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SYNTHESIS AND HYBRIDIZING PROPERTY OF OLIGONUCLEOTIDES INCLUDING 2′-*C*,4′-*C*-ETHYLENEOXY-BRIDGED 2′-DEOXYADENOSINE WITH AN EXOCYCLIC METHYLENE UNIT

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Dedicated to Professor Dr. Kaoru Fuji on the occasion of his 80th birthday

Abstract – 2′,4′-Bridged nucleic acids (2′,4′-BNAs) are of interest because oligonucleotides that include them have excellent duplex-forming capability and high nuclease resistance compared to natural oligonucleotides. We have recently developed 2′-*C*,4′-*C*-ethyleneoxy-bridged thymidine with an exocyclic methylene unit (methylene-EoDNA-T) as a novel 2′,4′-BNA analog. Oligonucleotides that include methylene-EoDNA-T have marked hybridizing capability, nuclease resistance, and *in vitro* gene-silencing potency. In the present study, we designed and synthesized a 2′-deoxyadenosine analog of methylene-EoDNA (methylene-EoDNA-A), and incorporated it into oligonucleotides. The results of melting temperature (T_m) analysis of duplexes formed from methylene-EoDNA-A-modified oligonucleotides indicated that the hybridizing capability with regard to complementary DNA was almost the same or slightly higher than that of natural DNA. Moreover, methylene-EoDNA-A:methylene-EoDNA-T base pairs increased the thermal stability of DNA duplexes compared to that of DNA duplexes containing methylene-EoDNA-A- or methylene-EoDNA-T-modification in one strand.

INTRODUCTION

Oligonucleotides have been used in gene diagnosis, therapeutic agents, and nanotechnologies.[1–7] However, the use of natural DNA or RNA for oligonucleotide-based technologies is problematic because they generally lack binding affinity with complementary single strands and are easily degraded by nucleases. Numerous chemically modified oligonucleotides have been developed to address these issues. Of these, conformationally constrained oligonucleotides—including modified nucleotides with bicyclic carbohydrate moieties—improve the stability of duplexes formed from complementary single strands. In particular, oligonucleotides modified by 2′,4′-bridged nucleic acids (2′,4′-BNAs)[8,9]/locked nucleic acids (LNAs)[10,11] have received significant attention because LNA-modification provides good duplex-forming capability. Moreover, LNA-modified oligonucleotides have improved nuclease resistance owing to the steric hindrance of the bridge moiety. Consequently, various LNA derivatives have been synthesized with the objective of producing an ideal material for practical oligonucleotides.[2,12–14]

We recently developed a methylene-EoDNA-T molecule with a six-membered bridge comprising a 6′-oxygen atom and an 8′-exocyclic methylene group (Figure 1).[15] The methylene-EoDNA-modified oligonucleotides had excellent hybridizing affinity with complementary RNA, and extremely high resistance to nucleases compared to natural oligonucleotides. Furthermore, the *in vitro* gene-silencing potency of methylene-EoDNA-T-modified oligonucleotides is comparable to that of LNA-modified oligonucleotides, which have already been used in therapeutic applications.[16] However, only thymidine analogs can restrict the application, other nucleoside analogs are required. On the other hand, duplexes including LNA:LNA base pairs are exceptionally stable compared to other duplexes.[17] For example, the T_m value of a fully LNA-modified 9-mer duplex was more than 60 °C higher than that of the corresponding natural DNA duplex. Moreover, oligonucleotides modified by 2′,4′-BNA^{NC} or 2′,4′-BNA^{COC}, which are 2′,4′-BNA analogs, can form markedly stable homoduplexes.[18,19] Therefore, we designed a 2′-deoxyadenosine analog of methylene-EoDNA (methylene-EoDNA-A, Figure 1), because we became interested in the stability of duplexes that include base pairs formed between methylene-EoDNA-A and methylene-EoDNA-T. Herein, we describe the synthesis of methylene-EoDNA-A and the evaluation of the effect of the methylene-EoDNA-T:methylene-EoDNA-A base pair on the stability of DNA duplexes.

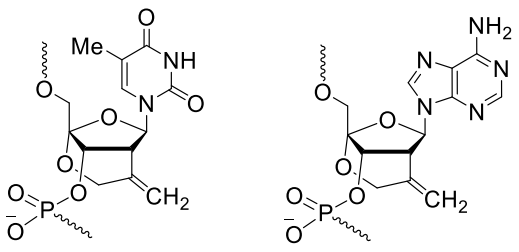
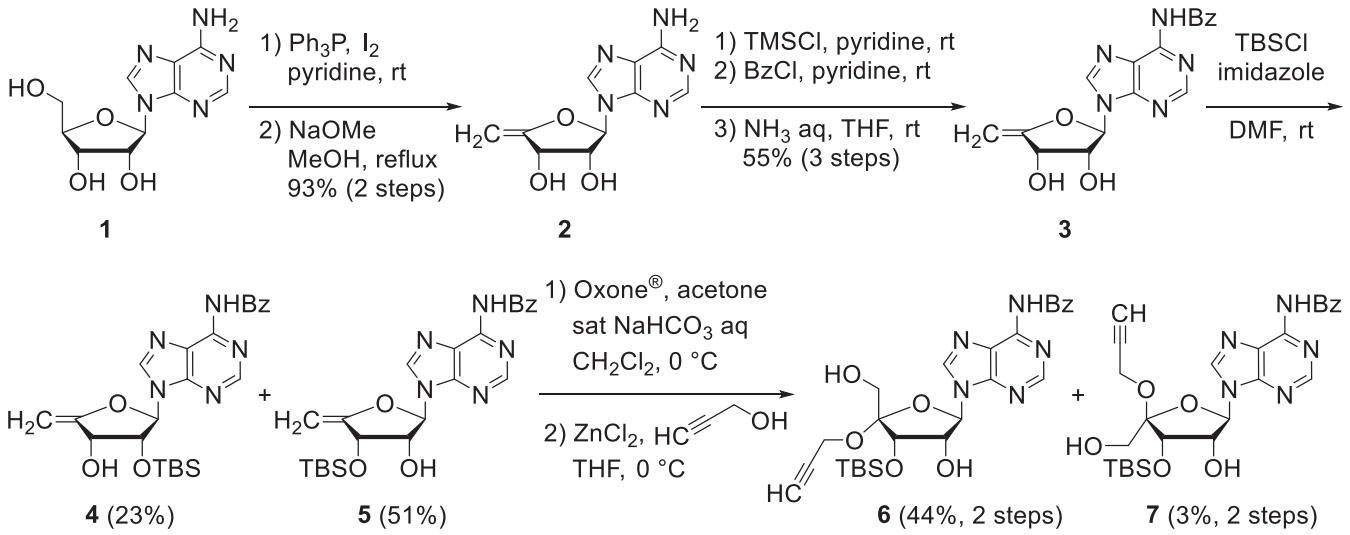


Figure 1. Structures of methylene-EoDNA-T and methylene-EoDNA-A

RESULTS AND DISCUSSION

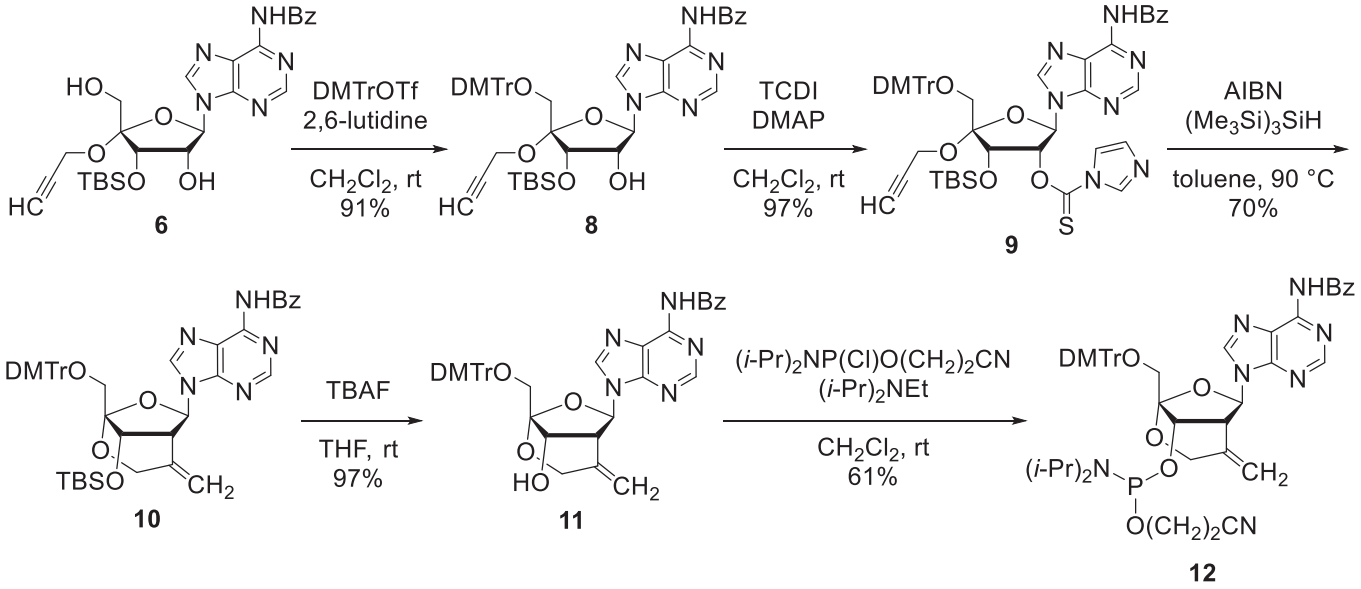
The synthesis of methylene-EoDNA-A began with olefin **2**,[20] prepared in two steps from adenosine **1** (Scheme 1). After protecting the 2′- and 3′-OH groups in compound **2** *in situ* with trimethylsilyl (TMS) groups, we acylated the NH_2 group at the C6 position of adenine with two equivalents of benzoyl chloride, then treated the product with aqueous ammonia to produce N^6-benzoyladenosine derivative **3** at a yield of 55% in three steps from **2**. The 3′-OH group in **3** was protected by a TBS group to give **5** at a yield of 51%, although 2′-*O*-TBS compound **4** was also obtained (23% yield). Epoxidation of **5** by *in situ*-generated dimethyldioxirane using Oxone® and acetone followed by $ZnCl_2$-mediated propargyloxylation at the C4′ position produced the desired 4′-*C*-propargyloxyadenosine **6** (44% yield) and α-lyxofuranosyl derivative **7** (3% yield).



Scheme 1. Synthesis of 4′-*C*-propargyloxyadenosine derivative **6**

After protecting the 5′-OH group of diol **6** using DMTrOTf,[21] we converted compound **8** to radical precursor **9** using 1,1′-thiocarbonyldiimidazole (TCDI), as shown in Scheme 2. Intramolecular radical cyclization using AIBN and $(Me_3Si)_3SiH$ afforded the desired cyclized product **10** in 70% yield. Methylene-EoDNA-A-phosphoramidite **12**, which is a building block for automated DNA synthesis, was synthesized by the removal of the TBS group in **10** using TBAF followed by phosphitylation of the obtained **11** using $(i\text{-Pr})_2NP(Cl)O(CH_2)_2CN$. Oligonucleotide synthesis was carried out on an automated DNA synthesizer using common phosphoramidite chemistry with a prolonged coupling time of 10 min (*cf.* 32 s for coupling of natural phosphoramidites) for the introduction of methylene-EoDNA-A **12** and methylene-EoDNA-T **13**[15] (Figure 2). To avoid the decomposition of the exocyclic methylene group, 1 M *t*-BuOOH in toluene,[22] instead of 0.02 M iodine solution, was used as an oxidizing agent in

oligonucleotide synthesis. Under these conditions, all the desired oligonucleotides (**ON1**–**7**, Figure 4) were obtained successfully.



Scheme 2. Synthesis of methylene-EoDNA-A phosphoramidite **12**

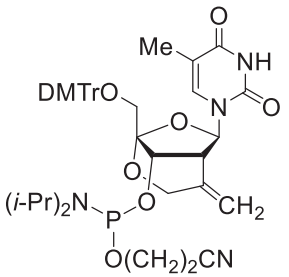


Figure 2. Structure of methylene-EoDNA-T phosphoramidite **13**

We evaluated the hybridization properties of methylene-EoDNA-A- and methylene-EoDNA-T-modified oligonucleotides **ON1**–**7** with regard to complementary DNA by UV-melting experiments, and compared the results to those obtained with natural DNA duplexes (**ON8** and **ON9**). Representative UV-melting profiles are shown in Figure 3, and the results of our experiments are summarized in Figure 4. The duplex-forming capability of the methylene-EoDNA-T-modified oligonucleotides **ON4**–**7** (**duplexes 4**–**7**, ΔT_m/mod. values ranged from −1 °C to +2 °C) was similar to or slightly higher than that of the natural oligonucleotide (**ON9**, T_m = 48 °C). These results are similar to those of our previous study on the duplex-forming capability of homopyrimidine 14-mer oligonucleotides, including those with methylene-EoDNA-T-modifications (ΔT_m/mod. ranged from −2 °C to +1 °C).[15]

Methylene-EoDNA-A-modified **ON1–3** (**duplexes 1–3**, ΔT_m/mod. values ranged from +1 °C to +2 °C) produced similar results to those for methylene-EoDNA-T-modified **ON4–7** (**duplexes 4–7**).

We carried out T_m analysis of **duplexes 8–11** to evaluate the thermal stability of DNA duplexes containing methylene-EoDNA-A and methylene-EoDNA-T modifications in each strand. Incorporation of a single base pair formed between methylene-EoDNA-A and methylene-EoDNA-T into a DNA duplex (**duplex 8**) resulted in a ΔT_m/mod. value of +0.5 °C, and a methylene-EoDNA-A:methylene-EoDNA-T base pair produced no significant stabilization. Moreover, **duplex 9** (ΔT_m/mod. = +1 °C), which includes methylene-EoDNA-A and methylene-EoDNA-T modification in each strand, did not exhibit increased stability relative to **duplex 1** (ΔT_m/mod. = +1 °C) or **duplex 5** (ΔT_m/mod. = +1 °C). A molecular thermodynamic study of duplexes with LNA-modifications implied that the additional stabilizing effect of LNA-modifications in both strands depends on the location of the two oppositely oriented LNA:DNA base pairs in the duplex.[23] Similarly this LNA study, the thermal stability of duplexes including methylene-EoDNA modifications in both strands might be affected by the positions of the modifications in the oligonucleotides, although further investigation is required. Interestingly, **duplexes 10** and **11**, which contain two methylene-EoDNA-A:methylene-EoDNA-T base pairs per duplex, exhibited synergistically increased stability compared to **duplex 8**, which includes a single methylene-EoDNA base pair. For instance, the ΔT_m/mod. value of **duplex 11** was +2.5 °C, which was significantly higher than that of **duplex 8** (ΔT_m/mod. = +0.5 °C). The synergistic stabilization resulting from the introduction of multiple methylene-EoDNA base pairs has also been observed in duplexes containing multiple 2′,4′-BNA^{NC}:2′,4′-BNA^{NC} base pairs.[18] Moreover, DNA duplexes that have been fully modified by LNA,[17] 2′,4′-BNA^{NC},[18] or 2′,4′-BNA^{COC},[19] have markedly high melting temperatures (T_m > 93 °C). The highly stabilizing effect on the duplex of multiple methylene-EoDNA base pairs might be a general effect of 2′,4′-BNA analogs with constrained sugar moieties that restrict the sugar conformation to a suitable form for duplex formation.

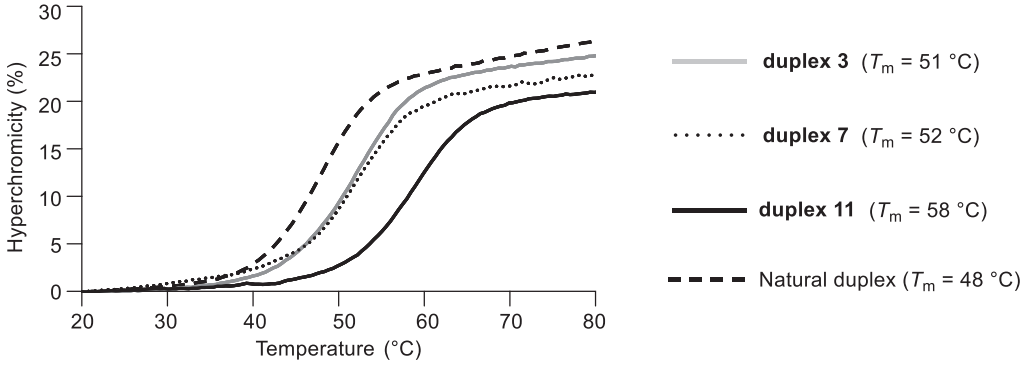


Figure 3. The representative data for UV-melting analysis of the modified oligonucleotides

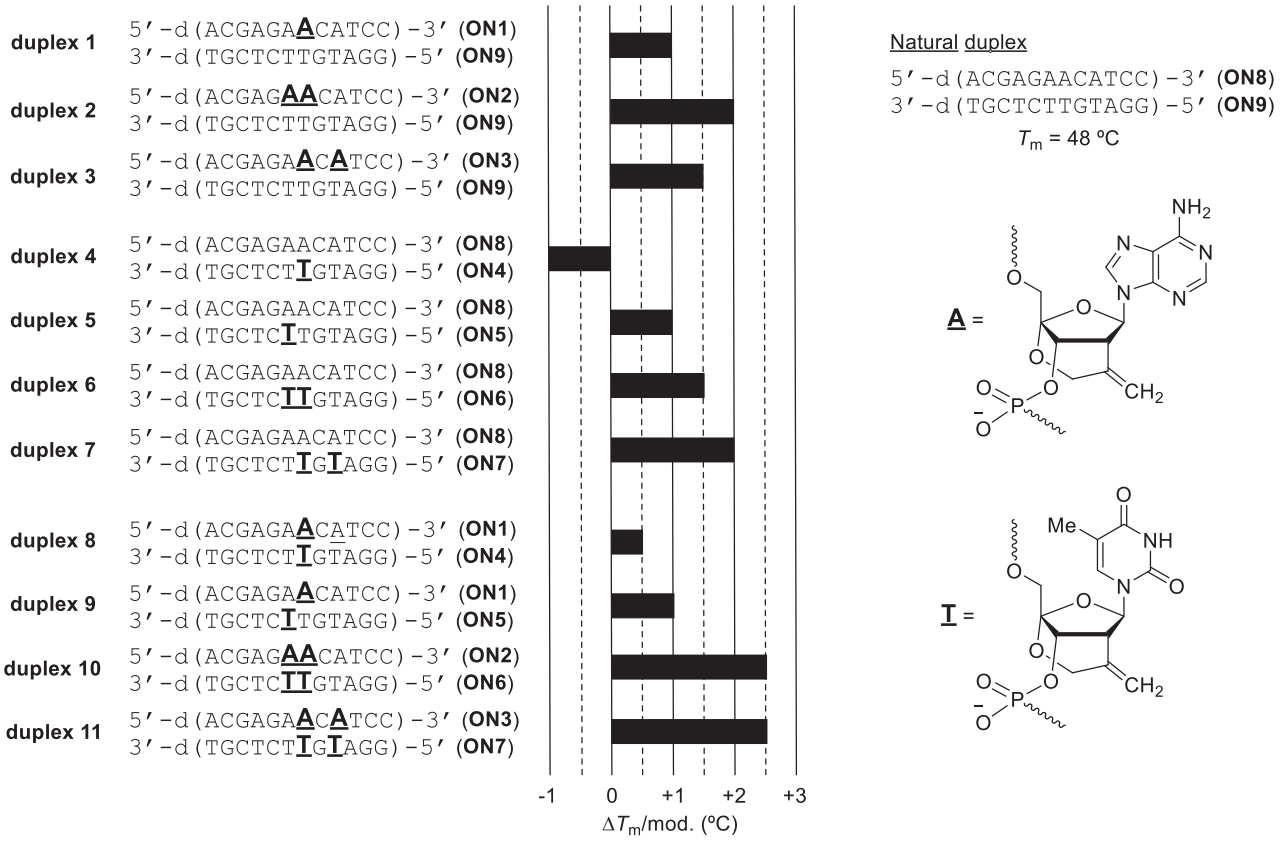


Figure 4. ΔT_m/mod. values of DNA duplexes formed by methylene-EoDNA-modified oligonucleotides (**ON1**–**7**). Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, and 2.5 μM of each oligonucleotide. ΔT_m/mod.: The change in T_m value per modification compared with natural DNA duplex (**ON8** and **ON9**, T_m = 48 °C).

CONCLUSIONS

In the present study, we synthesized the phosphoramidite of methylene-EoDNA-A and incorporated it into oligonucleotides. The results of the UV-melting experiments indicated that the thermal stability of a DNA duplex formed from methylene-EoDNA-A- and methylene-EoDNA-T-modified oligonucleotides was similar to or slightly higher than that of natural DNA. As a result of the evaluation of the stability of DNA duplexes including methylene-EoDNA-A:methylene-EoDNA-T base pairs, it seems that the incorporation of two methylene-EoDNA base pairs significantly increases the thermal stability of the duplexes compared to the thermal stability of duplexes including methylene-EoDNA modification in one strand. The accumulation of properties such as hybridizing capability through the development of various 2′,4′-BNA analogs might facilitate the discovery of useful materials for oligonucleotide-based technologies.

EXPERIMENTAL

General Methods. All moisture-sensitive reactions were conducted in well-dried glassware under an Ar atmosphere. IR spectra were recorded on a JASCO FT/IR-4200 spectrometer. NMR experiments were performed on Varian or Agilent Marcury 300 MHz, Varian or Agilent MarcuryPlus 300 MHz, and Bruker AVANCE III HD 500 MHz spectrometer equipped with a BBO cryoprobe. ^{1}H NMR spectra were recorded at 300 MHz and 500 MHz, ^{13}C NMR spectra were recorded at 75 MHz and 125 MHz, and ^{31}P NMR spectra were recorded at 202 MHz. All ^{13}C and ^{31}P NMR spectra were proton-decoupled. Chemical shift values are reported in parts per million downfield from internal tetramethylsilane (δ = 0.00 ppm) for ^{1}H NMR, residual $CDCl_3$ (δ = 77.0 ppm) for ^{13}C NMR, and external 5% H_3PO_4 (δ = 0.0 ppm) for ^{31}P NMR. For column chromatography, silica gel PSQ 60B (Fuji Silycia) was used. The progress of the reaction was monitored by analytical thin-layer chromatography (TLC) on pre-coated glass sheets (Silica gel 60 F_{254} by Merck). For high performance liquid chromatography (HPLC), JASCO EXTREMA (PU-4180, CO-4060 and UV-4075) with a fraction collector CHF122SC (ADVANTEC) was used. ESI-TOF mass spectra were recorded on Waters SYNAPT G2-Si HDMS mass spectrometer. UV melting experiments were performed on JASCO UV-730 UV/VIS spectrophotometer equipped with a T_m analysis accessory.

2′,3′-*O*-Dehydro-5-deoxyadenosine (2)[20]

Ph_3P (11.8 g, 44.9 mmol) and I_2 (11.4 g, 44.9 mmol) were added to a solution of adenosine **1** (10.0 g, 37.4 mmol) in anhydrous pyridine (40 mL) at 0 °C. The reaction mixture was stirred at room temperature for 14 h. After being quenched with MeOH, the resulting solution was concentrated *in vacuo*. The residue (39.0 g) was purified by column chromatography (silica gel 150 g, $CHCl_3$:MeOH = 30:1 to 5:1) to give mixtures of 5′-iodo-5′-deoxyadenosine as a brown powder (15.0 g). Under Ar atmosphere, the obtained product was dissolved in anhydrous MeOH (40 mL). Then, NaOMe (28% in MeOH, 37 mL, 185 mmol) was added to the solution. The reaction mixture was refluxed for 12 h. After being neutralized with NH_4Cl (10.0 g, 187 mmol) in water (40 mL), the resulting solution was concentrated *in vacuo*. Water (30 mL) was added to the residue (38.8 g), and the precipitate was collected by filtration and washed with water to give **3** (8.68 g, 93% for 2 steps) as a brown powder. The NMR spectral data of **2** were identical to those reported in the literature.[20]

9-(4,5-Dehydro-5-deoxy-β-D-ribofuranosyl)-6*N*-benzoyladenine (3)

Under Ar atmosphere, TMSCl (13.6 mL, 108 mmol) was added to a solution of compound **2**[20] (8.95 g, 36 mmol) in anhydrous pyridine (150 mL) at 0 °C. The reaction mixture was stirred at room temperature for 24 h. Then, BzCl (8.3 mL, 72 mmol) was added to this solution at 0 °C. The reaction mixture was stirred at room temperature for 1 h. After being quenched with sat. $NaHCO_3$ aq., the solution was diluted with

EtOAc. The solution was washed with H_2O and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The obtained residue (50.3 g) was dissolved in THF (150 mL), then 28% NH_3 aq. (20 mL) was added to this solution at room temperature. The reaction mixture was stirred at room temperature for 10 min. The solution was concentrated *in vacuo*. The obtained residue (61.0 g) was purified by column chromatography (silica gel 200 g, $CHCl_3$/MeOH = 30:1 to 10:1) to give compound **3** as a yellow powder (6.93 g, 55%, 3 steps from **2**). IR ν_{max} (ATR): 3486, 3302, 3227, 3140, 3090, 1710, 1687, 1617, 1582, 1529, 1460, 1401, 1389, 1362, 1332, 1305, 1292, 1286, 1258, 1238, 1208 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6) δ: 4.26 (s, 1H), 4.36 (s, 1H), 4.73 (t, J = 5.5 Hz, 1H), 4.91 (q, J = 5.5 Hz, 1H), 5.65 (d, J = 5.5 Hz, 1H), 5.82 (d, J = 5.5 Hz, 1H), 6.31 (d, J = 5.5 Hz, 1H), 7.53–7.68 (m, 3H), 8.04–8.06 (m, 2H), 8.75 (s, 1H), 8.79 (s, 1H), 11.28 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ: 69.7, 72.1, 85.0, 87.9, 125.9, 128.5, 132.5, 133.3, 143.5, 150.6, 152.0, 152.3, 162.1, 165.7. HRMS (ESI): Calcd for $C_{17}H_{15}N_5NaO_4$ [MNa^+] 376.1022, found 376.1025.

9-(2-*O*-*tert*-Butyldimethylsilyl-4,5-dehydro-5-deoxy-β-D-ribofuranosyl)-6*N*-benzoyladenine (4) and 9-(3-*O*-*tert*-butyldimethylsilyl-4,5-dehydro-5-deoxy-β-D-ribofuranosyl)-6*N*-benzoyladenine (5)

Under Ar atmosphere, imidazole (1.27 g, 19 mmol) and TBSCl (1.40 g, 9.3 mmol) were added to a solution of compound **3** (3.0 g, 8.5 mmol) in anhydrous DMF (40 mL). The reaction mixture was stirred at room temperature for 2 h. After being quenched with MeOH, the solution was diluted with EtOAc. The solution was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The obtained residue (4.28 g) was purified by column chromatography (silica gel 200 g, *n*-hexane/EtOAc = 1:1 to 1:2) to give compound **4** as a white powder (913 mg, 23%) and compound **5** as a white powder (2.02 g, 51%), respectively. Compound **4**: IR ν_{max} (ATR): 3481, 3285, 3091, 3070, 2929, 2857, 1698, 1609, 1581, 1512, 1487, 1470, 1455, 1408, 1349, 1329, 1293, 1251, 1228, 1216 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.01 (s, 3H), 0.07 (s, 3H), 0.89 (s, 9H), 2.72 (d, J = 6.5 Hz, 1H), 4.52 (s, 1H), 4.69 (s, 1H), 4.74 (dd, J = 3.0, 6.5 Hz, 1H), 5.02 (t, J = 3.0 Hz, 1H), 6.17 (d, J =3.0 Hz, 1H), 7.51–7.53 (m, 2H), 7.60–7.63 (m, 1H), 8.02–8.05 (m, 3H), 8.82 (s, 1H), 9.13 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: −5.1, −4.9, 18.0, 25.6, 70.0, 73.9, 87.5, 89.6, 123.5, 127.8, 128.9, 132.9, 133.5, 141.1, 149.8, 151.3, 153.0, 160.3, 164.5. HRMS (ESI): Calcd for $C_{23}H_{29}N_5NaO_4$ [MNa^+] 490.1887, found 490.1889. Compound **5**: IR ν_{max} (ATR): 3482, 3282, 3104, 3007, 2954, 2929, 2896, 2886, 2857, 1693, 1611, 1582, 1514, 1487, 1471, 1457, 1409, 1359, 1331, 1288, 1252, 1217 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.24 (s, 3H), 0.24 (s, 3H), 0.99 (s, 9H), 3.41 (d, J = 5.5 Hz, 1H), 4.35 (s, 1H), 4.63 (s, 1H), 4.82–4.85 (m, 1H), 5.16 (d, J = 5.0 Hz, 1H), 6.19 (d, J = 3.5 Hz, 1H), 7.50–7.53 (m, 2H), 7.59–7.62 (m, 1H), 8.01–8.03 (m, 2H), 8.10 (s, 1H), 8.76 (s, 1H), 9.18 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: −4.8, −4.5, 18.2, 25.7, 70.9, 73.4, 87.2, 89.7, 123.6, 127.9, 128.8,

132.8, 133.5, 141.8, 149.8, 151.4, 152.8, 160.3, 164.6. HRMS (ESI): Calcd for $C_{23}H_{29}N_5NaO_4$ [MNa^+] 490.1887, found 490.1887.

9-[3-*O*-*tert*-Butyldimethylsilyl-4-*C*-(2-propyn-1-yloxy)-β-D-ribofuranosyl]-6*N*-benzoyladenine (6) and 9-[3-*O*-*tert*-butyldimethylsilyl-4-*C*-(2-propyn-1-yloxy)-α-L-lyxofuranosyl]-6*N*-benzoyladenine (7)

Acetone (16 mL) and sat. $NaHCO_3$ (80 mL) aq. were added to a solution of compound **5** (800 mg, 1.7 mmol) in CH_2Cl_2 (24 mL). Then, Oxone® (3.16 g, 5.1 mmol) in H_2O (30 mL) was dropwise added to this solution at 0 °C. The reaction mixture was stirred at 0 °C for 0.5 h. The resulting solution 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 obtained crude residue (851 mg) was dissolved in anhydrous THF (20 mL) under Ar atmosphere, then 2-propyn-1-ol (1.0 mL, 17 mmol) and $ZnCl_2$ (2M in 2-methyltetrahydrofuran, 0.85 mL, 1.7 mmol) were added to this solution at −78 °C. The reaction mixture was stirred at 0 °C for 1 h. After being quenched with sat. $NaHCO_3$ aq. at 0 °C, the whole mixture was filtered through a pad of Celite®, and the filtrate was diluted with EtOAc. The solution was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The obtained residue (1.03 g) was purified by column chromatography (silica gel 40 g, *n*-hexane:EtOAc = 1:1 to 1:3) to give compound **6** as a white powder (407 mg, 44%, 2 steps from **5**) and **7** as a white powder (30 mg, 3%, 2 steps from **5**), respectively. Compound **6**: IR ν_{max} (ATR): 3409, 3288, 3106, 3070, 3007, 2953, 2930, 2884, 2857, 1702, 1610, 1582, 1510, 1485, 1456, 1417, 1409, 1391, 1361, 1329, 1300, 1250 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.21 (s, 3H), 0.25 (s, 3H), 0.99 (s, 9H), 2.45 (t, *J* = 2.5 Hz, 1H), 3.14 (d, *J* = 9.5 Hz, 1H), 3.69 (t, *J* = 11.5 Hz, 1H), 4.01 (d, *J* = 11.5 Hz, 1H), 4.39 (d, *J* = 2.5 Hz, 2H), 4.71 (d, *J* = 6.0 Hz, 1H), 4.88 (ddd, *J* = 6.0, 6.5, 9.5 Hz, 1H), 5.39 (d, *J* = 11.5 Hz, 1H), 5.94 (d, *J* = 6.5 Hz, 1H), 7.52–7.55 (m, 2H), 7.61–7.64 (m, 1H), 8.01–8.03 (m, 2H), 8.08 (s, 1H), 8.75 (s, 1H), 9.07 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: −5.2, −4.3, 18.5, 25.9, 51.7, 64.3, 73.8, 73.8, 74.2, 88.0, 92.2, 108.4, 124.4, 127.9, 128.9, 133.0, 133.4, 142.7, 150.3, 150.6, 152.3, 164.4. HRMS (ESI): calcd for $C_{26}H_{33}N_5NaO_6$ [MNa^+] 562.2098, found 562.2100. Compound **7**: IR ν_{max} (ATR): 3393, 3348, 3303, 3066, 3007, 2953, 2929, 2897, 2885, 2857, 1701, 1613, 1583, 1510, 1486, 1471, 1457, 1403, 1391, 1360, 1351, 1330, 1301, 1252, 1217 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 0.24 (s, 3H), 0.24 (s, 3H), 1.00 (s, 9H), 2.32 (s, 1H), 2.47 (t, *J* = 2.5 Hz, 1H), 3.86–3.96 (m, 3H), 4.20 (dd, *J* = 2.5, 16.0 Hz, 1H), 4.27 (dd, *J* = 2.5, 16.0 Hz, 1H), 4.44 (d, *J* = 4.5 Hz, 1H), 5.14 (dd, *J* = 4.5, 5.5 Hz, 1H), 6.17 (d, *J* = 5.5 Hz, 1H), 7.53 (t, *J* = 7.5 Hz, 2H), 7.62 (t, *J* = 7.5 Hz, 1H), 8.02 (d, *J* = 7.5 Hz, 2H), 8.22 (s, 1H), 8.75 (s, 1H), 9.08 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: −5.1, −4.5, 18.3, 25.8, 50.3, 58.9, 74.8, 74.8, 76.0, 79.8, 89.6, 111.1, 123.1, 127.9, 128.9, 132.9, 133.4, 141.5, 149.5, 151.8, 152.7, 164.6. HRMS (ESI): Calcd for $C_{26}H_{33}N_5NaO_6$ [MNa^+] 562.2098, found 562.2099.

9-[3-*O*-*tert*-Butyldimethylsilyl-5-*O*-(4,4′-dimethoxytrityl)-4-*C*-(2-propyn-1-yloxy)-β-D-ribofuranosyl]-6*N*-benzoyladenine (8)

Under Ar atmosphere, 2,6-lutidine (0.23 mL, 2.0 mmol) and DMTrOTf[21] (1.3 mmol) in anhydrous CH_2Cl_2 (6 mL) were added to a solution of compound **6** (350 mg, 0.65 mmol) in anhydrous CH_2Cl_2 (4 mL) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. After being quenched with MeOH at 0 °C, the whole mixture was diluted with $CHCl_3$. The solution was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The obtained residue (820 mg) was purified by column chromatography (silica gel 20 g, *n*-hexane:EtOAc = 1:1 to 1:2) to give compound **8** as a white foam (498 mg, 91%). IR (ATR) ν_{max}: 3546, 3295, 3005, 2953, 2931, 2900, 2884, 2857, 2838, 1703, 1608, 1581, 1547, 1508, 1490, 1456, 1416, 1328, 1299, 1249, 1212 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: 0.07 (s, 3H), 0.12 (s, 3H), 0.89 (s, 9H), 2.40 (t, *J* = 2.5 Hz, 1H), 3.17 (d, *J* = 10.5 Hz, 1H), 3.39 (*J* = 9.0 Hz, 1H), 3.60 (d, *J* = 10.5 Hz, 1H), 3.77 (s, 3H), 3.78 (s, 3H), 4.23 (dd, *J* = 2.5, 16.0 Hz, 1H), 4.34 (dd, *J* = 2.5, 16.0 Hz, 1H), 4.78 (ddd, *J* = 3.5, 6.0, 9.0 Hz, 1H), 5.02 (d, *J* = 6.0 Hz, 1H), 6.25 (d, *J* = 3.5 Hz, 1H), 6.75–6.79 (m, 4H), 7.19–7.37 (m, 9H), 7.50–7.64 (m, 3H), 8.02 (d, *J* = 7.5 Hz, 2H), 8.24 (s, 1H), 8.81 (s, 1H), 9.01 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: −5.0, −4.7, 18.2, 25.7, 51.0, 55.2, 55.2, 62.2, 72.2, 73.8, 74.1, 80.1, 86.8, 90.7, 107.2, 113.1, 113.1, 123.5, 126.9, 127.8, 127.8, 128.0, 128.8, 129.9, 130.0, 132.7, 133.5, 135.1, 135.2, 142.2, 144.0, 149.7, 151.4, 152.8, 158.5, 158.6, 164.5. HRMS (ESI): Calcd for $C_{47}H_{51}N_5NaO_8Si$ [MNa^+] 864.3405, found 864.3399.

9-[3-*O*-*tert*-Butyldimethylsilyl-5-*O*-(4,4′-dimethoxytrityl)-2-*O*-(1-imidazolylthiocarbonyl)-4-*C*-(2-propyn-1-yloxy)-β-D-ribofuranosyl]-6*N*-benzoyladenine (9)

Under Ar atmosphere, 1,1′-thiocarbonyldiimidazole (324 mg, 1.8 mmol) and DMAP (37 mg, 0.30 mmol) were added to a solution of compound **8** (510 mg, 0.61 mmol) in anhydrous CH_2Cl_2 (10 mL) at room temperature. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was then concentrated *in vacuo*. The obtained residue (880 mg) was purified by column chromatography (silica gel 40 g, *n*-hexane:EtOAc = 1:2 to 1:4) to give compound **9** as a white foam (559 mg, 97%). IR (ATR) ν_{max}: 3003, 2953, 2931, 2900, 2884, 2857, 2836, 1703, 1607, 1581, 1508, 1488, 1457, 1393, 1330, 1287, 1247, 1231 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: 0.00 (s, 3H), 0.02 (s, 3H), 0.76 (s, 9H), 2.35 (t, *J* = 2.5 Hz, 1H), 3.19 (*J* = 10.0 Hz, 1H), 3.61 (d, *J* = 10.0 Hz, 1H), 3.76 (s, 3H), 3.77 (s, 3H), 4.23 (dd, *J* = 2.5, 15.5 Hz, 1H), 4.37 (dd, *J* = 2.5, 15.5 Hz, 1H), 5.61 (d, *J* = 6.0 Hz, 1H), 6.47 (d, *J* = 2.5 Hz, 1H), 6.52 (dd, *J* = 2.5, 6.0 Hz, 1H), 6.73–6.77 (m, 4H), 7.03 (s, 1H), 7.19–7.36 (m, 9H), 7.50–7.61 (m, 3H), 7.72 (s, 1H), 8.02 (d, *J* = 7.5 Hz, 2H), 8.22 (s, 1H), 8.45 (s, 1H), 8.85, (s, 1H), 9.07 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: −5.0, −4.9, 17.8, 25.4, 51.2, 55.1, 55.2, 61.8, 71.4, 74.1, 80.1, 80.9, 113.1, 113.1, 118.3, 123.6, 126.9, 127.8, 127.8, 128.0, 128.8, 129.9, 130.0, 131.0, 132.8, 133.4, 135.0, 135.1, 137.3, 142.3, 144.0, 149.9, 151.4,

152.9, 158.5, 158.5, 164.6, 183.2. HRMS (ESI): Calcd for $C_{51}H_{53}N_7NaO_8SSi$ [MNa^+] 974.3343, found 974.3336.

9-[(1*R*,5*R*,6*R*,8*S*)-8-*tert*-Butyldimethylsilyloxy-1-(4,4′-dimethoxytrityloxy)methyl-2,7-dioxabicyclo[3.2.1]octan-6-yl]-6*N*-benzoyladenine (10)

Under Ar atmosphere, AIBN (45 mg, 0.27 mmol) and tris(trimethylsilyl)silane (0.84 mL, 2.7 mmol) were added to a solution of compound **9** (520 mg, 0.55 mmol) in anhydrous toluene (10 mL) at 90 °C. The reaction mixture was stirred at 90 °C for 1 h. The mixture was then concentrated *in vacuo*. The obtained residue (705 mg) was purified by column chromatography (silica gel 30 g, *n*-hexane:EtOAc = 2:1 to 1:2) to give compound **10** as a white foam (315 mg, 70%). IR (ATR) ν_{max}: 3003, 2953, 2930, 2902, 2896, 2885, 2856, 1703, 1608, 1579, 1490, 1454, 1407, 1332, 1298, 1249, 1215 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: −0.11 (s, 3H), 0.04 (s, 3H), 3.14 (d, *J* = 10.0 Hz, 1H), 3.53 (d, *J* = 10.0 Hz, 1H), 3.71 (d, *J* = 5.0 Hz, 1H), 3.76 (s, 6H), 3.75 (d, *J* = 14.5 Hz, 1H), 4.54 (d, *J* = 14.5 Hz, 1H), 4.94 (d, *J* = 5.0 Hz, 1H), 5.09 (s, 1H), 5.10 (s, 1H), 6.37 (s, 1H), 6.77–6.80 (m, 4H), 7.18–7.33 (m, 9H), 7.50–7.64 (m, 3H), 8.02–8.04 (m, 2H), 8.63 (s, 1H), 8.90 (s, 1H), 9.13 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: −5.0, −4.4, 17.8, 25.5, 51.3, 55.1, 61.0, 64.9, 68.4, 86.4, 86.6, 105.4, 112.5, 113.0, 113.1, 123.3, 127.0, 127.7, 127.8, 128.2, 128.8, 130.0, 130.0, 132.7, 133.6, 135.0, 135.2, 139.1, 141.6, 143.8, 149.5, 150.8, 152.9, 158.5, 164.5. HRMS (ESI): Calcd for $C_{47}H_{51}N_5NaO_7Si$ [MNa^+] 848.3455, found 848.3474.

9-[(1*R*,5*R*,6*R*,8*S*)-1-(4,4′-Dimethoxytrityloxy)methyl-8-hydroxy-2,7-dioxabicyclo[3.2.1]octan-6-yl]-6*N*-benzoyladenine (11)

TBAF (1 M in THF, 0.25 mL, 0.25 mmol) was added to a solution of compound **10** (190 mg, 0.23 mmol) in anhydrous THF (5 mL) at room temperature. The reaction mixture was stirred at room temperature for 18 h. The mixture was then concentrated *in vacuo*. The obtained residue (260 mg) was purified by column chromatography (silica gel 10 g, *n*-hexane:EtOAc = 1:2 to 1:5) to give compound **11** as a white foam (159 mg, 97%). IR (ATR) ν_{max}: 3398, 3286, 3127, 3061, 3005, 2957, 2935, 2837, 1703, 1607, 1580, 1508, 1490, 1453, 1398, 1352, 1334, 1297, 1247, 1215 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 2.04 (d, *J* = 10.0 Hz, 1H), 3.42 (s, 2H), 3.71 (d, *J* = 5.0 Hz, 1H), 3.78 (s, 6H), 4.32 (d, *J* = 14.0 Hz, 1H), 4.57 (d, *J* = 14.0 Hz, 1H), 5.29 (s, 2H), 6.39 (s, 1H), 6.82–6.84 (m, 4H), 7.21–7.42 (m, 9H), 7.52–7.56 (m, 2H), 7.61–7.65 (m, 1H), 8.02–8.04 (m, 2H), 8.54 (s, 1H), 8.85 (s, 1H), 9.01 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 51.7, 55.2, 61.5, 65.4, 68.7, 85.8, 87.1, 105.1, 113.3, 115.9, 123.3, 127.1, 127.8, 128.0, 128.1, 128.9, 130.0, 130.0, 132.8, 133.7, 135.1, 135.3, 136.9, 140.9, 144.0, 149.5, 150.8, 152.9, 158.6, 164.4. HRMS (ESI): Calcd for $C_{41}H_{37}N_5NaO_7Si$ [MNa^+] 734.2591, found 734.2593.

9-[(1*R*,5*R*,6*R*,8*S*)-8-[2-Cyanoethoxy(diisopropylamino)phosphinoxy]-1-(4,4′-dimethoxytrityloxy)-methyl-2,7-dioxabicyclo[3.2.1]octan-6-yl]-6*N*-benzoyladenine (12)

Under Ar atmosphere, (i-Pr)$_2$NP(Cl)O(CH$_2$)$_2$CN (0.11 mL, 0.49 mmol) and diisopropylethylamine (0.14 mL, 0.82 mmol) were added to a solution of compound **11** (116 mg, 0.16 mmol) in anhydrous CH_2Cl_2 (5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. The reaction was then quenched with sat. $NaHCO_3$ aq. and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The obtained residue (270 mg) was purified by column chromatography (silica gel 10 g, *n*-haxane/EtOAc = 1:2) to give compound **12** as a white foam (91 mg, 61%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.98–1.32 (m, 12H), 2.30–2.35 (m, 0.8H), 2.52 (t, J = 6.0 Hz, 1.2H), 3.28–3.31 (m, 1H), 3.50–3.89 (m, 12H), 4.31–4.37 (m, 1H), 4.53–4.61 (m, 1H), 4.91 (dd, J = 5.0, 8.0 Hz, 0.6H), 4.99 (dd, J = 5.0, 10.0 Hz, 0.4H), 5.14–5.23 (m, 2H), 6.39 (s, 0.4H), 6.42 (s, 0.6H), 6.78–6.84 (m, 4H), 7.15–7.64 (m, 12H), 8.01–8.04 (m, 2H), 8.61 (s, 0.6H), 8.61 (s, 0.4H), 8.87 (s, 0.4H), 8.88 (s, 0.6H), 9.09 (s, 1H). ^{31}P NMR (202 MHz, $CDCl_3$) δ: 149.0, 150.2. HRMS (ESI): calcd for $C_{50}H_{54}N_7NaO_8P$ [MNa^+] 934.3669, found 934.3665.

Synthesis of oligonucleotides

Phosphoramidites **12** and **13**, dT-phosphoramidite (Sigma), Ac-dC-phosphoramidite (Glen Research), Bz-dA-phosphoramidite (Glen Research), and iBu-dG-phosphoramidite (Glen Research) were dissolved in anhydrous MeCN to a final concentration of 0.1 M, respectively. The synthesis of **ON1**–**7** 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. Phosphoramidites **12** and **13** were incorporated into oligonucleotides at prolonged coupling times of 10 min. The oligonucleotides synthesized in trityl-on mode were cleaved from the CPG resin by treatment with 28% aqueous NH_3 at room temperature for 1.5 h. After all protecting groups of oligonucleotides were removed by treatment with 28% aqueous NH_3 at 55 °C for 16 h, removal of NH_3 was carried out *in vacuo*. The crude oligonucleotides were purified by Sep-Pak® Plus C18 cartridges (Waters) with the 5′-DMTr group being removed during purification using 1% (v/v) aqueous trifluoroacetic acid (TFA). The obtained oligonucleotides were further purified by reversed-phase HPLC (Waters XBridge® MS C18 Column 5 μm, 10 × 50 mm). The compositions of new oligonucleotides (**ON1**–**7**) were confirmed by ESI-TOF mass analysis. The deconvoluted ESI-TOF mass data [M] for **ON1**–**7** were as follows: **ON1**, found 3677.80 (calcd 3677.48); **ON2**, found 3732.00 (calcd 3731.53); **ON3**, found 3731.90 (calcd 3731.53); **ON4**, found 3721.70 (calcd 3721.47); **ON5**, found 3721.30 (calcd 3721.47); **ON6**, found 3376.00 (calcd 3775.52); **ON7**, found 3775.40 (calcd 3775.52).

UV-Melting experiments

For UV-melting experiments using the duplexes formed by **ON1**–**9**, oligonucleotides were dissolved in 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM NaCl to give a final concentration of 2.5 μM for each strand. The samples were annealed by heating at 100 °C followed by slow cooling down to room temperature. The melting profiles were recorded at 260 nm from 20 °C to 80 °C at a scan rate of

0.5 °C/min. The two-point average method was employed to obtain the T_m values, and the final values were determined by averaging three independent measurements, which were accurate within a 1 °C range.

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