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BRIDGED NUCLEOSIDES AS BUILDING BLOCKS OF OLIGONUCLEOTIDES: SYNTHESIS AND PROPERTIES

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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.

CONTENTS

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.

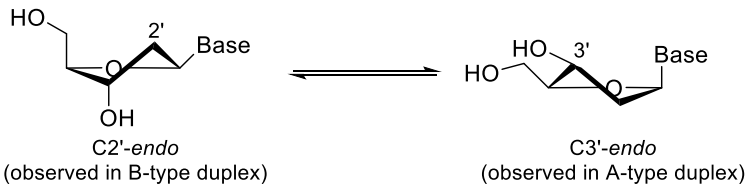


Figure 1. Equilibrium between C2′-*endo* and C3′-*endo* conformations of nucleosides

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.[1-3] 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.[4-18] 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 2′,4′-bridged nucleosides is still in demand.

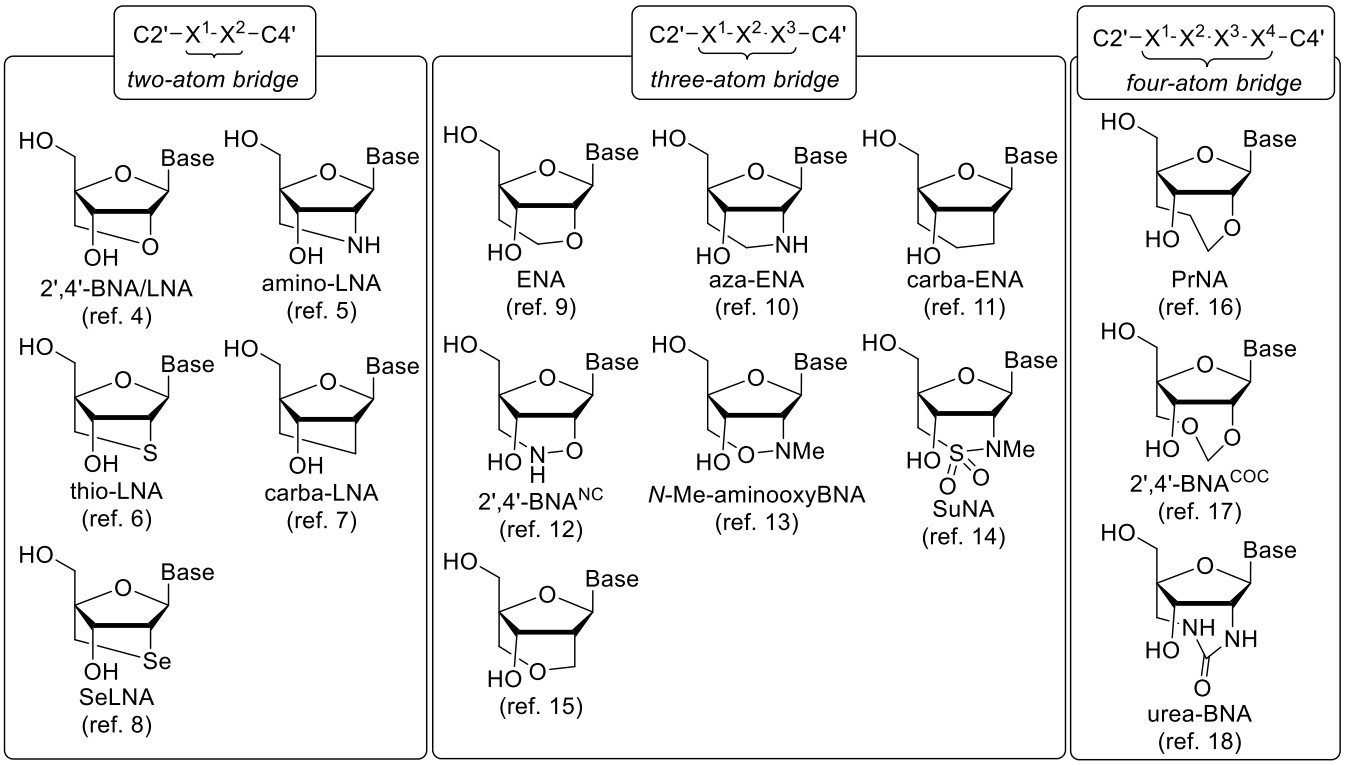


Figure 2. Typical examples of 2′,4′-bridged nucleosides, used for chemical modification of oligonucleotides, classified by bridge sizes

To construct the bridge of 2′,4′-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 S_N2 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 2′,4′-bridged nucleosides containing any heteroatom in the bridge, which account for the great majority of 2′,4′-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 2′- and 4′-positions. In 2006, Nielsen's group reported 2′,4′-bridge construction by means of ring-closing metathesis,[11] and various derivatives of the 2′,4′-carbocyclic nucleoside (carba-ENA) with a three-atom (C-C-C) bridge were synthesized.[11,19] Intramolecular radical cyclization of the 2′-carbon radicals of nucleosides with terminal alkene units was also employed to construct 2′,4′-carbocyclic nucleosides with two- and three-atom bridges (C2′-C-C-C4′ and C2′-C-C-C-C4′) by Chattopadhyaya's group in 2007.[20] It is well-known that the 2′-carbon radicals can be generated via deoxygenation by the reaction of 2′-*O*-thiocarbonyl nucleosides, like 2′-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.[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.[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[25-27] were synthesized (*see* Section 3) and the development of several 2′,4′-bridged nucleosides[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).[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[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 anomeric effect on the 4′-carbon induced by the alkoxy oxygen and the furanose oxygen.[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.[35] Thus, we designed 2′-*O*,4′-*C*-ethyleneoxy-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).[24] EoNA-T is the first example of bridged nucleosides replacing 6′-carbon by any heteroatom.

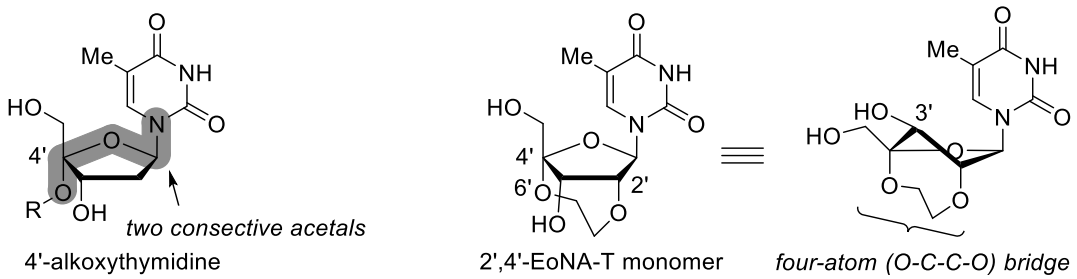
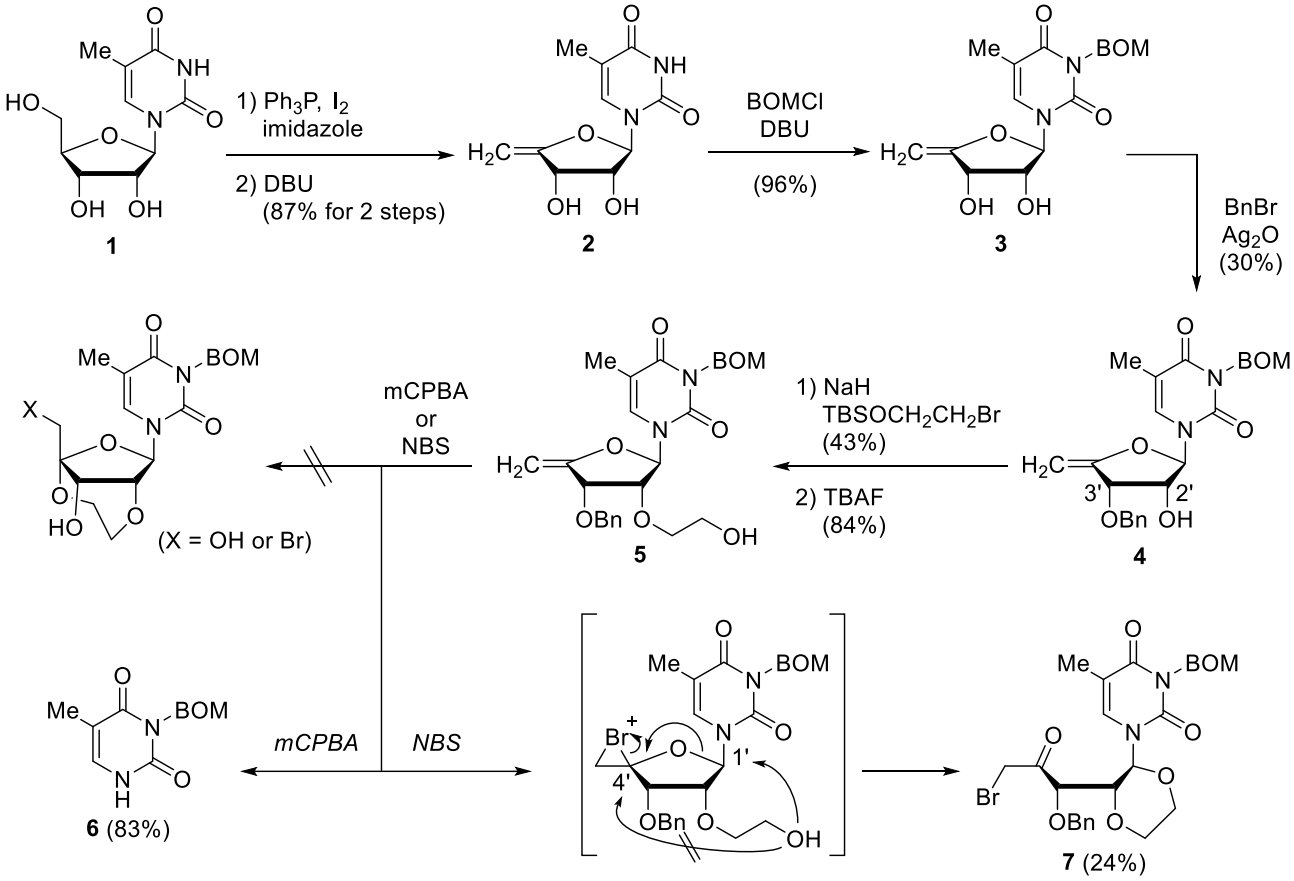


Figure 3. Structures of 4′-alkoxynucleosides and EoNA-T monomer

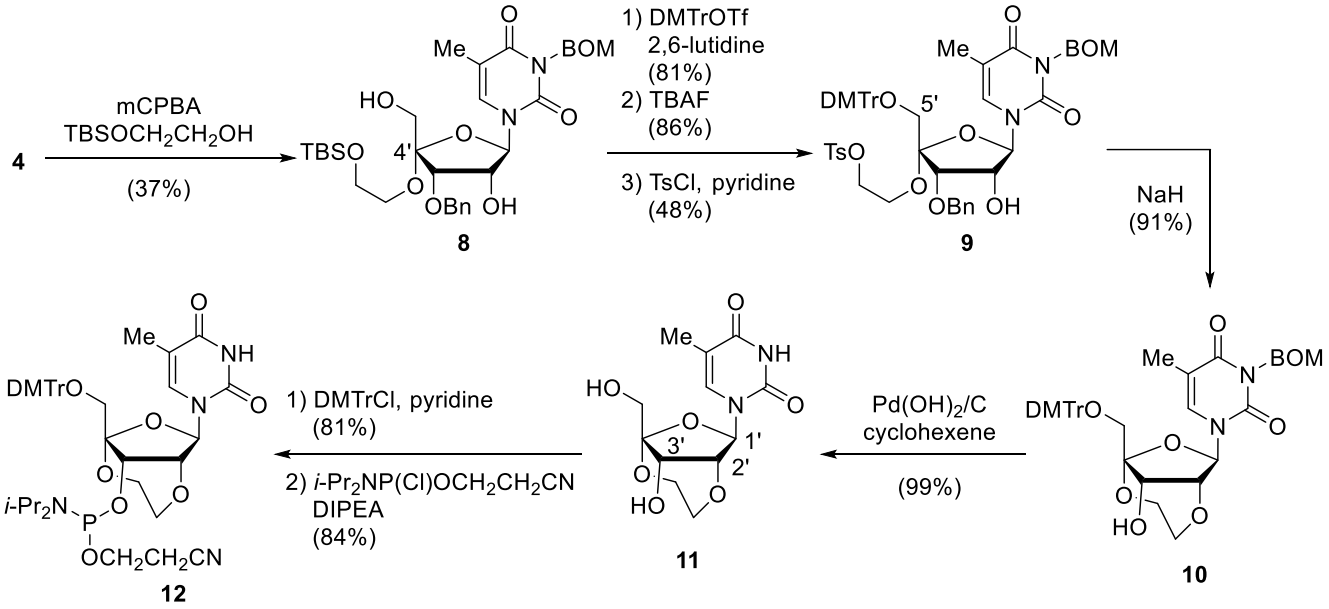
The synthesis from 5-methyluridine **1** was performed as shown in Scheme 1. Conversion of the 5′-hydroxyl group in **1** into iodide followed by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) produced compound **2** in 87% for two steps, which underwent benzyloxymethyl (BOM)-protection of the 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, Bn_2SnO and BnBr, and Ag_2O and BnBr. The reaction using Ag_2O 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.



Scheme 1. Synthesis of intermediate **5** and attempts to construct bridge structure

As an alternative route, a 2-hydroxylethyl unit was introduced into the 4′-carbon position in **4** by treatment with freshly prepared anhydrous mCPBA and $TBSOCH_2CH_2OH$ in a solvent-free system. In this reaction, stereoselective epoxidation followed by an S_N2 reaction of the siloxy alcohol at the 4′-carbon proceeded to afford **8** as a sole isomer. Dimethoxytritylation of 5′-hydroxyl group in **8** using dimethoxytrityl trifluoromethanesulfonate (DMTrOTf),[36] 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 2′,4′-BNA^{COC}-T monomer shown in Figure 4. This result implies that the sugar conformation of EoNA-T would be the same as that of 2′,4′-BNA^{COC}-T, C3′-*endo* conformation,[17] with the same four-atom bridge between 2′- and 4′-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. When the EoNA-T phosphoramidite was introduced into the oligonucleotide using an oligonucleotide synthesizer, a prolonged coupling time (20 min) gave an

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**

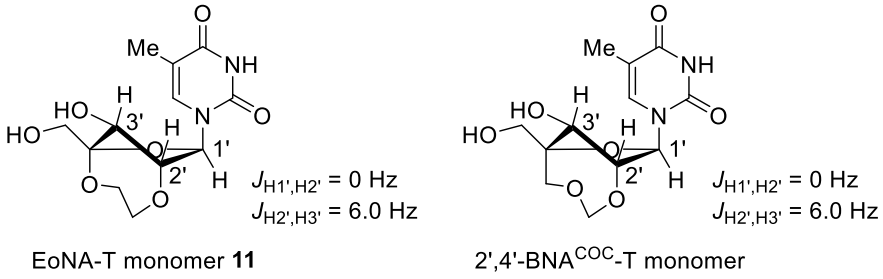


Figure 4. The $J_{H1',H2'}$ values of EoNA-T and 2′,4′-BNA^{COC}-T monomers

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 ΔT_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 (ΔT_m/mod. = 0.5 °C).[16] EoNA-T, which is the 6′-carbon analog of PrNA, increased the ΔT_m/mod. values of the duplexes by up to 3.6 °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′-T<u>C</u>TT<u>C</u>TTTTT<u>C</u>T<u>C</u>T-3′	51 °C	50 °C	31 °C
5′-T<u>C</u>TT<u>C</u>TT**T**TT<u>C</u>T<u>C</u>T-3′	52 °C (+1.0 °C)	48 °C (−2.0 °C)	32 °C (+1.0 °C)
5′-T<u>C</u>TT<u>C</u>T**TT**TT<u>C</u>T<u>C</u>T-3′	59 °C (+2.7 °C)	47 °C (−1.0 °C)	37 °C (+2.0 °C)
5′-T<u>C</u>TT<u>C</u>**T**TT**T**T<u>C</u>T<u>C</u>T-3′	60 °C (+3.0 °C)	47 °C (−1.0 °C)	41 °C (+3.3 °C)
5′-T<u>C</u>T**T**<u>C</u>**T**TT**T**T<u>C</u>**T**<u>C</u>T-3′	69 °C (+3.6 °C)	48 °C (−0.4 °C)	50 °C (+3.8 °C)
5′-T<u>C</u>TT<u>C</u>TT***T***TT<u>C</u>T<u>C</u>T-3′	52 °C (+1.0 °C)	48 °C (−2.0 °C)	31 °C (0 °C)
5′-T<u>C</u>TT<u>C</u>T***TTT***T<u>C</u>T<u>C</u>T-3′	57 °C (+2.0 °C)	45 °C (−1.7 °C)	31 °C (0 °C)
5′-T<u>C</u>TT<u>C</u>***T***T***T***T<u>C</u>T<u>C</u>T-3′	57 °C (+2.0 °C)	45 °C (−1.7 °C)	33 °C (+0.7 °C)
5′-T<u>C</u>T***T***<u>C</u>***T***T***T***T***T***<u>C</u>T<u>C</u>T-3′	62 °C (+2.2 °C)	42 °C (−1.6 °C)	33 °C (+0.4 °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^{COC}-T. <u>C</u> = 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(GCGTCTT-CTTTTTCTCTGCC)-3′, respectively. The changes in T_m values per modification (ΔT_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′-*S*p, a (3′*S*)-phosphorothioate linkage with high nuclease resistance, and was comparable with that of 2′,4′-BNA^{COC} (Figure 5a). In the modification at the 3′-end, the EoNA-modified oligonucleotide rather than the 2′,4′-BNA^{COC}-modified oligonucleotide significantly suppressed nuclease degradation (Figure 5b).

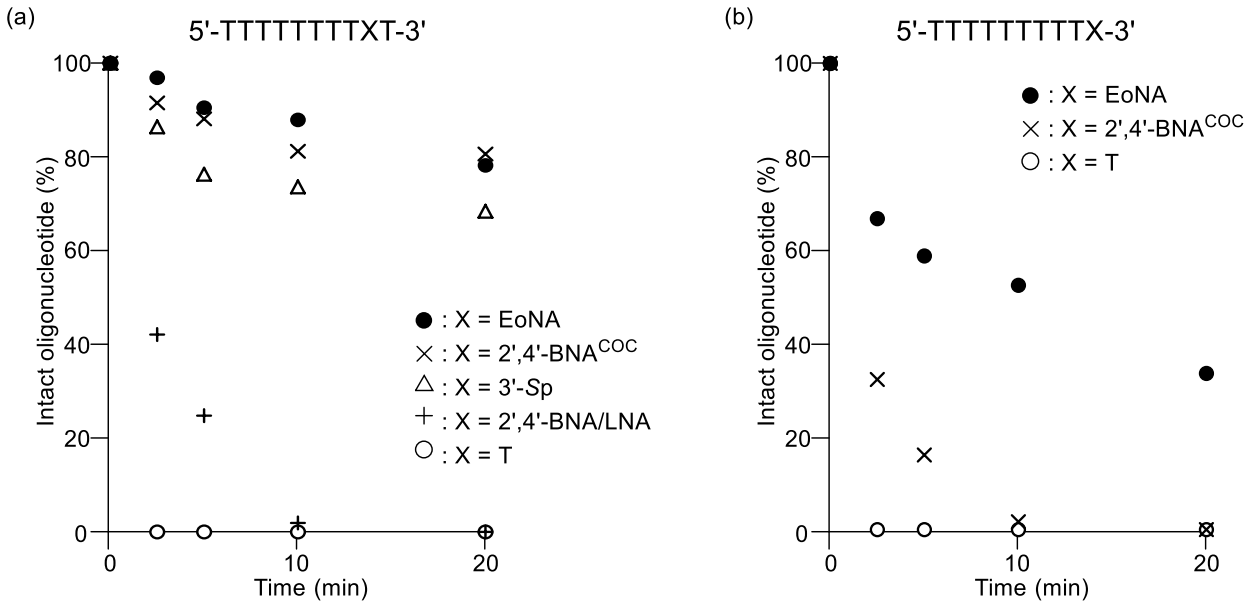


Figure 5. Degradation experiments by 3′-exonuclease. Conditions: 4 μg/mL *Crotalus admanteus* venom phosphodiesterase, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 7.5 μM of oligonucleotide at 37 °C.

Although EoNA has the same four-atom bridge with 2′,4′-BNA^{COC}, EoNA-T clearly improved the properties of oligonucleotides as compared with 2′,4′-BNA^{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[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 *iso*DNA or *iso*RNA, which tend to selectively and stably bind to ssRNA rather than ssDNA. However, there are few modifications[37] for *iso*DNA (or *iso*RNA) 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.

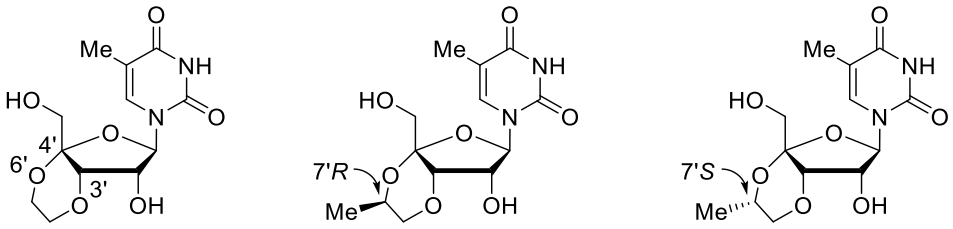
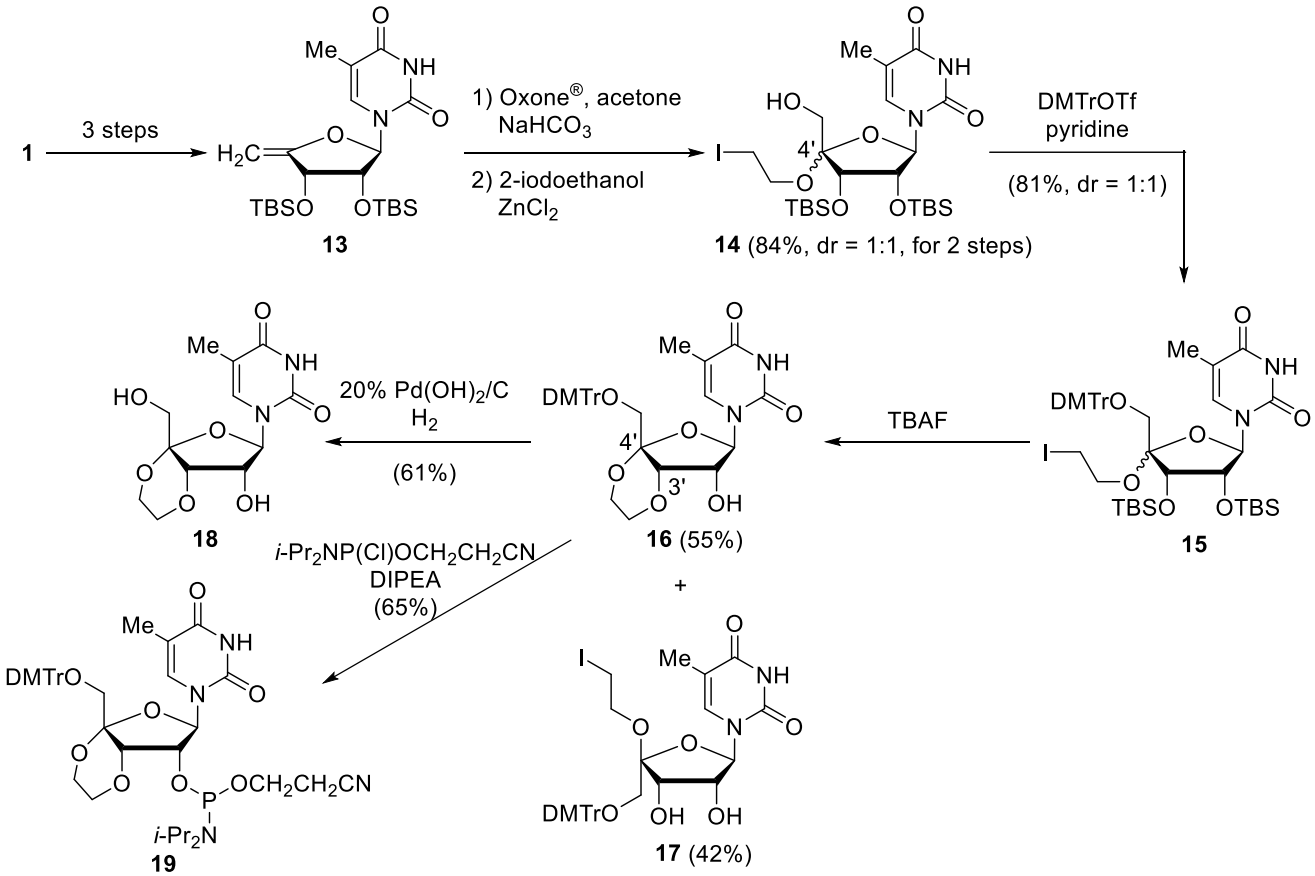


Figure 6. Structures of 3′,4′-EoNA-T, (*R*)-Me-3′,4′-EoNA-T, and (*S*)-Me-3′,4′-EoNA-T monomers

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 $ZnCl_2$-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_3OD by ^1H 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**

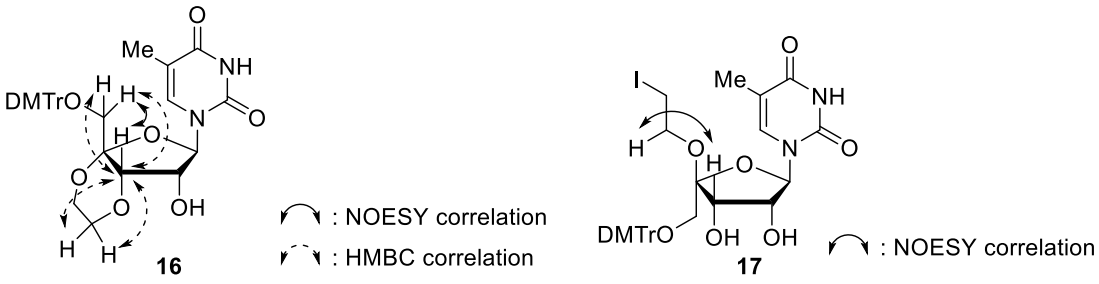


Figure 7. NOESY and HMBC correlations of **16** and NOESY correlations of **17**

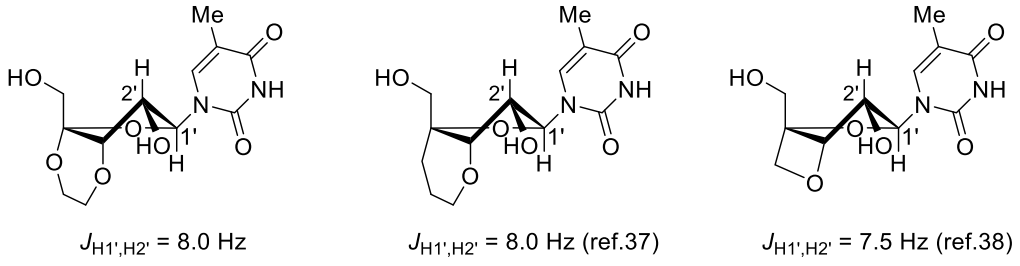
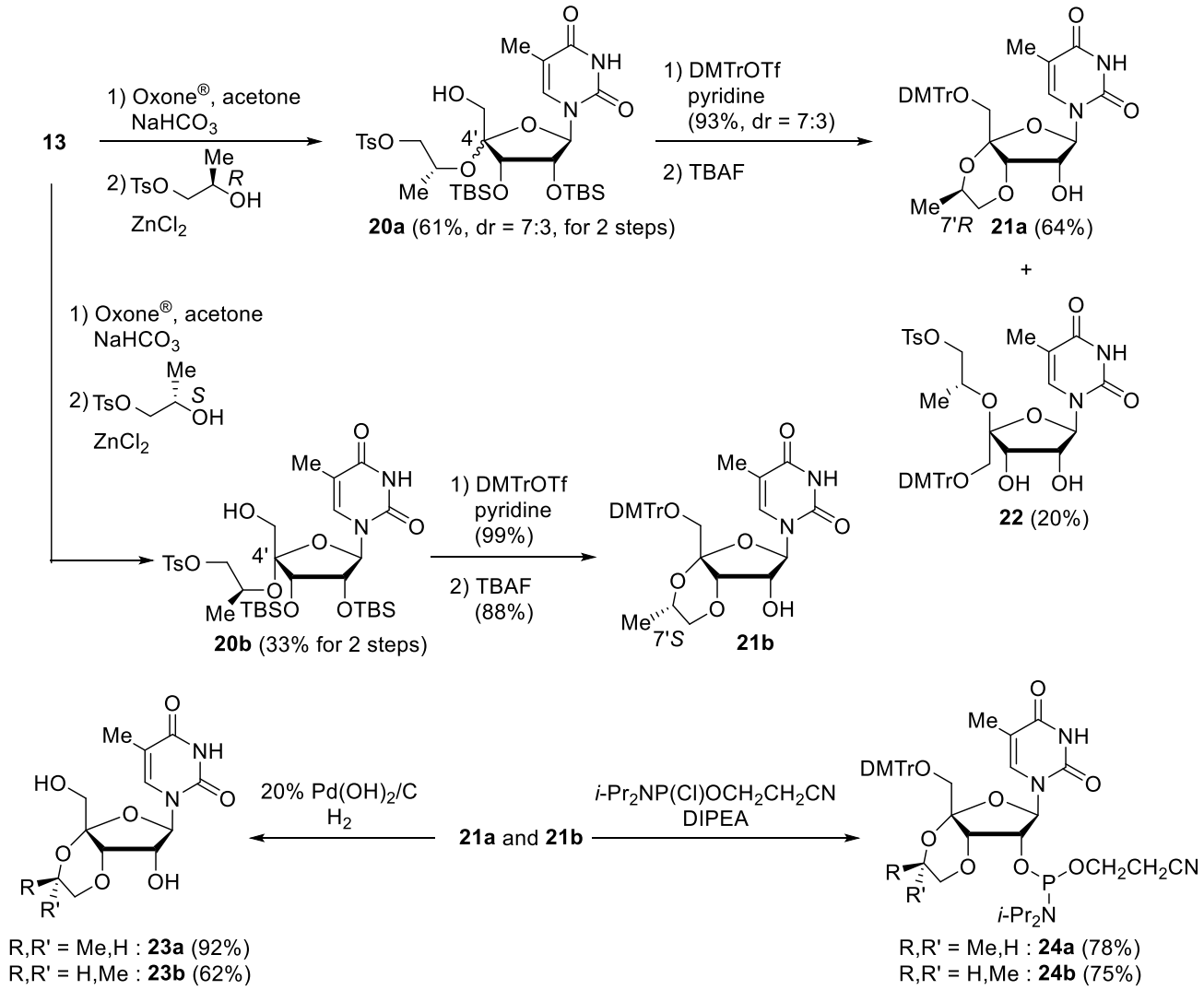


Figure 8. The $J_{H1',H2'}$ values of 3′,4′-bridged nucleosides

Synthesis of the 7′-methylated 3′,4′-EoNA-T phosphoramidites, shown in Scheme 4, was accomplished by the same method as that of unsubstituted 3′,4′-EoNA-T. Epoxidation of *exo*-olefin **13** followed by $ZnCl_2$-mediated ring-opening using a chiral alcohol with *R*-configuration produced **20a** as a 7:3 inseparable mixture. Methylated 3′,4′-EoNA-T **21a** with 7′*R*-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. 5′-*O*-Dimethoxytritylated (*S*)-Me-3′,4′-EoNA-T **21b** was prepared via two steps from **20b**. Analogously to the 7′*R*-isomer, **23b** and **24b** were also easily prepared. The $J_{H1',H2'}$ values of **23a** and **23b** in CD_3OD 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 3′,4′-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-3′,4′-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 (ΔT_m/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 (ΔT_m/mod. = 0 °C to 2.3 °C).

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

	with ssRNA	with ssDNA
5′-GGATGTTCTCGT-3′	47 °C	47 °C
5′-GGATG**T**TCTCGT-3′	45 °C (−2.0 °C)	37 °C (−10.0 °C)
5′-GGATG**TT**CTCGT-3′	44 °C (−1.5 °C)	29 °C (−9.0 °C)
5′-GGA**T**G**T**TC**T**CGT-3′	42 °C (−1.7 °C)	21 °C (−8.7 °C)
5′-GGATG***T***TCTCGT-3′	45 °C (−2.0 °C)	41 °C (−6.0 °C)
5′-GGATG***TT***CTCGT-3′	44 °C (−1.5 °C)	34 °C (−6.5 °C)
5′-GGA***T***G***T***TC***T***CGT-3′	42 °C (−1.7 °C)	29 °C (−6.0 °C)

Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, and 2.5 μM of each oligonucleotide. **T** = 3′,4′-EoNA-T. ***T*** = 2′,5′-T. 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.

Duplex- and triplex-forming abilities of *iso*DNAs containing 3′,4′-EoNA-T, (*R*)-Me-3′,4′-EoNA-T, and (*S*)-Me-3′,4′-EoNA-T, were examined. The modified *iso*DNAs 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 *iso*DNAs with two consecutive

3′,4′-EoNA-T modifications and its methyl congeners had almost the same duplex stability as unmodified *iso*DNA, 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 (ΔT_m/mod. = 2.0 °C to 2.3 °C) in comparison with (*S*)-Me-3′,4′-EoNA-T (ΔT_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 *iso*DNA targeting ssRNA.

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

	T_m	ΔT_m/mod.
5′-GGATGTTCTCGT-2′	41 °C	–
5′-GGATG**T**TCTCGT-2′	43 °C	+2.0 °C
5′-GGATG**TT**CTCGT-2′	40 °C	−0.5 °C
5′-GGA**T**G**T**TC**T**CGT-2′	48 °C	+2.3 °C
5′-GGATG***T***TCTCGT-2′	43 °C	+2.0 °C
5′-GGATG***TT***CTCGT-2′	41 °C	0 °C
5′-GGA***T***G***T***TC***T***CGT-2′	48 °C	+2.3 °C
5′-GGATG**<u>T</u>**TCTCGT-2′	42 °C	+1.0 °C
5′-GGATG**<u>TT</u>**CTCGT-2′	41 °C	0 °C
5′-GGA**<u>T</u>**G**<u>T</u>**TC**<u>T</u>**CGT-2′	45 °C	+1.3 °C

Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, 2.5 μM of each oligonucleotide. **T** = 3′,4′-EoNA-T, ***T*** = (*R*)-Me-3′,4′-EoNA-T, **<u>T</u>** = (*S*)-Me-3′,4′-EoNA-T. The sequences of ssRNA and ssDNA are 5′-ACGAGAACAUCC-3′ and 5′-ACGAGAACATCC-3′, respectively. ΔT_m/mod.: The changes in T_m values per modification relative to the unmodified *iso*DNA/RNA duplex.

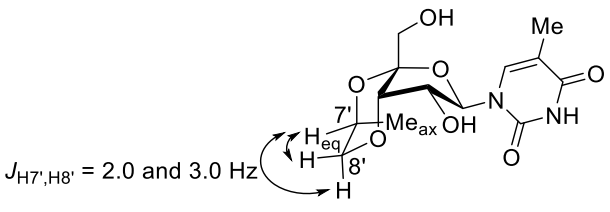
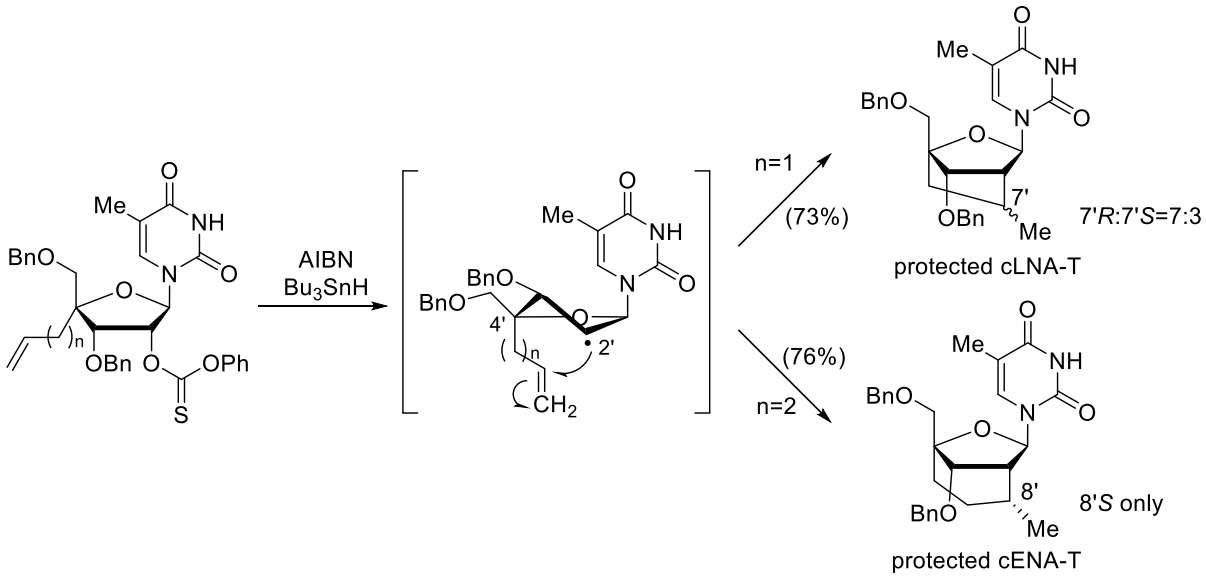


Figure 9. The bridge conformation of (*S*)-Me-3′,4′-EoNA-T monomer **23b**

4. 2′-*C*,4′-*C*-ETHYLENEOXY-BRIDGED THYMIDINES AND 2′-DEOXYADENOSINE[28-31]

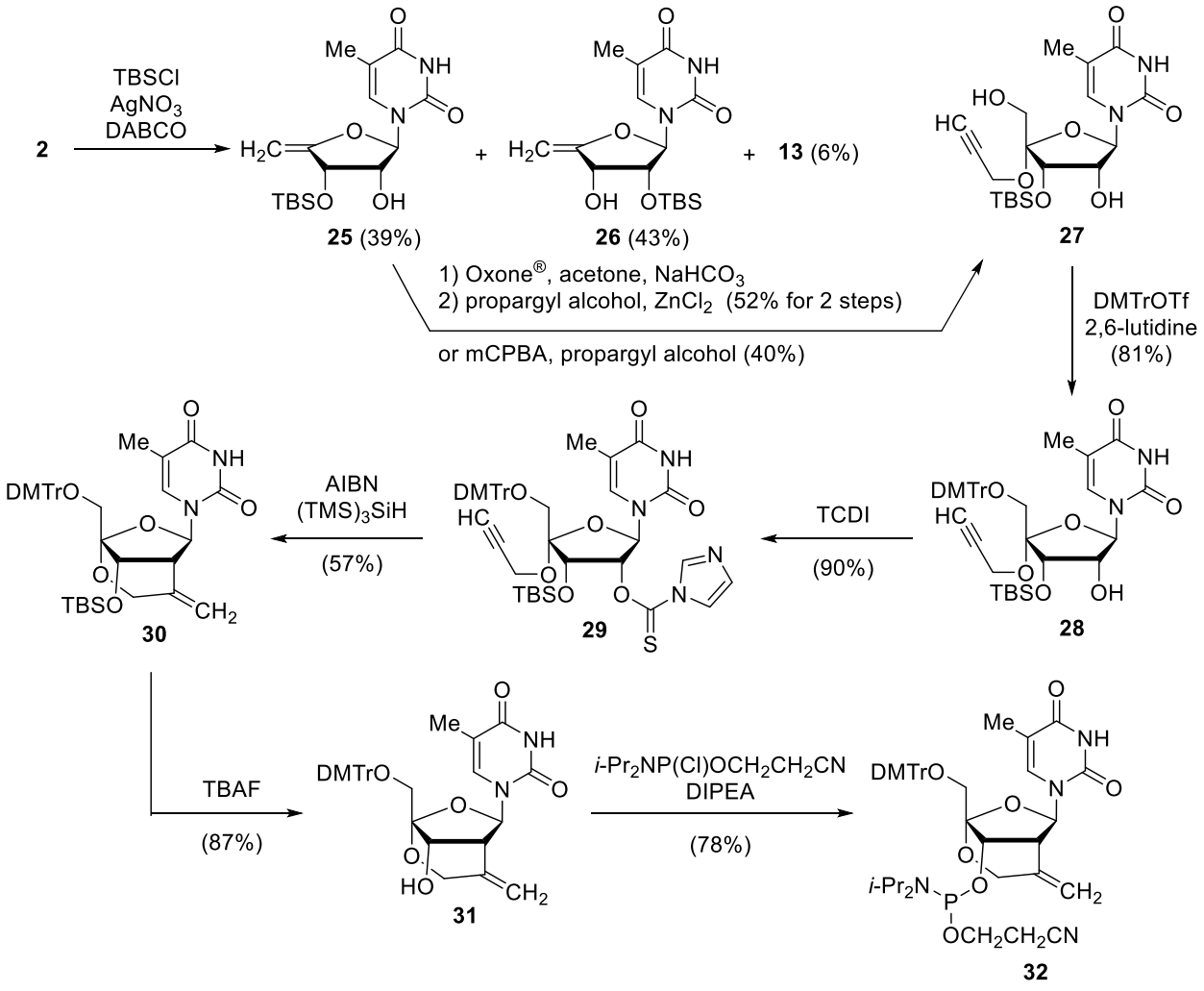
2′-*C*,4′-*C*-Ethyleneoxy-bridged nucleosides (EoDNAs) contain not only a 6′-oxygen atom but also a smaller three-atom (C-C-O) bridge than that of EoNA (Figure 10). In particular, methylene-EoDNAs were designed because it was reported by Seth's group that the exocyclic methylene moiety on methylene-cLNA can act as a bio-isostere of the 2′-oxygen.[23] Bond formation between 2′-carbon and 8′-carbon by intramolecular radical cyclization using 2′-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[20] (Scheme 5) and the bridges were limited to two- or three-carbon linkages.

Figure 10. Structures of EoDNA-T monomers



Scheme 5. Construction of 2′,4′-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 mono-silylation of nucleosides,[40] olefin **2** synthesized from 5-methyuridine for two steps was treated with TBSCl, $AgNO_3$, 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 dimethyldioxirane oxidation of 3′-*O*-TBS **25**, followed by $ZnCl_2$-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 S_N2-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*-$Pr_2NP(Cl)OCH_2CH_2CN$. 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**

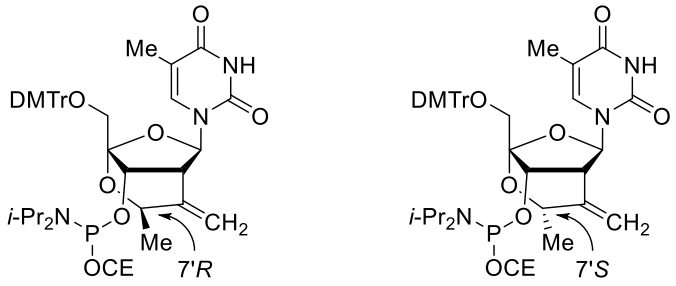
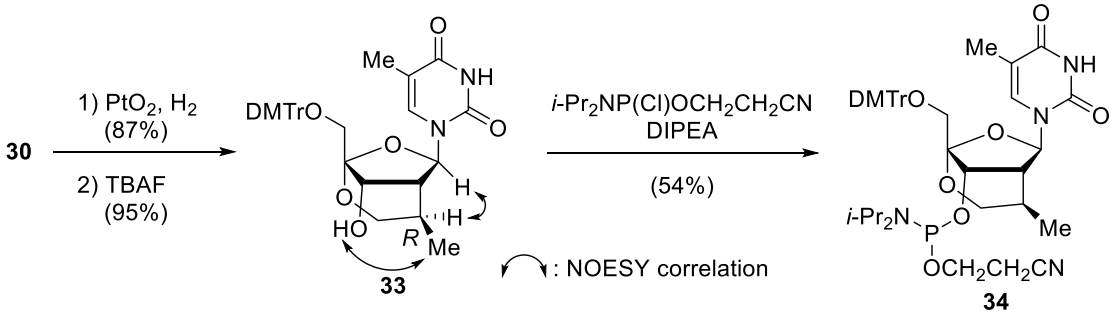


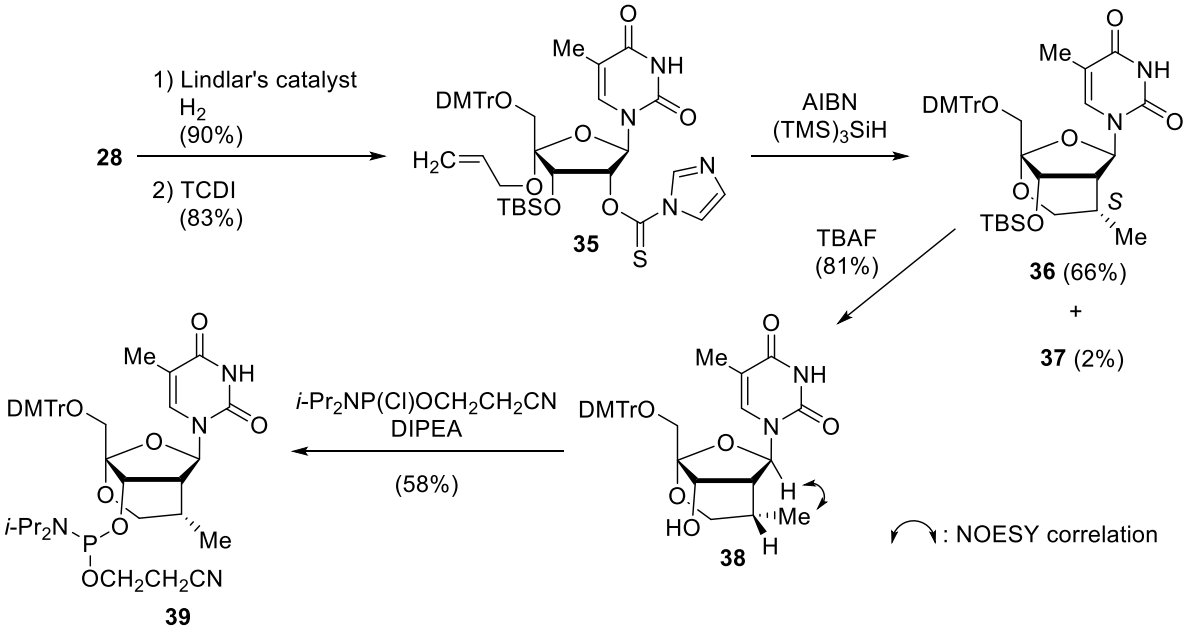
Figure 11. Structures of (*R*)- and (*S*)-Me-methylene-EoDNA-T phosphoramidites

The synthesis of (*R*)-Me-EoDNA-T phosphoramidite is shown in Scheme 7. Hydrogenolysis of **30** using H_2 and PtO_2 led to stereoselective reduction, producing an 8′-methyl compound with *R*-configuration. Then, treatment with TBAF afforded a 3′-hydroxyl compound **33**. Stereochemistry of the 8′-methyl group was determined by NOESY correlations between H1′ and H8′, and between 3′-hydroxyl and 8′-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 H_2 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**

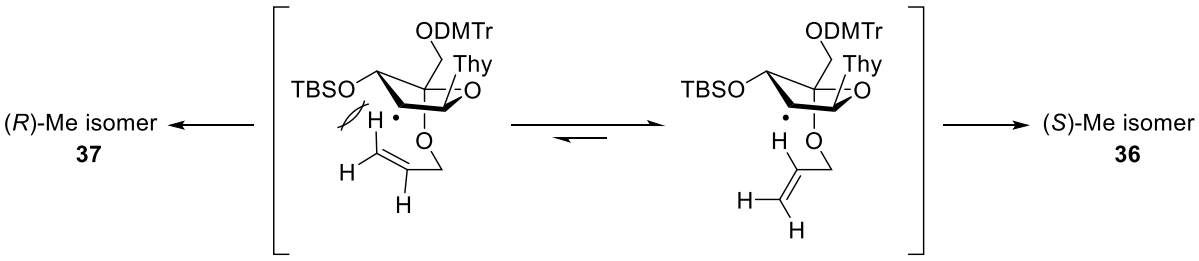
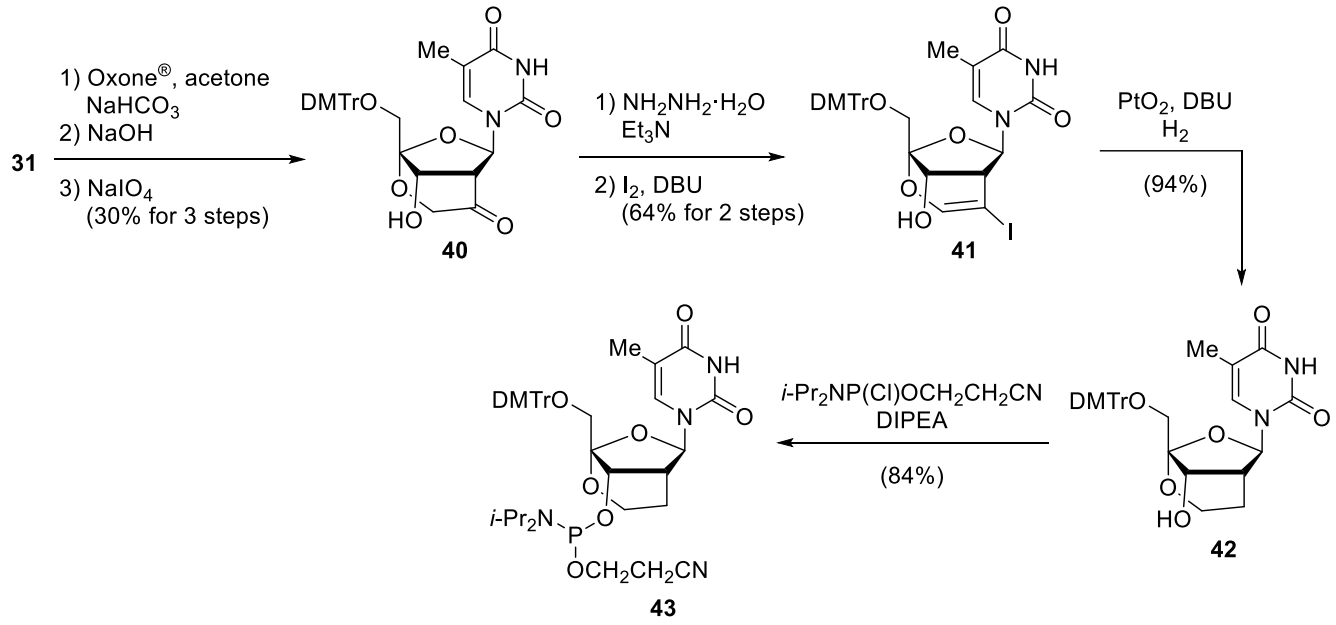


Figure 12. Stereoselectivity of intramolecular radical cyclization

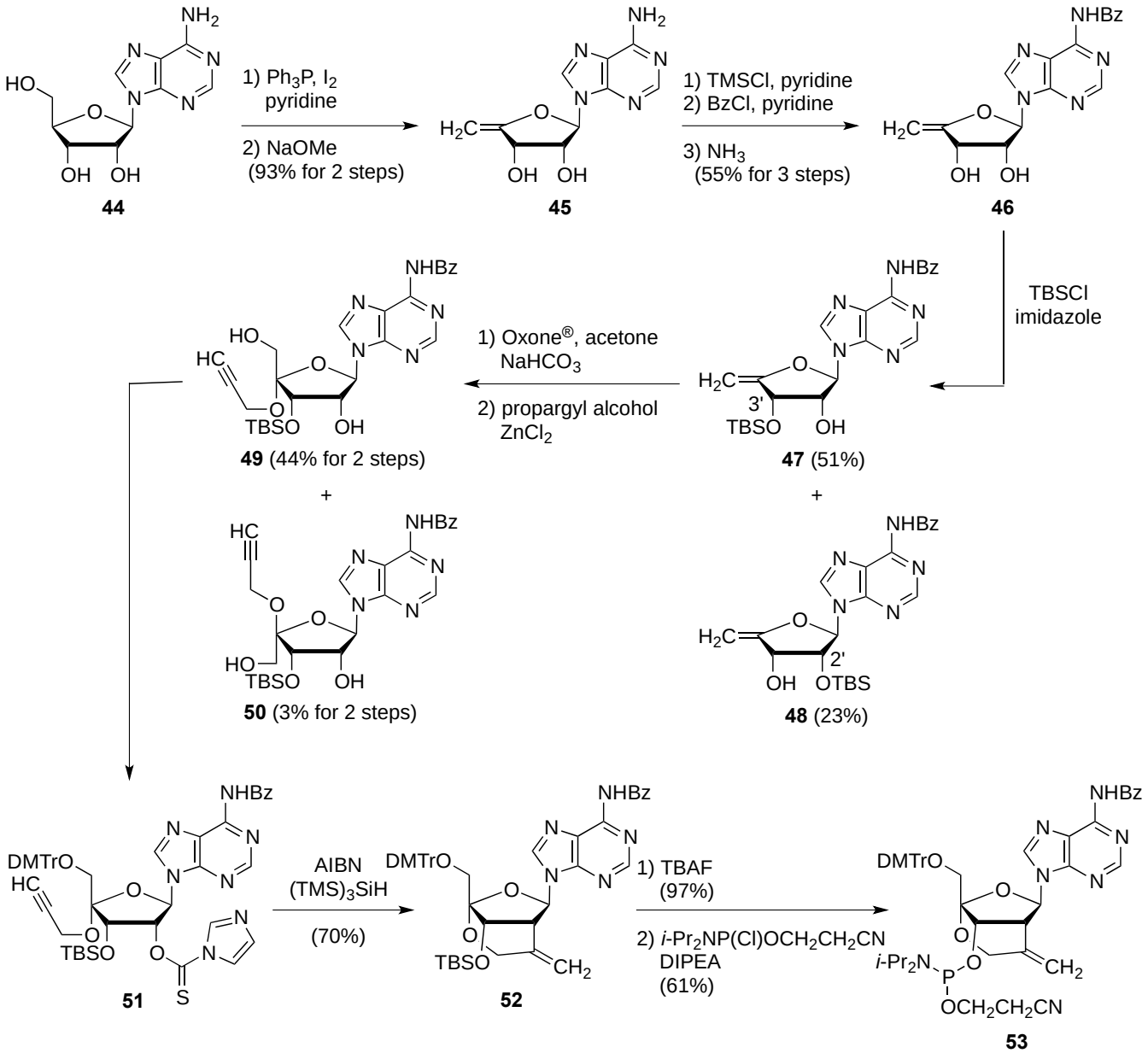
Unsubstituted EoDNA-T phosphoramidite was synthesized according to Scheme 9. 5′-*O*-Dimethoxytritylated methylene-EoDNA-T monomer **31** was treated with *in situ* generated dimethyldioxirane, and ring-opening of the obtained epoxide by NaOH followed by $NaIO_4$-oxidation yielded ketone **40** in 30% yield, via a three-step process. On the other hand, the oxidative cleavage of **31** using K_2OsO_4 and $NaIO_4$ 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*-$Pr_2NP(Cl)OCH_2CH_2CN$ successfully produced desired EoDNA-T phosphoramidite **43**.



Scheme 9. Synthesis of unsubstituted 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 N^6-benzoylated compound **46** was obtained via *exo*-olefin **45**. Compound **46** was subjected to

treatment with TBSCl 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 ΔT_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 ΔT_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 4. T_m Values of duplexes obtained by UV-melting experiments

	with ssRNA	with ssDNA
5′-T<u>C</u>TT<u>C</u>TTTTT<u>C</u>T<u>C</u>T-3′	51 °C	50 °C
5′-T<u>C</u>TT<u>C</u>T**XXX**T<u>C</u>T<u>C</u>T-3′	62 °C (+3.7 °C)	51 °C (+0.3 °C)
5′-T<u>C</u>TT<u>C</u>**X**T**X**T**X**<u>C</u>T<u>C</u>T-3′	65 °C (+4.7 °C)	50 °C (0 °C)

5′-T<u>C</u>T**X**<u>C</u>**X**T**X**T**X**<u>C</u>**X**<u>C</u>T-3′	75 °C (+4.8 °C)	55 °C (+1.0 °C)
5′-T<u>C</u>TT<u>C</u>T***XXX***T<u>C</u>T<u>C</u>T-3′	62 °C (+3.7 °C)	49 °C (−0.3 °C)
5′-T<u>C</u>TT<u>C</u>***X***T***X***T***X***<u>C</u>T<u>C</u>T-3′	65 °C (+4.7 °C)	49 °C (−0.3 °C)
5′-T<u>C</u>T***X***<u>C</u>***X***T***X***T***X***<u>C</u>***X***<u>C</u>T-3′	73 °C (+4.4 °C)	54 °C (+0.8 °C)
5′-T<u>C</u>TT<u>C</u>T<u>***XXX***</u>T<u>C</u>T<u>C</u>T-3′	63 °C (+4.0 °C)	48 °C (−0.7 °C)
5′-T<u>C</u>TT<u>C</u><u>***X***</u>T<u>***X***</u>T<u>***X***</u><u>C</u>T<u>C</u>T-3′	66 °C (+5.0 °C)	49 °C (−0.3 °C)
5′-T<u>C</u>T<u>***X***</u><u>C</u><u>***X***</u>T<u>***X***</u>T<u>***X***</u><u>C</u><u>***X***</u><u>C</u>T-3′	76 °C (+5.0 °C)	54 °C (+0.8 °C)
5′-T<u>C</u>TT<u>C</u>T**YYY**T<u>C</u>T<u>C</u>T-3′	61 °C (+3.3 °C)	50 °C (0 °C)
5′-T<u>C</u>TT<u>C</u>**Y**T**Y**T**Y**<u>C</u>T<u>C</u>T-3′	63 °C (+4.0 °C)	49 °C (−0.3 °C)
5′-T<u>C</u>T**Y**<u>C</u>**Y**T**Y**T**Y**<u>C</u>**Y**<u>C</u>T-3′	72 °C (+4.2 °C)	52 °C (+0.4 °C)
5′-T<u>C</u>TT<u>C</u>T***YYY***T<u>C</u>T<u>C</u>T-3′	56 °C (+1.7 °C)	45 °C (−1.7 °C)
5′-T<u>C</u>TT<u>C</u>***Y***T***Y***T***Y***<u>C</u>T<u>C</u>T-3′	60 °C (+3.0 °C)	44 °C (−2.0 °C)
5′-T<u>C</u>T***Y***<u>C</u>***Y***T***Y***T***Y***<u>C</u>***Y***<u>C</u>T-3′	67 °C (+3.2 °C)	47 °C (−0.6 °C)
5′-T<u>C</u>TT<u>C</u>T<u>***YYY***</u>T<u>C</u>T<u>C</u>T-3′	60 °C (+3.0 °C)	48 °C (−0.7 °C)
5′-T<u>C</u>TT<u>C</u><u>***Y***</u>T<u>***Y***</u>T<u>***Y***</u><u>C</u>T<u>C</u>T-3′	62 °C (+3.7 °C)	46 °C (−1.3 °C)
5′-T<u>C</u>T<u>***Y***</u><u>C</u><u>***Y***</u>T<u>***Y***</u>T<u>***Y***</u><u>C</u><u>***Y***</u><u>C</u>T-3′	71 °C (+4.0 °C)	50 °C (0 °C)

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. <u>***X***</u> = (*S*)-Me-methylene-EoDNA-T. **Y** = EoDNA-T. ***Y*** = (*R*)-Me-EoDNA-T. <u>***Y***</u> = (*S*)-Me-EoDNA-T. <u>C</u> = 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.

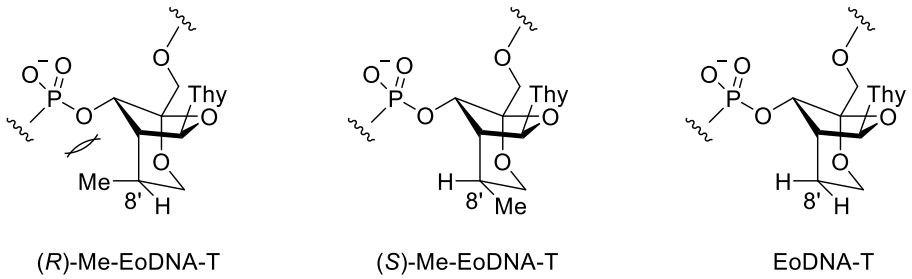


Figure 13. 1,3-Diaxial repulsion between 8′-methyl group and 3′-phosphodiester moiety in (*R*)-Me-EoDNA

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,[42] 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 ΔT_m/mod. values ranged from −1.0 °C to 2.0 °C. DNA duplexes including both single methylene-EoDNA-A and single methylene-EoDNA-T did not show a significant change in the ΔT_m/mod. values (ΔT_m/mod. = 0.5−1.0 °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 °C to 2.0 °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 °C.

Table 5. T_m Values of duplexes obtained by UV-melting experiments

	T_m	ΔT_m/mod.
5′-ACGAGAACATCG-3′ 3′-TGCTCTTGTAGG-5′	48 °C	–
5′-ACGAG**A**ACATCG-3′ 3′-TGCTCTTGTAGG-5′	49 °C	+1.0 °C
5′-ACGAG**AA**CATCG-3′ 3′-TGCTCTTGTAGG-5′	52 °C	+2.0 °C

5′-ACGAGAA**C**A**T**CG-3′ 3′-TGCTCTTGTAGG-5′	51 °C	+1.5 °C
5′-ACGAGAACATCG-3′ 3′-TGCTCT**T**GTAGG-5′	47 °C	−1.0 °C
5′-ACGAGAACATCG-3′ 3′-TGCTC**T**TGTAGG-5′	49 °C	+1.0 °C
5′-ACGAGAACATCG-3′ 3′-TGCTC**TT**GTAGG-5′	51 °C	+1.5 °C
5′-ACGAGAACATCG-3′ 3′-TGCTCT**T**G**T**AGG-5′	52 °C	+2.0 °C
5′-ACGAGA**A**CATCG-3′ 3′-TGCTCT**T**GTAGG-5′	49 °C	+0.5 °C
5′-ACGAGA**A**CATCG-3′ 3′-TGCTC**T**TGTAGG-5′	50 °C	+1.0 °C
5′-ACGAG**AA**CATCG-3′ 3′-TGCTC**TT**GTAGG-5′	58 °C	+2.5 °C
5′-ACGAGAA**C**A**T**CG-3′ 3′-TGCTCT**T**G**T**AGG-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 (ΔT_m/mod.) relative to the natural duplexes are shown in parentheses.

5. 2′-*C*,4′-*C*-METHYLENEOXY-BRIDGED THYMIDINES[32]

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).

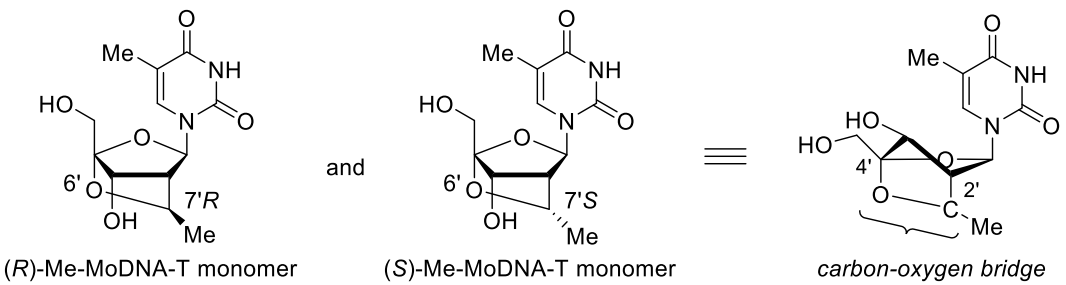
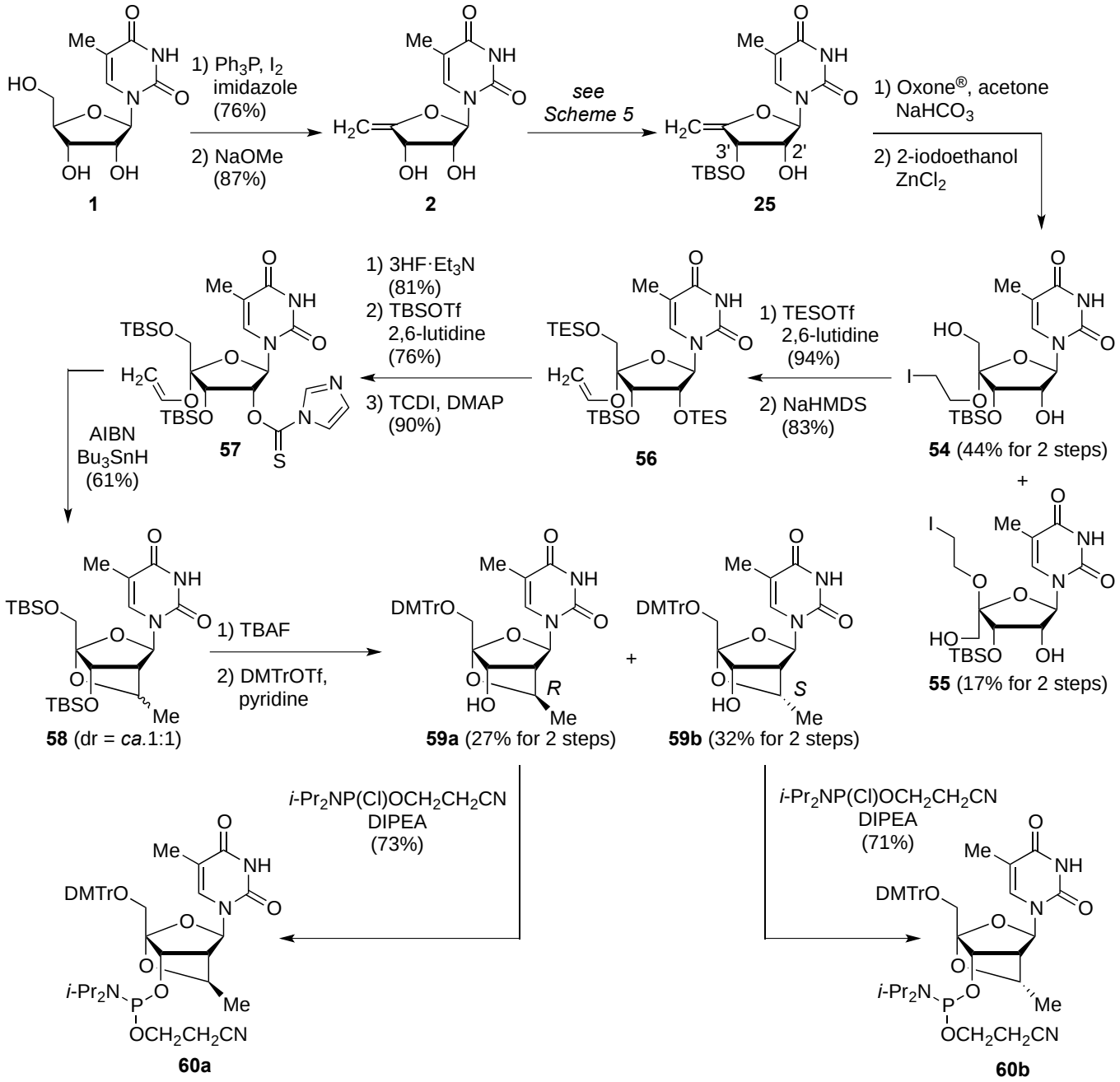


Figure 14. Structures of (*R*)-Me-MoDNA-T and (*S*)-Me-MoDNA-T monomers

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),[23] in the current procedure, 5′-iodide compound could be precipitated using 2.5% solution of MeOH in $CHCl_3$, 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).[43] Epoxidation of **25** with dimethyldioxirane *in situ* generated from Oxone® and acetone followed by $ZnCl_2$-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 $HF{\cdot}Et_3N$, TBS-protection of primary alcohol in the obtained diol followed by reaction with TCDI afforded radical precursor **57**. When a solution of AIBN and Bu_3SnH 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 Bu_3SnH 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**

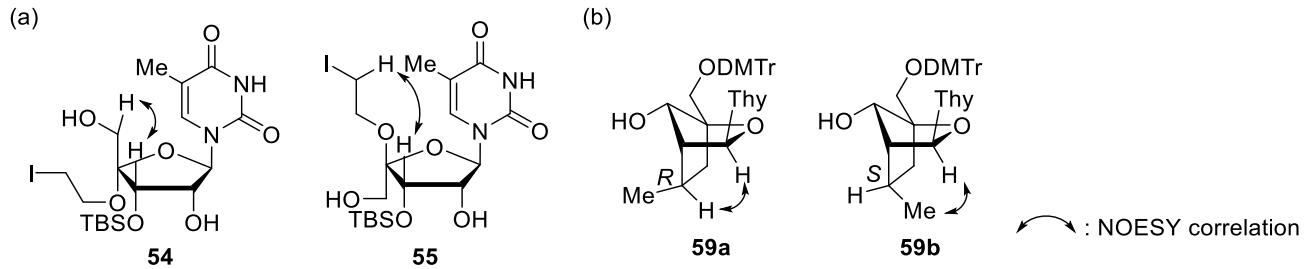


Figure 15. NOESY correlations of compounds **54**, **55**, **59a**, and **59b**

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 (ΔT_m/mod. = 6.0 °C) was comparable to that of 2′,4′-BNA/LNA-T (ΔT_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 it 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 ΔT_m/mod. values of 4.5 °C. The difference between the ΔT_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 6. T_m Values of duplexes obtained by UV-melting experiments

	with ssRNA	with ssDNA
5′-GGATGTTCTCGT-3′	47 °C	47 °C
5′-GGATG**T**TCTCGT-3′	52 °C (+5.0 °C)	48 °C (+1.0 °C)
5′-GGATG**TT**CTCGT-3′	57 °C (+5.0 °C)	51 °C (+2.0 °C)
5′-GGA**T**G**T**TCTCGT-3′	58 °C (+5.5 °C)	50 °C (+1.5 °C)
5′-GGATG***T***TCTCGT-3′	53 °C (+6.0 °C)	47 °C (0 °C)

5′-GGATG***TT***CTCGT-3′	59 °C (+6.0 °C)	50 °C (+1.5 °C)
5′-GGA***T***G***T***TC*T*CGT-3′	59 °C (+6.0 °C)	51 °C (+2.0 °C)
5′-GGATG***T***TCTCGT-3′	54 °C (+7.0 °C)	49 °C (+2.0 °C)
5′-GGATG***TT***CTCGT-3′	60 °C (+6.5 °C)	52 °C (+2.5 °C)
5′-GGA***T***G***T***TC*T*CGT-3′	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*** = 2′,4′-BNA/LNA-T.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.

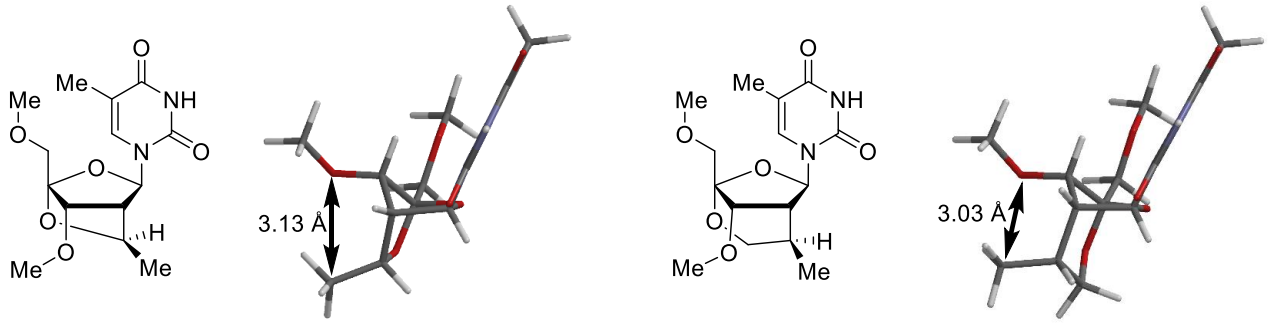


Figure 16. *Ab initio* calculations (B3LYP/6-31G*) of 3′,5′-*O*-bismethyl analogs of (*R*)-Me-MoDNA-T and (*R*)-Me-EoDNA-T

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 2′,4′-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 2′,4′-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 (7′*S*)-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 3′-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 (7′*R*)-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.

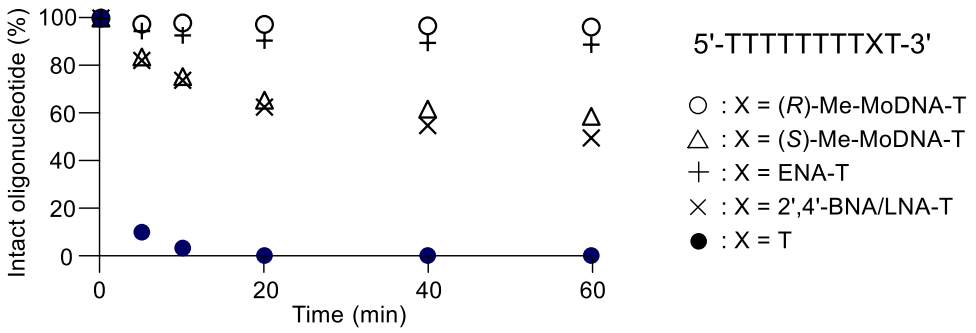


Figure 17. Degradation experiments by 3′-exonuclease. Conditions: 0.05 unit/mL *Crotalus admanteus* venom phosphodiesterase, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 7.5 μM of oligonucleotide at 37 °C.

6. 2′-*O*,4′-*C*-ETHYLENE-BRIDGED 5-METHYLURIDINE [33]

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.[44] 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.[45] 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.

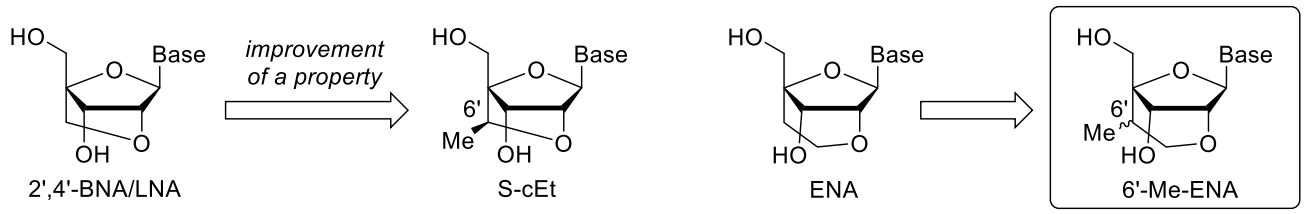
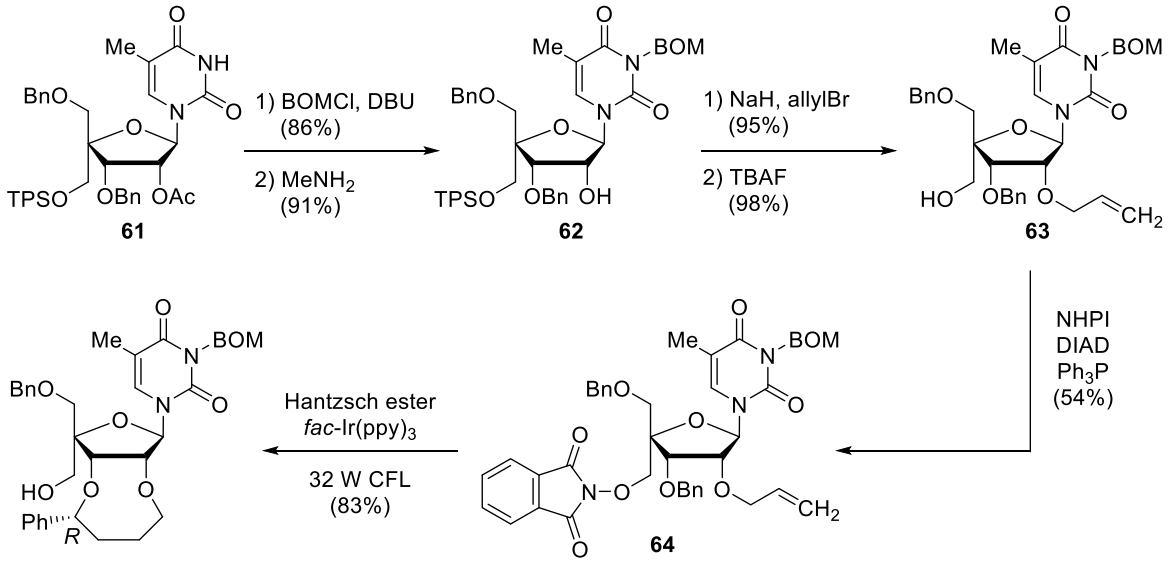


Figure 18. Structure of 6′-Me-ENA monomer

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 $MeNH_2$ afforded **62**, which was treated with allyl bromide and NaH 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,[44] 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

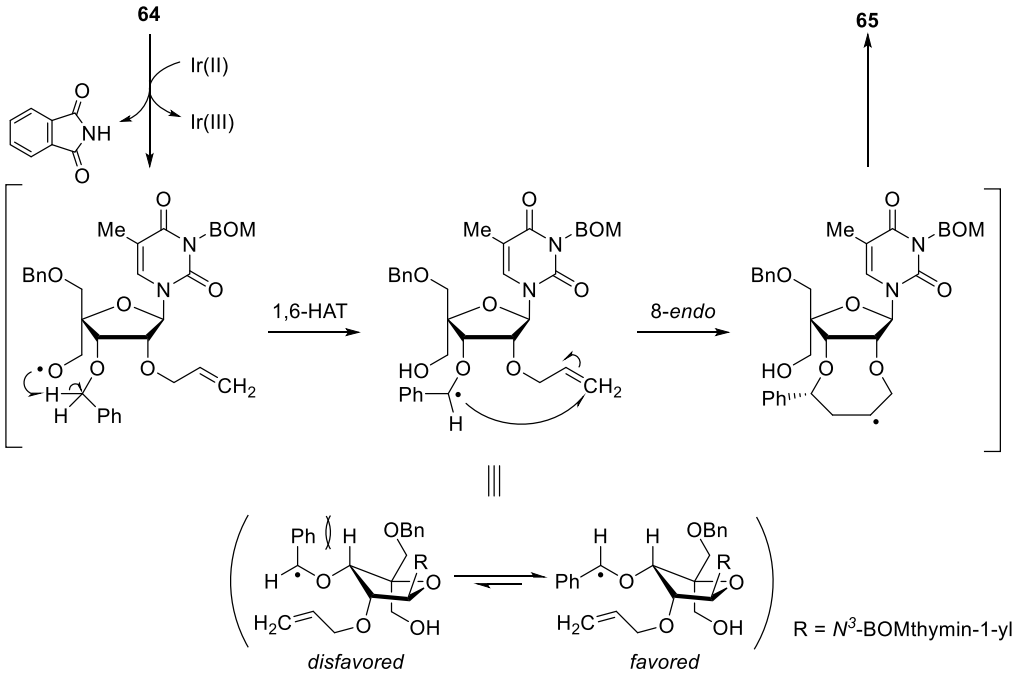
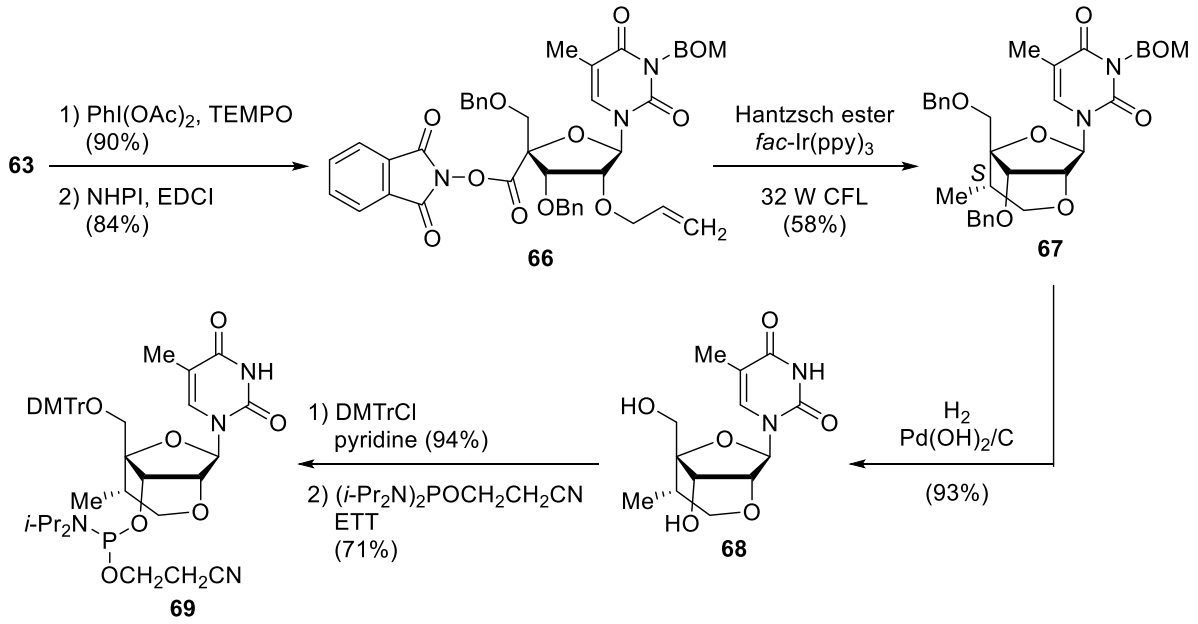


Figure 19. Plausible reaction mechanism for producing **65**

Decarboxylative radical generation on the 4′-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 4′-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 4′-carbon radical intermediate. The stereochemistry of the methyl group can be explained by steric repulsion between the 3′-benzyloxy group and alkene moiety, as shown in Figure 20. Moreover, the stereochemistry was determined by NOESY measurements (Figure 20). After hydrogenolysis using H_2 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 dimethoxyltritylation 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.



Scheme 13. Synthesis of (*S*)-Me-ENA-T phosphoramidite **69**

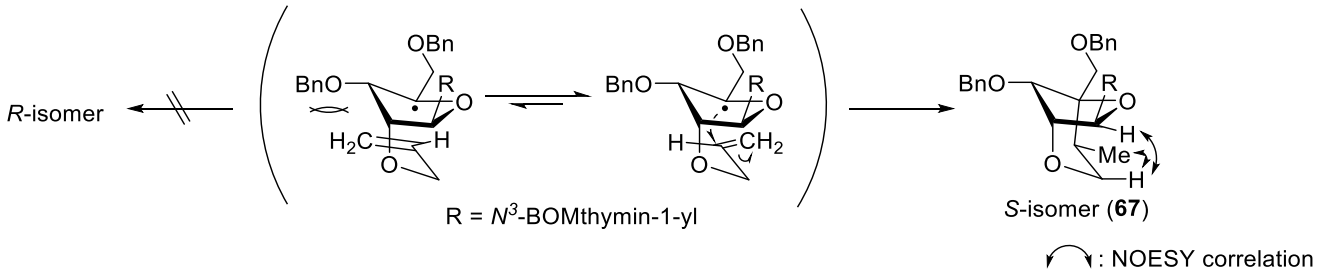


Figure 20. Interpretation of stereoselectivity in intramolecular radical cyclization

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 ΔT_m/mod. values ranging

from 3.0 °C to 4.7 °C, though the stabilization ability was slightly less than those of ENA and 2′,4′-BNA/LNA modifications.

Table 7. T_m Values of duplexes and triplexes obtained by UV-melting experiments

	duplex with ssRNA	duplex with ssDNA	triplex with dsDNA
5′-T<u>C</u>TT<u>C</u>TTTTT<u>C</u>T<u>C</u>T-3′	47 °C	46 °C	27 °C
5′-T<u>C</u>TT<u>C</u>TT**T**TT<u>C</u>T<u>C</u>T-3′	50 °C (+3.0 °C)	43 °C (−3.0 °C)	30 °C (+3.0 °C)
5′-T<u>C</u>TT<u>C</u>T**TT**TT<u>C</u>T<u>C</u>T-3′	60 °C (+4.3 °C)	45 °C (−0.3 °C)	37 °C (+3.3 °C)
5′-T<u>C</u>TT<u>C</u>**T**TT**T**T<u>C</u>T<u>C</u>T-3′	63 °C (+5.3 °C)	47 °C (+0.3 °C)	41 °C (+4.7 °C)
5′-T<u>C</u>TT<u>C</u>TT***T***TT<u>C</u>T<u>C</u>T-3′	51 °C (+4.0 °C)	46 °C (0 °C)	30 °C (+3.0 °C)
5′-T<u>C</u>TT<u>C</u>T***TTT***T<u>C</u>T<u>C</u>T-3′	61 °C (+4.7 °C)	49 °C (+1.0 °C)	42 °C (+5.0 °C)
5′-T<u>C</u>TT<u>C</u>***T***T***T***T<u>C</u>T<u>C</u>T-3′	64 °C (+5.7 °C)	48 °C (+0.7 °C)	45 °C (+6.0 °C)
5′-T<u>C</u>TT<u>C</u>TT<u>***T***</u>TT<u>C</u>T<u>C</u>T-3′	52 °C (+5.0 °C)	48 °C (+2.0 °C)	32 °C (+5.0 °C)
5′-T<u>C</u>TT<u>C</u>T<u>***TTT***</u>T<u>C</u>T<u>C</u>T-3′	61 °C (+4.7 °C)	49 °C (+1.0 °C)	40 °C (+4.3 °C)
5′-T<u>C</u>TT<u>C</u><u>***T***</u>T<u>***T***</u>T<u>***T***</u><u>C</u>T<u>C</u>T-3′	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 $MgCl_2$, and 1.5 μM of each oligonucleotide for triplex. **T** = (*S*)-Me-ENA-T. ***T*** = ENA-T. <u>***T***</u> = 2′,4′-BNA/LNA-T. <u>C</u> = 2′-deoxy-5-methylcytidine. The sequences of ssRNA, ssDNA, and dsDNA are 5′-r(AGAGAAAAAGAAGA)-3′, 5′-d(AGAGAAAAAGAAGA)-3′, and 5′-d(GGCAGAGAAAAAGA-AGACGC)-spacer18-d(GCGTCTTCTTTTTCTCTGCC)-3′, 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 3′-exonuclease was also obviously higher than those of ENA-T and 2′,4′-BNA/LNA-T (data not shown). This was likely due to the inhibition of access of nuclease to 3′-phosphodiester moiety by steric hindrance of the methyl group. If an oligonucleotide including the (6′*R*)-methyl analog was synthesized, the (6′*R*)-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, 2′,4′-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 2′,4′-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 2′,4′-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 6′-oxygen in the bridge improved the properties of oligonucleotides. Moreover, radical cyclization using 4′-carbon radical can allow us to develop useful 2′,4′-bridged nucleosides because there are few examples on the synthesis of nucleosides using 4′-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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