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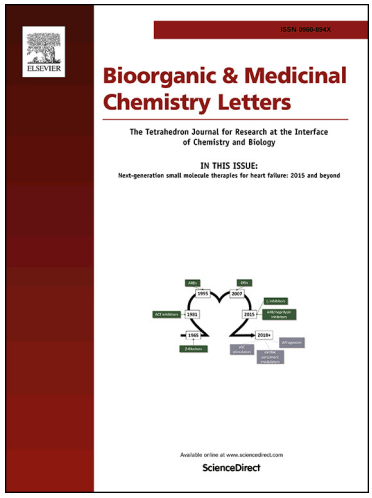

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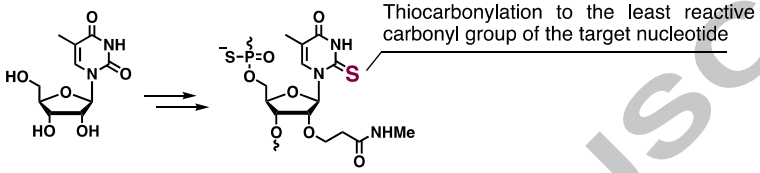

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Synthesis of 2′-*O*-(*N*-methylcarbamoylethyl) 5-methyl-2-thiouridine and its application to splice-switching oligonucleotides

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ABSTRACT

The effect of 2′-*O*-(*N*-methylcarbamoyl)ethyl (MCE) modification on splice-switching oligonucleotides (SSO) was systematically evaluated. The incorporation of five MCE nucleotides at the 5′-termini of SSOs effectively improved the splice switching effect. In addition, the incorporation of 2′-*O*-(*N*-methylcarbamoylethyl)-5-methyl-2-thiouridine (s^2T_{MCE}), a duplex-stabilizing nucleotide with an MCE modification, into SSOs further improved splice switching. These SSOs may be useful for the treatment of genetic diseases associated with splicing errors.

Alternative splicing allows the production of multiple isoforms from a single gene and provides an additional layer of regulation. Alternative splicing occurs in nearly 95% of mammalian genes.[1] Thus, it may not be surprising that at least 15% and perhaps 50% of human genetic diseases result from the disruption of the formal splicing process by genetic mutations.[2] Mutations affecting the splicing process, such as cryptic splicing, exon inclusion, exon exclusion, and intron retention, could disrupt the open reading frame and/or cause a premature stop codon, resulting in a loss of inherent protein function.[3] For example, Duchenne muscular dystrophy (DMD) is caused by mutations in the dystrophin gene, which induces the exclusion of exons during the splicing process and results in an out-of-frame mutation that produces nonfunctional dystrophin.[4] To treat these difficult genetic diseases, splice-switching oligonucleotides (SSOs) are promising.[5-6]

SSOs are short synthetic oligonucleotides that bind to specific pre-mRNAs and include/exclude exons of interest via the inhibition of splicing factor binding and/or splicing complex formation. Since the modulation of splicing by synthetic SSOs was initially demonstrated by Dominski and Kole,[7] SSO-mediated splicing correction has been widely applied for the treatment of genetic diseases. In the case of DMD, additional exon exclusion mediated by SSO, so-called exon skipping, can restore the reading frame, which results in a shortened but functional dystrophin production. In 2016, two SSO drugs, eteplirsen for DMD treatment and nusinersen for spinal muscular atrophy, were approved by the FDA.[8-9]

Various modifications have been explored to improve the efficiency of SSOs. Obika et al. demonstrated effective design of SSOs by using LNA-DNA mixmer.[10] Goyenvalle et al. successfully improved exon skipping efficacy by using tricyclo-DNA.[11] Le et al reported partial incorporation of LNA residues into 2′-*O*-methyl SSO showed improvement of exon skipping efficacy.[12] In contrast, influence of acyclic 2′-*O*-modification has been less studied. Yang et al. reported 2′-*O*-methoxyethyl(MOE) modification instead of 2′-*O*-methyl (Me) modification for exon 23 skipping in the mdx mouse, a model mouse of DMD.[13] Although the most effective SSO was 25mer MOE-modified one, the efficacy of 20mer Me-modified SSO and 20mer MOE-modified SSO were almost same, suggesting no improvement.

We have been studied the introduction of 2′-*O*-(*N*-methylcarbamoyl)ethyl (MCE)-modified nucleotides into oligonucleotides.[14] Although MCE-modified oligonucleotides do not exhibit improved binding affinities to their complementary RNAs compared to those of the corresponding 2′-*O*-methyl modified oligonucleotides, they exhibit a significantly elongated half-life toward enzymatic digestion.[15] Interestingly, our preliminary study of exon skipping treatment for DMD revealed

that an SSO containing 2′-*O*-(*N*-methyl-carbamoyl)ethyl (MCE) uridine (U_{MCE}) instead of 2′-*O*-methyl uridine, with a 6-nt replacement out of the 30-mer 2′-*O*-methyl SSO, showed significant improvement in exon skipping efficiency.[14] This result motivated us to investigate the effect of MCE modification.

In addition, we investigated the effect of binding affinity of MCE modified SSO, which is one of the most important determinants of the exon skipping efficiency.[10, 16-18] Among chemical modifications that improve binding affinity, the nucleobase 2-thiothymine is a good candidate; it is a non-canonical nucleobase found in naturally occurring RNAs. It is well known that 2-thiothymine considerably stabilizes Watson–Crick base pairing, resulting in improved binding affinity to target RNAs. In fact, we have reported that SSOs containing 2′-*O*-methyl-5-methyl-2-thiouridine (s^2T_m) instead of 2′-*O*-methyl uridine show more stable duplex formation with complementary RNA and significantly enhance the exon skipping efficiency.[19] Therefore, we hypothesized that the combination of 2-thiothymine and MCE modification has the potential to improve the exon skipping effect of SSOs.

Synthesis of SSO containing 2′-O-(N-methylcarbamoylethyl) -5-methyl-2-thiouridine (s^2T_{MCE})

For the synthesis of 2′-*O*-(*N*-methylcarbamoylethyl)-5-methyl-2-thiouridine (s^2T_{MCE}), it is essential to perform selective thiocarbonylation at the 2-position of the thymine nucleobase. However, Lawesson's reagent-mediated thiocarbonylation with 2′-*O*-(*N*-methylcarbamoylethyl)-5-methyluridine (rT_{MCE}) quantitatively proceeded on the carbonyl group of the MCE moiety (see Supplementaly Figure 1 for more details). In addition, thiocarbonylation on the thymine base selectively proceeds on the 4-carbonyl group, not on the 2-carbonyl group.[20] Taken together, the 2-carbonyl group on the thymine base is the least reactive carbonyl group in rT_{MCE} toward thiocarbonylation. Previously, Okamoto et al. reported that protection of the 4-carbonyl group with 2,6-dimethyl phenol allows the synthesis of the 2-thiothymine derivatives.[21] Importantly, ester groups are compatible for this method. Thus, we assumed that a 2′-*O*-(methoxycarbonylethyl)-4-*O*-(2,6-dimethylphenyl)-5-methyl uridine derivative would be an adequate precursor of 2-thiocarbonylation.

We used 3′, 5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-5-methyluridine **1** as starting material (Scheme 1). Triisopropylbenzene-sulfonyl chloride was selectively reacted with the 4-carbonyl group. The subsequent replacement with dimethyl phenol gave compound **2** in 64% (2 steps). The *oxa*-Michael reaction was performed using Saneyoshi's conditions.[22] The reaction of compound **2** with methyl acrylate in t-butyl alcohol in the presence of cesium carbonate at room temperature for 15 h gave the desired compound **3** in 65%. Then, thiocarbonylation of compound **3** with Lawesson's reagent successfully proceeded and provided the desired product **4** (yield 77%). It has been reported that 2-thiocarbonylation results in a 1′-proton chemical shift to lower magnetic fields.[21] Based on a comparison of ^{1}H-NMR spectra of compounds **3** and **4**, the 1′-proton spectrum shifted by 0.5 ppm to a lower magnetic field upon the thiocarbonylation reaction, whereas that of the alpha position and methyl group in the 2-methoxycarbonylethyl moiety did not change (Figure 2). This chemical shift supported selective 2-thiocarbonylation (see Supplementary Figure 2).

The 2,6-dimethylphenoxy group was removed by *syn*-*o*-nitrobenzaldoxime treatment. Then, methyl ester was converted to methyl amide by methylamine treatment to give the desired product **5** in 44% (2 steps). The silyl protecting group was

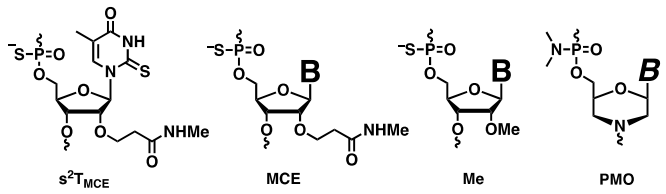
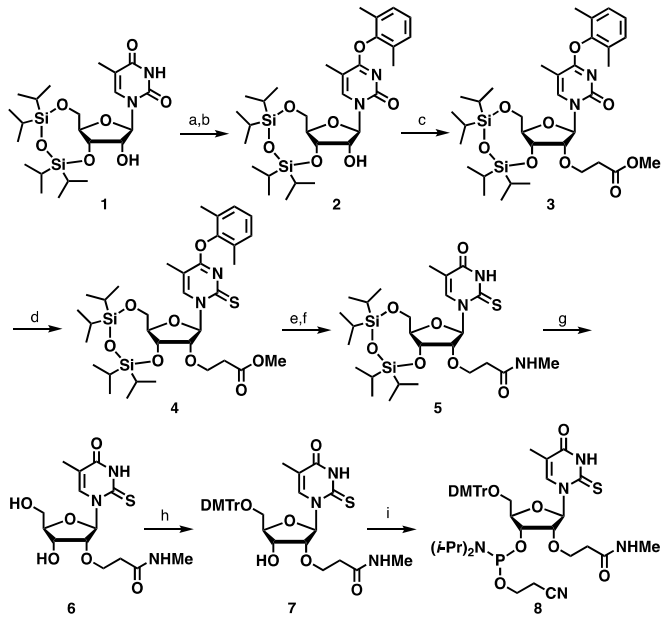


Figure 1. Chemical structures used in this study. B denotes A, U, G, C nucleobases and B denotes A, T, G, C nucleobases.



Scheme 1. Synthesis of s^2T_{MCE} phosphoramidite derivative **8**. Reagents and conditions: (a) 2,4,6-triisopropylbenzenesulfonyl chloride, triethylamine (TEA), *N,N*-dimethyl-4-aminopyridine, CH_3CN, room temperature (r.t.), 4 h; (b) 2,6-dimethylphenol, TEA, 1,4-diazabicyclo[2,2,2]octane, CH_3CN, r.t., 30 min, 64% (2 steps); (c) methyl acrylate, Cs_2CO_3, *t*-BuOH, r.t., 15 h, 65%; (d) Lawesson's reagent, $PhCH_3$, reflux, 2 h, 77%; (e) *syn*-*o*-nitrobenzaldoxime, 1,1,3,3-tetramethylguanidine, CH_3CN, r.t., 24 h; (f) 20% CH_3NH_2 in CH_3OH, r.t., 5 h, 44% (2 steps); (g) TEA·3HF, TEA, THF, r.t., 3 h, 90%; (h) 4,4′-dimethoxytrityl chloride, pyridine, r.t., 3 h, 82%; (i) $(i\text{-}Pr_2N)_2POCH_2CH_2CN$, 1*H*-tetrazole, diisopropylamine, CH_3CN, r.t., 5 h, 62%.

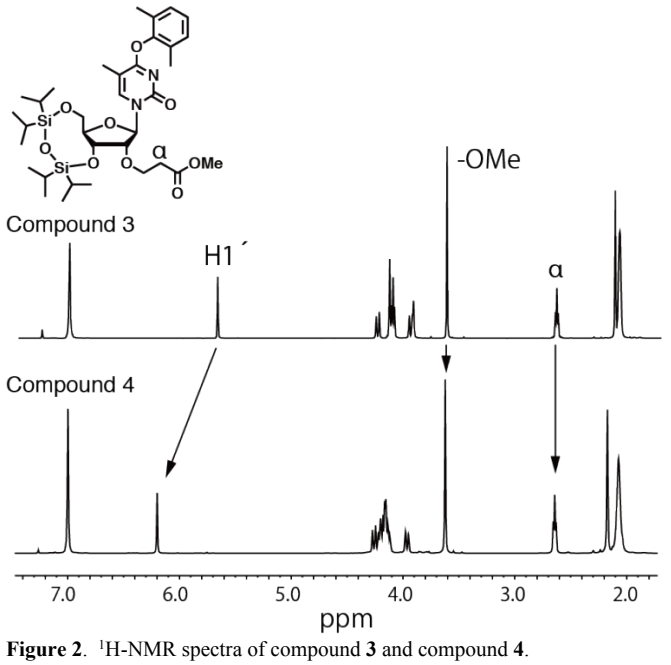


Figure 2. ^{1}H-NMR spectra of compound **3** and compound **4**.

removed by triethylamine trihydrofluoride to give 2′-*O*-(*N*-methylcarbamoylethyl)-5-methyl-2-thiouridine **6**. Compound **7** was synthesized by the dimetoxytritylation of the 5′-hydroxyl

group. Phosphitylation of the 3′-hydroxyl group with 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphordiamidite gave the s^2T_{MCE} phosphoramidite **8**. Then, phosphoramidite **8** was incorporated into SSOs using a DNA synthesizer. The synthesized SSOs were purified by HPLC and identified by MALDI-TOF-mass spectrometry. The synthesized SSO was the murine B30 sequence, referred to as the mB30 sequence (5′-CUCCAACAGCAAAGAAGAUGGCAUUUCUAG), which targets exon 51 of the mouse *Dmd* gene and induces exon skipping.[23-24] The chemical modifications of the SSOs are shown in Figure 3. **Me** denotes the 2′-*O*-methyl phosphorothioate oligonucleotide. **3′-MCE** denotes the phosphorothioate oligonucleotide in which 5 nucleotides counted from the 3′-terminus of **Me** were replaced with MCE nucleotides. Similarly, **5′-MCE** and **3′,5′-MCE** denote phosphorothioate oligonucleotides in which 5 nucleotides counted from the 5′-terminus or both termini of **Me** were replaced, respectively. **full-MCE** denotes the fully MCE modified phosphorothioate oligonucleotide. **sT-MCE** denotes the phosphorothioate oligonucleotide in which every MCE uridine of **full-MCE** was replaced with s^2T_{MCE}. **PMO** denotes the phosphorodiamidate morpholino oligomer.

Binding affinity of SSOs

To evaluate the binding affinity, UV-melting experiments were performed and the apparent melting temperature (T_m) was obtained. The observed T_m value of **Me** with its complementary RNA was 79 ± 1.4°C (Figure 4a). Similarly, the T_m values of **5′-MCE**, **3′-MCE**, **3′, 5′-MCE**, and **full-MCE** were 74 ± 0.3°C, 75 ± 0.5°C, 76 ± 0.3°C, and 76 ± 1.4°C, respectively. The average change in T_m was −0.1 to −1.0°C per MCE modification. The introduction of MCE modifications to phosphorothioate oligonucleotides resulted in slight decreases in binding affinity. The T_m value of SSO containing s^2T_{MCE} (**sT-MCE**) was 87 ± 0.7°C, which was the highest T_m value among tested samples. The average effect of the 2-thiothymine nucleobase on T_m values was +1.8°C per modification, based on comparisons with T_m values of **full-MCE** and **sT-MCE**. As expected, the incorporation of the s^2T_{MCE} nucleotide significantly improved the binding affinity. It is known that 2-thiocarbonyl modifications considerably stabilize the Watson–Crick base pairing by the stabilization of stacking interactions and the preorganization effect of sugar puckering. Our result agreed with those of previous reports.[19, 21, 25-29] The T_m of PMO was 71 ± 0.7°C, which indicated that PMO has the weakest binding affinity toward its complementary RNA among SSOs tested in this study. Taken together, the order of observed binding affinity is as follows: **sT-MCE** > **Me** > **full-MCE** ≈ **3′,5′-MCE** ≈ **3′-MCE** ≈ **5′-MCE** > **PMO**.

Splice switching effect of SSOs

Next, the splice switching effects of the synthesized SSOs were evaluated based on the exon skipping efficiency of exon 51 of *Dmd* in *C2C12* cells. Endo-porter, which is an amphiphilic peptide and does not interact with the cargo,[30] was used for the delivery of SSOs to *C2C12* cells. The exon skipping efficiency was evaluated by RT-PCR of the total RNAs as described previously.[14] In short, the relative gel band intensity of PCR product, [skipped band] / ([skipped band]+[unskipped band]), were quantified by ImageJ and used as the exon skipping efficiency. The exon skipping efficiency induced by **Me** was 14 ± 2.1% (Figure 4b). Similarly, those of **5′-MCE** and **3′-MCE** were 38 ± 4.2% and 25 ± 6.1%, respectively. Even only 5-nucleotide replacements at the terminal positions of **Me** can improve the exon skipping efficiencies. In the case of **3′,5′-MCE** and **full-MCE**, the observed exon skipping efficiencies were 39 ± 2.4% and 42 ± 2.1%, respectively. **full-MCE** showed a slightly improved exon skipping effect. We also

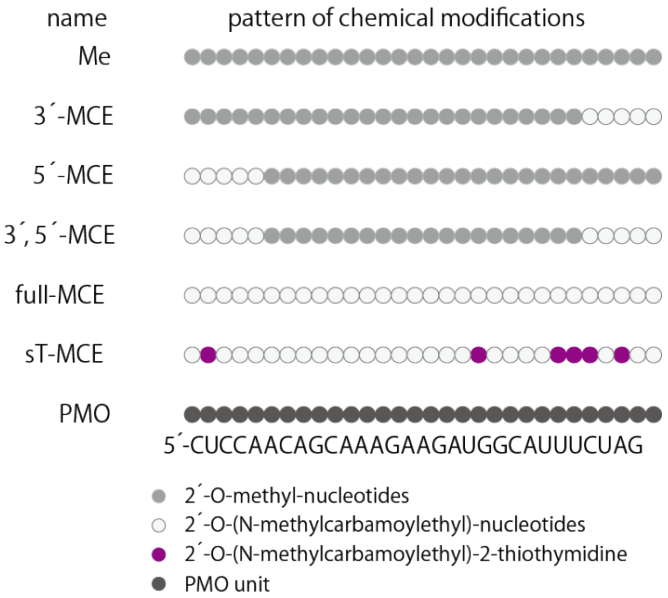


Figure 3. Summary of chemical modifications examined in this study. Nucleobases for 2′-*O*-methyl-nucleoside and 2′-*O*-(*N*-methylcarbamoylethyl) -nucleosides are uracil, adenine, cytosine, and guanine bases. Nucleobases for PMO units are thymine, adenine, cytosine, and guanine bases.

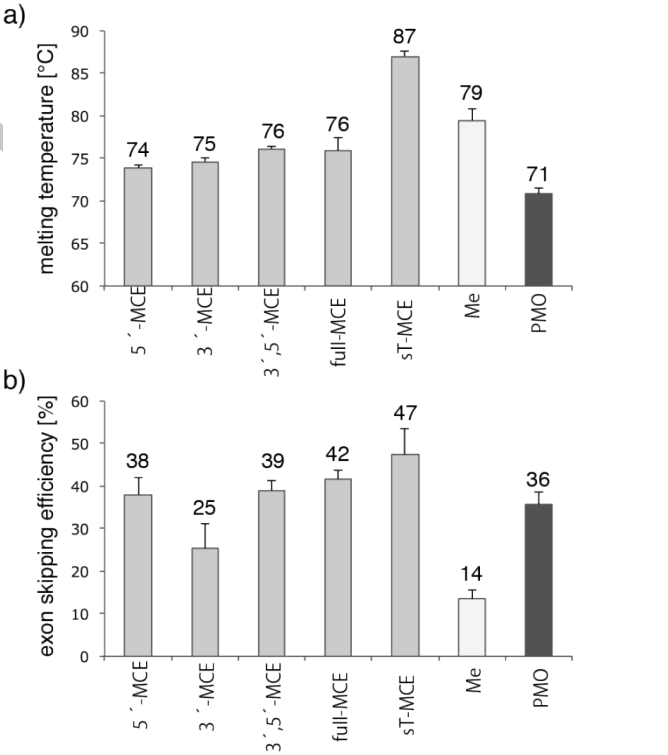


Figure 4. Properties of SSOs. a) Melting temperatures of each SSO with complementary RNA. b) Observed exon skipping efficiency induced by each SSO.

quantified gel bands by using Experion™, which also showed higher activity of the MCE modified ASOs than the Me modified one (Supplementary Figure 5 and Figure 6).

Importantly, the corresponding melting temperature of MCE-modified SSO with complementary RNA was lower than that of **Me**, which indicates that the mechanism underlying the improvement by MCE modification was different from that for the improvement of duplex stability. Interestingly, **5′-MCE** and **full-MCE** showed similar exon skipping efficiencies, whereas **3′-MCE** was less effective. These observations indicated the effect of MCE modification was position-dependent. We previously

found that MCE modification can improve resistance to nuclease digestion.[15] However, nuclease resistance was not a key factor because **full-MCE** did not result in improved exon skipping compared to **5′-MCE**. Previous studies have suggested that exon skipping can be induced by the inhibition of the binding of exonic splicing enhancers (ESEs) to the exon sequence of target pre-mRNA.[16, 31] ESE binding motifs in mice were predicted using RESCUE-ESE.[32-33] In the complementary sequence of ASO, there are four ESE binding motifs (see supplementary information). Interestingly, the complementary sequence of MCE-modified nucleotides in 5′-MCE overlapped with two of these binding motifs, whereas there was no overlap for **3′-MCE**. Thus, it might be possible that ESE binding could be effectively inhibited by the incorporation of MCE modifications in **5′-MCE**. Further detailed investigations are necessary to understand the precise effects of MCE modifications. It should be noted that the exon skipping efficiency of **PMO** was 36 ± 2.8%, which is comparable to that of **5′-MCE**. Even a five-nucleotide substitution could greatly improve the exon skipping efficiency compared with that of **Me**. The exon skipping efficiency induced by MCE-modified SSO containing s^2T_{MCE} (**sT-MCE**) was 47 ± 6.1%. The SSO with s^2T_{MCE} was the most effective among the evaluated SSOs. This tendency was consistent with the results of our previous report.[19] Since the incorporation of MCE-modified nucleotides results in a slight decrease in duplex stability, additional stabilizing modifications can further improve the exon skipping effect.

In summary, we successfully synthesized duplex stabilizing nucleotides with an MCE modification, 2′-*O*-(*N*-methylcarbamoylethyl)-5-methyl-2-thiouridine (s^2T_{MCE}). Selective 2-thiocarbonylation was achieved with the proper precursor, which had a protection group at the 4-position and ester group at the 2′-modification to avoid undesired thiocarbonylation. The synthesized s^2T_{MCE} phosphoramidite derivative could be incorporated into the oligonucleotide following standard methods. MCE modification and s^2T_{MCE} residues in SSOs effectively induced the exon skipping effect. Combining duplex stabilizing modifications with the inhibitory modification of splicing factors will be a useful strategy for the development of SSOs.

Acknowledgments

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Supplementary Material

Supplementary material can be found online at https://doi.org/10.1016/j.bmcl.####.##.###..