those of natural RNAs and 2′-*O*-methyl RNAs. When 2′-*O*-MCE-U was incorporated into all U's of U_{12} (ORN 1, entry 2), the T_m value of the duplex of 2′-*O*-MCE-U_{12} with the complementary RNA strand was 12 °C higher than that of the duplex derived from natural RNA (entry 1). The ΔT_m value was 1 °C per modification. The degree of this T_m increase was the same as that of 2′-*O*-Me-U_{12}.[17] As opposed to this result, when we incorporated 2′-*O*-MCE-A into all A's of A_{12} (entry 4), we did not observe such a significant T_m increase. Next, the effect of one point 2′-*O*-MCE-ribonucleoside modification on the thermostabilities of the duplexes was examined. We incorporated 2′-*O*-MCE-G, A, and C into the fifth G (ORN 6, entry 6), sixth A (ORN 7, entry 7), and seventh C (ORN 8, entry 8), respectively, of 5′-(2′-*O*-Me)-CGUAGACUAUCUC-3′ (ORN 5, entry 5). The differences in T_m between the modified and unmodified duplexes were −2, +1, and 0 °C for the complementary RNA strand, respectively. Meanwhile, the ΔT_m

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Table 3. Hybridization Affinity of 2′-*O*-MCE-Oligonucleotides

entry	sequence	T_m (°C) vs RNA[a]	T_m (°C) vs DNA[a]
1	5′-UUU UUU UUU UUU-3′	14	9
2	5′-**UUU** **UUU** **UUU** **UUU**-3′ (ORN 1)	26	n.d.
3	5′-AAA AAA AAA AAA-3′	14	n.d.
4	5′-**AAA** **AAA** **AAA** **AAA**-3′ (ORN 4)	15	n.d.
5	5′-(2′-*O*-Me)-CGU AGA CUA UCU C-3′ (ORN 5)	67	49
6	5′-(2′-*O*-Me)-CGU A**G**A CUA UCU C-3′ (ORN 6)	65	45
7	5′-(2′-*O*-Me)-CGU AG**A** CUA UCU C-3′ (ORN 7)	68	48
8	5′-(2′-*O*-Me)-CGU AGA **C**UA UCU C-3′ (ORN 8)	67	48
9	5′-(2′-*O*-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3′ (PO backbone, ORN 9)	85	74
10	5′-(2′-*O*-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3′ (PS backbone, ORN 10)	81	69
11	5′-(2′-*O*-Me)-C**U**C CAA CAG CAA AGA AGA **U**GG CA**U** **UU**C **U**AG-3′ (PO backbone, ORN 11)	85	71
12	5′-(2′-*O*-Me)-C**U**C CAA CAG CAA AGA AGA **U**GG CA**U** **UU**C **U**AG-3′ (PS backbone, ORN 12)	81	73

[a] T_m values were measured in 2 μM oligonucleotide, 10 mM sodium phosphate buffer (pH 7.0), 100 mM NaCl, and 0.1 mM EDTA.

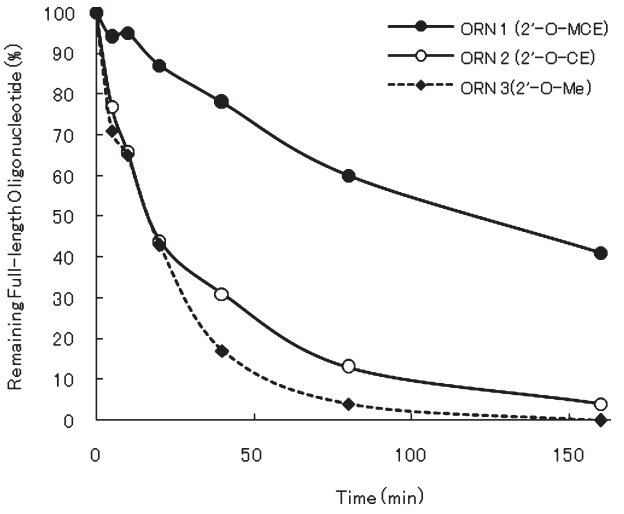

Figure 3. Time courses of degradation of 2′-*O*-MCE, 2′-*O*-CE, and 2′-*O*-methyl oligonucleotides by snake venom phosphodiesterase.

values of the same 2′-*O*-MCE oligonucleotides for the cDNA strand were −4, −1, and −1 °C, respectively. The 2′-*O*-MCE oligonucleotides exhibited stronger binding affinities for RNAs than DNAs, as shown in Table 3 similar to the 2′-*O*-methyl RNAs. Six-point modification of ORNs 9 and 10 with 2′-O-MCE-U showed these modified ORNs 11 and 12 could maintain the original binding affinity for the complementary RNA and DNA strands, as shown in entries 9−12. This property should be suitable for oligonucleotide therapeutics targeting RNAs.

Nuclease Resistance of 2′-*O*-MCE RNA. We examined the 3′-exonuclease resistance of 2′-*O*-MCE oligonucleotides using snake venom phosphodiesterase (SVPD).[32] The 2′-*O*-MCE-oligonucleotide ORN 1 was subjected to the digestion reaction using SVPD. The time course of the remaining full-length ORN 1 was measured by RP-HPLC, as shown in Figure 3. The 3′-exonuclease resistance properties of 2′-*O*-CE oligonucleotide (ORN 2) and 2′-*O*-Me oligonucleotide (ORN 3) were also studied to compare with that of ORN 1. As a result, the 3′-exonuclease resistance of ORN 1 was much higher than those of ORN 2 and ORN 3. Although $t_{1/2}$ of ORN 1 was about 120 min, it was six times larger than those of ORN 2 and ORN 3.

Exon-Skipping Activity of 2′-*O*-MCE RNAs in the Splicing of the Pre-mRNA of Mouse Dystrophin. Because the T_m analysis and nuclease resistance assay of 2′-*O*-MCE oligonucleotides described above demonstrated their potential availability for oligonucleotide therapy targeting specific RNAs, we examined the biological activities of the exon skipping of pre-mRNA of mouse dystrophin.[33] Exon skipping is currently the most promising approach to the oligonucleotide therapy of Duchenne muscular dystrophy.[34] Because antisense oligonucleotides for exon skipping must be delivered to the cellular nucleus in order to modulate the splicing of pre-mRNA, they must possess sufficient stabilities against nucleases in blood and cytoplasm. Once antisense oligonucleotides are delivered into the nucleus successfully, they must hybridize with the targeting pre-mRNA selectively. It was reported that phosphorothioate (PS) oligonucleotides with nuclease resistance[35] were easily delivered into the nucleus once they permeated the cells.[36] We considered that 2′-*O*-MCE-oligonucleotides with phosphorothioate backbones would improve the efficacy of exon skipping by adding further nuclease resistance to the phosphorothioate 2′-*O*-methyl-oligonucleotides and improve the binding toward the complementary RNA strands. Recently, 2′-*O*-methyl-PS-oligonucleotides and morpholino oligonucleotides have been proven to be effective for exon skipping, as exemplified by successful *mdx* mice *in vivo* experiments. Therefore, we synthesized a 2′-*O*-MCE-PS-oligonucleotide of ORN 12 that corresponded to a 30 base sequence (mB30) reported by Arechevala-Gomeza et al.[37] The mB30 sequence was reported to be effective for exon skipping when incorporated into not only 2′-*O*-methyloligoribonucleotides with phosphorothioate linkages but also morpholino oligonucleotides. We incorporated 2′-*O*-MCE-Us into the U residues of 2′-*O*-methyl-phosphorothioate ORN 10. There are a total of 6 uridine residues in mB30, and they are mainly localized at the 3′-end of the strand. To evaluate the influence of the phosphorothioate backbone, we also synthesized phosphate derivatives ORNs 9 and 11. We purchased the antisense morpholino ORN 13 from Gene Tools.

ORNs 9−13 were administrated to the exon 52-deleted *mdx* mouse (*mdx*52) by intramuscular injection.[38] We evaluated the efficacy of exon skipping by the RT-PCR of the total RNAs as described previously.[39] Figure 4 displays the results. The experiments were performed two times. As shown in part a, six-point substitution of 2′-*O*-MCE-U (ORN 12, exon-skipping efficacy = 62%) dramatically enhanced the exon-skipping efficacy compared with that of the 2′-*O*-methyl-PS oligonucleotides (ORN 10, exon-skipping efficacy = 34%). Its efficacy was almost the

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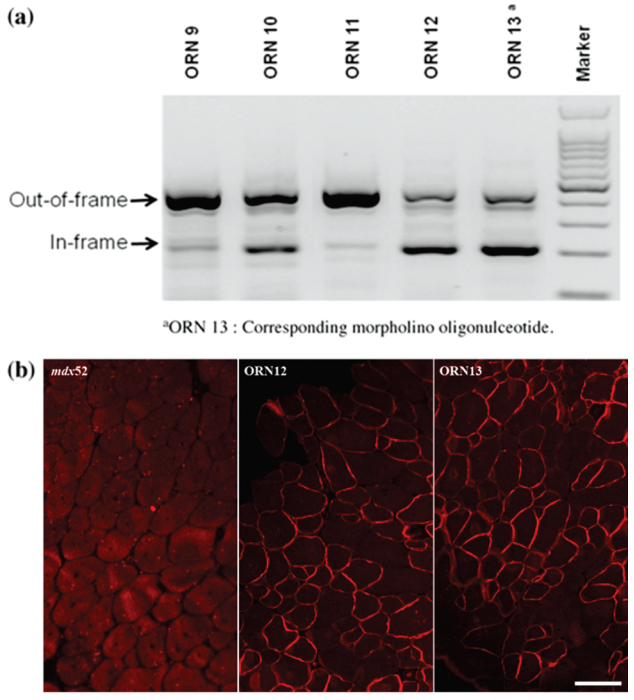

Figure 4. (a) One canonical example of the detection of antisence-mediated exon-skipping in *mdx52* mice by reverse transcriptase polymerase chain reaction (RT-PCR). (b) Immunohistochemical staining of dystrophin in TA muscle of untreated and treated *mdx*52 mice (ORN 12 and ORN 13). There was no expression of dystrophin around the rim of any muscle fibers from untreated *mdx*52 mice. In contrast, restoration of dystrophin around the rim of muscle fibers was observed in both muscles from treated *mdx*52 by ORN12 and ORN 13. Dystrophin was detected with with dystrophin C-terminal antibody (*Dys-2*). Bar = 100 μm.

same as that of the corresponding morpholino nucleic acid (ORN 13, exon-skipping efficacy = 65%). We also evaluated the efficacy of ORN 12 by immunohistochemistry with dystrophin C-terminal antibody (*Dys-2*) as shown in part b. The number of dystrophin positive fibers was almost the same as that which resulted from ORN 13. These results indicated that the incorporation of 2′-*O*-MCE-Us into antisense RNA was effective for exon skipping.

■ CONCLUSIONS

In conclusion, we have developed an effective method for the synthesis of 2′-*O*-alkoxycarbonyl-ribonucleosides via Michael addition by using acrylate esters. We easily converted the MOCE group to the chemically stable MCE group by aminolysis. The amide group of the MCE group was not detrimental to the hybridization and enhanced metabolic stability. The phosphorothioate derivative of a 2′-*O*-Me-oligonucleotide incorporating six 2′-*O*-MCE-Us with a base sequence of mB30 proved to exhibit effective exon skipping in the splicing of the pre-mRNA of mouse dystrophin.

■ EXPERIMENTAL SECTION

Materials and Methods. All reagents and solvents obtained from commercial suppliers were used without further purification. All reactions were carried out under argon atmosphere in oven-dried glassware. NMR chemical shifts are given in parts per million (ppm); *J* values are given in hertz (Hz). ^{1}H and ^{13}C spectra were internally referenced to the appropriate residual undeuterated solvent. ^{31}P NMR was externally referenced with 85% H_3PO_4. Column chromatography was performed with silica gel C-200. Reversed phase HPLC on a C18 column was performed using a linear gradient of acetonitrile in 0.1 M ammonium acetate buffer. Anion-exchange HPLC was performed using a linear gradient of 0−100% solution A (20% CH_3CN in 0.5 M KH_2PO_4) in solution B (20% CH_3CN in 0.005 M KH_2PO_4). Thin-layer chromatography was performed using 60F-254 (0.25 mm). MALDI-TOF was measured in reflectron positive mode. The matrix condition was 3-hydroxypicolinic acid (HPA) 50 mg/mL in MeCN:H_2O (1:1, v/v), diammonium hydrogen citrate (AHC) 100 mg/mL in H_2O.

***N*^{3}-Benzoyl-2′-*O*-(2-methoxycarbonylethyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine (6).** To a solution of N^3-benzoyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine (**5**) (1.80 g, 3.03 mmol) in *t*-BuOH (15 mL) were added methyl acrylate (5.40 mL, 60.2 mmol) and cesium carbonate (489 mg, 1.39 mmol). The solution was stirred at ambient temperature for 4 h. The solution was extracted with EtOAc and saturated NH_4Cl. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/EtOAc (9:1, v/v) gave compound **6** (1.47 g, 2.17 mmol, 71%) as a white foam: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.00 (d, J = 8.0 Hz, 1H), 7.94−7.48 (m, 5H), 5.77 (d, J = 8.0 Hz, 1H), 5.75 (s, 1H), 4.27−4.04 (m, 6H), 4.00 (dd, J = 2.5, 13.5 Hz, 1H), 3.88 (d, J = 4.0 Hz, 1H), 3.62 (s, 3H), 2.60−2.58 (m, 2H), 1.11−0.97 (m, 28H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.7, 168.9, 162.3, 149.1, 139.3, 135.4, 131.4, 130.7, 129.3, 101.6, 89.2, 82.6, 81.9, 68.2, 66.7, 59.5, 51.7, 35.2, 17.6, 17.4, 17.3, 17.1,17.0, 13.6, 13.2, 13.0, 12.6. HRMS m/z calcd for $C_{32}H_{49}N_2O_{10}Si_2$ $[M + H]^+$ 677.2926, found 677.2931.

***N*^{3}-Benzoyl-2′-*O*-(2-ethoxycarbonylethyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine (7).** To a solution of compound **5** (120 mg, 0.20 mmol) in *t*-BuOH (1 mL) were added cesium carbonate (65 mg, 0.20 mmol) and ethyl acrylate (0.43 mL, 4.0 mmol). The solution was vigorously stirred at ambient temperature for 18 h. The solution was extracted with EtOAc and saturated NH_4Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/EtOAc (5:1, v/v) gave compound **7** (83 mg, 0.12 mmol, 60%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.00 (d, J = 8.0 Hz, 1H), 7.93 (d, J = 7.5 Hz, 2H), 7.65 (dd, J = 7.5, 7.5 Hz, 1H), 7.50 (dd, J = 7.5, 7.5 Hz, 1H), 5.77 (d, J = 8.0 Hz, 1H), 5.75 (s, 1H), 4.09−3.88 (m, 9H), 2.65−2.55 (m, 2H), 1.09−0.85 (m, 31H). HRMS m/z calcd for $C_{33}H_{51}N_2O_{10}Si_2$ $[M + H]^+$ 691.3078, found 691.2997.

***N*^{3}-Benzoyl-2′-*O*-[2-[(2,2,2-trifluoroethoxy)carbonyl]ethyl]-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine (8).** To a solution of compound **5** (9.00 g, 15.23 mmol) in *t*-BuOH (75 mL) were added 2,2,2-trifluoroethyl acrylate (30.2 g, 304.67 mmol) and cesium carbonate (4.96 g, 15.23 mmol). The solution was stirred at ambient temperature for 2 h. Then the solution was extracted with EtOAc and saturated NH_4Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica. Elution with hexane/EtOAc (6:1, v/v) gave compound **8** (8.29 g, 11.12 mmol, 73%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.02 (d, J = 7.5 Hz, 2H), 7.85 (d, J = 8.0 Hz, 1H), 7.78 (dd, J = 7.0 Hz, 2H), 7.60 (dd, J = 7.5, 2 Hz), 5.82 (d, J = 8.0, 1 Hz), 5.64 (s, 1H), 4.66 (dd, J = 9.0, 13.0 Hz, 2H), 4.28−4.25 (m, 1H), 4.19−4.17 (m, 2H), 3.99−3.93 (m, 4H), 2.72 (dd, J = 6.0, 2 Hz), 1.05−0.94 (m, 27H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.5, 169.4, 161.9, 148.6, 140.1, 135.5, 131.1, 130.3, 129.4, 100.6, 89.0, 81.2, 68.4, 65.9, 59.7, 59.5, 59.4, 34.3, 17.3, 17.2, 17.1, 17.0, 16.9, 16.84, 16.82, 16.7, 12.8, 12.3, 12.2, 11.9. HRMS m/z calcd for $C_{33}H_{48}F_3N_2O_{10}Si_2$ $[M + H]^+$ 745.2798, found 745.2781.

2′-*O*-(2-Methoxycarbonylethyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine (9). To a solution of compound **6**

(1.71 g, 2.53 mmol) in anhydrous THF (20 mL) was added *n*-$PrNH_2$ (1.5 mL, 18.0 mmol). The solution was vigorously stirred for 4 h. The solution was evaporated under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/EtOAc (1:4, v/v) gave compound **9** (1.03 g, 1.79 mmol, 71%) as a white foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.49 (s, 1H), 7.89 (d, J = 5.0 Hz, 1H), 5.73 (s, 1H), 5.67 (d, J = 5.0 Hz, 1H), 4.24−4.09 (m, 6H), 3.96−3.84 (m, 1H), 3.84 (s, 1H), 2.66−2.65 (m, 1H), 1.09−0.92 (m, 28H). HRMS m/z calcd for $C_{25}H_{45}N_2O_9Si_2$ $[M + H]^+$ 573.2663, found 573.2619.

2′-*O*-[2-(*N*-Methylcarbomoyl)ethyl]-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)uridine (10). To a solution of compound **5** (1.80 g, 3.03 mmol) in *t*-BuOH (15 mL) were added cesium carbonate (489 mg, 1.39 mmol) and methyl acrylate (5.4 mL, 60.2 mmol). The solution was vigorously stirred at ambient temperature for 4 h. The suspension was diluted with EtOAc, and extracted with EtOAc and saturated $NaHCO_3$ aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was treated with 40% $MeNH_2$ in MeOH (30 mL) at ambient temperature for 2 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/EtOAc (1:1, v/v) gave compound **10** (1.14 g, 66%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.90 (br, 1H), 7.91 (d, J = 8.0 Hz, 1H), 6.90 (br, 1H), 5.73−5.71 (m, 2H), 4.29−3.85 (7H, m), 2.81−2.79 (m, 3H), 2.63−2.47 (m, 2H). HRMS m/z calcd for $C_{25}H_{46}N_3O_8Si_2$ $[M + H]^+$ 572.2823, found 572.2826.

2′-*O*-[2-(*N*,*N*-Dimethylcarbamoyl)ethyl]3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine (11). To a solution of compound **8** (240 mg, 0.32 mmol) in THF (3 mL) was added 2 M Me_2NH (2 mL). The solution was stirred for 2 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 5% MeOH in $CHCl_3$ gave compound **11** (152 mg, 0.26 mmol, 81%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.56 (br, 1H), 7.85 (d, J = 5.0 Hz, 1H), 5.74 (s, 1H), 5.65 (d, J = 5.0 Hz, 1H), 4.19−4.06 (m, 5H), 3.92 (m, 1H), 3.85 (s, 1H), 3.03 (s, 3H), 2.91 (s, 3H), 1.01−0.90 (m, 28H). HRMS m/z $C_{26}H_{48}N_3O_8Si_2$ $[M + H]^+$ 586.2980, found 586.2921.

2′-*O*-[2-(*N*-Methylcarbomoyl)ethyl]uridine (13). A solution of compound **10** (563 mg, 0.98 mmol) in dry THF (10 mL) was treated with triethylamine trihydrofluoride (570 μL, 3.50 mmol) and triethylamine (253 μL, 1.80 mmol) at ambient temperature for 1 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 10% MeOH in $CHCl_3$ gave compound **13** (250 mg, 0.75 mmol, 76%): ^{1}H NMR (500 MHz, D_2O) δ 5.95 (d, J = 4.5 Hz, 1H), 5.92 (d, J = 8.5 Hz, 1H), 4.33 (t, J = 5.5 Hz, 1H), 4.17−4.12 (m, 2H), 3.98−3.88 (m, 3H), 3.79 (dd, J = 4.5, 13.0, 1H), 2.72−2.68 (m, 3H), 2.57−2.53 (m, 2H); ^{13}C NMR (125 MHz, D_2O) δ 175.0, 166.7, 152.0, 142.3, 102.9, 88.2, 85.1, 81.8, 68.9, 67.2, 61.1, 36.5, 26.4. HRMS m/z calcd for $C_{13}H_{20}N_3O_7$ $[M + H]^+$ 330.1301, found 330.1307.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(methoxycarbonyl)ethyl]uridine (15). To a solution of compound **9** (780 mg, 1.37 mmol) in anhydrous THF (13 mL) was added $Et_3N\cdot 3HF$ (780 μL, 4.77 mmol). The solution was stirred at ambient temperature for 2 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 10% MeOH in $CHCl_3$ gave compound **12** as an intermediate, which was coevaporated three times with dry pyridine and finally dissolved in dry pyridine (13 mL). To the solution was added DMTrCl (510 mg, 1.50 mmol). The solution was stirred vigorously for 4 h. Then the solution was extracted with EtOAc and brine. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with EtOAc containing 0.5% Et_3N gave compound **15** (780 mg, 1.23 mmol, 90%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.78 (br, 1H), 7.95 (d, J = 8.0 Hz, 1H), 7.28−7.21 (m, 9H), 6.82−6.80 (m, 4H), 5.91 (d, J = 1.5 Hz, 1H), 5.26 (d, J = 8.0 Hz, 1H), 4.49−4.45 (m, 1H), 4.04−3.92 (m, 4H), 3.76 (s, 6H), 3.68 (s, 3H), 3.50−3.45 (m, 3H), 2.70−2.56 (m, 2H). HRMS m/z calcd for $C_{34}H_{36}N_2NaO_{10}$ $[M + Na]^+$ 655.2268, found 655.2269.

(4,4′-Dimethoxytrityl)-2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]-uridine (16). To a solution of compound **13** (553 mg, 1.67 mmol) in dry pyridine (15 mL) was added DMTrCl (622 mg, 1.84 mmol). The solution was stirred at ambient temperature for 4 h. The solution was diluted with EtOAc and washed with saturated $NaHCO_3$ aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 2% MeOH in $CHCl_3$ containing 0.5% triethylamine gave compound **16** (740 mg, 1.17 mmol, 70%) as a white foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.95 (d, J = 8.0 Hz, 1H), 7.39−7.22 (9H, m), 6.86−6.83 (m, 4H), 5.93 (d, J = 3.0 Hz, 1H), 5.86 (br, 1H), 5.23 (d, J = 8.0 Hz, 1H), 3.97−3.90 (m, 2H), 3.79 (s, 3H), 3.54−3.48 (m, 2H), 2.81 (m, 3H), 2.61−2.43 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.2, 163.6, 158.8, 150.8, 144.5, 140.3, 135.5, 135.3, 130.3, 130.2, 128.1, 127.3, 113.4, 102.4, 87.9, 87.2, 83.7, 82.9, 69.2, 66.6, 62.1, 55.4, 35.9, 26.5. HRMS m/z calcd for $C_{34}H_{37}N_3NaO_9$ $[M + Na]^+$ 654.2427, found 654.2446.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(*N*,*N*-dimethylcarbamoyl)-ethyl]uridine (17). To a solution of compound **11** (3.61 g, 6.18 mmol) in THF (60 mL) was added $Et_3N\cdot 3HF$ (3.4 mL, 21.6 mmol). The solution was vigorously stirred for 4 h. The solution was evaporated under reduced pressure. The residue was chromatographed on silica gel. Elution with 10% MeOH in $CHCl_3$ gave compound **14** as an intermediate, which was coevaporated three times with anhydrous pyridine three times. The residue was dissolved in anhydrous pyridine (60 mL). To the solution was added DMTrCl (2.30 g, 6.80 mmol). The solution was vigorously stirred overnight. The solution was evaporated under reduced pressure. The residue was chromatographed on silica gel. Elution with 5% MeOH in $CHCl_3$ containing 0.5% Et_3N gave compound **17** (2.78 g, 4.32 mmol, 70%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.72 (br, 1H), 7.92 (d, J = 8.0 Hz, 1H), 7.29−7.21 (m, 9H), 6.84−6.82 (m, 4H), 5.96 (d, J = 3.5 Hz, 1H), 5.36 (d, J = 3.5 Hz, 1H), 5.28 (d, J = 8.0 Hz, 1H), 4.59−4.56 (m, 1H), 4.11−4.07 (m, 2H), 4.00−3.98 (m, 1H), 3.90−3.87 (m, 1H), 3.79 (s, 6H), 3.54−3.44 (m, 2H), 3.00 (s, 3H), 2.96 (s, 3H), 2.78−2.75 (m, 1H), 2.45−2.41 (m, 1H). HRMS m/z calcd for $C_{35}H_{39}N_3NaO_9$ $[M + Na]^+$ 668.2584, found 668.2582.

Triethylamine Salt of 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(2-carboxyethyl)uridine (18). To a solution of compound **15** (10 mg, 0.015 mmol) in pyridine (1 mL) was added 0.1 M NaOH (1 mL). The solution was stirred at ambient temperature for 30 min. The solution was extracted with $CHCl_3$ and 10% citric acid in H_2O. To the organic solution was added Et_3N (1 mL). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to give compound **18** (7 mg, 0.0097 mmol, 65%): ^{1}H NMR ($CDCl_3$, 500 MHz) 8.08−7.16 (m, 14H), 6.83 (d, J = 10.0 Hz, 1H), 6.00 (d, J = 5.0 Hz, 1H), 5.31 (d, J = 10.0 Hz, 1H), 4.52−4.53 (m, 1H) 4.10−3.92 (m, 5H), 3.78 (s, 6H), 3.64−3.63 (m, 2H), 3.12−3.08 (m, 6H), 2.67−2.51 (m, 2H), 1.29−1.27 (m, 9H). HRMS m/z calcd for $C_{33}H_{34}N_2NaO_{10}$ $[M + Na]^+$ 641.2111, found 641.2174.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(2-carbamoylethyl)uridine-(19). To a solution of compound **15** (10 mg, 0.015 mmol) in pyridine (1 mL) was added 28% NH_4OH (1 mL). The solution was stirred at ambient temperature for 30 min. The solution was concentrated under reduced pressure to give compound **19** (10 mg, 0.015 mmol, 99%): ^{1}H NMR (CD_3CN, 500 MHz) δ 7.79 (d, J = 8.0 Hz, 1H), 7.53−7.34 (m, 11H), 6.98−6.96 (m, 11H), 6.50 (br, 1H), 5.99 (br, 1H), 5.89 (d, J = 4.0 Hz, 1H), 5.53 (s, 1H), 5.38 (d, J = 8.0 Hz, 1H), 4.48−4.46 (m, 1H), 4.08−4.05 (m, 2H), 3.92−3.86 (m, 2H), 3.50 (s, 6H), 3.41−3.39 (m, 2H), 2.60−2.51 (m, 2H); ^{13}C NMR (CD_3CN, 125 MHz) δ 174.9, 163.9, 159.7, 159.7, 151.4, 145.8, 141.1, 136.6, 136.4, 131.0, 131.0, 129.0, 128.9, 127.9, 118.3, 114.1, 114.1, 102.5, 88.3, 87.5, 84.2, 82.8, 70.0, 66.9, 63.4, 55.9, 35.7. HRMS m/z $C_{33}H_{35}N_3NaO_9$ $[M + Na]^+$ 640.2271, found 640.2257.

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2′-*O*-[2-(Methoxycarbonyl)ethyl]-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (26). A solution of 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (**24**) (10.0 g, 19.6 mmol) in *t*-BuOH (100 mL) was treated with cesium carbonate (6.4 g, 19.6 mmol) at ambient temperature for 30 min. To the suspension was added methyl acrylate (35.5 mL, 392 mmol). The suspension was vigorously stirred for 9 h. The suspension was extracted with EtOAc and saturated NH_4Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromagraphed on silica gel. Elution with 0.5% MeOH in $CHCl_3$ gave compound **26** (8.40 g, 14.1 mmol, 72%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.19 (s, 1H), 8.07 (s, 1H), 7.35−7.25 (br, 2H), 5.92 (s, 1H), 4.33 (dd, J = 4.0 Hz, 6.5 Hz, 1H), 4.47 (d, J = 4.0 Hz, 1H), 4.04−4.02 (m, 3H), 3.92−3.90 (m, 3H), 2.65−2.55 (m, 2H), 1.06 −0.95 (m, 28 H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.3, 156.0, 152.5, 148.5, 139.2, 119.2, 87.5, 81.3, 80.6, 70.0, 66.5, 60.3, 51.2, 34.7, 17.2, 17.14, 17.10, 17.0, 16.9, 16.8, 16.7, 12.7, 12.3, 12.1, 12.1. HRMS m/z calcd for $C_{26}H_{45}N_5NaO_7Si_2$ $[M + Na]^+$ 618.2758, found 618.2772.

2′-*O*-[2-(Methoxycarbonyl)ethyl]-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)cytidine (27). To the solution of 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)cytidine (**25**) (5.0 g, 10.3 mmol) in *t*-BuOH (50 mL) was added cesium carbonate (3.33 g, 10.3 mmol). The suspension was stirred at ambient temperature for 30 min. To the suspension was added methyl acrylate (22.5 mL, 392 mmol). The suspension was vigorously stirred for 6 h. The suspension was extracted with EtOAc and saturated NH_4Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 2% MeOH in $CHCl_3$ gave compound **27** (4.41 g, 7.73 mmol, 75%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.68 (d, J = 7.5 Hz, 1H), 7.19 (br, 1H), 5.68 (d, J = 7.5 Hz, 1H), 5.59 (s, 1H), 4.16−4.12 (m, 2H), 4.01−3.83 (m, 5H), 3.58 (s, 3H), 2.63−2.60 (t, J = 6.5 Hz, 2H), 1.06−0.92 (m, 28H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.1, 166.3, 155.8, 140.8, 93.9, 89.4, 82.4, 81.4, 68.1, 66.5, 59.7, 51.7, 35.2, 17.6, 17.6, 17.4, 17.4, 17.2, 17.14, 17.11, 17.0, 13.5, 13.1, 13.0, 12.7. HRMS m/z calcd for $C_{25}H_{46}N_3O_8Si_2$ $[M + H]^+$ 572.2832, found 572.2738.

2′-*O*-[2-(Methoxycarbonyl)ethyl]-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-aminoadenosine (29). To a solution of 3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-aminoadenosine (**28**) (11.0 g, 21.0 mmol) in *t*-BuOH (110 mL) were added cesium carbonate (7.03 g, 21.6 mmol) and methyl acrylate (39.1 mL, 431 mmol). The solution was stirred at ambient temperature for 18 h. The suspension was extracted with $CHCl_3$ and saturated NH_4Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ and absorbed with silica gel. The silica gel was subjected to a column. Elution with 3% MeOH in $CHCl_3$ gave compound **29** (10.1 g, 16.5 mmol, 79%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.74 (s, 1H), 6.82−6.78 (br, 2H), 5.75 (s, 1H), 5.72 (s, 2H), 4.56 (dd, J = 5.0, 10 Hz, 1H), 4.30 (d, J = 5.0 Hz, 1H), 4.06−4.02 (m, 2H), 3.93−3.88 (m, 3H), 3.55 (s, 3H), 2.63−2,60 (m, 2H), 1.08−0,94 (m, 28H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.3, 160.3, 156.1, 151.0, 134.5, 113.3, 86.3, 81.2, 80.5, 69.9, 66.3, 60.2, 51.3, 34.7, 17.3, 17.2, 17.1, 17.0, 16.9, 16.87, 16.80, 12,8, 12.4, 12.2, 12.1. HRMS m/z calcd for $C_{26}H_{47}N_6O_7Si_2$ $[M + H]^+$ 611.3039, found 611.3038.

6-*N*-Acetyl-2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]adenosine (33). To a solution of compound **26** (4.70 g, 7.90 mmol) in EtOH (90 mL) was added 40% $MeNH_2$ in MeOH (90 mL). The solution was stirred at ambient temperature for 18 h. Then the solution was concentrated under reduced pressure. The residue was coevaporated three times with dry pyridine and finally dissolved in dry pyridine (90 mL). To the solution was added acetyl chloride (790 μL, 11.1 mmol). The solution was vigorously stirred for 6 h. The solution was diluted with EtOAc, and then extracted with EtOAc and saturated $NaHCO_3$ aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure to give compound **31** as an intermediate. The residue was coevaporated with toluene and dissolved in dry THF. To the solution were added triethylamine and triethylamine trihydrofluoride. The solution was vigorously stirred for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel without aqueous workup. Elution with 3.5% MeOH in $CHCl_3$ gave compound **33** (1.87 g, 4.74 mmol, 60%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.75−10.65 (br, 1H), 8.69 (s, 1H), 8.65 (s, 1H), 7.83 (d, J = 4.5 Hz, 1H), 6.06 (d, J = 6.0 Hz, 1H), 5.34 (d, J = 3.5 Hz, 1H), 5.18 (dd, J = 5.0 Hz), 4.57 (dd, J = 6.0 Hz, 1H), 4.41 (d, J = 3.0 Hz, 1H), 3.99 (d, J = 3.0 Hz, 1H), 3.08−3.76 (m, 1H), 3.69−3.33 (m, 3H), 2.51−2.52 (d, J = 4.5 Hz, 3H), 2.33−2.30 (m, 2H), 2.25 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.8, 168.8, 151.7, 151.6, 149.6, 142.7, 123.7, 86.1, 85.6, 80.9, 68.9, 65.6, 61.2, 35.2, 25.4, 24.3. HRMS m/z calcd for $C_{16}H_{22}N_6NaO_6$ $[M + Na]^+$ 417.1499, found 417.1498.

4-*N*-Acetyl-2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]cytidine (34). Compound **27** (2.90 g, 5.08 mmol) was treated with 40% $MeNH_2$ in MeOH (50 mL) at ambient temperature for 6 h. The solution was concentrated under reduced pressure and coevaporated three times with dry pyridine. The residue was dissolved with dry pyridine (50 mL), and to the solution was added acetyl chloride (398 μL, 5.58 mmol). The solution was vigorously stirred for 4 h. The solution was concentrated under reduced pressure, and coevaporated with toluene to give compound **32** as an intermediate. The residue was dissolved in THF, and to the solution were added TEA (1.25 mL, 8.63 mmol) and TEA·3HF (2.92 mL, 17.77 mmol). The solution was vigorously stirred for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 7% MeOH in $CHCl_3$ gave compound **34** (994 mg, 2.68 mmol, 53%) as white foam: ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.88 (s, 1H), 8.43 (d, J = 7.5 Hz, 1H), 7.84 (d, J = 4.0 Hz, 1H), 7.18 (d, J = 7.5 Hz, 1H), 5.80 (d, J = 2.5 Hz, 1H), 5.21 (dd, J = 5.0 Hz, 1H), 5.17 (d, J = 6.0 Hz, 1H), 4.08 (dd, J = 4.0, 6.0 Hz, 1H), 3.86−3.39 (m, 6H), 2.56 (d, J = 4.0 Hz, 1H), 2.39−2.35 (m, 2H), 2.09 (s, 3H). HRMS m/z calcd for $C_{15}H_{22}N_4O_7$ $[M + H]^+$ 371.1556, found 371.1516.

6-*N*-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]adenosine (35). To a solution of compound **33** (1.50 g, 3.80 mmol) in dry pyridine was added DMTrCl (1.05 g, 3.24 mmol). The solution was stirred at ambient temperature for 5 h. The solution was extracted with $CHCl_3$ and brine. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 3% MeOH in $CHCl_3$ containing 0.5% Et_3N gave compound **35** (1.95 g, 2.80 mmol, 73%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.70 (br, 1H), 8.57 (s, 1H), 8.56 (s, 1H), 7.83−7.82 (m, 1H), 7.33−7.17 (m, 9H), 6.84−6.80 (m, 4H), 6.09 (d, J = 5.0 Hz, 1H), 5.38 (d, J = 5.0 Hz, 1H), 4.71−4.69 (m, 1H), 4.49−4.47 (m, 1H), 4.09−4.08 (m, 1H), 3.82−3.67 (m, 8H), 3.21−3.16 (m, 2H), 2.50 (s, 3H), 2.33−2.25 (m, 2H), 2.11 (s, 3H). HRMS m/z calcd for $C_{37}H_{41}N_6O_8$ $[M + H]^+$ 697.2986, found 697.2946.

4-*N*-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]cytidine (36). To a solution of compound **34** (374 mg, 1.00 mmol) in dry pyridine (6 mL) was added DMTrCl (244 mg, 0.72 mmol). The solution was stirred at ambient temperature for 5 h. The solution was extracted with $CHCl_3$ and brine. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 6% MeOH in $CHCl_3$ containing 0.5% Et_3N gave compound **36** (350 mg, 0.52 mmol, 52%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.49 (br, 1H), 8.48 (d, J = 7.5 Hz, 1H), 7.41−7.23 (m, 9H), 6.85−6.83 (m, 4H), 6.52 (br, 1H), 5.89 (s, 1H), 4.49−4.45 (m, 2H), 4.15−3.92 (m, 4H), 3.79 (s, 3H), 3.79 (s, 3H), 3.54−3.54 (m, 2H), 2.76 (d, 3H),

2.62−2.39 (m, 2H), 2.18 (s, 3H). HRMS m/z calcd for $C_{36}H_{40}N_4NaO_9$ $[M + Na]^+$ 695.2693, found 695.2694.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(*N*-methylcarbamoyl)-ethyl]uridine-3-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (20). To a solution of compound **16** (490 mg, 0.77 mmol) in dry MeCN (2 mL) were added diisopropylammonium 1*H*-tetrazolide (101 mg, 1.19 mmol) and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (358 mg, 1.19 mmol). The solution was stirred under argon atmosphere at ambient temperature for 8 h. The solution was diluted with EtOAc and washed with saturated $NaHCO_3$ aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 1% MeOH in $CHCl_3$ containing 0.5% triethylamine gave compound **20** (384 mg, 0.46 mmol, 60%) as a white foam: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.09−8.01 (m, 1H), 7.47−7.27 (m, 9H), 6.86−6.82 (m, 4H), 6.49 (br, 1H), 5.90 (m, 1H), 5.28−5.21 (m, 1H), 4.61−4.48 (m, 1H), 4.25−3.86 (m, 4H), 3.80 −3.79 (m, 1H), 3.75−3.43 (m, 6H), 2.80−2.77 (m, 3H), 2.64−2.42 (m, 4H), 1.29−1.03 (m, 12H); ^{31}P NMR (125 MHz, $CDCl_3$) δ 151.32, 150.48. HRMS m/z calcd for $C_{43}H_{55}N_5O_{10}P$ $[M + H]^+$ 832.3687, found 832.3650.

6-*N*-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]adenosine-3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (21). To a solution of compound **35** (1.00 g, 1.44 mmol) in dry MeCN (150 mL) were added 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (920 μL, 2.87 mmol) and diisopropylammonium 1*H*-tetrazolide (147 mg, 0.86 mmol) at ambient temperature for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 3% MeOH in $CHCl_3$ gave compound **21** (980 mg, 1.09 mmol, 76%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.63−8.61 (m, 2H), 8.26−8.22 (m, 1H), 7.42−7.21 (m, 9H), 6.82−6.79 (m, 4H), 6.41−6.38 (m, 1H), 6.16−6.15 (m, 1H), 4.69−4.59 (m, 2H), 4.40−4.33 (m, 1H), 4.04−3.97 (m, 1H), 3.90−3.85 (m, 2H), 3.79−3.62 (m, 6H), 3.60−3.52 (s, 4H), 3.39−3.35 (m, 1H); ^{31}P NMR (125 MHz, $CDCl_3$) δ 151.34, 151.30. HRMS m/z calcd for $C_{46}H_{58}N_8O_9P$ $[M + H]^+$ 897.4064, found 897.4022.

4-*N*-Acetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]cytidine-3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (22). To a solution of compound **36** (480 mg, 0.71 mmol) in dry MeCN (2 mL) were added diisopropylammonium 1*H*-tetrazolide (92 mg, 0.54 mmol) and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (343 μL, 1.07 mmol). The solution was stirred at ambient temperature for 10 h. The solution was extracted with $CHCl_3$ and brine. The organic layer was dried, collected over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 1.5% MeOH in $CHCl_3$ containing 0.5% Et_3N gave compound **22** (300 mg, 0.34 mmol, 48%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.70−9.67 (m, 1H), 8.63−8.56 (m, 1H), 7.34−6.86 (m. 11H), 5.93 (s, 1H), 4.57−4.48 (m, 1H), 4.30−4.02 (m 4H), 3.84−3.83 (m, 6H), 3.73−3.46 (m, 6H), 2.81−2.62 (m, 4H), 2.43−2.37 (m, 2H), 2.26 (s, 3H), 1.27−1.03 (m, 12H); ^{31}P NMR (125 MHz, $CDCl_3$) δ 151.43, 150.23. HRMS m/z calcd for $C_{45}H_{58}N_6O_{10}P$ $[M + H]^+$ 873.3952, found 872.3911.

2′-*O*-[2-(*N*-Methylcarbamoyl)ethyl]-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-aminoadenosine (37). To a solution of compound **29** (1.0 g, 1.64 mmol) in EtOH (8 mL) was added 40% methylamine in MeOH (8 mL) at ambient temperature for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 4% MeOH in $CHCl_3$ gave compound **37** (770 mg, 1.26 mmol, 77%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.76−7.74 (m, 2H), 6.82−6.76 (br, 2H), 5.75 (s, 1H), 5.73 (s, 2H), 4.54 (dd, J = 5.0, 8.0 Hz, 1H), 4.27 (d, J = 5.0 Hz, 1H), 4.06−3.98 (m, 2H), 3.94−3.90 (m, 2H), 3.87−3.82 (m, 1H), 2.53 (d, J = 4.0 Hz, 3H), 2.37 (dd, J = 6 Hz, 2H), 1.04−1.02 (m, 28H). HRMS m/z calcd for $C_{26}H_{48}N_7O_6Si_2$ $[M + H]^+$ 610.3198, found 610.3183.

2′-*O*-[2-(*N*-Methylcarbamoyl)ethyl]-2-*N*-phenoxyacetyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-aminoadenosine (38). To a solution of compound **37** (500 mg, 0.82 mmol) in dry pyridine (20 mL) at −10 °C was dropwise added phenoxyacetyl chloride (124 μL, 0.90 mmol) over 5 min. The solution was vigorously stirred at −10 °C for 2 h and then at ambient temperature for 1 h. The solution was diluted with $CHCl_3$ and extracted with brine. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. Elution with 5% MeOH in $CHCl_3$ gave compound **38** (442 mg, 0.60 mmol, 73%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.00 (s, 1H), 8.04 (s, 1H), 7.68 (d, J = 4.0 Hz, 1H), 7.50−7.38 (br, 2H), 7.29−7.26 (m, 2H), 6.37−6.91 (m, 3H), 5.88 (s, 1H), 5.02 (s, 2H), 4.53 (dd, J = 5.0, 8 Hz, 1H), 4.38 (d, J = 8.0 Hz, 1H), 4.11−4.08 (m, 1H), 4.03−3.86 (m, 4H), 2.49 (m, 3H), 2.33 (dd, J = 7.0, 7.0 Hz, 2H), 1.04−0.93 (m, 28H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.1, 158.0, 156.1, 152.5, 149.4, 137.2, 129.4, 120.8, 116.1, 114.5, 86.8, 81.0, 80.9, 69.7, 67.4, 67.1, 60.4, 40.0, 36.1, 25.3, 17.3, 17.16, 17.12, 17.0, 16.9 16.8, 12.7, 12.3, 12,1, 12.0. HRMS m/z calcd for $C_{34}H_{53}N_7NaO_8Si_2$ $[M + Na]^+$ 766.3392, found 766.3400.

2′-*O*-[2-(*N*-Methylcarbamoyl)ethyl]-2-*N*-phenoxyacetylguanosine (30). To a stirred solution of compound **38** (1.00 g, 1.34 mmol) in AcOH (100 mL) were added H_2O (40 mL) and $NaNO_2$ (752 mg, 10.75 mmol). After 30 min another portion of $NaNO_2$ (752 mg, 10.75 mmol) was added. The solution was stirred at ambient temperature for 48 h. The solution was concentrated under reduced pressure to give a yellow syrup. The syrup was extracted once with brine and three times with saturated $NaHCO_3$ aq. The organic layer was dried, collected over sodium sulfate, and concentrated under reduced pressure to give compound **39** as an intermediate. The residue was coevaporated with dry pyridine. The residue was dissolved in anhydrous THF (130 mL). To the solution were added triethylamine (340 μL) and triethylamine trihydrofluoride (650 μL). The solution was stirred vigorously at ambient temperature for 6 h. The solution was concentrated under reduced pressure. The residue was extracted with H_2O and $CHCl_3$. After several minutes, white precipitates appeared in the aqueous layer. The aqueous layer was concentrated to reduce its volume. The precipitate was collected by filtrtion and dried. Finally, it was dried by vacuum and gentle heating to give compound **30** (490 mg, 0.97 mmol, 71%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.84 (s, 1H), 11.78 (s, 1H), 8.29 (s, 1H), 7.89 (d, J = 4.0 Hz, 1H), 7.32−7.29 (m, 2H), 6.99−6.97 (m, 3H), 5.87 (d, J = 6.5 Hz, 1H), 5.30 (d, J = 3.5 Hz, 1H, diminished with D_2O), 5.09 (dd, J = 5.5, 5.5 Hz, 1H, diminished with D_2O), 4.86 (s, 2H), 4.44−4.42 (m, 1H), 4.38−4.37 (m, 1H), 3.95 (d, J = 2.0 Hz, 1H), 3.79−3.76 (m, 1H), 3.66−3.55 (m, 3H), 2.54 (d, J = 4.0 Hz, 3H), 2.33 (s, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.05, 171.01, 157.6, 154.8, 148.7, 147.4, 137.8, 129.5, 121.3, 120.3, 114.5, 86.1, 84.5, 81.2, 68.9, 66.2, 65.4, 61.3, 35.1, 25.0. HRMS m/z calcd for $C_{22}H_{26}N_6NaO_8$ $[M + Na]^+$ 525.1710, found 525.1715.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(*N*-methylcarbamoyl)-ethyl]-2-*N*-phenoxyacetylguanosine (40). To a solution of compound **30** (330 mg, 0.66 mmol) in pyridine (6 mL) was added DMTrCl (244 mg, 0.72 mmol). The solution was stirred at ambient temperature for 3 h. The solution was diluted with $CHCl_3$ and extracted with saturated $NaHCO_3$ aq. The organic layer was dried, collected over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with TEA/MeOH/$CHCl_3$ (0.5:2:98, v/v/v) gave compound **40** (401 mg, 0.50 mmol, 75%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.95−11.85 (m, 2H), 8.13 (s, 1H), 7.89 (d, J = 4.5 Hz, 1H), 7.36−7.21 (m, 11H), 7.00−6.97 (m, 3H), 6.85−6.82 (m, 4H), 5.92 (d, J = 5.5 Hz, 1H), 5.36 (d, J = 4.5 Hz, 1H, diminished with D_2O), 4.86 (dd, J = 15.5, 22.5 Hz, 2H), 4.50 (dd, J = 5.5, 10.0 Hz, 1H), 4.39−4.36 (m, 1H), 4.05−4.04 (m, 1H), 3.84−3.82 (m, 1H), 3.76−3.68 (m, 7H), 3.30−3.27 (m, 1H), 3.17−3.15 (m, 1H), 2.55 (d, J = 4.5 Hz, 3H), 2.38−2.34 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.42, 170.78, 158.49, 156.97, 148.39, 144.44, 137.31, 135.53,

135.48, 130.00, 129.61, 128.08, 127.80, 126.88, 122.16, 121.26, 114.64, 114.53, 113.12, 86.50, 84.19, 82.24, 69.67, 66.94, 66.18, 63.34, 60.33, 55.12, 45.98, 35.61, 26.22, 14.12, 9.03. HRMS *m*/*z* calcd for $C_{43}H_{45}N_6O_{10}$ $[M + H]^+$ 805.3119, found 805.3112.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-[2-(*N*-methylcarbamoyl)-ethyl]-2-*N*-phenoxyacetylguanosine-3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (23). To a solution of compound **40** (500 mg, 0.62 mmol) in dry MeCN (50 mL) were added 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (477 μL, 1.49 mmol) and diisopropylammonium 1*H*-tetrazolide (64 mg, 0.37 mmol). The solution was stirred at ambient temperature for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 2% MeOH in $CHCl_3$ gave compound **23** (498 mg, 0.49 mmol, 80%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.91−7.87 (m, 1H), 7.47−7.26, (m, 13H), 7.23−7.20, (m, 1H), 7.07−7.04 (m, 1H), 6.99−6.95 (m, 2H), 6.83−6.80 (m, 2H), 6.16−6.00 (m, 1H), 5.99−5.93 (m, 1H), 4.74−4.70 (m, 2H), 4.46−3.84 (m, 3H), 3.78−3.77 (m, 6H), 3.67−3.32 (m, 4H), 2.74−2.73 (m, 3H), 2.66−2.35 (m, 5H), 1.29−1.00 (m, 12H). HRMS *m*/*z* calcd for $C_{52}H_{61}N_8O_{11}PNa$ $[M + Na]^+$ 1027.4095, found 1027.4098.

Oligonucleotide Synthesis. RNA oligonucleotides (Table 2) were synthesized on an Applied Biosystems 392 oligonucleotide synthesizer on a 1 μmol scale, using a 0.1 M solution each of **20**, **21**, **22**, and **23** with commercially available 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-methyl-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite monomers of uridine, 4-*N*-benzoylcytidine, 6-*N*-benzoyladenosine (ABz), and 2-*N*-isobutyrylguanosine (GiBu). A 0.25 M solution of 5-ethiothio-1*H*-tetrazole in dry acetonitrile or a 0.25 M solution of 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole in dry acetonitrile was used as the activator for the coupling reaction. Coupling time was set to be 10 min. [(Dimethylaminomethylidene)amino]-3*H*-1,2,4-dithiazole-3-thione (DDTT)[42] was used as the sulfurization agent for the phosphorothioate synthesis. After completion of the synthesis, the release of 2′-*O*-modified oligonucleotides from the support and deprotection of all protecting groups were simultaneously carried out in NH_4OH (1 mL) at ambient temperature for 2 h, and then CPG was filtrated. The filtrate was heated at 55 °C for 5 h. The filtrate was concentrated under reduced pressure and then subjected to C-18 cartridge purification. Each product was purified by using anion-exchange chromatography.

T_m Experiments. Each oligonucleotide (1.0 nmol) was dissolved in 10 mM sodium phosphate buffer (pH 7.0, containing 150 mM NaCl and 0.1 mM EDTA) (500 μL) to arrange the final concentration of each oligonucleotide to be 2 μM. The solution was separated into quartz cells (10 mm) and incubated at 85 °C. After 10 min, the solution was cooled to 5 °C at a rate of annealing and melting, the absorption at 260 nm was recorded and used to draw UV-melting curves. T_m values were calculated as the temperature that gave the maximum of the first derivative of the UV−melting curve.

Nuclease Resistance Assay. Each oligonucleotide (10.0 nmol) was dissolved in Tris-HCl buffer (at pH 8.5, 72 mM NaCl, 14 mM $MgCl_2$ at 37 °C) (990 μL). To the solution was added snake venom phosphodiesterase (5×10^{-4} unit/mL) (10 μL) to arrange the final concentration of each oligonucleotide to be 10 μM. Aliquots of the reaction solution were removed at indicated times, quenched by heating at 100 °C for 2 min. The mixture was analyzed by anion exchange HPLC.

Delivery Method. Eight weeks old *mdx52* mice were used. As antisense oligonucleotides against exon 51 of dystrophin gene, ORNs 9−12 were used. Each antisense oligonucleotide (20 μg) was dissolved in saline. Ten micrograms of antisense oligonucleotide was injected into each tibialis anterior muscle with lipofectamine 2000. Two weeks after injection, mice were sacrificed and muscles were dissected. Total RNA was extracted from frozen tissue and 50 ng of total RNA was used for one-step RT-PCR according to the manufacturer's instructions. The primer sequences were Ex50F 5′-TTTACTTCGGGAGCTGAGGA-3′ and Ex53R 5′-ACCTGTTCGGCTTCTTCCTT-3′ for amplification of cDNA from exons 50−53. The PCR conditions were 95 °C for 4 min, then 35 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 7 min. The intensity of PCR bands was analyzed by using ImageJ software (http://rsbweb.nih.gov/ij/), and skipping efficiency was calculated by using the following formula [(the intensity of skipped band)/(the intensity of skipped band + the intensity of unskippedband)], after the resulting PCR bands were extracted using a gel extraction kit.

Immunohistochemistry. Eight micrometer cryosections were cut from flash-frozen muscle, then placed on poly-L-lysine-coated slides and air-dried. The sections were stained with monoclonal mouse antibody *Dys*-2 against C-terminus of cardiac muscular dystrophin from normal mouse used as a secondary antibody. 4′,6-Diamidino-2-phenylindole containing a mounting agent was used for nuclear counterstaining.

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR charts. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*Email: msekine@bio.titech.ac.jp.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published to the Web on March 23, 2011, with errors in schemes 1 and 3. These errors were fixed when the paper was published to the Web on March 29, 2011.