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SYNTHESIS AND PROPERTIES OF OLIGONUCLEOTIDES CONTAINING 3′-*O*,4′-*C*-ETHYLENEOXY-BRIDGED 5-METHYLURIDINES

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Dedicated to Professor Dr. Kiyoshi Tomioka on the occasion of his 70th birthday

Abstract – A phosphoramidite of 3′-*O*,4′-*C*-ethyleneoxy-bridged 5-methyluridine (3′,4′-EoNA-T) was successfully incorporated into oligonucleotides, and their abilities to form duplexes with RNA and DNA as well as triplexes with double-stranded DNA were evaluated. The stabilities of the duplexes formed between RNA and 3′,4′-EoNA-modified oligonucleotides were only slightly lower than those of the natural DNA–RNA duplex, while the duplexes formed between 3′,4′-EoNA-modified oligonucleotides and DNA were drastically less stable than the natural DNA–DNA duplex. Moreover, under the same conditions as the UV-melting experiments of the duplexes, 3′,4′-EoNA-modified oligonucleotides were unable to form triplexes with dsDNA. These results showed that oligonucleotides containing 3′,4′-EoNA-T had excellent RNA selectivity.

INTRODUCTION

During the past few decades, chemically modified oligonucleotides have been developed for gene diagnostics[1–4] and gene therapy.[5–8] In particular, oligonucleotides with 2′,5′-phosphodiester linkages (*iso*DNA and *iso*RNA) have interesting physical properties, such as high resistance towards nuclease digestion.[9–11] Moreover, these oligonucleotides can selectively hybridize with single-stranded RNA (ssRNA) rather than single-stranded DNA (ssDNA); however, the thermal stabilities of the duplexes formed between ssRNA and *iso*DNA or *iso*RNA are generally similar to or lower than those of the

corresponding DNA–RNA and RNA–RNA duplexes.[9,10] Several reports have been published regarding 3′,5′-linked oligonucleotides containing 2′,5′-phosphodiester linkages.[12–15] For example, the incorporation of 3′,4′-bridged nucleic acid analogs **1**–**3** forming 2′,5′-linkages (Figure 1) into natural 3′,5′-linked oligonucleotides led to moderate or large decreases in the affinities towards ssDNA as compared to unmodified DNA, whereas the affinity towards ssRNA did not change appreciably.[13,14] Accordingly, oligonucleotides containing 3′,4′-bridged nucleic acid analogs **1**–**3** could form duplexes with ssRNA in an RNA-selective manner.

On the other hand, oligonucleotides that contain 2′,4′-bridged nucleic acids adopting an N-type sugar conformation are known to form stable complexes with both ssRNA and dsDNA.[16] Recently, we reported 2′,4′-bridged nucleic acid analogs with oxygen atoms at the 6′-positions.[17–19] The presence of the 6′-oxygen atom could positively affect the duplex- and triplex-forming abilities of the oligonucleotides. In addition, we synthesized a 3′,4′-ethyleneoxy-bridged 5-methyluridine monomer,[20] which had an oxygen atom at the 6′-position (3′,4′-EoNA-T, Figure 1). The 3′,4′-EoNA is capable of forming 2′,5′-linkages in oligonucleotides. Therefore, to further this line of research, we were interested in evaluating the duplex- and triplex-forming ability of 3′,5′-linked oligonucleotides that contain 3′,4′-EoNA-T. Here, we report on the synthesis of 3′,5′-linked oligonucleotides modified with 3′,4′-EoNA-T, and their binding affinities towards ssRNA, ssDNA, and dsDNA.

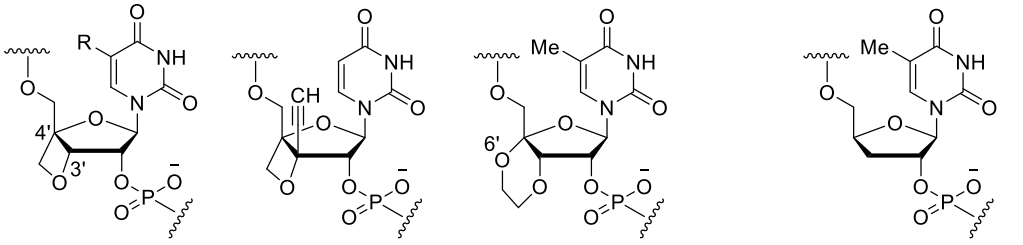
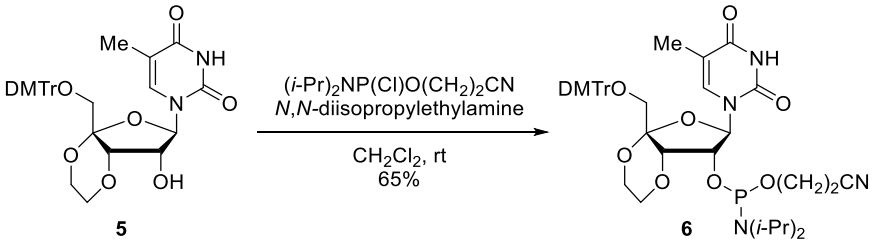


Figure 1. Structures of 3′,4′-bridged nucleic acids **1-4**

RESULTS AND DISCUSSION

Phosphoramidite **6** of 3′,4′-EoNA-T was obtained in 65% yield by phosphitylation of **5**[20] previously reported (Scheme 1). In addition, the phosphoramidite of 3′-deoxy-5-methyluridine (2′,5′-T) was prepared from 5-methyluridine according to reported procedures.[21,22] The synthesis of oligonucleotides (**ON1**–**14**, Table 1) was accomplished on an automated DNA synthesizer using common phosphoramidite chemistry with a prolonged coupling time of 5 min for incorporation of 3′,4′-EoNA-T. The purity and molecular weights of **ON1**–**14** were determined using reversed-phase HPLC and ESI-TOF (or MALDI-TOF) mass analyses, respectively.



Scheme 1. Synthesis of 3′,4′-EoNA-T-phosphoramidite **6**

The duplex-forming abilities of homopyrimidine oligonucleotides **ON2**–**4** containing 3′,4′-EoNA-T towards complementary ssRNA and ssDNA were evaluated using UV-melting experiments, and were compared to those of the natural congener **ON1** and 2′,5′-T-modified oligonucleotides **ON5**–**7**. The melting temperatures (T_m), and changes in T_m values per modification (ΔT_m/mod.) relative to the natural **ON1** (T_m = 49 °C for ssRNA and 46 °C for ssDNA, respectively) are summarized in Table 1. 3′,4′-EoNA-T only slightly reduced the stability of the duplex with ssRNA (ΔT_m/mod. = −2.0 °C to −1.7 °C, **ON2**–**4**), while a drastic decrease in the stability with ssDNA was observed (ΔT_m/mod. = −11.0 °C to −9.0 °C, **ON2**–**4**). The duplex stability of 3′,4′-EoNA-modified **ON2**–**4** with ssRNA was almost equal to that modified with 2′,5′-T (ΔT_m/mod. = −2.0 °C to −1.0 °C, **ON5**–**7**). In contrast, 3′,4′-EoNA (ΔT_m/mod. = −11.0 °C to −9.0 °C, **ON2**–**4**) significantly destabilized duplexes with ssDNA in comparison to 2′,5′-T (ΔT_m/mod. = −5.7 °C to −5.0 °C, **ON5**–**7**).

Table 1. T_m values of duplexes formed with ssRNA and ssDNA by **ON1**–**14**[a]

	ssRNA	ssDNA
5′-d(TCTTCTTTTTCTCT)-3′ (**ON1**)	49 °C	46 °C
5′-d(TCTTCTT**T**TTCTCT)-3′ (**ON2**)	47 °C (−2.0 °C)	35 °C (−11.0 °C)
5′-d(TCTTCT**TTT**TCTCT)-3′ (**ON3**)	43 °C (−2.0 °C)	19 °C (−9.0 °C)
5′-d(TCTTC**T**T**T**T**T**CTCT)-3′ (**ON4**)	44 °C (−1.7 °C)	19 °C (−9.0 °C)
5′-d(TCTTCTT*T*TTCTCT)-3′ (**ON5**)	47 °C (−2.0 °C)	41 °C (−5.0 °C)
5′-d(TCTTCT*TTT*TCTCT)-3′ (**ON6**)	46 °C (−1.0 °C)	29 °C (−5.7 °C)
5′-d(TCTTC*T*T*T*T*T*CTCT)-3′ (**ON7**)	45 °C (−1.3 °C)	30 °C (−5.3 °C)
5′-d(GGATGTTCTCGT)-3′ (**ON8**)	47 °C	47 °C
5′-d(GGATG**T**TCTCGT)-3′ (**ON9**)	45 °C (−2.0 °C)	37 (−10.0 °C)
5′-d(GGATG**TT**CTCGT)-3′ (**ON10**)	44 °C (−1.5 °C)	29 (−9.0 °C)
5′-d(GGA**T**G**T**TC**T**CGT)-3′ (**ON11**)	42 °C (−1.7 °C)	21 (−8.7 °C)
5′-d(GGATG*T*TCTCGT)-3′ (**ON12**)	45 °C (−2.0 °C)	41 (−6.0 °C)
5′-d(GGATG*TT*CTCGT)-3′ (**ON13**)	44 °C (−1.5 °C)	34 (−6.5 °C)

5′-d(GGA*TGTTCTCGT*)-3′ (**ON14**)	42 °C (−1.7 °C)	29 (−6.0 °C)

[a]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* = 3′-deoxy-5-methyluridine (2′,5′-T), C = 2′-deoxy-5-methylcytidine. The sequences of ssRNA used are 5′-r(AGAGAAAAAGAAGA)-3′ and 5′-r(ACGAGAACAUCC)-3′. The sequences of ssDNA used are 5′-r(AGAGAAAAAGAAGA)-3′ and 5′-d(ACGAGAACATCC)-3′. The change in T_m value per modification (ΔT_m/mod.) compared with the natural **ON1** and **ON8**, and ΔT_m/mod. values are shown in parentheses.

To confirm the generality of the hybridization ability of 3′,4′-EoNA-modified oligonucleotides, the duplex-forming abilities of oligonucleotides with mixed-base sequence, *i.e.*, **ON8**–**11**, were evaluated (Table 1 and Figure 2). The ΔT_m/mod. values of **ON9**–**14** compared to **ON8** (T_m = 47 °C for ssRNA and 47 °C for ssDNA, respectively). Similar to the results obtained with **ON1**–**7**, the 3′,4′-EoNA-modified oligonucleotides **ON9**–**11** rather than natural **ON8** and 2′,5′-T-modified **ON12**–**14** exhibited high selectivity towards ssRNA.

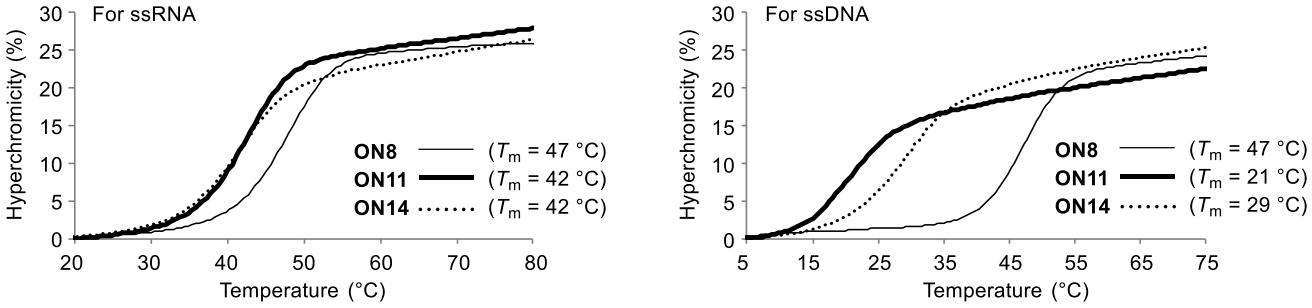


Figure 2. Representative UV-melting curves of duplexes

Next, the triplex-forming ability of **ON1**–**ON7** with dsDNA was evaluated (Table 2). Under the same conditions as the UV-melting analysis of the duplexes, only the melting transitions of dsDNA were observed, while no triplexes were observed. Therefore, the conditions for the UV melting experiments were modified to include a high concentration of $MgCl_2$ (50 mM) in order to increase the stability of the triplexes. 2′,5′-T modification slightly stabilized the triplexes with dsDNA (ΔT_m/mod. = 0 °C to +2.3 °C, **ON5**–**7**), which was consistent with literature data.[15] On the other hand, triplex formation of **ON3** with three successive 3′,4′-EoNA-Ts was not observed (ΔT_m/mod. = < −4.3 °C), although in the case of **ON2** and **ON4**, the stabilities of the triplexes were fairly similar to that obtained with **ON1** (ΔT_m/mod. = +1.0 °C and 0 °C, respectively). The results of UV-experiments shown in Table 1 and Table 2 imply that modification with 3′,4′-EoNA-T promoted the binding selectivity with ssRNA over ssDNA and dsDNA as compared to modification with 2′,5′-T.

Table 2. T_m values of triplexes formed between dsDNA and **ON1**–**7**[a]

	no $MgCl_2$	50 mM $MgCl_2$
5′-d(TCTTCTTTTTCTCT)-3′ (**ON1**)	nd	28 °C
5′-d(TCTTCTT**T**TTCTCT)-3′ (**ON2**)	nd	29 °C (+1.0 °C)
5′-d(TCTTCT**TTT**TCTCT)-3′ (**ON3**)	nd	<15 °C (<−4.3 °C)
5′-d(TCTTC**T**TT**T**TCTCT)-3′ (**ON4**)	nd	28 °C (±0 °C)
5′-d(TCTTCTT*T*TTCTCT)-3′ (**ON5**)	nd	30 °C (+2.0 °C)
5′-d(TCTTCT*TTT*TCTCT)-3′ (**ON6**)	nd	28 °C (±0 °C)
5′-d(TCTTC*T*TTT*T*CTCT)-3′ (**ON7**)	nd	35 °C (+2.3 °C)

[a]Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, 0 mM or 50 mM $MgCl_2$, and 1.5 μM of each oligonucleotide. **T** = 3′,4′-EoNA-T, *T* = 3′-deoxy-5-methyluridine (2′,5′-T), C = 2′-deoxy-5-methylcytidine. The sequence of dsDNA used is 5′-d(GGCAGAAGAAAAAGAGACGC)-spacer18-d(GCGTCTCTTTTTCTTCTGCC)-3′. ΔT_m/mod. values are shown in parentheses. nd: Not detected.

The duplex formation ability of oligonucleotides containing other 3′,4′-bridged nucleosides **1**–**3** (Figure 1) with ssDNA and ssRNA was previously reported.[13,14] The stability appeared to be highly dependent on the sequence and the modification pattern. 3′,4′-BNA-T **1** and 3′,4′-BNA-U **2** showed ΔT_m/mod. values of −1.3 °C to +4.0 °C towards ssRNA as compared to the corresponding natural DNA, while in most cases the ΔT_m/mod. values towards ssDNA ranged from −6.5 °C to 0 °C.[13] Concerning **3**, the ΔT_m/mod. values towards ssDNA and ssRNA were generally −9.5 °C to −1.5 °C and −4.0 °C to +3.5 °C, respectively.[14] Consecutive modifications of oligonucleotides using **1**–**3** led to further reduction in stability. Based on the results obtained with **1**–**4** (Figure 1) and 2′,5′-T, 3′,4′-EoNA resulted in a highly RNA-selective binding ability with almost no dependency on the sequence or modification pattern, regardless of whether consecutive modifications were made or not. Therefore, it was concluded that the high RNA-selectivity of 3′,4′-EoNA was due to the drastic decrease in duplex stability with ssDNA. This may imply that the hydration of the duplex with ssDNA was disrupted by the presence of the 6′-oxygen atom and/or the bulky six-membered ring in 3′,4′-EoNA; however, further investigations are required to clarify this issue.

CONCLUSION

In this study, oligonucleotides containing 3′,4′-EoNA-T were synthesized, and their hybridization with ssRNA, ssDNA and dsDNA was evaluated. The results demonstrated that 3′,4′-EoNA-T resulted in a higher RNA-selective binding ability than natural DNA, 2′,5′-T, and previously reported 3′,4′-bridged nucleic acids. Since RNA plays a key role in regulating numerous cellular pathways and processes,[23] the development of chemically modified oligonucleotides as tools for detecting and modulating RNA is significant. In this light, 3′,4′-EoNA might be a promising modification for an oligonucleotide probe

detecting ssRNA. Furthermore, not only the development of various 3′,4′-bridged nucleic acid analogues but also understanding their hybridization properties might contribute to the discovery of useful materials for oligonucleotide-based technologies.

EXPERIMENTAL

General methods

All moisture-sensitive reactions were conducted in a well-dried glassware under an argon atmosphere. NMR experiments were performed on a Bruker AVANCE III HD 500 MHz spectrometer equipped with a BBO cryoprobe. ^{1}H NMR spectra were recorded at 500 MHz, and ^{31}P NMR spectra were recorded at 202 MHz. Chemical shift values were reported in parts per million downfield from internal tetramethylsilane (δ = 0.00 ppm) for ^{1}H NMR, and external 5% H_3PO_4 (δ = 0.00 ppm) for ^{31}P NMR. For column chromatography, silica gel PSQ60B (Fuji Silycia) was used. For thin-layer chromatography, silica gel 60 F_{254} glass plates (Merck) were used. High performance liquid chromatography (HPLC) was performed on a JASCO EXTREMA (PU-4180, CO-4060, and UV-4075) with a fraction collector CHF122SC (ADVANTEC). ESI-TOF mass spectra were recorded on a Waters SYNAPT G2-Si HDMS mass spectrometer, and MALDI-TOF mass spectra were recorded on a Bruker Daltonics Autoflex II mass spectrometer. UV melting experiments were performed on a JASCO UV-730 UV/VIS spectrophotometer equipped with a T_m analysis accessory.

1-[2-*O*-(2-Cyanoethoxy)(diisopropylamino)phosphino-5-*O*-(4,4′-dimethoxytrityl)-3-*O*,4-*C*-ethyleneoxy-β-D-ribofuranosyl]thymine (6)

Under an argon atmosphere, *N*,*N*-diisopropylethylamine (0.14 mL, 0.83 mmol) and *i*-$Pr_2NP(Cl)O(CH_2)_2CN$ (74 μL, 0.33 mmol) were added to a solution of compound **5**[20] (100 mg, 0.17 mmol) in anhydrous CH_2Cl_2 (5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 5 h. After being quenched with sat. $NaHCO_3$ aq., the reaction mixture was extracted with $CHCl_3$. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (172 mg) was purified by silica gel column chromatography (silica gel 10 g, hexane:EtOAc = 1:1) to give compound **6** as a white powder (87 mg, 65%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.04 (d, *J* = 7.5 Hz, 3H), 1.14–1.24 (m, 12H), 2.51–2.73 (m, 2H), 3.16–3.19 (m, 1H), 3.32–3.37 (m, 2H), 3.57–3.90 (m, 12H), 4.01–4.06 (m, 1H), 4.13 (d, *J* = 3.5 Hz, 0.5H), 4.25 (d, *J* = 4.0 Hz, 0.5H), 4.98–5.04 (m, 1H), 6.63–6.66 (m, 1H), 6.84–6.87 (m, 4H), 7.26–7.40 (m, 9H), 7.65 (s, 0.5H), 7.66 (s, 0.5H), 8.27 (s, 1H). ^{31}P NMR (202 MHz, $CDCl_3$) δ: 151.4, 152.4. HRMS (ESI): Calcd for $C_{42}H_{51}N_4NaO_{10}P$ [MNa^+] 825.3241, found 825.3239.

Synthesis of oligonucleotides

Phosphoramidites were dissolved in anhydrous MeCN to obtain a final concentration of 0.1 M. The

synthesis of **ON1**–**14** was performed on a 0.2-μmol scale using an automated DNA synthesizer (Gene Design nS-8II) with 0.25 M 5-(ethylthio)-1*H*-tetrazole in MeCN as an activator. Modified phosphoramidite **6** was introduced into the oligonucleotides at a prolonged coupling time of 5 min. The synthesis was carried out in trityl-on mode. The cleavage of the oligonucleotide from the CPG resin was accomplished using concentrated ammonium hydroxide at room temperature for 1.5 h. All protecting groups of oligonucleotides were removed by treatment with concentrated ammonium hydroxide at room temperature for 4 h (for **ON1**–**7**) and at 55 °C for 16 h (for **ON8**–**14**). Ammonia was removed *in vacuo*. The crude oligonucleotides were purified using Sep-Pak® Plus C18 cartridges (Waters); during purification, the 5′-DMTr group was removed using 1% (v/v) aqueous trifluoroacetic acid (TFA). The obtained oligonucleotides were further purified using reversed-phase HPLC (Waters XBridge® MS C18 column 5 μm, 10 × 50 mm). The compositions of the purified oligonucleotides (**ON2**–**14**) were confirmed by ESI-TOF mass analysis or MALDI-TOF mass analysis. The deconvoluted ESI-TOF mass data [M] for **ON2**–**8** were as follows: **ON2**, found 4251.50 (calcd 4250.86); **ON3**, found 4367.00 (calcd 4366.94); **ON4**, found 4367.00 (calcd 4366.94); **ON5**, found 4191.70 (calcd 4192.83); **ON6**, found 4192.50 (calcd 4192.83); **ON7**, found 4191.70 (calcd 4192.83); **ON8**, found 3667.60 (calcd 3667.42). The MALDI-TOF mass data ($[M-H]^-$) for **ON9**–**14** were as follows: **ON9**, found 3724.22 (calcd 3724.45); **ON10**, found 3781.84 (calcd 3782.49); **ON11**, found 3840.67 (calcd 3840.52); **ON12**, found 3666.44 (calcd 3666.42); **ON13**, found 3666.73 (calcd 3666.42); **ON14**, found 3667.04 (calcd 3666.42).

UV-melting experiments

For the UV-melting experiments using the duplexes, the oligonucleotides were dissolved in 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM NaCl to obtain a final concentration of 2.5 μM for each strand. The samples were annealed by heating at 100 °C, followed by slow cooling to room temperature. The melting profiles were recorded at 260 nm from 20 °C to 80 °C for ssRNA and from 5 °C to 75 °C for ssDNA at a scan rate of 0.5 °C/min. The two-point average method was employed to calculate the T_m values, and the final values were determined by averaging three independent measurements, which were accurate within 1 °C.

For the UV-melting experiments using the triplexes, oligonucleotides and dsDNA were dissolved in 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM NaCl and 0 mM or 50 mM $MgCl_2$ to obtain a final concentration of 1.5 μM for each strand. The samples were annealed by heating at 100 °C followed by slow cooling to room temperature. The melting profiles were recorded at 260 nm from 5 °C to 95 °C at a scan rate of 0.5 °C/min. The two-point average method was employed to calculate the T_m values, and the final values were determined by averaging three independent measurements, which were accurate within 1 °C.

ACKNOWLEDGEMENTS

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