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Synthesis and Properties of 2′-*O*,4′-*C*-Ethyleneoxy Bridged 5-Methyluridine

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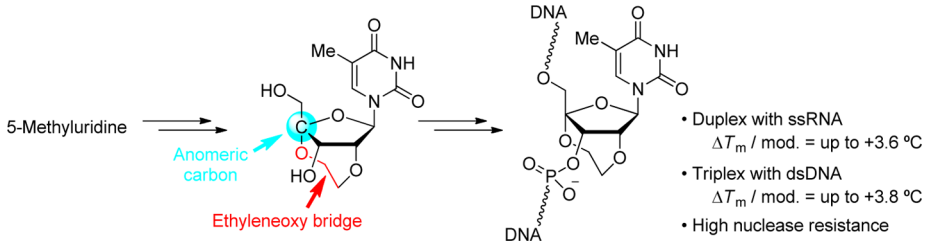
ABSTRACT



2′-*O*,4′-*C*-Ethyleneoxy bridged 5-methyluridine (EoNA-T), possessing a seven-membered linkage and an anomeric 4′-carbon, was synthesized and introduced into oligonucleotides by using an automated DNA synthesizer. The EoNA-modified oligonucleotides significantly stabilized the duplexes with single-stranded RNA and triplexes with double-stranded DNA relative to the natural oligonucleotide and oligonucleotides modified by another seven-membered bridged 5-methyluridine, 2′,4′-BNA^{COC}-T. In addition, EoNA-T showed excellent nuclease resistance.

Artificial nucleic acids that stabilize complexes with target nucleic acids are useful materials for various nucleic acid technologies such as gene therapy and genetic diagnosis. Among numerous analogs developed to date, nucleic acids bridged between the 2′- and 4′-positions generally lead to an increased affinity to single-stranded RNA (ssRNA) or double-stranded DNA (dsDNA), or both.[1−6] Moreover, the bridged nucleic acids have increased resistance to nuclease degradation when compared with a natural nucleic acid. The bridge size between the 2′- and 4′-positions is considered to crucially affect the binding affinity and nuclease resistance. 2′,4′-Methylene-bridged nucleic acid (BNA/LNA) with a five-membered bridge has outstanding high-binding affinities to ssRNA and dsDNA, as well as improved

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nuclease resistance.[2] In contrast, seven-membered bridge analogs such as PrNA,[3] 2′,4′-BNA^{COC},[4] and urea-BNA[5] have excellent nuclease resistance because of the sterically large bridge structure, although they lack the high binding affinities probably due to fixation of their sugar conformation being incomplete (Figure 1).

4′-Alkoxynucleosides tend toward the N-type sugar conformation presumably because of the anomeric effect on the 4′-carbon atom.[7,8] Recently, Rosenberg's group reported that oligonucleotides modified by 4′-alkoxythymidines increased the T_m value of duplexes with ssRNA by approximately 1 °C per modification.[8] Thus, we designed a 2′-*O*,4′-*C*-ethyleneoxy bridged nucleic acid (EoNA) with a seven-membered bridged structure between the 2′- and 4′-positions and an anomeric carbon at the 4′-position. Its sugar conformation is anticipated to be sufficiently restricted to the N-form by the additional anomeric effect despite the large, seven-membered bridge (Figure 1). This can contribute not only to high nuclease resistance but also to acquisition of high duplex- or triplex-forming ability. Furthermore, as far as we know, there are no reports on 2′,4′-bridged nucleic acids with an additional heteroatom on the 4′-carbon atom, which motivated us to conduct the present study. Here, 2′-*O*,4′-*C*-ethyleneoxy bridged 5-methyluridine (EoNA-T) was synthesized, and evaluation of its oligonucleotides was carried out.

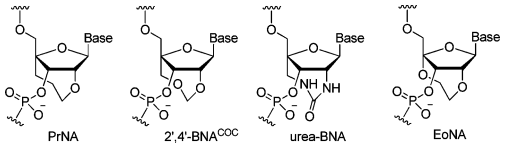
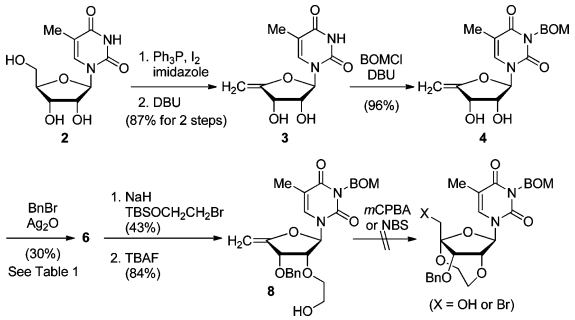


Figure 1. Structures of PrNA, 2′,4′-BNA^{COC}, urea-BNA, and EoNA designed in the present study.

The synthesis of EoNA-T phosphoramidite **1** was examined (Schemes 1 and 3). After conversion from commercially available 5-methyluridine **2** into *exo*-olefin **3**, **4** was obtained by BOM-protection. Benzylation of **4** was examined (Table 1). A conventional method using NaH and BnBr was used for the preferential 2′-*O*-benzylation reaction leading to the 7:1 separable mixture of 2′-*O*-benzylated **5** and 3′-*O*-benzylated **6**. Dibenzylated compound **7** was also obtained in 30% yield. In contrast, a reaction system using Bu_2SnO/BnBr or Ag_2O/BnBr proceeded in a monobenzylation reaction without regioselectivity. Consequently, in a reaction using Ag_2O/BnBr, the desired product **6** was isolated in 30% yield after purification by silica gel column chromatography (Scheme 1).

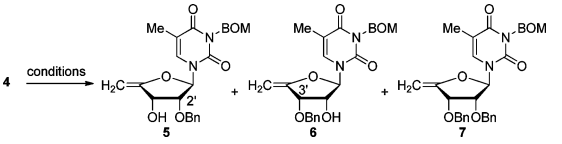
After bonding of the 2-hydroxyethyl unit to 2′-oxygen of **6** via two steps, **8** was treated with *m*CPBA or NBS to

Scheme 1. Synthesis of Intermediates and Attempted Bridge Construction



construct the bridged structure.[9] In the former reaction, 3-benzyloxymethylthymine (BOM-T) was the only isolated product (83% yield). The reaction using NBS afforded dioxane **9** in 24% yield via attack of hydroxyl group on the 1′-carbon, as shown in Scheme 2. In both cases, no desired product was detected at all. These results imply that construction of the bridged structure by the attack on the 4′-carbon is difficult.

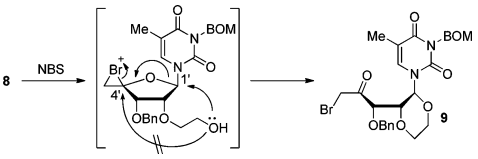
Table 1. Benzylation of **4**[a]



conditions	yield of **5** and **6** (**5**:**6**)	yield of **7**
NaH, BnBr, DMF, 0 °C, 0.5 h	45% (7:1)	30%
Bu_2SnO, BnBr, DMF, 80 °C, 19 h	47% (8:10)	3%
Ag_2O, BnBr, CH_2Cl_2, rt, 16 h	75% (9:10)	6%

[a] Recovery yields of starting material were 19% in NaH/BnBr, 28% in Bu_3SnO/BnBr, or 17% in Ag_2O/BnBr.

Scheme 2. Possible Reaction Mechanism



As an alternative route, construction of the bridged structure after introduction of the 2-hydroxyethoxy unit

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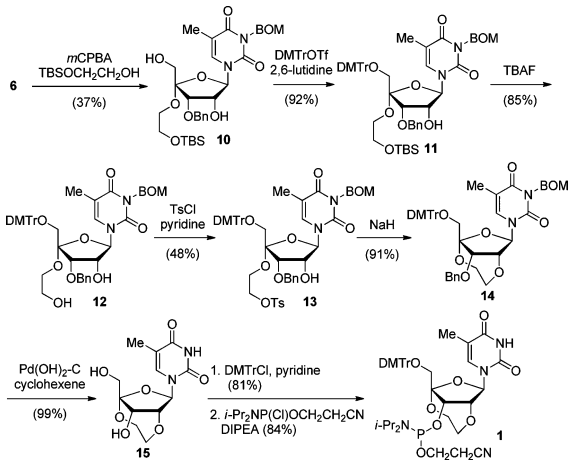
Scheme 3. Synthesis of Phosphoramidite **1**



on the 4′-carbon was investigated (Scheme 3). Treatment of **6** with *m*CPBA and an excess amount of siloxyethanol in a solvent-free system led to compound **10** as the sole isolated product. This stereoselectivity might be caused by the less hindered β-face attack of *m*CPBA on the olefin. Protection of the primary alcohol of **10** by 4,4′-dimethoxytrityl triflate (DMTrOTf)[10] followed by desilylation of **11** afforded diol **12**. Although no ring closure of **12** proceeded under Mitsunobu conditions using TMAD and Bu_3P,[11] the tosylated compound **13** prepared from **12** was treated with NaH to give the desired product **14** in 91% yield. All protecting groups were removed by hydrogenolysis to obtain EoNA-T monomer **15**. The ^{1}H NMR measurement demonstrated that the $J_{1',2'}$ and $J_{2',3'}$ values of **15** were 0 and 6 Hz, respectively, which coincided with those of 2′,4′-BNA^{COC}-T monomer.[4a,12] Afterward, the desired phosphoramidite **1** was obtained according to common methods to prepare a suitable building block for oligonucleotide synthesis. The oligonucleotide synthesis was performed on an automated DNA synthesizer using common phosphoramidite chemistry with a prolonged coupling time (20 min) for the introduction of the analog **1**.[13] Concerning the oligonucleotide **18** with three consecutive modifications shown in Table 1, successful synthesis was achieved using double-coupling cycles[14] together with the prolonged coupling time.[13]

The duplex- and triplex-forming abilities of the modified oligonucleotides **17**–**20** with ssDNA, ssRNA, and dsDNA were evaluated by UV melting experiments and compared with those of the corresponding natural counterparts **16** and 2′,4′-BNA^{COC}-modified oligonucleotides **21**–**24** (Table 2; see Figure 1 for the structure of 2′,4′-BNA^{COC}). The duplex-forming ability of **17**–**20** with ssDNA and ssRNA showed the same tendency as that of the 2′,4′-BNA^{COC}-modified congeners **21**–**24**; the duplexes with ssDNA were destabilized relative to that of **16**, and the duplexes with ssRNA were stabilized. However, this modification rather than the 2′,4′-BNA^{COC} modification enabled the stable formation of the duplexes with ssRNA. Interestingly, stabilization by this modified nucleic acid was apparently synergistic, and the quintuple-modified oligonucleotide **20** stabilized the duplex with ssRNA by +3.6 °C per modification, the T_m value of which was 69 °C. In triplexes formed with dsDNA, 2′,4′-BNA^{COC}-modified oligonucleotides **21**–**24** showed almost no stabilization. In contrast, oligonucleotides **17**–**20** showed significant stabilization of the triplexes formed, and up to +3.8 °C per modification was observed. These results may imply that the N-type sugar conformation constrained by not only the bridge structure but also an anomeric effect contributes to significant stabilization of the duplexes and triplexes formed with ssRNA and dsDNA, as expected.

Table 2. Duplex- and Triplex-Forming Abilities of Modified Oligonucleotides with ssDNA, ssRNA, and dsDNA[a]

oligonucleotide	ssDNA T_m (°C)	ssRNA T_m (°C)	dsDNA T_m (°C)
5′-TCTTCTTTTTCTCT-3′ (**16**)	50	51	31
5′-TCTTCTTT**T**TCTCT-3′ (**17**)	48 (−2.0)	52 (+1.0)	32 (+1.0)
5′-TCTTCT**TTT**TCTCT-3′ (**18**)	47 (−1.0)	59 (+2.7)	37 (+2.0)
5′-TCTTCT**T**TTTCTCT-3′ (**19**)	47 (−1.0)	60 (+3.0)	41 (+3.3)
5′-TCTTCT**T**TTTCT**C**T-3′ (**20**)	48 (−0.4)	69 (+3.6)	50 (+3.8)
5′-TCTTCTT*T*TTCTCT-3′ (**21**)	48 (−2.0)	52 (+1.0)	31 (0)
5′-TCTTCT*TTT*TCTCT-3′ (**22**)	45 (−1.7)	57 (+2.0)	31 (0)
5′-TCTTCT*T*TTT*C*TCT-3′ (**23**)	45 (−1.7)	57 (+2.0)	33 (+0.7)
5′-TC*T**C*TTTT*C**C*T-3′ (**24**)	42 (−1.6)	62 (+2.2)	33 (+0.4)

[a] 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. C = 2′-deoxy-5-methylcytidine. The sequences of ssDNA, ssRNA, and dsDNA are 5′-d(AGAGAAAAAGAAGA)-3′, 5′-r(AGAGAAAAAGAAGA)-3′, and 5′-d(GGCAGAAGAAAAAGAGACGC)-spacer18-d(GCGTCTTCTTTTTCTCTGCC)-3′, respectively.

The enzymatic stability of the modified oligonucleotides was evaluated using 3′-exonuclease. A comparison of oligonucleotides **25**–**29** is shown in Figure 2a. Although the 2′,4′-BNA/LNA-modified compound **28** and the natural compound **29** were quickly degraded, **25**, which had this analog, showed high resistance against the nuclease, as we expected. The ability was comparable to that observed by 2′,4′-BNA^{COC} modification (**26**) and was better than that of **27**, which had a chiral phosphorothioate linkage, i.e., an S_p-isomer possessing a highly nuclease-resistant property.[15] Moreover, examination of the oligonucleotides modified at the 3′-terminus demonstrated that modification by this analog significantly suppressed degradation of

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(11) Tsunoda, T.; Otsuka, J.; Yamamiya, Y.; Itô, S. *Chem. Lett.* **1994**, 539.
(12) The $J_{1',2'}$ value (0 Hz) indicates that the sugar conformation of EoNA-T monomer **15** adopts the N-form. Altona, C.; Sundaralingam, M. *J. Am. Chem. Soc.* **1973**, *95*, 2333.
(13) By means of monitoring the DMTr cation, the average coupling yield for incorporation of **1** into oligonucleotides was estimated to be 90–95%.
(14) A double-coupling and waiting cycle was carried out prior to the oxidation step.
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the 5′-site by the nuclease compared with 2′,4′-BNA^{COC}, although the reason for this suppression is unclear (Figure 2b).

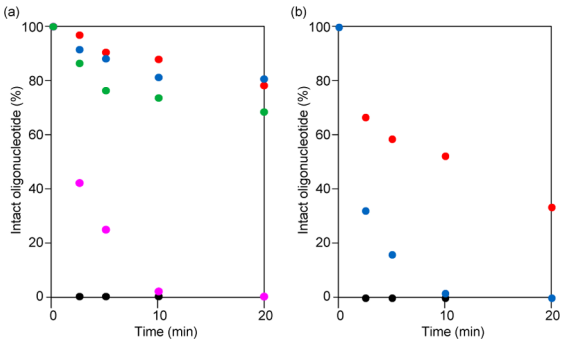

Figure 2. Degradation experiments by nuclease. Conditions: 2 μg/mL *Crotalus admanteus* venom phosphodiesterase (CAVP), 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.0), 7.5 μM each oligonucleotide at 37 °C. (a) 5′-TTTTTTTTTT-3′ [T = EoNA-T (**25**, red), 2′,4′-BNA^{COC}-T (**26**, blue), 3′-S_p-phosphorothioate-T (**27**, green), 2′,4′-BNA/LNA-T (**28**, pink), and natural T (**29**, black)]. (b) 5′-TTTTTTTTTT-3′ [T = EoNA-T (**30**, red), 2′,4′-BNA^{COC}-T (**31**, blue), and natural T (**29**, black)].

In conclusion, we designed and synthesized a 2′-*O*,4′-*C*-ethyleneoxy bridged 5-methyluridine. The synthetic process for the desired phosphoramidite was short (12 steps from 5-methyluridine). The modified oligonucleotides showed stabilization of complexes with ssRNA and dsDNA and increased stability against nuclease degradation. These properties were superior to those of 2′,4′-BNA^{COC} that are most excellent among a series of seven-membered bridged nucleic acids. These modified oligonucleotides are advantageous in the development of applications in which mRNA and genomic DNA are targeted. Moreover, this result suggests that the design concept of addition of a heteroatom at the 4′-carbon atom in the bridged structure is valuable to the development of prominent tools for nucleic acid based technologies.

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Supporting Information Available. Full experimental details, representative UV melting data, representative HPLC data of nuclease experiments, ^{1}H, ^{13}C, and ^{31}P spectra of all new compounds, and HPLC charts and MALDI-TOF-MS spectra of new oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.