Abstract – Bridging between the 2’- and 4’-carbons in a nucleoside restricts the furanose ring to C3’-endo conformation, which coincides with the sugar conformation in an oligonucleotide forming a duplex with single-stranded RNA (ssRNA) and a triplex with double-stranded DNA (dsDNA). Therefore, oligonucleotides modified by 2’,4’-bridged nucleosides generally increase hybridization ability with ssRNA and dsDNA when compared with the natural oligonucleotide. Till date, a large number of 2’,4’-bridged nucleosides with additional two-atom to four-atom bridges between 2’- and 4’-carbons have been developed by many research groups including our group. For this, ionic cyclization, ring-closing metathesis, and radical cyclization have been used so far as the synthetic strategies of bridge constructions. Based on such a background, we recently proposed a 2’,4’-bridged nucleoside possessing 6’-oxygen founded on a new design concept and several types of analogs including 2’-O,4’-C-ethyleneoxy-bridged 5-methyluridine with a four-atom bridge have been developed. In addition, as a new strategy of bridge construction, radical cyclization using the 4’-carbon radical of a nucleoside was exemplified and a promising 2’,4’-bridged nucleoside, the 6’-methyl analog of 2’-O,4’-C-ethylene-bridged 5-methyluridine, was found. This review mainly focuses on our recent results on bridged nucleosides used for chemically modified oligonucleotides. It describes the design and synthesis of the bridged nucleosides, along with the properties of oligonucleotides including bridged nucleosides.

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1. INTRODUCTION

Chemical modification of oligonucleotides is a powerful strategy for the improvement of binding affinity to target nucleic acids, nuclease resistance, and so on. In particular, a sugar moiety as a modification site has attracted immense attention because control of flexible conformation of the furanose ring in a nucleoside is possible. In fact, although nucleic acids can form various structures based on hydrogen-bond formation between nucleobases, the sugar moiety is necessarily required to make the conformation acceptable for each structure. For example, while C2'-endo conformation is adopted in a B-type duplex (like DNA/DNA duplex), the sugar conformation in an A-type duplex (like DNA/RNA or RNA/RNA duplexes) adopts C3'-endo (Figure 1). The small structural changes of the sugar ring have great influence on the composite structure of nucleic acids.

![Equilibrium between C2'-endo and C3'-endo conformations of nucleosides](image)

**Figure 1.** Equilibrium between C2'-endo and C3'-endo conformations of nucleosides

Introduction of bridge structure into the sugar moiety can lead to restrict of the conformation. Especially, 2',4'-bridge modifications rigidly restrict the sugar conformation to C3'-endo, which would be entropically advantageous to duplex formation with single-stranded RNA (ssRNA) as well as triplex formation with double-stranded DNA (dsDNA) since oligonucleotides adopt a C3'-endo conformation in both the duplex and the triplex. Therefore, a large number of 2',4'-bridged nucleosides, differing in the size and composition of the bridge and substituent on the bridge, have been developed as building blocks introduced into oligonucleotides targeting ssRNA or dsDNA. In particular, 2',4'-bridged nucleosides with two-, three-, and four-atom bridge between 2'-carbon and 4'-carbon in the furanose ring have been actively studied and it has been shown that their modified oligonucleotides have improved properties. The typical analogs are shown in Figure 2. To date, properties of oligonucleotides including 2',4'-bridged nucleosides have been investigated; however, sufficient understanding of the influence of the bridge...
structure on the properties has not been attained and a new class of 2ʹ,4ʹ-bridged nucleosides is still in demand.

**Figure 2.** Typical examples of 2ʹ,4ʹ-bridged nucleosides, used for chemical modification of oligonucleotides, classified by bridge sizes

To construct the bridge of 2ʹ,4ʹ-bridged nucleosides, three types of synthetic strategies, namely, (i) ionic cyclization, (ii) ring-closing metathesis, and (iii) intramolecular radical cyclization, have been adapted. Ionic cyclizations, including an intramolecular SN2 reaction and an addition-elimination reaction, have most widely been used and heteroatom-carbon bonds can be formed by the reactions. Thus, ionic cyclizations have been applied for the synthesis of 2ʹ,4ʹ-bridged nucleosides containing any heteroatom in the bridge, which account for the great majority of 2ʹ,4ʹ-bridged nucleosides.\(^1\) Ring-closing metathesis is a useful strategy for carbon-carbon bond formation to construct the bridge, though terminal alkenes are required at both sites of the 2ʹ- and 4ʹ-positions. In 2006, Nielsen’s group reported 2ʹ,4ʹ-bridge construction by means of ring-closing metathesis,\(^1\)\(^1\) and various derivatives of the 2ʹ,4ʹ-carbocyclic nucleoside (carba-ENA) with a three-atom (C-C-C) bridge were synthesized.\(^1\)\(^1\),\(^19\) Intramolecular radical cyclization of the 2ʹ-carbon radicals of nucleosides with terminal alkene units was also employed to construct 2ʹ,4ʹ-carbocyclic nucleosides with two- and three-atom bridges (C2ʹ-C-C-C4ʹ and C2ʹ-C-C-C-C4ʹ) by Chattopadhyaya’s group in 2007.\(^2\)\(^0\) It is well-known that the 2ʹ-carbon radicals can be generated via deoxygenation by the reaction of 2ʹ-O-thiocarbonyl nucleosides, like 2ʹ-xanthate, with
trialkyltin radical, which has often been used for the synthesis of 2'-deoxyribonucleosides from ribonucleosides. After this report, 2',4'-carbocyclic nucleosides with various substituents on the bridges have been developed by means of radical addition to oximes or alkynes by 2'-carbon radicals.\textsuperscript{7,21-23} With this background, we have also engaged in the development of 2',4'-bridged nucleosides and proposed a new design concept of nucleosides with 6'-oxygen in addition to the 2',4'-bridge.\textsuperscript{24} The nucleosides designed have unique two consecutive acetals, O6'-C4'-O4'-C1'-N1, and in 2013, a 2',4'-bridged nucleoside consisting of a four-atom bridge, C2'-O-C-C-O-C4', was synthesized (see Section 2). After that, 3',4'-bridged congeners\textsuperscript{25-27} were synthesized (see Section 3) and the development of several 2',4'-bridged nucleosides\textsuperscript{28-32} was also achieved based on a similar design concept (see Sections 4 and 5). Furthermore, the synthesis of a 2',4'-bridged nucleoside was achieved by intramolecular radical cyclization using an uncommon 4'-carbon radical (see Section 6).\textsuperscript{33} This review mainly summarizes our recent results pertaining to the synthesis of 2',4'-bridged nucleosides and the properties of their modified oligonucleotides.

2. 2'-O,4'-C-ETHYLENEOXY-BRIDGED 5-METHYLURIDINE\textsuperscript{24}

Although many 2',4'-bridged nucleosides have been developed as shown in Figure 2, we realized that the 6'-atom attached to the 4'-carbon in 2',4'-bridged nucleosides was limited to carbon atoms only. However, if the 6'-atom were any heteroatom like oxygen, it was considered that the stability of a 2',4'-bridged nucleoside could be problematic because of including two consecutive acetals, O6'-C4'-O4'-C1'-N1. Moreover, there might also be difficulty in synthesis because the hydroxyl group attached to the 4'-carbon of a nucleoside is spontaneously decomposed to eliminate the nucleobase. On the other hand, 4'-alkoxynucleosides preferentially adopt N-type sugar conformations, presumably due to the anomic effect on the 4'-carbon induced by the alkoxy oxygen and the furanose oxygen.\textsuperscript{34,35} In addition, Rosenberg’s group reported in 2011 that the two consecutive acetals in 4'-alkoxynucleosides were stable to reaction conditions used for oligonucleotide synthesis and oligonucleotides containing 4'-methoxy- or 4'-(2-methoxyethoxy)-thymidines stabilized the duplexes with ssRNA, showing an increased $T_m$ value of approximately 1 °C per modification.\textsuperscript{35} Thus, we designed 2'-O,4'-C-ethylenedioxy-bridged 5-methyluridine (EoNA-T) as a nucleoside possessing four-atom (O-C-C-O) 2',4'-bridge as well as a 6'-oxygen atom (Figure 3).\textsuperscript{24} EoNA-T is the first example of bridged nucleosides replacing 6'-carbon by any heteroatom.
Figure 3. Structures of 4′-alkoxynucleosides and EoNA-T monomer

The synthesis from 5-methyluridine 1 was performed as shown in Scheme 1. Conversion of the 5′-hydroxyl group in 1 into iodide followed by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) produced compound 2 in 87% for two steps, which underwent benzyloxymethyl (BOM)-protection of the imido nitrogen afforded compound 3. Regioselective monobenzylation at the 3′ position was carried out under several conditions such as in the presence of NaH and BnBr, Bn2SnO and BnBr, and Ag2O and BnBr. The reaction using Ag2O gave the best results though the isolated yield of 4 was poor (30% yield). Introduction of 2-hydroxyethyl unit into 2′-oxygen in 4 was accomplished via two steps. Compound 5 was subjected to m-chloroperoxybenzoic acid (mCPBA) or N-bromosuccinimide (NBS) for making bridge between 2′- and 4′-positions; however, no bridged compound was detected at all in both reactions. Reaction with mCPBA gave 3-benzyloxymethylthymine 6 as the only isolated product with 83% yield, which might imply that the 4′-hydroxyl compound was produced leading to decomposition of the sugar moiety. In contrast, treatment with NBS produced unexpected dioxane 7 via nucleophilic attack by the hydroxyl group to 1′-carbon. It was considered that bridge construction by the hydroxyl group of 2′-O-hydroxylethyl moiety was quite difficult because the hydroxyl group attacked the 1′-carbon rather than the 4′-carbon.
As an alternative route, a 2-hydroxylethyl unit was introduced into the 4ʹ-carbon position in 4 by treatment with freshly prepared anhydrous mCPBA and TBSOCH₂CH₂OH in a solvent-free system. In this reaction, stereoselective epoxidation followed by an S_N2 reaction of the siloxy alcohol at the 4ʹ-carbon proceeded to afford 8 as a sole isomer. Dimethoxytritylation of 5ʹ-hydroxyl group in 8 using dimethoxytrityl trifluoromethanesulfonate (DMTrOTf), removal of a TBS group, and tosylation of the primary alcohol furnished 9, treatment of which with NaH resulted in a ring-closing reaction leading to compound 10 with an EoNA skeleton in excellent yield. Hydrogenolysis of 10 produced protection-free EoNA-T monomer 11 in 99% yield. The J_{H1',H2'} and J_{H2',H3'} values of 11 by ^1H NMR measurement were 0 Hz and 6 Hz, respectively, which coincided with those of the 2ʹ,4ʹ-BNACOC-T monomer shown in Figure 4. This result implies that the sugar conformation of EoNA-T would be the same as that of 2ʹ,4ʹ-BNACOC-T, C3ʹ-endo conformation, with the same four-atom bridge between 2ʹ- and 4ʹ-carbons.

The phosphoramidite 12 was prepared by a common procedure toward oligonucleotide synthesis, that is dimethoxytritylation and phosphitylation. The synthesis of EoNA-T phosphoramidite 12 from 5-methyluridine was achieved via twelve steps.
acceptable yield of 90-95%. Using a double-coupling cycle, that performs coupling step twice prior to the next step, with the prolonged coupling time was effective for consecutive introduction of the EoNA-T phosphoramidite (wherein, each coupling yield was 90-95%).

![Scheme 2. Synthesis of EoNA-T phosphoramidite 12](image)

**Figure 4.** The $J_{H1',H2'}$ values of EoNA-T and 2',4'-BNA COC-T monomers

UV-melting experiments of the duplexes formed with ssRNA and ssDNA by oligonucleotides modified by EoNA-T were performed and the $T_m$ values were compared with those of 2',4'-BNA COC-T (Table 1). EoNA-modified oligonucleotides significantly stabilized the duplexes with RNA though a slight destabilization of those with ssDNA was observed. This tendency was similar to the oligonucleotides modified by 2',4'-BNA COC-T. As a result, in comparison to 2',4'-BNA COC-T, EoNA-T had higher stabilization ability of duplexes with ssRNA. It was also found that EoNA-T had higher stabilization ability of triplexes with dsDNA than 2',4'-BNA COC-T. Interestingly, stabilization of complexes with ssRNA and dsDNA by EoNA-T modification was apparently synergistic, and increasing the number of
modifications led to an increase in the $\Delta T_{m/mod.}$ values. Meanwhile, it was reported by Koizumi’s group that PrNA with a four-atom (O-C-C-C) bridge, as shown in Figure 1, slightly decreased the stability of the duplex formed with ssRNA ($\Delta T_{m/mod.} = 0.5 ^\circ C$).$^{16}$ EoNA-T, which is the 6'-carbon analog of PrNA, increased the $\Delta T_{m/mod.}$ values of the duplexes by up to 3.6 $^\circ C$, which suggests that the 6'-oxygen in a four-atom bridged nucleoside could be a key factor for the stabilization of duplexes with ssRNA.

| Table 1. $T_m$ Values of duplexes and triplexes obtained by UV-melting experiments |
|---------------------------------|-----------------|-----------------|-----------------|
| duplex with ssRNA               | duplex with ssDNA | triplex with dsDNA |
| 5′-TCTTCTTTTTCTCT-3′            | 51 $^\circ$C     | 50 $^\circ$C     | 31 $^\circ$C     |
| 5′-TCTTCTTTTTCTCT-3′            | 52 $^\circ$C (+1.0 $^\circ$C) | 48 $^\circ$C (−2.0 $^\circ$C) | 32 $^\circ$C (+1.0 $^\circ$C) |
| 5′-TCTTCTTTTTCTCT-3′            | 59 $^\circ$C (+2.7 $^\circ$C) | 47 $^\circ$C (−1.0 $^\circ$C) | 37 $^\circ$C (+2.0 $^\circ$C) |
| 5′-TCTTCTTTTTCTCT-3′            | 60 $^\circ$C (+3.0 $^\circ$C) | 47 $^\circ$C (−1.0 $^\circ$C) | 41 $^\circ$C (+3.3 $^\circ$C) |
| 5′-TCTTCTTTTTCTCT-3′            | 69 $^\circ$C (+3.6 $^\circ$C) | 48 $^\circ$C (−0.4 $^\circ$C) | 50 $^\circ$C (+3.8 $^\circ$C) |
| 5′-TCTTCTTTTTCTCT-3′            | 52 $^\circ$C (+1.0 $^\circ$C) | 48 $^\circ$C (−2.0 $^\circ$C) | 31 $^\circ$C (0 $^\circ$C) |
| 5′-TCTTCTTTTTCTCT-3′            | 57 $^\circ$C (+2.0 $^\circ$C) | 45 $^\circ$C (−1.7 $^\circ$C) | 31 $^\circ$C (0 $^\circ$C) |
| 5′-TCTTCTTTTTCTCT-3′            | 57 $^\circ$C (+2.0 $^\circ$C) | 45 $^\circ$C (−1.7 $^\circ$C) | 33 $^\circ$C (+0.7 $^\circ$C) |
| 5′-TCTTCTTTTTCTCT-3′            | 62 $^\circ$C (+2.2 $^\circ$C) | 42 $^\circ$C (−1.6 $^\circ$C) | 33 $^\circ$C (+0.4 $^\circ$C) |

Conditions: 10 mM sodium cacodylate buffer (pH 7.2), 140 mM KCl, and 4 μM of each oligonucleotide for duplex; and 10 mM sodium cacodylate buffer (pH 7.2), 140 mM KCl, 5 mM MgCl$_2$, and 1.5 μM of each oligonucleotide for triplex. $T = $ EoNA-T. $T = $ 2′,4′-BNA$^{\text{COC}}$-T. $C = $ 2′-deoxy-5-methylcytidine. The sequences of ssRNA, ssDNA, and dsDNA are 5′-r(AGAGAAAAAGAAGA)-3′, 5′-d(AGAGAAAAAGAAGA)-3′, and 5′-d(GGCAGAGAAAAAGAAGACGC)-spacer18-d(GCGTCTTTTTCTCTGCC)-3′, respectively. The changes in $T_m$ values per modification ($\Delta T_{m/mod.}$) relative to the natural duplexes are shown in parentheses.

Nuclease resistance of EoNA modification against 3′-exonuclease was examined (Figure 5). EoNA was shown to have excellent stability against nuclease degradation. When oligonucleotides modified at the second position from the 3′-end were used, the stability of EoNA was superior to those of 2′,4′-BNA/LNA as well as 3′-Sp, a (3′S)-phosphorothioate linkage with high nuclease resistance, and was comparable with that of 2′,4′-BNA$^{\text{COC}}$ (Figure 5a). In the modification at the 3′-end, the EoNA-modified oligonucleotide rather than the 2′,4′-BNA$^{\text{COC}}$-modified oligonucleotide significantly suppressed nuclease degradation (Figure 5b).
Although EoNA has the same four-atom bridge with 2',4'-BNA\textsuperscript{COC}, EoNA-T clearly improved the properties of oligonucleotides as compared with 2',4'-BNA\textsuperscript{COC}-T. A structural difference would be the presence of 6'-oxygen, which might cause the improvement of properties. Therefore, introduction of heteroatom into the 6'-position could be useful as the design concept of 2',4'-bridged modification.

3. 3'-O,4'-C-ETHYLENEOXY-BRIDGED 5-METHYLURIDINES\textsuperscript{25-27}

We were interested in bridged nucleosides possessing 6'-oxygen, other than the 2',4'-bridged nucleoside, and designed a 3'-O,4'-C-ethyleneoxy-bridged 5-methyluridine (3',4'-EoNA-T) and its 7'-methyl congeners, (R)-Me-3',4'-EoNA-T and (S)-Me-3',4'-EoNA-T (Figure 6). These 3',4'-EoNA-T modifications form 2',5'-phosphodiester linkages in oligonucleotides. In general, oligonucleotides comprised from 2',5'-phosphodiester linkages are called isoDNA or isoRNA, which tend to selectively and stably bind to ssRNA rather than ssDNA. However, there are few modifications\textsuperscript{37} for isoDNA (or isoRNA) that are capable of significant stabilization of duplexes with ssRNA, thus rendering the development of a new nucleoside capable of forming a 2',5'-phosphodiester linkage is necessary. From this viewpoint, features of 3',4'-EoNA-T and its 7'-methyl congeners as modification of oligonucleotides were also quite interesting.
The synthesis of 3’,4’-EoNA-T is shown in Scheme 3. When compound 13 prepared from 5-methyluridine (via three steps) was subjected to mCPBA and 2-iodoethanol in a solvent-free system, unlike the 2'-hydroxyl congener as described in Section 4 (Scheme 3), a complex mixture was yielded without production of desired 14. However, two-step reaction, that is, epoxidation with in situ generated dimethyldioxirane, and ZnCl₂-mediated ring-opening in the presence of 2-iodoethanol, led to the formation of a 4'-iodoethoxy compound 14 in a 1:1 inseparable mixture. After conversion of 14 into 5’-O-DMTr 15, treatment of 15 with TBAF was conducted. Interestingly, the removal of two TBS groups and intramolecular 1,4-dioxane ring formation occurred simultaneously to afford 16 with a 3’,4’-EoNA skeleton in 55% yield, together with α-L-lyxofuranose analog 17 (42% yield). The stereochemistry of 4'-carbon and 1,4-dioxane structure in 16 were confirmed by NOESY and HMBC correlations, respectively (Figure 7). Structure of 17 was also confirmed by NOESY correlations shown in Figure 7. Hydrogenolysis of 16 gave protection-free monomer 18, the $J_{H1',H2'}$ value of which in CD$_3$OD by $^1$H NMR measurement was 8 Hz. This means that the sugar conformation of 3’,4’-EoNA adopts S-form (DNA-like conformation) similar to other 3’,4’-BNA analogs$^{38,39}$ shown in Figure 8. In addition, 18 including two consecutive acetals was stable under acidic conditions (80% AcOH aq. at room temperature). The 3’,4’-EoNA-T phosphoramidite 19 was successfully prepared from 17 and the number of synthetic steps was only eight steps from 5-methyluridine 1.
Scheme 3. Synthesis of 3',4'-EoNA-T phosphoramidite 19

Figure 7. NOESY and HMBC correlations of 16 and NOESY correlations of 17

Figure 8. The $J_{H1',H2'}$ values of 3',4'-bridged nucleosides
Synthesis of the 7ʹ-methylated 3ʹ,4ʹ-EoNA-T phosphoramidites, shown in Scheme 4, was accomplished by the same method as that of unsubstituted 3ʹ,4ʹ-EoNA-T. Epoxidation of exo-olefin 13 followed by ZnCl₂-mediated ring-opening using a chiral alcohol with R-configuration produced 20a as a 7:3 inseparable mixture. Methylated 3ʹ,4ʹ-EoNA-T 21a with 7ʹR-configuration was obtained together with undesired 22, by dimethoxytritylation of 20a and treatment with TBAF. Compound 21a was successfully converted into protection-free 23a and the phosphoramidite 24a. In the case of using chiral alcohol with S-configuration, 20b was isolated as a sole isomer. 5ʹ-O-Dimethoxytritylated (S)-Me-3ʹ,4ʹ-EoNA-T 21b was prepared via two steps from 20b. Analogously to the 7ʹR-isomer, 23b and 24b were also easily prepared. The JH1',H2' values of 23a and 23b in CD3OD by ¹H NMR measurement were also 8 Hz, which indicated that 23a and 23b could adopt a similar sugar conformation as unsubstituted 18. Introduction of unsubstituted, (R)-methyl, and (S)-methyl 3ʹ,4ʹ-EoNA-T phosphoramidites into oligonucleotides could be achieved in a coupling yield of over 95% with prolonged coupling time (5 min or 10 min).

Scheme 4. Synthesis of (R)- and (S)-Me-3ʹ,4ʹ-EoNA-T phosphoramidites 24a and 24b
Duplex-forming ability of 3’,4’-EoNA-T-modified oligonucleotides with ssRNA and ssDNA was investigated by UV-melting experiment, and a part of the obtained \( T_m \) values is summarized in Table 2. 3’,4’-EoNA-T within oligonucleotides consisting of natural 3’,5’-phosphodiester linkages slightly destabilized duplexes with ssRNA compared to the natural oligonucleotide, and the \( T_m \) reduction was 1.5–2 °C per modification. The duplex stability was almost equal to that of 3’-deoxy-5-methyluridine (2’,5’-T), forming the same 2’,5’-phosphodiester linkage as 3’,4’-EoNA-T. On the other hand, duplexes formed with ssDNA were drastically destabilized by 3’,4’-EoNA-T modification (\( \Delta T_m/\text{mod.} = \) approximately −9 °C) while 2’,5’-T modification reduced the \( T_m \) value per modification by approximately 6 °C. These results demonstrated that oligonucleotides modified by 3’,4’-EoNA-T had high selectivity toward ssRNA during duplex formation, as compared with 2’,5’-T and natural thymidine. With regard to triplexes formed with dsDNA using a homopyrimidine oligonucleotide, single or alternate 3’,4’-EoNA-T modification had almost no effect on the stability; however, no triplex formation was observed by three consecutive 3’,4’-EoNA-Ts within oligonucleotides. In contrast, 2’,5’-T slightly stabilized the triplexes (\( \Delta T_m/\text{mod.} = 0 \) °C to 2.3 °C).

Table 2. \( T_m \) Values of duplexes obtained by UV-melting experiments

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<tr>
<th>Oligonucleotide</th>
<th>with ssRNA ( T_m )</th>
<th>with ssDNA ( T_m )</th>
</tr>
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<tbody>
<tr>
<td>5’-GGATGTTCTCGT-3’</td>
<td>47 °C</td>
<td>47 °C</td>
</tr>
<tr>
<td>5’-GGATGTTCTCGT-3’</td>
<td>45 °C (−2.0 °C)</td>
<td>37 °C (−10.0 °C)</td>
</tr>
<tr>
<td>5’-GGATGTTCTCGT-3’</td>
<td>44 °C (−1.5 °C)</td>
<td>29 °C (−9.0 °C)</td>
</tr>
<tr>
<td>5’-GGATGTTCTCGT-3’</td>
<td>42 °C (−1.7 °C)</td>
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<td>41 °C (−6.0 °C)</td>
</tr>
<tr>
<td>5’-GGATGTTCTCGT-3’</td>
<td>44 °C (−1.5 °C)</td>
<td>34 °C (−6.5 °C)</td>
</tr>
<tr>
<td>5’-GGATGTTCTCGT-3’</td>
<td>42 °C (−1.7 °C)</td>
<td>29 °C (−6.0 °C)</td>
</tr>
</tbody>
</table>

Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, and 2.5 μM of each oligonucleotide. \( T = 3’,4’-\text{EoNA-T} \). \( T = 2’,5’-\text{T} \). The sequences of ssRNA and ssDNA are 5’-ACGAGAACAUCC-3’ and 5’-ACGAGAACATCC-3’, respectively. The changes in \( T_m \) values per modification (\( \Delta T_m/\text{mod.} \)) relative to the natural duplexes are shown in parentheses.

Duplex- and triplex-forming abilities of isoDNAs containing 3’,4’-EoNA-T, (R)-Me-3’,4’-EoNA-T, and (S)-Me-3’,4’-EoNA-T, were examined. The modified isoDNAs formed duplexes only with ssRNA, while duplex with ssDNA and triplex with dsDNA could not be observed because of the low stability. A part of the results of duplexes with ssRNA is shown in Table 3. Although isoDNAs with two consecutive
3’,4’-EoNA-T modifications and its methyl congeners had almost the same duplex stability as unmodified isoDNA, single or three non-consecutive modifications increased the stability. In particular, 3’,4’-EoNA-T and (R)-Me-3’,4’-EoNA-T significantly stabilized the duplex ($\Delta T_m$/mod. = 2.0 °C to 2.3 °C) in comparison with (S)-Me-3’,4’-EoNA-T ($\Delta T_m$/mod. = 1.0 °C to 1.3 °C). The low stabilization by (S)-Me-3’,4’-EoNA-T might be caused by structural distortion of the duplex by the axial 7’-methyl group (Figure 9). Consequently, 3’,4’-EoNA-T and (R)-Me-3’,4’-EoNA-T were found to be useful chemical modifications for isoDNA targeting ssRNA.

Table 3. $T_m$ Values of duplexes with ssRNA obtained by UV-melting experiments

<table>
<thead>
<tr>
<th></th>
<th>$T_m$</th>
<th>$\Delta T_m$/mod.</th>
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<tbody>
<tr>
<td>5’-GGATGTTCCTCGT-2’</td>
<td>41 °C</td>
<td></td>
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<tr>
<td>5’-GGATGTTCCTCGT-2’</td>
<td>43 °C</td>
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<td>+2.3 °C</td>
</tr>
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<td>+2.0 °C</td>
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Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, 2.5 μM of each oligonucleotide. $\text{T} = 3’,4’$-EoNA-T, $\text{T} = (R)$-Me-3’,4’-EoNA-T, $\text{T} = (S)$-Me-3’,4’-EoNA-T. The sequences of ssRNA and ssDNA are 5’-ACGAGAACAUCC-3’ and 5’-ACGAGAAACATCC-3’, respectively. $\Delta T_m$/mod.: The changes in $T_m$ values per modification relative to the unmodified isoDNA/RNA duplex.

Figure 9. The bridge conformation of (S)-Me-3’,4’-EoNA-T monomer 23b
4. 2ʹ-C,4ʹ-C-ETHYLENEOXY-BRIDGED THYMIDINES AND 2ʹ-DEOXYADENOSINE\textsuperscript{28-31}

2ʹ-C,4ʹ-C-Ethyleneoxy-bridged nucleosides (EoDNAs) contain not only a 6ʹ-oxygen atom but also a smaller three-atom (C-C-O) bridge than that of EoNA (Figure 10). In particular, methylene-EoDNAs were designed because it was reported by Seth’s group that the exocyclic methylene moiety on methylene-cLNA can act as a bio-isostere of the 2ʹ-oxygen.\textsuperscript{23} Bond formation between 2ʹ-carbon and 8ʹ-carbon by intramolecular radical cyclization using 2ʹ-carbon radical would be effective for construction of an EoDNA skeleton. In fact, Chattopadhyaya’s group reported that cLNA, cENA, and their derivatives were synthesized by the intramolecular radical cyclization\textsuperscript{20} (Scheme 5) and the bridges were limited to two- or three-carbon linkages.

![Figure 10. Structures of EoDNA-T monomers](image)

**Figure 10.** Structures of EoDNA-T monomers

![Scheme 5. Construction of 2ʹ,4ʹ-carbocyclic skeletons by the intramolecular radical cyclization](image)

**Scheme 5.** Construction of 2ʹ,4ʹ-carbocyclic skeletons by the intramolecular radical cyclization
The synthesis of a methylene-EoDNA-T monomer was shown in Scheme 6. According to the report on 3′-O-selective monosilylation of nucleosides, olefin 2 synthesized from 5-methyuridine for two steps was treated with TBSCI, AgNO₃, and 1,4-diazabicyclo[2.2.2]octane (DABCO). Although the reaction gave unsatisfied selectivity, desired 3′-O-TBS compound 25 was isolated in 39% yield, along with the 2′-O-TBS 26 (43% yield) and 2′,3′-bis-O-TBS 13 (6% yield). Analogously to the synthesis of 3′,4′-EoNA-T shown in Scheme 3, in situ generated dimethylidioxirane oxidation of 3′-O-TBS 25, followed by ZnCl₂-mediated ring-opening afforded compound 27, in 52% yield, as the sole diastereoisomer. The result suggests that β-selective epoxidation proceeded by masking the α-face of the olefin by the 3′-TBSO group and propargyl alcohol made an SN₂-type nucleophilic attack on the 4′-carbon atom. Treatment of 25 with anhydrous mCPBA freshly prepared in propargyl alcohol also produced 27 (40% yield) with complete diastereoselectivity in one step. Dimethoxytritylation of 27 produced 28, which reacted with 1,1′-thiocarbonyldiimidazole (TCDI) to furnish 29. Compound 29 was subjected to treatment with (TMS)₃SiH, in the presence of a catalytic amount of 2,2'-azobis(isobutyronitrile) (AIBN), and 6-exo radical cyclization exclusively occurred to produce 30 with a methylene-EoDNA skeleton in 57% yield. Deprotection of the 3′-O-TBS group in 30 gave 31, which was phosphitylated to give the desired phosphoramidite 32, using i-Pr₂NP(Cl)OCH₂CH₂CN. In the synthetic route shown in Scheme 5, the methylene-EoDNA-T phosphoramidite was prepared from 5-methyluridine via nine or ten steps. Using commercially available (R)-3-butyn-2-ol and the S-isomer, 7′-methylated methylene-EoDNA-T phosphoramidites, (R)-Me-methylene-EoDNA-T and (S)-Me-methylene-EoDNA-T ones (Figure 11), could be synthesized, respectively.
Scheme 6. Synthesis of methylene-EoDNA-T phosphoramidite 32

Figure 11. Structures of (R)- and (S)-Me-methylene-EoDNA-T phosphoramidites

The synthesis of (R)-Me-EoDNA-T phosphoramidite is shown in Scheme 7. Hydrogenolysis of 30 using H2 and PtO2 led to stereoselective reduction, producing an 8ʹ-methyl compound with R-configuration. Then, treatment with TBAF afforded a 3ʹ-hydroxyl compound 33. Stereochemistry of the 8ʹ-methyl group was determined by NOESY correlations between H1ʹ and H8ʹ, and between 3ʹ-hydroxyl and 8ʹ-methyl groups in 33. Desired phosphoramidite 34 was obtained by phosphitylation of 33.
Scheme 7. Synthesis of (R)-Me-EoDNA-T phosphoramidite 34

The (S)-Me-EoDNA-T phosphoramidite was synthesized according to Scheme 8. 4’-Propargyloxy 28 was reduced with H₂ in the presence of Lindlar’s catalyst to afford 4’-allyloxy compound in 90% yield, treatment of which with TCDI gave radical precursor 35 in 83% yield. Compound 35 underwent a radical reaction in the presence of AIBN and (TMS)₃SiH to yield (S)-methyl compound 36 and the R-isomer 37 in 66% and 2% yields, respectively, through deoxygenation and intramolecular radical cyclization. The high stereoselectivity was considered to be due to the steric repulsion between the 3’-O-TBS group and the alkene moiety, as shown in Figure 12. Similarly, the radical cyclization for bridge construction of cENA with a C-C-C linkage exhibited the same configuration with exclusive stereoselectivity (Scheme 5).²⁰ Compound 36 was converted into desilylated 38, NOESY measurement of which led to determination of the configuration of the 8’-methyl group. Compound 38 was phosphitylated to afford the phosphoramidite 39 with a (S)-methyl EoDNA skeleton.

Scheme 8. Synthesis of (S)-Me-methylene-EoDNA-T phosphoramidite 39
Unsubstituted EoDNA-T phosphoramidite was synthesized according to Scheme 9. 5′-O-Dimethoxytritylated methylene-EoDNA-T monomer 31 was treated with in situ generated dimethyldioxirane, and ring-opening of the obtained epoxide by NaOH followed by NaIO₄-oxidation yielded ketone 40 in 30% yield, via a three-step process. On the other hand, the oxidative cleavage of 31 using K₂OsO₄ and NaIO₄ did not proceed at all. After 40 was converted into the corresponding hydrazone, treatment with iodine and DBU led to the formation of iodoalkene 41, hydrogenolysis of which afforded 42 with an EoDNA skeleton. Phosphitylation of 42 using i-Pr₂NP(Cl)OCH₂CH₂CN successfully produced desired EoDNA-T phosphoramidite 43.

As a purine analog of methylene-EoDNA, the phosphoramidites bearing adenine protected by a benzoyl group on the 6-amino group was synthesized (Scheme 10). Adenosine 44 was used as a starting material, and an N⁶-benzoylated compound 46 was obtained via exo-olefin 45. Compound 46 was subjected to
treatment with TBSCI and imidazole, leading to the formation of 3'-O-TBS 47 (51% yield) along with 2'-O-TBS 48 (23% yield). Epoxidation of 47 by *in situ* generated dimethyldioxirane followed by introduction of propargyl alcohol afforded desired 49 in 44% yield, with high diastereoselectivity, though 50 was by-produced in 3% yield for two steps. After conversion of 49 into radical precursor 51, the radical reaction gave fully protected EoDNA-A monomer 52. The phosphoramidite 53 was successfully obtained from 52 via two steps.

**Scheme 10.** Synthesis of methylene-MoDNA-A phosphoramidite 53

Oligonucleotide synthesis using methylene-EoDNA-T, methylated methylene-EoDNA-T, EoDNA-T, methylated EoDNA-T, and methylene-EoDNA-A phosphoramidites adopted prolonged coupling time
(from 25 s to 10 min) for sufficiently incorporating modified phosphoramidites. Particularly, in the case of oligonucleotides including methylene-EoDNA derivatives, 1 M BuOOH in toluene, instead of a common 0.02 M iodine solution, was required as an oxidizing reagent to avoid the decomposition of the methylene moiety.

The $T_m$ values of duplexes including multiple modification by six EoDNA-T derivatives (methylene-EoDNA-T, (R)-Me-methylene-EoDNA-T, (S)-Me-methylene-EoDNA-T, EoDNA-T, (R)-Me-EoDNA-T, and (S)-Me-EoDNA-T) are shown in Table 4. All EoDNA-T derivatives stabilized the duplexes formed with ssRNA, while slightly lower stability of duplexes with ssDNA was shown when compared with that of the natural DNA duplex depending on the number and position of modification. In the case of EoDNA-T derivatives with exocyclic methylene groups, regardless of whether a 7’-methyl group exists or not, an apparent increase in $\Delta T_m$/mod. values was observed by up to 5.0 °C. It was shown that the 7’-methyl group in (R)-Me-methylene-EoDNA-T and (S)-Me-methylene-EoDNA-T might have no effect on the duplex structure including the hydrogen bonding network in the minor groove.41

Unsubstituted EoDNA-T modification apparently had a slightly lower stabilization ability of duplexes with ssRNA than did methylene-EoDNA-T modification. Among EoDNA-T derivatives without exocyclic methylene group, stabilization ability of (R)-Me-EoDNA-T was lowest though a $T_m$ increase of 3.2 °C per modification was shown using the oligonucleotide containing five modifications. This result suggested that the 8’-methyl group in (R)-Me-EoDNA-T affected the stability of the duplexes with ssRNA, which might be caused by structural distortion based on steric repulsion between the 3’-phosphodiester moiety and the (R)-methyl group at the 8’ position (Figure 13). Chattopadhyaya’s group already reported duplex-forming ability of oligonucleotides modified by 2’,4’-carbocyclic-ENA-T and its 8’-methyl analogs with ssRNA by UV-melting experiments.22 As the results, 2’,4’-carbocyclic-ENA-T, (8’R)-methyl 2’,4’-carbocyclic-ENA-T, and a 4:5 mixture of (8’R)-methyl and (8’S)-methyl 2’,4’-carbocyclic-ENA-T increased the $\Delta T_m$/mod. values by approximately 1.4 °C, 0.3 °C, and 0.5 °C, respectively. The values were much lower than those of the 6’-oxygen analogs, namely, EoDNA-T, (R)-Me-EoDNA-T, and (S)-Me-EoDNA-T, which also demonstrated that the 6’-oxygen could be important for stabilization of the duplexes with ssRNA.

<table>
<thead>
<tr>
<th>Table 4. $T_m$ Values of duplexes obtained by UV-melting experiments</th>
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<tr>
<td>5’-TCTTCTTTTTCTCTTCTTCTCT-3’ with ssRNA</td>
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</tr>
<tr>
<td>5’-TCTTCTXTXCTXCTCTCT-3’ with ssRNA</td>
</tr>
<tr>
<td>Oligonucleotide</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>5'-TCTXCTXTXCTCT-3'</td>
</tr>
<tr>
<td>5'-TCTTCTXXTCTCT-3'</td>
</tr>
<tr>
<td>5'-TCTTCXTXTXCTCT-3'</td>
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<tr>
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<td>5'-TCTTCTYYTCTCT-3'</td>
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<tr>
<td>5'-TCTTCTYYTCTCT-3'</td>
</tr>
<tr>
<td>5'-TCTTCTYYTCTCT-3'</td>
</tr>
</tbody>
</table>

Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 140 mM KCl, and 4 μM of each oligonucleotide. X = methylene-EoDNA-T. X = (R)-Me-methylene-EoDNA-T. X = (S)-Me-methylene-EoDNA-T. Y = EoDNA-T. Y = (R)-Me-EoDNA-T. Y = (S)-Me-EoDNA-T. C = 2'-deoxy-5-methylcytidine. The sequences of ssRNA and ssDNA are 5'-ACGAGAACAUCC-3' and 5'-ACGAGAACATCC-3', respectively. The changes in T_m values per modification (ΔT_m/mod.) relative to the natural duplexes are shown in parentheses.

Figure 13. 1,3-Diaxial repulsion between 8'-methyl group and 3'-phosphodiester moiety in (R)-Me-EoDNA

Degradation of oligonucleotides using 3'-exonuclease demonstrated that methylene-EoDNA-T was the most labile against nuclease amongst the six EoDNA-T derivatives (data not shown). However, in terms of the resistance against nuclease, even methylene-EoDNA-T with three-atom (C-C-O) bridge was
superior to EoNA-T with four-atom bridge, although a large bridge size is generally considered to have higher stability against nuclease degradation. This result may imply that the carbon attached to 2’-carbon is a key factor for improving nuclease resistance. Substituents on the 8’-carbon also had a significant influence on the nuclease resistance, which decreased in the following order: (R)-methyl group, (S)-methyl group, non-substituent, and methylene group. With regard to the 7’-substituents in methylene-EoDNA-T derivatives, nuclease resistance decreased in the order of (R)-methyl group, (S)-methyl group, and non-substituent. From these results, the methyl groups, like (7’R)-methyl or (8’R)-methyl ones, located close to the 3’-phosphodiester moiety were considered to disturb the nuclease degradation by steric hindrance. In addition, EoDNA-T exhibited high resistance to nuclease, as compared with ENA, thus indicating that the presence of 6’-oxygen might also contribute to increased nuclease resistance in the case of 2’,4’-bridged modifications with three-atom bridges.

Evaluation of apoB (apolipoprotein B) mRNA inhibitory activity of gapmer oligonucleotides, including six EoDNA-T derivatives, using Huh-7 cells was performed (data not shown). All EoDNA-T derivatives significantly reduced apoB mRNA expression levels in a concentration-dependent manner and provided comparable potency to LNA-T. It suggested that these EoDNA-T modifications could be promising building blocks for antisense oligonucleotides.

We were also interested in the stability of DNA duplex including base pairs between methylene-EoDNA-A and methylene-EoDNA-T and the stability was examined by UV-melting experiments (Table 5). In the case of DNA duplexes including either methylene-EoDNA-A or methylene-EoDNA-T modifications, the $\Delta T_m$/mod. values ranged from $-1.0 \, ^\circ\text{C}$ to $2.0 \, ^\circ\text{C}$. DNA duplexes including both single methylene-EoDNA-A and single methylene-EoDNA-T did not show a significant change in the $\Delta T_m$/mod. values ($\Delta T_m$/mod. = $0.5$–$1.0 \, ^\circ\text{C}$). Interestingly, while the $T_m$/mod. values of duplexes including either two methylene-EoDNA-A or two methylene-EoDNA-T modifications ranged from $1.5 \, ^\circ\text{C}$ to $2.0 \, ^\circ\text{C}$, duplexes with two methylene-EoDNA-A:methylene-EoDNA-T base pairs were synergistically stabilized and showed increased $T_m$/mod. values of $2.5 \, ^\circ\text{C}$.

<table>
<thead>
<tr>
<th>Table 5. $T_m$ Values of duplexes obtained by UV-melting experiments</th>
</tr>
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<tbody>
<tr>
<td><strong>$T_m$</strong></td>
</tr>
<tr>
<td>5’-ACGAGAACATCG-3’ 3’TGCTCTTGTAGG-5’</td>
</tr>
<tr>
<td>5’-ACGAGAACATCG-3’ 3’TGCTCTCTTGTAGG-5’</td>
</tr>
<tr>
<td>5’-ACGAGAACATCG-3’ 3’TGCTCTTGTAGG-5’</td>
</tr>
</tbody>
</table>
5′-ACGAGAACATCG-3′
3′-TGCTCTTTGTAGG-5′

51 °C +1.5 °C

5′-ACGAGAACATCG-3′
3′-TGCTCTTTGTAGG-5′

47 °C −1.0 °C

5′-ACGAGAACATCG-3′
3′-TGCTCTTTGTAGG-5′

49 °C +1.0 °C

5′-ACGAGAACATCG-3′
3′-TGCTCTTTGTAGG-5′

51 °C +1.5 °C

5′-ACGAGAACATCG-3′
3′-TGCTCTTTGTAGG-5′

52 °C +2.0 °C

5′-ACGAGAACATCG-3′
3′-TGCTCTTTGTAGG-5′

49 °C +0.5 °C

5′-ACGAGAACATCG-3′
3′-TGCTCTTTGTAGG-5′

50 °C +1.0 °C

5′-ACGAGAACATCG-3′
3′-TGCTCTTTGTAGG-5′

58 °C +2.5 °C

5′-ACGAGAACATCG-3′
3′-TGCTCTTTGTAGG-5′

58 °C +2.5 °C

Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, and 2.5 μM of each oligonucleotide. \( A \) = methylene-EoNA-A. \( T \) = methylene-EoNA-T. The changes in \( T_m \) values per modification (Δ\( T_m \)/mod.) relative to the natural duplexes are shown in parentheses.

5. 2′,4′-C-METHYLENEOXY-BRIDGED THYMIDINES

As shown in Figure 1, many 2′,4′-bridged nucleosides for modifications of oligonucleotides have been developed to date; however, there was no previous report on a 2′,4′-bridged nucleoside with 6′-oxygen in addition to a two-atom bridge. Improvement of the properties of oligonucleotides by EoNA-T and EoDNA-T derivatives encouraged us to develop 2′-C,4′-C-methyleneoxy-bridged nucleosides, MoDNAs, with 6′-oxygen atom and a small two-atom (carbon-oxygen) bridge. Owing to their synthetic accessibility, the 7′-methyl analogs of MoDNA-T, (\( R \))-Me-MoDNA-T and (\( S \))-Me-MoDNA-T were designed (Figure 14).

\[ \text{carbon-oxygen bridge} \]

Figure 14. Structures of (\( R \))-Me-MoDNA-T and (\( S \))-Me-MoDNA-T monomers
The synthesis of (R)-Me-MoDNA-T and (S)-Me-MoDNA-T are shown in Scheme 11. Iodation of 5-methyluridine 1 followed by treatment with NaOMe afforded exo-olefin 2. Although our previous procedure needed silica gel column chromatography after each step (iodation and base treatment), in the current procedure, 5'-iodide compound could be precipitated using 2.5% solution of MeOH in CHCl₃, and the reaction using NaOMe, instead of DBU, also gave exo-olefin 2 without column purification. Consequently, chromatography-free isolation of 2 could be achieved. In terms of the synthesis of 3'-O-silylated compound 25 from 2, it was found that the common conditions using TBSCl and imidazole also afforded desired 25 with almost the same efficiency (42% yield). Epoxidation of 25 with dimethyldioxirane in situ generated from Oxone® and acetone followed by ZnCl₂-mediated ring-opening yielded desired 54, along with the diastereoisomer 55. The structures of 54 and 55 were determined by NOESY measurement (Figure 15a). After deprotection of two TES groups in 56 using HF·Et₃N, TBS-protection of primary alcohol in the obtained diol followed by reaction with TCDI afforded radical precursor 57. When a solution of AIBN and Bu₃SnH in toluene was slowly added dropwise to the reaction solution, intramolecular 5-exo radical cyclization exclusively proceeded to give cyclized product 58 as an inseparable 1:1 diastereomixture. In contrast, the one-portion addition of AIBN and Bu₃SnH unexpectedly led to decomposition of the precursor 57, without production of desired 58. After separation of each isomer in the step of compounds 59a and 59b, the stereochemistry of the 7'-methyl groups in 59a and 59b was determined by NOESY measurement (Figure 15b). Phosphitylation of 59a and 59b produced phosphoramidites 60a and 60b, which are suitable building blocks for oligonucleotide synthesis. Oligonucleotides modified by (R)-Me-MoDNA-T and (S)-Me-MoDNA-T were synthesized on an oligonucleotide synthesizer through a common phosphoramidite chemistry; however, the coupling time for efficient introduction of Me-MoDNA-T phosphoramidites 60a and 60b was prolonged from 25 s to 10 min.
Scheme 11. Synthesis of (R)- and (S)-Me-MoDNA-T phosphoramidites 60a and 60b

Figure 15. NOESY correlations of compounds 54, 55, 59a, and 59b
The $T_m$ values of duplexes of oligonucleotides including (R)-Me-MoDNA-T or (S)-Me-MoDNA-T with ssRNA or ssDNA are shown in Table 6. Me-MoDNA-T modifications increased the stability of duplexes with ssRNA. In particular, stabilization ability of (S)-Me-MoDNA-T ($\Delta T_m$/mod. = 6.0 °C) was comparable to that of 2',4'-BNA/LNA-T ($\Delta T_m$/mod. = 6.5–7.0 °C), which has been used for therapeutic oligonucleotides. With regard to the stability of duplexes formed with ssDNA, oligonucleotides modified by (R)-Me-MoDNA-T or (S)-Me-MoDNA-T exhibited lower $T_m$ values than 2',4'-BNA/LNA-modified oligonucleotides. Consequently, (S)-Me-MoDNA-T had high stabilization ability to duplexes formed with ssRNA in a RNA-selective fashion, as compared with 2',4'-BNA/LNA, which was due to low stabilization of duplexes with ssDNA by (S)-Me-MoDNA-T. In the case of Me-EoDNA-T with three-atom (C-C-O) bridge, the stereochemistry of 8'-methyl group affected the stability of duplexes with ssRNA, and 8'R-methyl group destabilized the duplexes by steric repulsion between the methyl group and 3'-phosphodiester backbone. In contrast, this case with two-atom (C-O) bridge demonstrated that the stereochemistry of the methyl group had almost no effect on the stability. Ab initio calculations of 3',5'-O-bismethyl analogs of (R)-Me-MoDNA-T and (R)-Me-EoDNA-T, which were used to simplify the calculations, showed that the distance between the methyl carbon and the 3'-oxygen in (R)-Me-MoDNA-T was farther than that in (R)-Me-EoDNA-T (Figure 16), which also supported the deduction that the methyl group of Me-MoDNA-T hardly affected the stability. Chattopadhyaya’s group reported that cENA-T, which is the 6'-carbon analog of Me-EoDNA-T, stabilized the duplexes with ssRNA by a $T_m$ increase of 3.1 °C per modification.21 Under the same conditions, 2',4'-BNA/LNA-T showed $\Delta T_m$/mod. values of 4.5 °C. The difference between the $\Delta T_m$/mod. values of cENA-T and 2',4'-BNA/LNA-T was −1.4 °C. In contrast, the difference between those of Me-EoDNA-T and 2',4'-BNA/LNA-T was slightly small when compared with that in the case of the 6'-carbon analog, cENA-T. This may also imply importance of the 6'-oxygen in the two-atom bridge in the duplex with ssRNA.

<table>
<thead>
<tr>
<th></th>
<th>with ssRNA</th>
<th>with ssDNA</th>
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<tbody>
<tr>
<td>5'-GGATGTTCCTCGT-3'</td>
<td>47 °C</td>
<td>47 °C</td>
</tr>
<tr>
<td>5'-GGATGTTCCTCGT-3'</td>
<td>52 °C (+5.0 °C)</td>
<td>48 °C (+1.0 °C)</td>
</tr>
<tr>
<td>5'-GGATGTTCCTCGT-3'</td>
<td>57 °C (+5.0 °C)</td>
<td>51 °C (+2.0 °C)</td>
</tr>
<tr>
<td>5'-GGATGTTCCTCGT-3'</td>
<td>58 °C (+5.5 °C)</td>
<td>50 °C (+1.5 °C)</td>
</tr>
<tr>
<td>5'-GGATGTTCCTCGT-3'</td>
<td>53 °C (+6.0 °C)</td>
<td>47 °C (0 °C)</td>
</tr>
</tbody>
</table>
5ʹ-GGATG
TTCTCGT-3ʹ  59 °C (+6.0 °C)  50 °C (+1.5 °C)
5ʹ-GGATG
TTCTCGT-3ʹ  59 °C (+6.0 °C)  51 °C (+2.0 °C)
5ʹ-GGATG
TTCTCGT-3ʹ  54 °C (+7.0 °C)  49 °C (+2.0 °C)
5ʹ-GGATG
TTCTCGT-3ʹ  60 °C (+6.5 °C)  52 °C (+2.5 °C)
5ʹ-GGATG
TTCTCGT-3ʹ  60 °C (+6.5 °C)  53 °C (+3.0 °C)

Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, and 2.5 μM of each oligonucleotide. T = (R)-Me-MoDNA-T. T = (S)-Me-MoDNA-T. T = 2ʹ,4ʹ-BNA/LNA-T. The sequences of ssRNA and ssDNA are 5ʹ-ACGAGAACAUCC-3ʹ and 5ʹ-ACGAGAACATCC-3ʹ, respectively. The changes in T_m values per modification (ΔT_m/mod.) relative to the natural duplexes are shown in parentheses.

Figure 16. Ab initio calculations (B3LYP/6-31G*) of 3ʹ,5ʹ-O-bismethyl analogs of (R)-Me-MoDNA-T and (R)-Me-EoDNA-T

Triplex-forming ability of 14-mer oligonucleotides containing (R)-Me-MoDNA-T or (S)-Me-MoDNA-T with dsDNA was also examined by UV-melting experiments and was compared to that containing 2ʹ,4ʹ-BNA/LNA-T (data not shown). Oligonucleotides modified by (R)-Me-MoDNA-T or (S)-Me-MoDNA-T formed significantly stable triplexes with dsDNA, as compared to the unmodified triplex formed, though triplexes with (R)-Me-MoDNA-T or (S)-Me-MoDNA-T modifications were less stable than those including 2ʹ,4ʹ-BNA/LNA-T. Contrary to the duplexes formed with ssRNA, (R)-Me-MoDNA-T was found to more stabilize the triplexes in comparison to (S)-Me-MoDNA-T. This result implies that the (7ʹR)-methyl group possibly caused steric repulsion in the triplex structure, which is less flexible and more crowded than the duplex.

Nuclease degradation experiments of Me-MoDNA-modified oligonucleotides using 3ʹ-exonuclease were carried out and the results are shown in Figure 17. As expected, Me-MoDNA modification increased the resistance of oligonucleotides against the nuclease compared to unmodification. Nuclease resistance of (R)-Me-MoDNA-T was superior to that of (S)-Me-MoDNA-T and was almost the same as that of ENA with a larger three-atom bridge. This indicated that the (7ʹR)-methyl group might disturb the access of
nuclease to the 3’-phosphodiester linkage because the methyl group is located close to the 3’-phosphodiester moiety.

**Figure 17.** Degradation experiments by 3’-exonuclease. Conditions: 0.05 unit/mL *Crotalus admanteus* venom phosphodiesterase, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, and 7.5 μM of oligonucleotide at 37 °C.

6. **2’-O,4’-C-ETHYLENE-BRIDGED 5-METHYLURIDINE**

The bridge construction in 2’,4’-bridged nucleosides by intramolecular radical cyclization was limited to the use of the 2’-carbon radical, which was generated via deoxygenation of ribonucleosides as mentioned in Introduction. In addition, because 2’,4’-bridged nucleosides synthesized using the 2’-carbon radical have a C2’-C bond in the bridge, no 2’-ribo-type of the bridged nucleosides could be synthesized. In 2018, we reported a facile generation method of 4’-carbon radical by photoredox-catalyzed deformylative reaction of 2’-deoxy-5’-O-phthalimidonucleosides and the short-step synthesis of various 5’-carbon analogs of nucleoside 5’-phosphates was achieved. This strategy might be applicable to the synthesis of 2’,4’-bridged nucleosides; in particular, 2’-ribo-type of 2’,4’-bridged nucleosides with a C2’-O bond in the bridge. Thus, 6’-methylated 2’-O,4’-C-ethylene-bridged 5-methyluridine, 6’-Me-ENA-T, was designed (Figure 18). There is a case of improving the property of oligonucleotides when 2’,4’-BNA/LNA modification was replaced by S-cEt, the (6’S)-methyl analog of 2’,4’-BNA/LNA. Thus, introduction of a methyl group onto the bridge moiety might be an effective modification for improvement of the properties of oligonucleotides modified by 2’,4’-bridged nucleoside without any substituent on the bridge.

**Figure 18.** Structure of 6’-Me-ENA monomer
The synthesis of a radical precursor, along with the radical reaction, is shown in Scheme 12. BOM-protection of thymine base in the known compound 61 followed by deacetylation using MeNH₂ afforded 62, which was treated with allyl bromide and NaN₃ and desilylated using TBAF to yield 63. Next, O-phthalimidation of 63 using N-hydroxyphthalimide (NHPI) under Mitsunobu conditions led to the 4'-carbon radical precursor 64. In the presence of fac-Ir(ppy)₃ as a photoredox catalyst and Hantzsch ester as a reductant and hydrogen source, irradiation of 64 with a 32 W compact fluorescent lamp (CFL) stereoselectively produced unexpected 2',3'-bridged nucleoside 65 in 83% yield. The plausible reaction mechanism is shown in Figure 19. Initially, an oxy radical generated by light irradiation underwent 1,6-hydrogen atom transfer (1,6-HAT) leading to a benzyl radical. Then, 8-endo cyclization of the benzyl radical proceeded to yield 65. The complete stereoselectivity was probably due to steric repulsion between the phenyl group and 3'-hydrogen atom in the transition state of 8-endo radical cyclization. Consequently, unlike 2'-deoxy-5'-O-phthalimidonucleosides, compound 64 exclusively underwent 1,6-HAT, instead of the desired 4'-carbon radical generation via deformylation.

Scheme 12. Synthesis of intermediate 64 and the radical reaction
Decarboxylative radical generation on the 4ʹ-carbon was tried because decarboxylation is much faster than deformylation in a radical reaction (Scheme 13). Thus, the alcohol of compound 63 was oxidized to the corresponding carboxylic acid, condensation of which with NHPI yielded radical precursor 66 for the 4ʹ-carbon radical generation via decarboxylation. The light-mediated radical reaction of 66 expectedly resulted in radical decarboxylation to stereoselectively lead to methylated ENA 67 with an S-configuration via the 4ʹ-carbon radical intermediate. The stereochemistry of the methyl group can be explained by steric repulsion between the 3ʹ-benzyloxy group and alkene moiety, as shown in Figure 20. Moreover, the stereochemistry was determined by NOESY measurements (Figure 20). After hydrogenolysis using H₂ and Pd(OH)₂/C yielding protection-free (S)-Me-ENA-T monomer 68 in 93% yield, the desired phosphoramidite 69 for oligonucleotide synthesis was synthesized by dimethoxytritylation followed by phosphitylation. Oligonucleotides modified by (S)-Me-ENA-T were synthesized on an oligonucleotide synthesizer through a common phosphoramidite chemistry and the coupling efficiency for introduction of (S)-Me-ENA-T phosphoramidites 69 was estimated to be over 95%, with a prolonged coupling time from 25 s to 10 min.
The $T_m$ values of duplexes and triplexes formed by oligonucleotides, including (S)-Me-ENA-T, ENA-T, and 2',4'-BNA/LNA-T, are summarized in Table 7. Oligonucleotides modified by (S)-Me-ENA-T had almost the same stability of duplexes with ssRNA as ENA- and 2',4'-BNA/LNA-modified oligonucleotides. In terms of duplexes with ssDNA, (S)-Me-ENA-T allowed a decrease in the $T_m$ values in comparison to ENA-T and 2',4'-BNA/LNA-T. These results demonstrated that the (6'S)-methyl group affected the duplex structure formed with ssDNA though the duplex with ssRNA were hardly affected. Consequently, differences in the $T_m$ values against ssRNA and ssDNA of (S)-Me-ENA-T were larger than those of ENA and 2',4'-BNA/LNA; for example, in the case of a single modification, (S)-Me-ENA-T had a $T_m$ difference of 7 °C while those of ENA and 2',4'-BNA/LNA modifications were 4 °C and 3 °C, respectively. This means that (S)-Me-ENA-T could have high ssRNA-selectivity in duplex formation. In triplex formed with dsDNA, (S)-Me-ENA-T increased the stability, with the $\Delta T_m$mod. values ranging...
from 3.0 °C to 4.7 °C, though the stabilization ability was slightly less than those of ENA and 2ʹ,4ʹ-BNA/LNA modifications.

| Table 7. $T_m$ Values of duplexes and triplexes obtained by UV-melting experiments |
|--------------------------------------|----------------|----------------|----------------|
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 47 °C          | 46 °C          | 27 °C          |
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 50 °C (+3.0 °C)| 43 °C (−3.0 °C)| 30 °C (+3.0 °C)|
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 60 °C (+4.3 °C)| 45 °C (−0.3 °C)| 37 °C (+3.3 °C)|
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 63 °C (+5.3 °C)| 47 °C (+0.3 °C)| 41 °C (+4.7 °C)|
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 51 °C (+4.0 °C)| 46 °C (0 °C)   | 30 °C (+3.0 °C)|
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 61 °C (+4.7 °C)| 49 °C (+1.0 °C)| 42 °C (+5.0 °C)|
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 64 °C (+5.7 °C)| 48 °C (+0.7 °C)| 45 °C (+6.0 °C)|
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 52 °C (+5.0 °C)| 48 °C (+2.0 °C)| 32 °C (+5.0 °C)|
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 61 °C (+4.7 °C)| 49 °C (+1.0 °C)| 40 °C (+4.3 °C)|
| 5ʹ-TCTTCTTTTTCTCT-3ʹ                  | 63 °C (+5.3 °C)| 51 °C (+1.7 °C)| 44 °C (+5.7 °C)|

Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM KCl, and 2.5 μM of each oligonucleotide for duplex; and 10 mM sodium cacodylate buffer (pH 7.4), 100 mM KCl, 10 mM MgCl₂, and 1.5 μM of each oligonucleotide for triplex. $T = (S)$-Me-ENA-T, $T = ENA-T$, $T = 2′,4′$-BNA/LNA-T. C = 2′-deoxy-5-methylcytidine. The sequences of ssRNA, ssDNA, and dsDNA are 5ʹ-r(AGAGAAAAAGAAGA)-3ʹ, 5ʹ-d(AGAGAAAAAGAAGA)-3ʹ, and 5ʹ-d(GCGTCTTCTTTTTCTCTGCC)-3ʹ, respectively. The changes in $T_m$ values per modification ($ΔT_m$/mod.) relative to the natural duplexes are shown in parentheses.

Nuclease degradation experiments of $(S)$-Me-ENA-modified oligonucleotide were also performed. Stability of $(S)$-Me-ENA-T against nuclease degradation using 3ʹ-exonuclease was also obviously higher than those of ENA-T and 2ʹ,4ʹ-BNA/LNA-T (data not shown). This was likely due to the inhibition of access of nuclease to 3ʹ-phosphodiester moiety by steric hindrance of the methyl group. If an oligonucleotide including the (6ʹR)-methyl analog was synthesized, the (6ʹR)-methyl analog would show higher resistance against nuclease degradation than $(S)$-Me-ENA-T in light of the results of nuclease degradation experiments of $(R)$-Me-EoDNA-T and $(S)$-Me-EoDNA-T.

7. CONCLUSION
Development of therapeutic oligonucleotides is receiving the increased attention and chemical modifications of oligonucleotides are growing in importance. In particular, 2ʹ,4ʹ-bridged nucleosides are
promising modifications for antisense oligonucleotides because there is no doubt that they can significantly increase not only the binding affinity of oligonucleotides to ssRNA but also resistance of oligonucleotides against nuclease degradation. Therefore, evaluation of oligonucleotides using various 2ʹ,4ʹ-bridged nucleosides would deepen the understanding of rational design of the oligonucleotide and accumulation of the data might allow us to efficiently develop therapeutic oligonucleotides. In this review, our recent results on 2ʹ,4ʹ-bridged nucleosides developed based on a new design concept or a new synthetic strategy were described. In many cases, it was found that the presence of 6ʹ-oxygen in the bridge improved the properties of oligonucleotides. Moreover, radical cyclization using 4ʹ-carbon radical can allow us to develop useful 2ʹ,4ʹ-bridged nucleosides because there are few examples on the synthesis of nucleosides using 4ʹ-carbon radical. The author expects that the contents mentioned in this review can contribute to development of practical and useful oligonucleotides.

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43. Unpublished data.


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