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# 2′-*O*,4′-*C*-Ethylene-Bridged Nucleic Acids (ENA): Highly Nuclease-Resistant and Thermodynamically Stable Oligonucleotides for Antisense Drug

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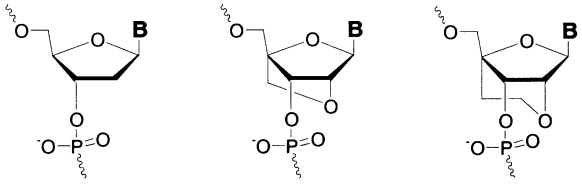
**Abstract**—To develop antisense oligonucleotides, novel nucleosides, 2′-*O*,4′-*C*-ethylene nucleosides and their corresponding phosphoramidites, were synthesized as building blocks. The $^1$H NMR analysis showed that the 2′-*O*,4′-*C*-ethylene linkage of these nucleosides restricts the sugar puckering to the *N*-conformation as well as the linkage of 2′-*O*,4′-*C*-methylene nucleosides which are known as bridged nucleic acids (BNA) or locked nucleic acids (LNA). The ethylene-bridged nucleic acids (ENA) showed a high binding affinity for the complementary RNA strand ($\Delta T_m = +5.2\,^\circ$C/modification) and were more nuclease-resistant than natural DNA and BNA/LNA. These results indicate that ENA have better properties as antisense oligonucleotides than BNA/LNA.


Antisense technology has attracted more interest as a target validation tool in the field of drug discovery through genomics,[1] and developing antisense oligonucleotides as nucleic acid drugs for clinical use could lead to a novel treatment of inveterate diseases such as cancer, inflammation, and viral diseases.[2] Phosphorothioate oligonucleotides, which are most widely used in antisense technology, exhibit high nuclease resistance, but have a number of limitations, such as non-sequence specific toxicity, immune stimulation and low RNA affinity.[3] Many studies have been focused on developing various types of modified oligonucleotides.[4–9] Recently, our and Wengel's group independently reported the synthesis of novel 2′-*O*,4′-*C*-methylene nucleosides whose sugar puckering is fixed in the *N*-conformation as in RNA, and that oligonucleotides containing these bridged nucleosides (BNA/LNA) showed much higher affinity toward their complementary RNA than any other modified oligonucleotides.[10–13] Moreover, Wahlestedt et al. showed that LNA and DNA copolymers were useful for in vivo antisense application.[14]




**DNA** **BNA/LNA** **ENA**

The 2′-*O*,4′-*C*-methylene linkage forms a five-membered ring with the furanose to give a bicyclic nucleoside. It occurred to us that adding one more carbon in the linkage could form a six-membered ring with less strain than a five-membered ring and the resulting nucleoside and corresponding oligonucleotide might show better properties. Recently, Wang et al. reported the synthesis of the 2′,4′-*C*-bridged 2′-deoxynuclosides, which has six-membered rings with 2′-deoxy-type sugars and an increase in $T_m$ values of their corresponding oligonucleotides.[15] In this report, we synthesized 2′-*O*,4′-*C*-ethylene nucleosides, which have six-membered rings with ribo-type sugars, containing all possible natural nucleobases (thymine, adenine, guanine, cytosine, uracil, 5-methylcytosine) and evaluated their basic properties as antisense oligonucleotides.

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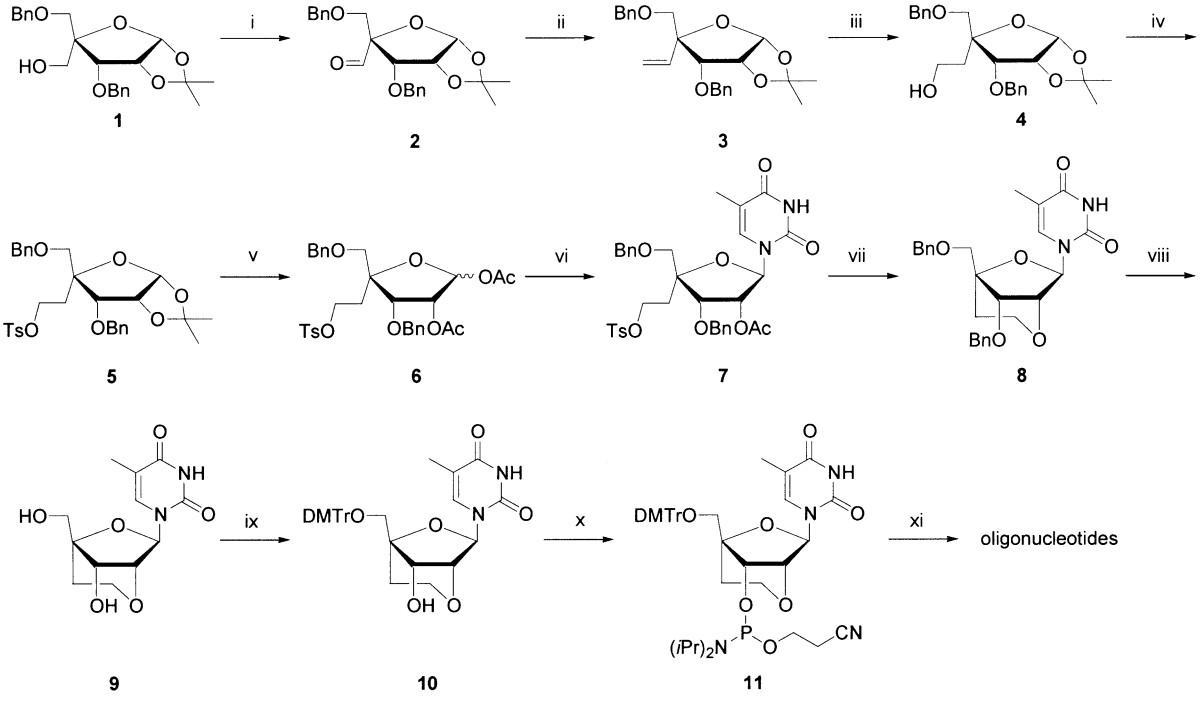
A novel nucleoside, 2′-*O*,4′-*C*-ethylene thymidine was synthesized as shown in Scheme 1. The hydroxymethyl group at the 4-position of compound **1**[16] was converted to a hydroxyethyl group via Swern oxidation, Wittig reaction and hydroboration followed by oxidation to give compound **4**. Tosylation of **4** afforded **5**, which was converted to diacetate **6** by treatment with AcOH and $Ac_2O$ in the presence of a catalytic amount of $H_2SO_4$. The resulting compound **6** was coupled with a silylated thymine by Vorbrüggen's method.[17] Then, base-induced ring closure by the treatment with 1 M NaOH/pyridine-$H_2O$ afforded 2′-*O*,4′-*C*-ethylene thymidine **8**. After debenzylation of compound **8**, the phosphoramidite building block **11** was prepared by a standard procedure. In the $^1$H NMR analysis of the bridged nucleosides (**8**–**11**),[18] the coupling constant ($J_{H1'-H2'}$) was 0 Hz just as that of 2′-*O*,4′-*C*-methylene-bridged nucleosides,[10] which indicated that the puckering of the furanose ring was restricted to the *N*-conformation.[19,20] Other nucleosides containing all possible natural nucleobases (adenine, cytosine, guanine, uracil and 5-methylcytosine) were synthesized by a similar procedure (K. Morita et al., in preparation). Using these phosphoramidites and natural DNA phosphoramidites, desired oligonucleotides were synthesized on a DNA synthesizer by the phosphoramidite method and purified by reverse-phase HPLC. These modified oligonucleotides were characterized by negative ion ESI mass spectroscopy.

First, we tested the affinity of an oligonucleotide containing six 2′-*O*,4′-*C*-ethylene thymidines (eT) toward its complementary RNA or DNA, and compared it to that of an oligonucleotide containing six 2′-*O*,4′-*C*-methylene thymidines (mT). The 2′-*O*,4′-*C*-ethylene-modified oligonucleotide showed an excellent $T_m$ value for RNA ($\Delta T_m$/modification = 5.2 °C), that was almost identical to that of the 2′-*O*,4′-*C*-methylene-modified oligonucleotide ($\Delta T_m$/modification = 5.5 °C, Table 1). Also, $T_m$ values of both modified oligonucleotides for DNA were identical. These results indicate that 2′-*O*,4′-*C*-ethylene nucleic acids (ENA) have a high affinity for DNA and RNA as do 2′-*O*,4′-*C*-methylene nucleic acids (BNA/LNA).

Next, we evaluated the effect on stability of a 2′-*O*,4′-*C*-ethylene modification against endonuclease using thymidine trimers (5′-TpXpT-3′). The middle nucleoside



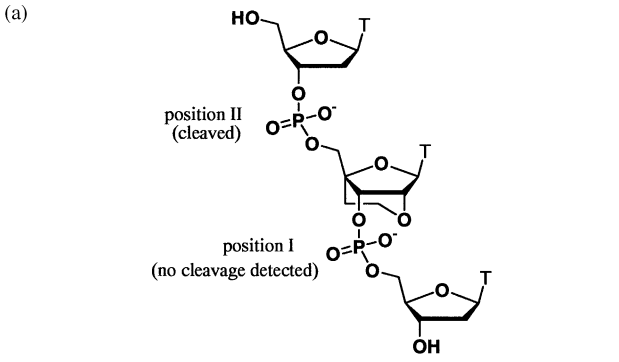

**Scheme 1.** (i) $(COCl)_2$, DMSO, $Et_3N$, $CH_2Cl_2$, −78 °C, 91%; (ii) $Ph_3P^+CH_3Br^-$, NaH, DMSO, 64%; (iii) 9-BBN, THF then $H_2O_2$, NaOH, 93%; (iv) TsCl, $Et_3N$, $CH_2Cl_2$ 97%; (v) $Ac_2O$, $H_2SO_4$, AcOH, 86% (α:β = 4:1); (vi) silylated thymine, TMSOTf, $ClC_2H_4Cl$, reflux, 67%; (vii) 1 N NaOH, pyridine–$H_2O$, 80%; (viii) $H_2$, $Pd(OH)_2$, MeOH, 77%; (ix) DMTrCl, pyridine, $CH_2Cl_2$, 81%; (x) $((iPr)_2N)_2P(OC_2H_4CN)$, *N*,*N*-diisopropylammonium tetrazolide, 89%; (xi) DNA/RNA synthesizer.

**Table 1.** $T_m$ values (°C) of the modified oligonucleotides towards complementary RNA and DNA

| Oligonucleotide 5′-d(GCGXXXXXXGCT)-3′ | RNA complement 5′-r(AGCAAAAAACGC)-3′ | | DNA complement 5′-d(AGCAAAAAACGC)-3′ | |
|---|---|---|---|---|
| | $T_m$ (°C) | $\Delta T_m$ (°C) /modification | $T_m$ (°C) | $\Delta T_m$ (°C)/modification |
| DNA (X = T) | 43 | | 48 | |
| BNA/LNA (X = mT) | 77 | 5.5 | 61 | 2.2 |
| ENA (X = eT) | 75 | 5.2 | 61 | 2.2 |

Duplex concentration: 4 μM. Buffer: 100 mM NaCl, 10 mM sodium phosphate buffer (pH 7.2); mT: 2′-*O*,4′-*C*-methylene thymidine, eT: 2′-*O*,4′-*C*-ethylene thymidine.

(X) of the trimer was substituted with 2′-*O*,4′-*C*-ethylene thymidine (eT) or 2′-*O*,4′-*C*-methylene thymidine (mT). The trimer has two phosphodiester linkages (position I and II, Fig. 1a) which can be cleaved by endonuclease, nuclease $P_1$. When the modified oligonucleotides (X = eT and mT) were incubated with nuclease $P_1$, the products (5′-TpX-3′ and thymidine-5′-monophosphate), which would be cleaved at position I, were never detected in the HPLC analysis even under high concentration as 62.5 μg/mL nuclease $P_1$ (data not shown). On the other hand, the products (thymidine and 5′-pXpT-3′), which were cleaved at position II, were detected at 1.3 μg/mL nuclease $P_1$. Under this condition, both phosphodiester linkages in natural 5′-TpTpT-3′ were completely hydrolyzed by nuclease $P_1$ within 5 min (Fig. 1b). Hydrolysis rates of the phosphodiester linkage at position II in 5′-TpXpT-3′ (X = eT and mT) were measured (Fig. 1b). The initial rate constants of hydrolysis of the trimers (X = eT and mT) were calculated as $4.6\times10^{-3}$ $min^{-1}$ and $1.8\times10^{-2}$ $min^{-1}$, respectively. From these results, we found that the 2′-*O*,4′-*C*-ethylene-modified trimer was approximately 4 times more stable than the 2′-*O*,4′-*C*-methylene-modified trimer.



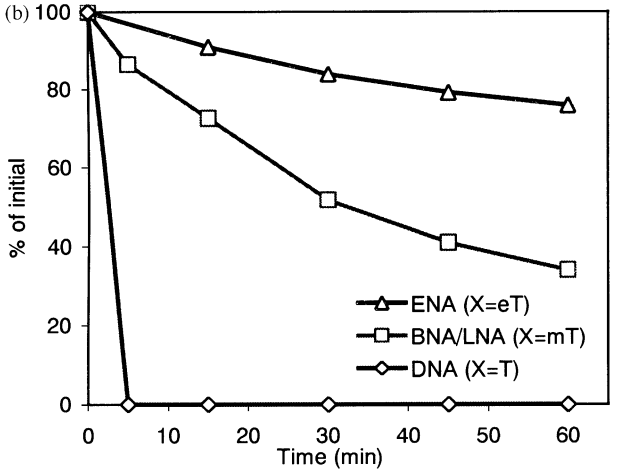




**Figure 1.** (a) Two positions of 5′-TXT-3′ (X = eT), which can be recognized and cleaved by nuclease $P_1$. (b) Stability of modified oligonucleotides 5′-TXT-3′ (X = T, mT or eT) at position II against nuclease $P_1$; oligo concentration: 6.4 μg/mL, enzyme concentration: 1.3 μg/mL; buffer: 20 mM sodium acetate buffer (pH 5.2), 0.1 mM $ZnCl_2$, at 37 °C.

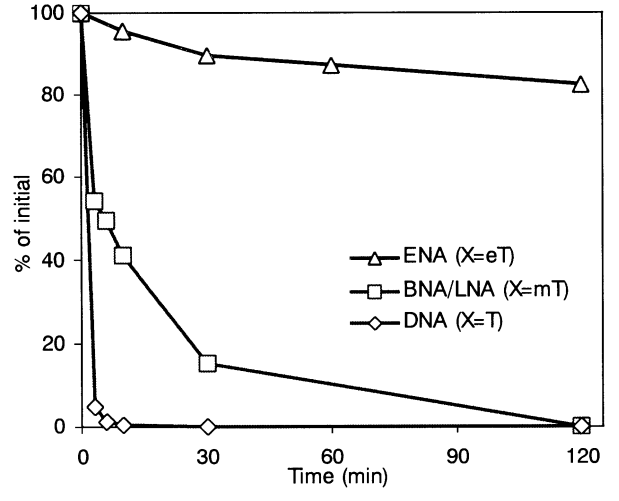



**Figure 2.** Stability of modified oligonucleotides (5′-d(TTTTTTTTTTXT)-3′ (X = T, mT or eT) against SVPD; oligo concentration: 26 μg/mL, enzyme concentration: 0.3 μg/mL; buffer: 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, at 37 °C.

Furthermore, we investigated the stability of the modified oligonucleotides against 3′-exonuclease (snake venom phosphodiesterase, SVPD). Using oligothymidylates, which contain a modified thymidine on the second nucleotide from the 3′-end (5′-d(TTTTTTTTTTXT)-3′, X = T, mT or eT), the amount of the first 3′-phosphodiester cleavage by 3′-exonuclease was quantified by HPLC analysis. Although natural oligothymidylates (X = T) and 2′-*O*,4′-*C*-methylene-modified oligonucleotides (X = mT) were rapidly hydrolyzed by 3′-exonuclease, approximately 82% of 2′-*O*,4′-*C*-ethylene-modified oligonucleotides still remained after 2 h (Fig. 2). We measured and calculated the observed rate constants ($k_{obs}$) of hydrolysis of the three oligonucleotides (DNA: $k_{obs} = > 7.5\times10^{-1}$ $min^{-1}$, BNA/LNA: $k_{obs} = 8.2\times10^{-2}$ $min^{-1}$, ENA: $k_{obs} = 1.5\times10^{-3}$ $min^{-1}$). These data show that one 2′-*O*,4′-*C*-ethylene modification resulted in more than 500 times higher stability than natural nucleotides, and approximately 55 times higher stability than the 2′-*O*,4′-*C*-methylene nucleotide.

In conclusion, we further optimized BNA/LNA by the addition of one more carbon in the linkage, and synthesized novel 2′-*O*,4′-*C*-ethylene-bridged nucleic acids (ENA) which have high affinity for RNA and high nuclease-resistance. Only one example of an in vivo BNA/LNA application in the cerebrospinal fluid system with very low levels of nuclease has already been reported.[14] More highly nuclease-resistant ENA than BNA/LNA would greatly contribute to the developments of antisense drugs. Further antisense studies are currently in progress.

## References and Notes

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