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SYNTHESIS AND HYBRIDIZATION PROPERTIES OF OLIGONUCLEOTIDES INCLUDING 2′-*N*-ALKOXYCARBONYL-2′-AMINO-LNA DERIVATIVES

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Dedicated to Professor Dr. Tohru Fukuyama on the occasion of his 70th birthday

Abstract – 2′-Amino-LNA is an attractive material that helps in realizing the practical use of oligonucleotides because it can stabilize complexes with complementary strands and various substituents can be introduced on the 2′-amino group. Here, 2′-*N*-alkoxycarbonyl derivatives of 2′-amino-LNA were newly designed, synthesized, and incorporated into oligonucleotides. The results of UV-melting analysis indicated that 2′-*N*-alkoxycarbonyl-modified 2′-amino-LNA could enhance the stability of the duplex formed with single-stranded RNA and the triplex formed with double-stranded DNA.

INTRODUCTION

Chemical modification of oligonucleotides is necessary for the application of oligonucleotides in gene diagnosis,[1,2] nanotechnology,[3,4] and medicinal science,[5–7] since natural DNA and RNA not only lack binding affinity with a complementary single strand but also can be easily cleaved by a nuclease. 2′,4′-BNA[8]/LNA[9] is a highly promising candidate toward the practical use of oligonucleotides because of the unprecedented high binding affinity toward single-stranded RNA (ssRNA). Moreover, LNA modification results in improved enzymatic stability to oligonucleotides due to the steric hindrance of the bridge moiety. Thus, various 2′,4′-bridged nucleic acids (2′,4′-BNAs) have been developed to discover an ideal material for realizing the practical applications of oligonucleotides.[10]

Among 2′,4′-BNAs, 2′-amino-LNA (Figure 1) with a nitrogen atom at the 2′-position can form remarkably stable duplexes with complementary ssRNA,[11] and 2′-amino-LNA-modification significantly enhances the stability of the triplex formed with double-stranded DNA (dsDNA).[12] Moreover, the

2′-nitrogen atom of 2′-amino-LNA offers a modification site for oligonucleotides, and thus, various 2′-*N*-alkyl- and 2′-*N*-acyl-modifications have been introduced into 2′-amino-LNA.[11–27] Studies on the thermal stability of the duplexes formed by 2′-*N*-modified 2′-amino-LNAs indicated that 2′-*N*-acyl modification lead to higher hybridization ability compared with the corresponding 2′-*N*-alkyl ones,[13] possibly due to the favorable hydration of the carbonyl oxygen atoms.

On the other hand, the alkoxycarbonyl group can be attached to a nitrogen atom. Alkoxycarbonyl modification of 2′-amino-LNA is expected to improve the hybridization properties of the modified oligonucleotides over acyl modification, since the presence of an additional oxygen atom might enhance the hydration network around the minor groove of the duplex.[28,29] Therefore, we became interested in the hybridization properties of oligonucleotides including 2′-*N*-alkoxycarbonyl derivatives of 2′-amino-LNA. Here, the synthesis of oligonucleotides including 2′-*N*-alkoxycarbonyl-modified 2′-amino-LNAs as well as the duplex- and triplex-forming abilities of the modified oligonucleotides are described.

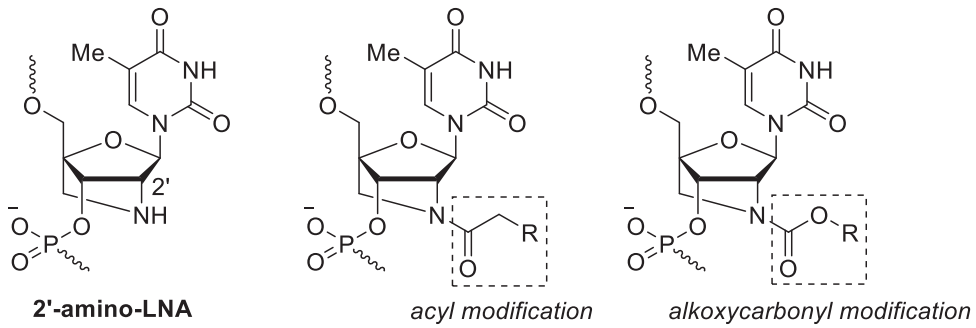
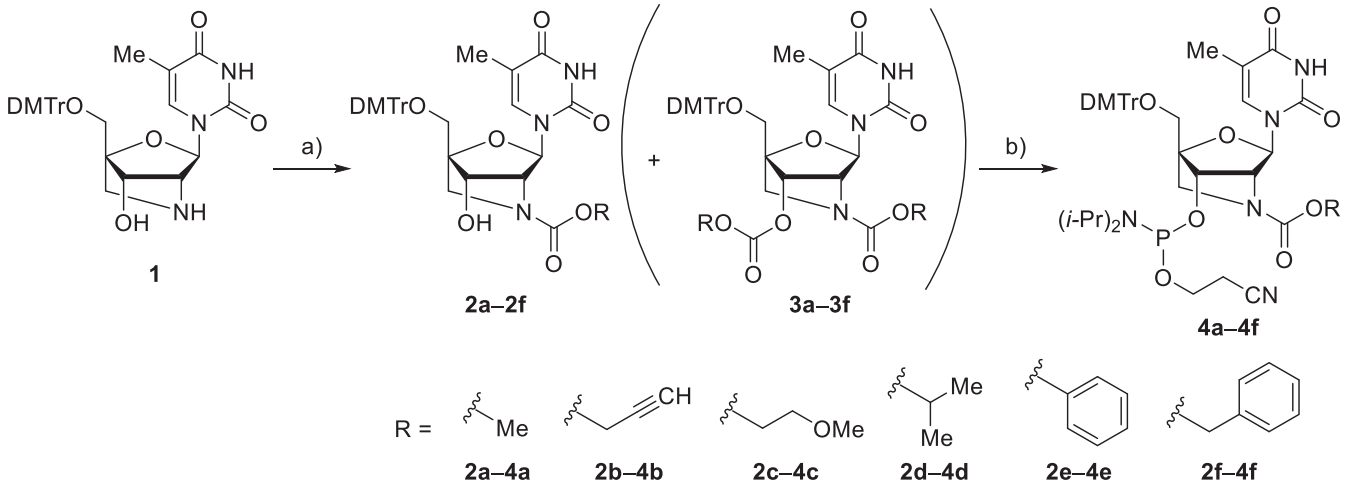


Figure 1. Structures of 2′-amino-LNA and its 2′-*N*-modifications

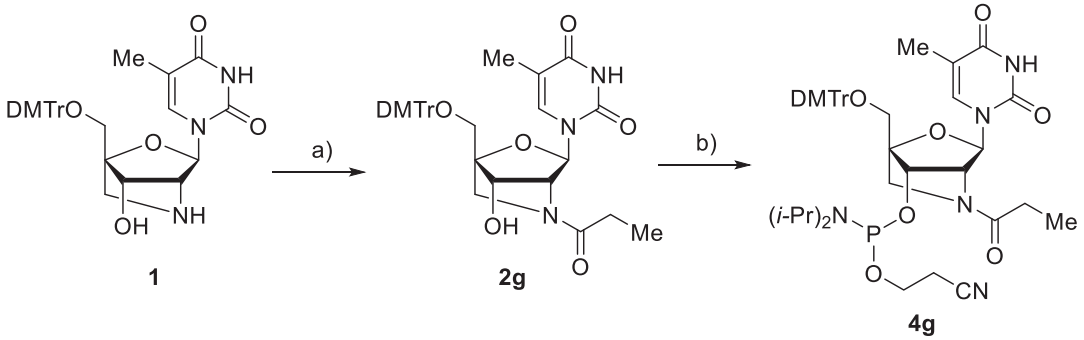
RESULTS AND DISCUSSION

The synthesis of 2′-*N*-alkoxycarbonyl-modified 2′-amino-LNAs was started from the known 2′-amino-LNA **1**[11] (Scheme 1). The 2′-amino group of **1** was protected by using various chloroformate and carbonate reagents to give the corresponding **2a**–**2f**, with the dialkoxycarbonyl derivatives of 2′-amino-LNA **3a**–**3f** as byproducts. Phosphitylation of **2a**–**2f** using $(i\text{-Pr})_2\text{NP(Cl)O(CH}_2)_2\text{CN}$ afforded phosphoramidites **4a**–**4f**, which act as the building blocks for oligonucleotide synthesis.

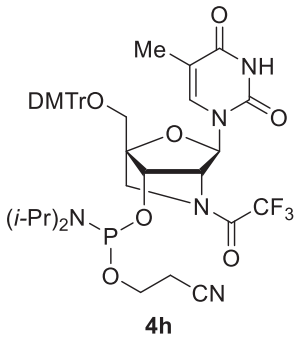


Scheme 1. Synthesis of 2′-*N*-alkoxycarbonyl-modified 2′-amino-LNA derivatives. Reagents and conditions: a) methyl chloroformate, Et_3N, CH_2Cl_2, 0 °C, 52% (**2a**), 38% (**3a**); 4-nitrophenyl propargyl carbonate, Et_3N, CH_2Cl_2, rt, 60% (**2b**), 31% (**3b**); 2-methoxyethyl chloroformate, Et_3N, CH_2Cl_2, rt, 57% (**2c**), 18% (**3c**); isopropyl chloroformate, Et_3N, CH_2Cl_2, 0 °C, 34% (**2d**), 48% (**3d**); phenyl chloroformate, Et_3N, CH_2Cl_2, rt, 35% (**2e**), 18% (**3e**); benzyl chloroformate, Et_3N, CH_2Cl_2, 0 °C, 55% (**2f**), 20% (**3f**); b) $(i\text{-Pr})_2NP(Cl)O(CH_2)_2CN$, *N*,*N*-diisopropylethylamine, CH_2Cl_2, rt, 80% (**4a**), 65% (**4b**), 55% (**4c**), 87% (**4d**), 88% (**4e**), 77% (**4f**).

To compare the effect of 2′-*N*-alkoxycarbonyl and 2′-*N*-acyl modification on the hybridization properties of oligonucleotides, the phosphoramidite of 2′-*N*-propionyl-2′-amino-LNA **4g** was prepared by acylation of **1**, followed by phosphitylation of **2g** (Scheme 2). Furthermore, for unmodified 2′-amino-LNA, 2′-*N*-trifluoroacetyl derivative **4h** (Figure 2) was synthesized according to the reported procedure.[11] The synthesis of modified oligonucleotides (**ON1**–**8**, **ON10**–**17**), shown in Table 1 and Table 2, was carried out on an automated DNA synthesizer by means of common procedures, except for the extended coupling time (10 min) for the incorporation of **4a**–**4h** (*cf.*, 25 s for coupling of the other phosphoramidites). After purification of the synthesized oligonucleotides by reversed-phase HPLC, the purity and molecular weights of the oligonucleotides were confirmed by analytical HPLC and mass spectrometric analyses, respectively.



Scheme 2. Synthesis of 2′-*N*-propionyl-2′-amino-LNA. Reagents and conditions: a) propionyl chloride, Et_3N, CH_2Cl_2, 0 °C, 62%; b) $(i\text{-Pr})_2NP(Cl)O(CH_2)_2CN$, *N*,*N*-diisopropylethylamine, CH_2Cl_2, rt, 53%.



4h

Figure 2. Structure of 2′-*N*-trifluoroacetyl-2′-amino-LNA phosphoramidite **4h**

The hybridization properties of 2′-*N*-alkoxycarbonyl-modified oligonucleotides **ON1**–**6** toward complementary ssDNA and ssRNA were evaluated by UV-melting experiments and compared with those of the carba analog of methoxycarbonyl derivative (acyl-modified oligonucleotide) **ON7**, unmodified 2′-amino-LNA **ON8**, and natural DNA **ON9**. The results of these experiments are summarized in Table 1 and the representative UV-melting profiles are shown in Figure 3. The thermal stability of the duplexes formed with ssDNA by **ON1**–**6** including 2′-*N*-alkoxycarbonyl derivatives (T_m = 47–49 °C) was comparable to that of **ON7**–**9** (T_m = 47–49 °C). In contrast, for the duplexes with complementary ssRNA, the T_m values of **ON1**–**6** ranged from 51 °C to 53 °C, which were much higher than that of natural **ON9** (T_m = 47 °C). These results indicated that 2′-*N*-alkoxycarbonyl-2′-amino-LNA showed significant stabilization ability for the DNA-RNA duplexes in an RNA-selective fashion. The RNA-selectivity was observed because introduction of 2′,4′-BNA analogs into DNA locally induced the structural change in the DNA strand towards an A-type, which was the same as for DNA-RNA duplex.[30] Among **ON1**–**8**, isopropyloxycarbonyl and phenoxycarbonyl modifications, possessing the branched α-carbon of the alkoxy oxygen atom, slightly decreased the stability of the duplex (**ON4**–**5**, T_m = 51–52 °C) relative to the unmodified 2′-amino-LNA **ON8** (T_m = 54 °C). This trend might be due to steric repulsion based on this branched α-carbon, in the minor groove of the duplex though the details are unclear. The other 2′-*N*-alkoxycarbonyl-2′-amino-LNA analogs (**ON1**–**3**, **6**, T_m = 53 °C) had similar stabilizing ability against duplexes with ssRNA as the carba analog **ON7** (T_m = 53 °C) and unmodified 2′-amino-LNA **ON8** (T_m = 54 °C). The T_m value of methoxycarbonyl **ON1** was the same as that of the carba analog, propionyl **ON7**, which unfortunately suggested that the oxygen atom of the alkoxy group does not affect the stability of the duplex, and no additional hydration may be formed.

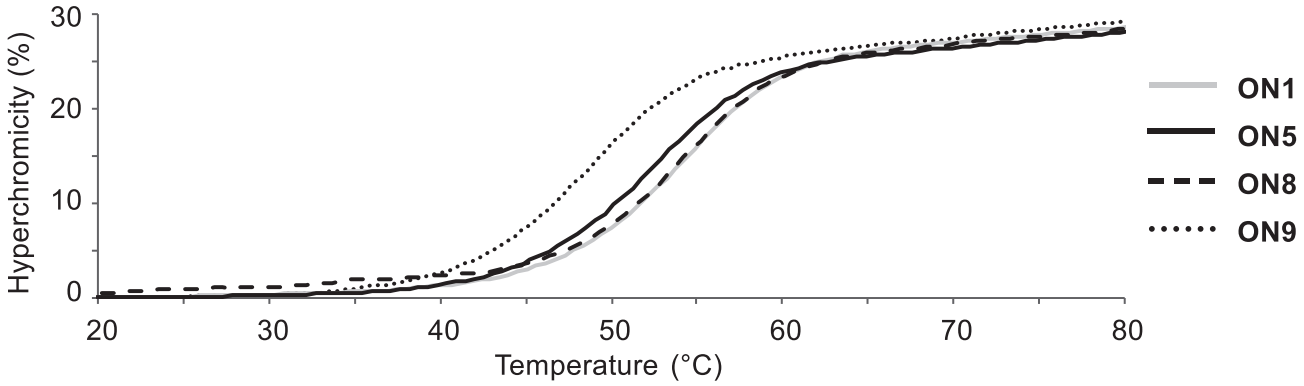
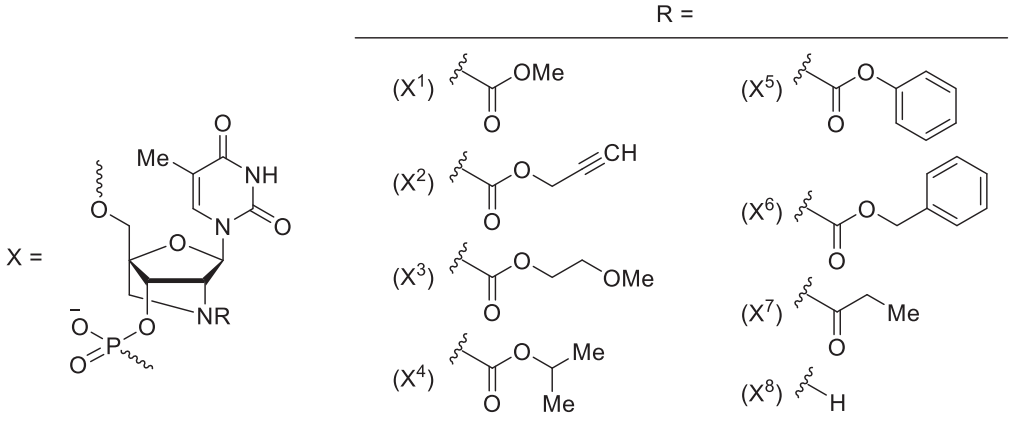


Figure 3. The representative UV-melting profiles of duplexes formed with target ssRNA

Table 1. Melting temperatures (T_ms) of duplexes formed by **ON1–9**

		vs ssDNA	vs ssRNA
		T_m (ΔT_m)	T_m (ΔT_m)
ON1	5′-d(GGATGX[1]TCTCGT)-3′	48 °C (+1 °C)	53 °C (+6 °C)
ON2	5′-d(GGATGX[2]TCTCGT)-3′	49 °C (+2 °C)	53 °C (+6 °C)
ON3	5′-d(GGATGX[3]TCTCGT)-3′	48 °C (+1 °C)	53 °C (+6 °C)
ON4	5′-d(GGATGX[4]TCTCGT)-3′	47 °C (±0 °C)	52 °C (+5 °C)
ON5	5′-d(GGATGX[5]TCTCGT)-3′	47 °C (±0 °C)	51 °C (+4 °C)
ON6	5′-d(GGATGX[6]TCTCGT)-3′	49 °C (+2 °C)	53 °C (+6 °C)
ON7	5′-d(GGATGX[7]TCTCGT)-3′	48 °C (+1 °C)	53 °C (+6 °C)
ON8	5′-d(GGATGX[8]TCTCGT)-3′	49 °C (+2 °C)	54 °C (+7 °C)
ON9	5′-d(GGATGTTCTCGT)-3′	47 °C	47 °C

Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM NaCl, and 2.5 μM of each oligonucleotide. The sequences of target ssDNA and target ssRNA are 5′-d(ACGAGAACATCC)-3′ and 5′-r(ACGAGAACAUCC)-3′, respectively. ΔT_m: Change in T_m value compared with that of natural **ON9**.



2′-*N*-Alkoxycarbonyl-2′-amino-LNA (**ON10**–**15**, Table 2) formed significantly stable triplexes with dsDNA (T_m = 29–31 °C) relative to the natural congener (**ON18**, T_m = 26 °C). In particular, 2′-*N*-alkoxycarbonyl-2′-amino-LNAs with small substituents [methoxy and propargyloxy groups (**ON10**–**11**), T_m = 31 °C] showed slightly higher stabilization ability of the triplex compared with the unsubstituted 2′-amino-LNA (**ON17**, T_m = 30 °C), consistent with a previous report on the triplex-forming ability of 2′-*N*-acetyl-2′-amino-LNA.[12] On the other hand, the results for **ON10** and **ON16** suggested that alkoxycarbonyl and acyl modifications led to almost no difference in the triplex-forming ability of oligonucleotides, as was the case for the duplex. In addition, incorporation of isopropyloxy and phenoxy groups (**ON13**, **14**, T_m = 29 °C) apparently destabilized the triplex relative to other modifications (**ON10**–**12**, **15**–**17**, T_m = 30–31 °C); this trend was consistent with the abovementioned results for the duplex, possibly due to steric hindrance. Moreover, lower hyperchromicity of triplexes formed by isopropyloxycarbonyl **ON13** and phenoxycarbonyl **ON14** was observed compared with that of other triplexes (Figure 4), which also suggesting the branched α-carbon of the alkoxy oxygen atom might distort the triplex structure.

The comparison between the phenoxycarbonyl modification (**ON5**, **14**) and benzyloxycarbonyl modification (**ON6**, **15**) indicated that the α-methylene unit of the alkoxy oxygen plays an important role in preserving the increased stability of the duplex and triplex. Moreover, **ON1**–**3** and **ON10**–**12** possessing linear alkoxy groups did not decrease the stability of the duplex and triplex. These results suggested that alkoxycarbonyl modifications with linear alkoxy groups could be used to attach various functional units to 2′-amino-LNA without loss of the duplex- and triplex-forming ability.

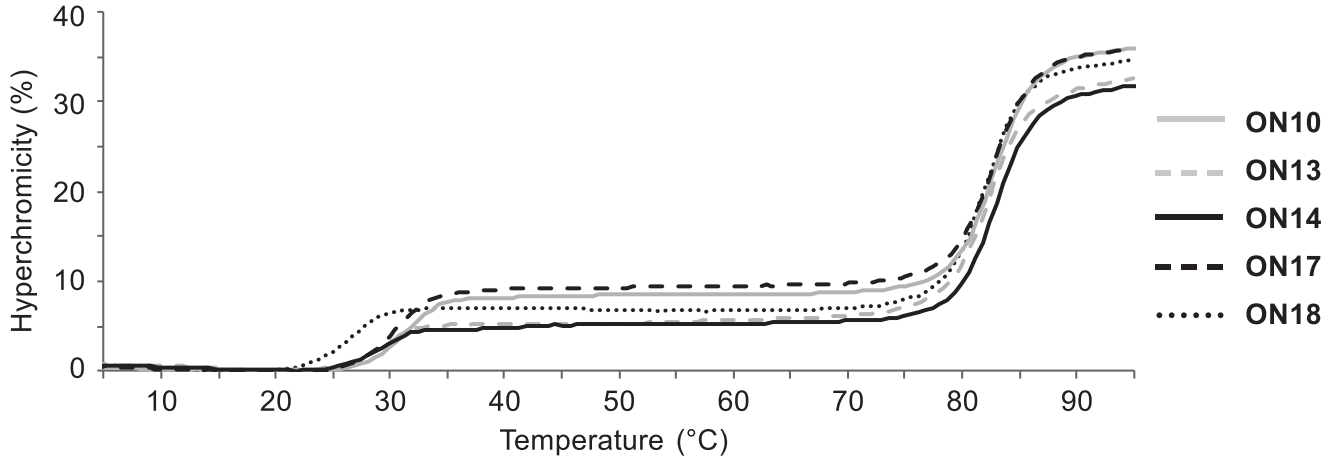
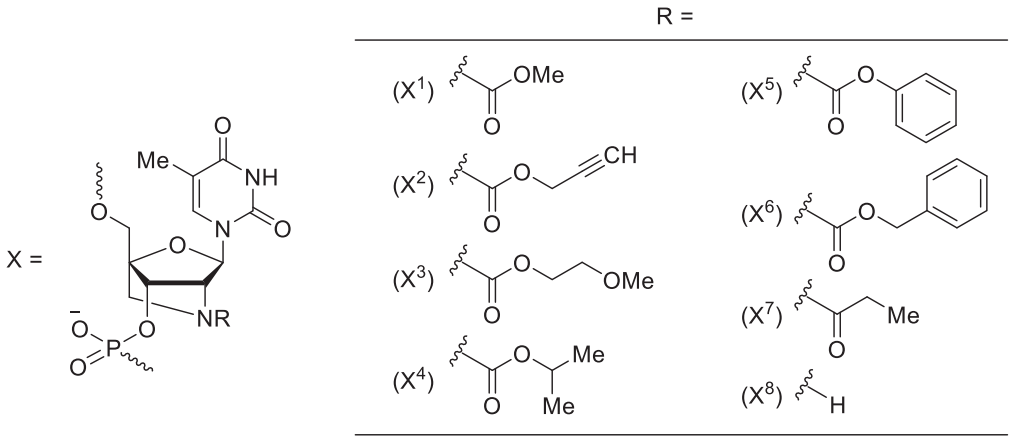


Figure 4. The representative UV-melting profiles of triplexes formed with target dsDNA

Table 2. Melting temperatures (T_ms) of triplexes formed by **ON10–18**

		T_m (ΔT_m)
ON10	5′-d(T^{m}CTT^{m}CTTX^{1}TT^{m}CT^{m}CT)-3′	31 °C (+5 °C)
ON11	5′-d(T^{m}CTT^{m}CTTX^{2}TT^{m}CT^{m}CT)-3′	31 °C (+5 °C)
ON12	5′-d(T^{m}CTT^{m}CTTX^{3}TT^{m}CT^{m}CT)-3′	30 °C (+4 °C)
ON13	5′-d(T^{m}CTT^{m}CTTX^{4}TT^{m}CT^{m}CT)-3′	29 °C (+3 °C)
ON14	5′-d(T^{m}CTT^{m}CTTX^{5}TT^{m}CT^{m}CT)-3′	29 °C (+3 °C)
ON15	5′-d(T^{m}CTT^{m}CTTX^{6}TT^{m}CT^{m}CT)-3′	30 °C (+4 °C)
ON16	5′-d(T^{m}CTT^{m}CTTX^{7}TT^{m}CT^{m}CT)-3′	31 °C (+5 °C)
ON17	5′-d(T^{m}CTT^{m}CTTX^{8}TT^{m}CT^{m}CT)-3′	30 °C (+4 °C)
ON18	5′-d(T^{m}CTT^{m}CTTTTT^{m}CT^{m}CT)-3′	26 °C

Conditions: 10 mM sodium cacodylate buffer (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, and 1.5 μM of each oligonucleotide. The sequence of target dsDNA is 5′-d(GGC*AGAAGAAAAAGAGA*CGC)-spacer18-d(GCG*TCTCTTTTTCTTCT*GCC)-3′ (spacer18: hexaethylene glycol linker), and the sequence shown in italic type was hybridized with **ON10–18** to form triplex. ^{m}C: 2′-deoxy-5-methylcytidine. ΔT_m: Change in T_m value compared with that of natural **ON18**.



CONCLUSION

2′-*N*-Alkoxycarbonyl derivatives of 2′-amino-LNA were synthesized and successfully incorporated into oligonucleotides. Their hybridization properties with ssDNA, ssRNA, and dsDNA were evaluated by UV-melting experiments. Isopropyloxycarbonyl and phenyloxycarbonyl modifications tended to decrease the stability of the duplexes and triplexes compared with the other alkoxycarbonyl modifications, perhaps due to the steric effect. However, in general, introduction of 2′-*N*-alkoxycarbonyl derivatives into oligonucleotides led to significant stabilization of the complexes formed with ssRNA and dsDNA, and the stabilizing ability was comparable with that of 2′-amino-LNA and its acyl modification. Unfortunately, no further improvement of the duplex- and triplex-forming abilities was observed by the presence of the

oxygen atom of alkoxy group, but the 2′-*N*-alkoxycarbonyl groups were identified to be useful as new modifications of 2′-amino-LNA. Since 2′-*N*-acyl-modified 2′-amino-LNAs have been used for various oligonucleotide-based technologies including antisense therapy[25] and gene diagnosis,[16,18,19,21] the results of this study suggest that 2′-*N*-alkoxycarbonyl-modified 2′-amino-LNA is a suitable material that would help realize the practical application of oligonucleotides. Furthermore, using oligonucleotides including 2′-amino-LNA with a highly reactive carbamate at the 2′-position may enable the direct construction of 2′-*N*-alkoxycarbonyl-2′-amino-LNA within oligonucleotides. Post-synthetic modification of 2′-*N*-alkoxycarbonyl-2′-amino-LNA is currently under investigation.

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 using a Bruker AVANCE III HD 500 MHz spectrometer equipped with a BBO cryoprobe. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were recorded at 500 MHz, 125 MHz, and 202 MHz, respectively. Chemical shift values are reported in parts per million downfield from the 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 reaction progress was monitored by analytical thin-layer chromatography (TLC) on pre-coated glass sheets (Silica gel 60 F_{254} by Merck). High-performance liquid chromatography (HPLC) was performed using a JASCO EXTREMA (PU-4180, CO-4060 and UV-4075) system with a fraction collector CHF122SC (ADVANTEC). ESI-TOF mass spectra were recorded on a Waters SYNAPT G2-Si HDMS spectrometer. UV melting experiments were performed on a JASCO UV-730 UV/VIS spectrophotometer equipped with a T_m analysis accessory.

(2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-(*N*-methoxycarbonyliminomethano)thymidine (2a) and (2′*R*)-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-methoxycarbonyl-2′,4′-(*N*-methoxycarbonyliminomethano)-thymidine (3a)

Under an Ar atmosphere, Et_3N (98 μL, 0.70 mmol) and methyl chloroformate (23 μL, 0.30 mmol) were added to a solution of **1**[11] (100 mg, 0.18 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 3 h. After quenching with sat. $NaHCO_3$ aq. and dilution with $CHCl_3$, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (116 mg) was purified by column chromatography (silica gel 4 g, $CHCl_3$:MeOH = 50:1 to 10:1) to give **2a** as a white powder (57 mg, 52%) and **3a** as a white powder (46 mg, 38%). Compound **2a**: IR (ATR) ν_{max}: 3392, 3194, 3064, 3034, 3007, 2953, 2931, 2836, 1706, 1686, 1607, 1582, 1508, 1456, 1395, 1351,

1297, 1272, 1250, 1213 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.59 (s, 3H), 2.37 (s, 0.5H), 3.34–3.78 (m, 13H), 4.26–4.34 (m, 1.5H), 4.61 (s, 0.5H), 4.79 (s, 0.5H), 5.52 (s, 0.5H), 5.59 (s, 0.5H), 6.82–6.86 (m, 4H), 7.20–7.47 (m, 9H), 7.61–7.65 (m, 1H), 9.69 (s, 0.5H), 9.78 (s, 0.5H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 29.6, 51.6, 51.9, 52.9, 55.2, 55.3, 59.4, 62.9, 62.9, 69.2, 69.2, 69.7, 86.6, 86.7, 87.0, 88.1, 88.6, 110.0, 110.2, 113.2, 127.0, 128.0, 128.0, 130.0, 130.1, 134.9, 135.2, 135.2, 135.3, 135.3, 135.4, 144.4, 149.9, 149.9, 150.1, 156.1, 156.3, 158.6, 164.5, 164.5. HRMS (ESI): Calcd for $C_{34}H_{35}N_3NaO_9$ [MNa^+] 652.2271, found 652.2271. Compound **3a**: IR (ATR) ν_{max}: 3191, 3064, 3013, 2955, 2930, 2837, 1759, 1687, 1607, 1582, 1508, 1455, 1444, 1392, 1353, 1314, 1274, 1249 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.59 (s, 2.1H), 1.62 (s, 0.9H), 3.37–3.61 (m, 4H), 3.71–3.80 (m, 12H), 4.86 (s, 0.7H), 4.94 (s, 0.3H), 5.04 (s, 0.7H), 5.07 (s, 0.3H), 5.62 (s, 0.7H), 5.70 (s, 0.3H), 6.83–6.86 (m, 4H), 7.23–7.43 (m, 9H), 7.64–7.66 (m, 1H), 8.83 (s, 0.3H), 9.21 (s, 0.7H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 51.9, 52.9, 53.1, 55.2, 55.5, 55.6, 58.5, 60.9, 61.2, 73.0, 86.9, 86.9, 87.1, 87.2, 110.7, 110.9, 113.3, 113.3, 127.2, 128.0, 128.1, 130.0, 130.0, 134.1, 134.1, 134.9, 135.0, 144.0, 149.5, 149.9, 154.1, 154.2, 155.1, 155.4, 158.7, 163.6, 163.8. HRMS (ESI): Calcd for $C_{36}H_{37}N_3NaO_{11}$ [MNa^+] 710.2326, found 710.2335.

(2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-(*N*-methoxycarbonyliminomethano)thymidine (4a)

Under an Ar atmosphere, *N*,*N*-diisopropylethylamine (72 μL, 0.41 mmol) and $(i\text{-Pr})_2NP(Cl)O(CH_2)_2CN$ (44 μL, 0.20 mmol) were added to a solution of **2a** (35 mg, 0.055 mmol) in anhydrous CH_2Cl_2 (3 mL) at 0 °C. The reaction mixture was stirred at room temperature for 6 h. After quenching with sat. $NaHCO_3$ aq. and dilution with EtOAc, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (74 mg) was purified by column chromatography (silica gel 7 g, hexane:EtOAc = 2:1 to 1:1) to give **4a** as a white powder (37 mg, 80%). ^1H NMR (500 MHz, $CDCl_3$) δ: 0.96–1.30 (m, 12H), 1.52–1.56 (m, 3H), 2.36–2.39 (m, 1H), 2.50–2.68 (m, 1H), 3.33–3.80 (m, 17H), 4.35–4.44 (m, 1H), 4.77–4.78 (m, 0.7H), 4.85 (s, 0.3H), 5.59 (s, 0.7H), 5.65 (s, 0.3H), 6.82–6.87 (m, 4H), 7.22–7.46 (m, 9H), 7.67–7.71 (m, 1H), 8.63 (s, 1H). ^{31}P NMR (202 MHz, $CDCl_3$) δ: 149.0, 149.1, 149.4. HRMS (ESI): Calcd for $C_{43}H_{52}N_5NaO_{10}P$ [MNa^+] 852.3349, found 852.3350.

(2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-(*N*-propargyloxycarbonyliminomethano)thymidine (2b) and (2′*R*)-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-propargyloxycarbonyl-2′,4′-(*N*-propargyloxycarbonylimino-methano)thymidine (3b)

Under an Ar atmosphere, Et_3N (99 μL, 0.71 mmol) and 4-nitrophenyl propargyl carbonate[31] (47 mg, 0.21 mmol) were added to a solution of **1**[11] (102 mg, 0.18 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at room temperature for 18 h. After quenching with sat. $NaHCO_3$ aq. and dilution with $CHCl_3$, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated

in vacuo. The residue (147 mg) was purified by column chromatography (silica gel 7 g, $CHCl_3$:MeOH = 50:1 to 20:1) to give **2b** as a white powder (70 mg, 60%) and **3b** as a white powder (41 mg, 31%). Compound **2b**: IR (ATR) ν_{max}: 3388, 3300, 3068, 3012, 2953, 2932, 2837, 1707, 1685, 1607, 1581, 1508, 1456, 1443, 1345, 1313, 1297, 1274, 1248, 1217 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.60 (s, 3H), 2.01 (s, 0.6H), 2.43 (s, 1H), 2.97 (s, 0.4H), 3.39 (d, *J* = 10.0 Hz, 1H), 3.47–3.64 (m, 3H), 3.78 (s, 6H), 4.31 (s, 0.4H), 4.34 (s, 0.6H), 4.65–4.77 (m, 3H), 5.54 (s, 0.4H), 5.59 (s, 0.6H), 6.83–6.86 (m, 4H), 7.22–7.46 (m, 9H), 7.62 (s, 0.6H), 7.65 (s, 0.4H), 9.12 (s, 0.4H), 9.32 (s, 0.6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 12.5, 51.7, 51.9, 53.3, 53.3, 55.2, 59.0, 59.2, 63.0, 69.3, 69.9, 74.8, 75.0, 77.9, 78.1, 86.8, 86.9, 87.0, 88.0, 88.5, 110.2, 110.3, 113.3, 127.1, 128.0, 130.0, 130.1, 134.8, 135.2, 135.3, 144.3, 149.8, 154.7, 158.7, 164.0, 164.1. HRMS (ESI): Calcd for $C_{36}H_{35}N_3NaO_9$ [MNa^+] 676.2271, found 676.2277. Compound **3b**: IR (ATR) ν_{max}: 3286, 3066, 3035, 2953, 2933, 2838, 1760, 1710, 1686, 1607, 1593, 1508, 1460, 1442, 1423, 1387, 1374, 1338, 1314, 1274, 1245 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.60 (s, 1.8H), 1.61 (s, 1.2H), 2.46–2.50 (m, 1H), 2.55–2.57 (m, 1H), 3.38–3.62 (m, 4H), 3.80 (s, 6H), 4.67–4.83 (m, 4H), 4.92 (s, 0.6H), 4.96 (s, 0.4H), 5.09 (s, 0.6H), 5.13 (s, 0.4H), 5.65 (s, 0.6H), 5.71 (s, 0.4H), 6.84–6.87 (m, 4H), 7.23–7.43 (m, 9H), 7.64 (s, 1H), 8.98 (s, 0.4H), 9.31 (s, 0.6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 51.9, 52.0, 53.3, 53.4, 55.2, 56.2, 56.3, 58.4, 60.9, 61.3, 73.2, 73.6, 74.9, 75.1, 76.0, 76.6, 76.6, 77.7, 77.9, 86.9, 86.9, 87.0, 87.2, 110.8, 111.0, 113.3, 113.3, 127.2, 127.9, 128.1, 130.0, 133.9, 133.9, 134.8, 134.9, 143.9, 149.5, 149.8, 152.9, 153.0, 153.5, 154.1, 158.7, 163.6, 163.8. HRMS (ESI): Calcd for $C_{40}H_{37}N_3NaO_{11}$ [MNa^+] 758.2326, found 758.2327.

(2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-(*N*-propargyloxycarbonyliminomethano)thymidine (4b)

Under an Ar atmosphere, *N*,*N*-diisopropylethylamine (0.38 mL, 2.2 mmol) and $(i\text{-Pr})_2NP(Cl)O(CH_2)_2CN$ (0.17 mL, 0.76 mmol) were added to a solution of **2b** (141 mg, 0.22 mmol) in anhydrous CH_2Cl_2 (3 mL) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. After quenching with sat. $NaHCO_3$ aq. and dilution with EtOAc, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (298 mg) was purified by column chromatography (silica gel 4 g, hexane:EtOAc = 1:1) to give **4b** as a white powder (119 mg, 65%). ^1H NMR (500 MHz, $CDCl_3$) δ: 0.97–1.31 (m, 12H), 1.51–1.55 (m, 3H), 2.37–2.39 (m, 1H), 2.42–2.47 (m, 1H), 2.54–2.68 (m, 1H), 3.37–3.68 (m, 8H), 3.79–3.80 (m, 6H), 4.37–4.45 (m, 1H), 4.67–4.88 (m, 3H), 5.60 (s, 0.6H), 5.66 (s, 0.4H), 6.82–6.87 (m, 4H), 7.23–7.46 (m, 9H), 7.68–7.70 (m, 1H), 8.39–8.55 (m, 1H). ^{31}P NMR (202 MHz, $CDCl_3$) δ: 149.2, 149.3, 149.4, 149.5. HRMS (ESI): Calcd for $C_{45}H_{52}N_5NaO_{10}P$ [MNa^+] 876.3349, found 876.3358.

(2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-[*N*-(2-methoxyethoxycarbonyl)iminomethano]thymidine (2c) and (2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(2-methoxyethoxycarbonyl)-2′,4′-[*N*-(2-methoxyethoxycarbonyl)iminomethano]thymidine (3c)

Under an Ar atmosphere, Et_3N (95 μL, 0.70 μmol) and 2-methoxyethyl chloroformate (24 μL, 0.20 mmol) were added to a solution of **1**[11] (98 mg, 0.17 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at room temperature for 18 h. After quenching with sat. $NaHCO_3$ aq. and dilution with $CHCl_3$, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (122 mg) was purified by column chromatography (silica gel 10 g, $CHCl_3$:MeOH = 50:1 to 20:1) to give **2c** as a white powder (66 mg, 57%) and **3c** as a white powder (24 mg, 18%). Compound **2c**: IR (ATR) ν_{max}: 3385, 3201, 3065, 3037, 3006, 2953, 2932, 2909, 2896, 2836, 1709, 1686, 1607, 1582, 1508, 1490, 1462, 1445, 1430, 1396, 1346, 1298, 1273, 1249, 1211 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.60 (s, 3H), 2.21 (s, 1H), 3.31–3.63 (m, 9H), 3.78 (s, 6H), 4.08–4.16 (m, 1H), 4.23–4.34 (m, 2H), 4.66 (s, 0.4H), 4.77 (s, 0.6H), 5.54 (s, 0.4H), 5.59 (s, 0.6H), 6.83–6.85 (m, 4H), 7.22–7.46 (m, 9H), 7.63 (s, 1H), 9.13 (s, 0.4H), 9.39 (s, 0.6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 51.7, 51.8, 58.7, 59.0, 59.2, 59.3, 62.9, 63.0, 64.4, 64.7, 69.2, 69.8, 70.7, 86.7, 86.7, 87.0, 88.2, 88.6, 110.2, 113.3, 127.1, 128.0, 128.1, 130.0, 130.1, 134.7, 134.8, 135.3, 135.4, 144.4, 149.9, 155.6, 155.7, 158.7, 164.1, 164.1. HRMS (ESI): Calcd for $C_{36}H_{39}N_3NaO_{10}$ [MNa^+] 696.2533, found 696.2530. Compound **3c**: IR (ATR) ν_{max}: 3202, 3069, 3004, 2953, 2931, 2893, 2837, 1757, 1709, 1687, 1607, 1581, 1508, 1458, 1445, 1423, 1395, 1388, 1371, 1346, 1272, 1247, 1201 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ: 1.57 (s, 1.8H), 1.60 (s, 1.2H), 3.36–3.66 (m, 14H), 3.80 (s, 6H), 4.21–4.39 (m, 4H), 4.87 (s, 0.6H), 4.94 (s, 0.4H), 5.09 (s, 0.6H), 5.10 (s, 0.4H), 5.63 (s, 0.6H), 5.70 (s, 0.4H), 6.84–6.86 (m, 4H), 7.23–7.42 (m, 9H), 7.65 (s, 1H), 8.70 (s, 0.4H), 8.84 (s, 0.6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 12.5, 51.9, 55.2, 58.6, 58.9, 58.9, 59.0, 59.0, 60.8, 61.2, 64.8, 64.8, 67.8, 69.8, 69.9, 70.5, 70.7, 73.0, 73.3, 86.9, 86.9, 86.9, 87.0, 87.0, 87.2, 110.6, 110.7, 113.3, 127.2, 128.0, 128.0, 130.0, 134.1, 134.9, 135.0, 144.0, 149.7, 149.6, 153.6, 153.7, 154.5, 154.9, 158.7, 163.6, 163.6. HRMS (ESI): Calcd for $C_{40}H_{45}N_3NaO_{13}$ [MNa^+] 798.2850, found 798.2852.

(2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-[*N*-(2-methoxyethoxycarbonyl)iminomethano]thymidine (4c)

Under an Ar atmosphere, *N*,*N*-diisopropylethylamine (0.32 mL, 1.9 mmol) and $(i\text{-Pr})_2NP(Cl)O(CH_2)_2CN$ (0.15 mL, 0.66 mmol) were added to a solution of **2c** (127 mg, 0.19 mmol) in anhydrous CH_2Cl_2 (3 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After quenching with sat. $NaHCO_3$ aq. and dilution with EtOAc, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (259 mg) was purified by column chromatography (silica gel 10 g, hexane:EtOAc = 1:1 to 1:3) to give **4c** as a white powder (91 mg, 55%). ^1H NMR (500 MHz, $CDCl_3$) δ:

0.96–1.30 (m, 12H), 1.51–1.55 (m, 3H), 2.36–2.39 (m, 1.2H), 2.51–2.66 (m, 0.8H), 3.35–3.66 (m, 13H), 3.79–3.80 (m, 6H), 4.15–4.44 (m, 3H), 4.78–4.85 (m, 1H), 5.59–5.60 (m, 0.6H), 5.65 (s, 0.4H), 6.82–6.87 (m, 4H), 7.23–7.46 (m, 9H), 7.68–7.70 (m, 1H), 8.18 (s, 1H). ^{31}P NMR (202 MHz, $CDCl_3$) δ: 149.1, 149.2, 149.3. HRMS (ESI): Calcd for $C_{45}H_{56}N_5NaO_{11}P$ [MNa^+] 896.3612, found 896.3602.

(2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-(*N*-isopropyloxycarbonyliminomethano)thymidine (2d) and (2′*R*)-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-isopropyloxycarbonyl-2′,4′-(*N*-isopropyloxycarbonylimino-methano)thymidine (3d)

Under an Ar atmosphere, Et_3N (0.15 mL, 1.0 mmol) and isopropyl chloroformate (51 μL, 0.45 mmol) were added to a solution of **1**[11] (150 mg, 0.26 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. After quenching with sat. $NaHCO_3$ aq. and dilution with $CHCl_3$, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (198 mg) was purified by column chromatography (silica gel 4 g, hexane:EtOAc = 1:1 to 1:3) to give **2d** as a white powder (59 mg, 34%) and **3d** as a white powder (93 mg, 48%). Compound **2d**: IR (ATR) ν_{max}: 3385, 3194, 3065, 3035, 3006, 2979, 2952, 2931, 2877, 2837, 1707, 1669, 1607, 1582, 1508, 1491, 1463, 1444, 1419, 1387, 1340, 1298, 1271, 1248, 1214 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.17–1.30 (m, 6H), 1.60 (s, 3H), 2.47 (s, 0.5H), 3.31–3.66 (m, 4.5H), 3.79 (s, 6H), 4.28 (s, 0.5H), 4.32 (s, 0.5H), 4.57 (s, 0.5H), 4.76–4.93 (m, 1.5H), 5.52 (s, 0.5H), 5.59 (s, 0.5H), 6.84–6.86 (m, 4H), 7.22–7.46 (m, 9H), 7.65 (s, 1H), 8.62 (s, 0.5H), 9.16 (s, 0.5H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 21.1, 29.7, 51.3, 51.8, 55.2, 59.1, 59.3, 62.8, 69.4, 69.5, 70.0, 70.5, 86.8, 87.0, 88.0, 88.6, 110.2, 113.3, 127.1, 128.1, 130.0, 130.1, 134.8, 135.3, 144.3, 149.7, 155.6, 158.7, 163.8, 164.0. HRMS (ESI): Calcd for $C_{36}H_{39}N_3NaO_9$ [MNa^+] 680.2584, found 680.2580. Compound **3d**: IR (ATR) ν_{max}: 3192, 3070, 2981, 2935, 2837, 1752, 1710, 1691, 1607, 1581, 1509, 1490, 1464, 1445, 1417, 1386, 1377, 1362, 1340, 1273, 1253, 1211 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.21–1.32 (m, 12H), 1.57 (s, 2.1H), 1.59 (s, 0.9H), 3.36–3.56 (m, 4H), 3.80 (s, 6H), 4.82–4.98 (m, 3H), 5.08 (s, 0.7H), 5.13 (s, 0.3H), 5.60 (s, 0.7H), 5.69 (s, 0.3H), 6.83–6.85 (m, 4H), 7.23–7.43 (m, 9H), 7.66 (s, 1H), 8.19 (s, 0.3H), 8.23 (s, 0.7H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 21.6, 21.6, 22.0, 22.1, 22.1, 51.8, 52.0, 55.2, 55.2, 58.7, 58.8, 60.8, 61.2, 69.3, 69.7, 72.7, 73.0, 73.5, 73.5, 86.8, 86.9, 87.0, 87.1, 87.3, 110.6, 110.6, 113.3, 113.3, 127.2, 128.1, 130.0, 130.1, 134.3, 134.9, 135.1, 144.0, 149.4, 149.5, 153.1, 153.2, 154.6, 154.9, 158.7, 163.3, 163.4. HRMS (ESI): Calcd for $C_{40}H_{45}N_3NaO_{11}$ [MNa^+] 766.2952, found 766.2960.

(2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-(*N*-isopropyloxycarbonyliminomethano)thymidine (4d)

Under an Ar atmosphere, *N*,*N*-diisopropylethylamine (0.25 mL, 1.4 mmol) and $(i\text{-Pr})_2NP(Cl)O(CH_2)_2CN$ (0.11 mL, 0.49 mmol) were added to a solution of **2d** (93 mg, 0.14 mmol) in anhydrous CH_2Cl_2 (2 mL) at

0 °C. The reaction mixture was stirred at room temperature for 1 h. After quenching with sat. $NaHCO_3$ aq. and dilution with EtOAc, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (199 mg) was purified by column chromatography (silica gel 5 g, hexane:EtOAc = 1:1 to 1:2) to give **4d** as a white powder (105 mg, 87%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.97–1.33 (m, 18H), 1.50–1.56 (m, 3H), 2.35–2.39 (m, 1H), 2.51–2.69 (m, 1H), 3.31–3.68 (m, 8H), 3.79–3.80 (m, 6H), 4.34–4.44 (m, 1H), 4.73–4.84 (m, 1H), 4.92–4.97 (m, 1H), 5.56–5.57 (m, 0.7H), 5.66 (s, 0.3H), 6.83–6.87 (m, 4H), 7.23–7.46 (m, 9H), 7.70–7.72 (m, 1H), 8.39–8.47 (m, 1H). ^{31}P NMR (202 MHz, $CDCl_3$) δ: 149.1, 149.3, 149.3. HRMS (ESI): Calcd for $C_{45}H_{56}N_5NaO_{10}P$ [MNa^+] 880.3662, found 880.3678.

(2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-(*N*-phenoxycarbonyliminomethano)thymidine (2e) and (2′*R*)-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-phenoxycarbonyl-2′,4′-(*N*-phenoxycarbonyliminomethano)-thymidine (3e)

Under an Ar atmosphere, Et_3N (15 μL, 0.10 mmol) and phenyl chloroformate (12 μL, 0.096 mmol) were added to a solution of **1**[11] (50 mg, 0.087 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at room temperature for 15 h. After quenching with sat. $NaHCO_3$ aq. and dilution with $CHCl_3$, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (67 mg) was purified by column chromatography (silica gel 6 g, hexane:EtOAc = 1:1 to 1:2) to give **2e** as a white powder (21 mg, 35%) and **3e** as a white powder (13 mg, 18%). Compound **2e**: IR (ATR) ν_{max}: 3217, 3060, 2951, 1687, 1607, 1508, 1461, 1442, 1410, 1249, 1204 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.59 (s, 1.5H), 1.60 (s, 1.5H), 2.82 (s, 0.5H), 3.29 (s, 0.5H), 3.43–3.55 (m, 3H), 3.61 (d, J = 11.0 Hz, 0.5H), 3.65 (d, J = 10.5 Hz, 0.5H), 3.78 (s, 6H), 4.23 (s, 0.5H), 4.32 (s, 0.5H), 4.78 (s, 0.5H), 4.83 (s, 0.5H), 5.64 (s, 0.5H), 5.69 (s, 0.5H), 6.84–6.85 (m, 4H), 7.08–7.45 (m, 14H), 7.51 (s, 0.5H), 7.63 (s, 0.5H), 8.94 (s, 0.5H), 9.29 (s, 0.5H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 12.6, 51.7, 52.4, 55.4, 59.0, 59.2, 63.1, 63.5, 69.4, 70.0, 86.7, 86.9, 87.0, 88.0, 88.5, 110.1, 110.3, 113.3, 113.4, 121.5, 125.4, 125.5, 127.1, 127.2, 128.0, 128.1, 129.2, 129.2, 130.0, 130.1, 134.6, 134.7, 135.2, 135.3, 135.4, 144.3, 144.3, 149.8, 150.8, 150.9, 153.7, 153.9, 158.7, 158.7, 163.9, 164.0. HRMS (ESI): Calcd for $C_{39}H_{37}N_3NaO_9$ [MNa^+] 714.2427, found 714.2421. Compound **3e**: IR (ATR) ν_{max}: 3194, 3066, 2954, 2928, 2850, 2838, 1767, 1731, 1693, 1607, 1509, 1494, 1460, 1446, 1411, 1352, 1293, 1274, 1250, 1207 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.58–1.59 (m, 3H), 3.50 (d, J = 11.5 Hz, 1H), 3.60–3.79 (m, 9H), 5.08 (s, 0.4H), 5.14 (s, 0.6H), 5.27 (s, 0.6H), 5.30 (s, 0.4H), 5.82–5.83 (m, 1H), 6.85–6.87 (m, 4H), 7.05–7.46 (m, 19H), 7.67 (s, 0.4H), 7.69 (s, 0.6H), 8.07–8.12 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 12.5, 29.6, 29.7, 52.2, 52.5, 55.2, 58.5, 61.2, 61.9, 73.5, 73.8, 87.0, 87.1, 87.1, 87.4, 110.9, 111.0, 113.4, 113.4, 120.5, 120.7, 121.4, 121.5, 125.7, 127.3, 128.1, 128.2, 129.3, 129.4, 129.6, 130.1, 130.1, 133.9, 134.8, 134.9,

143.9, 149.4, 149.5, 150.7, 150.7, 150.8, 152.1, 152.2, 153.0, 153.2, 158.8, 163.1, 163.1. HRMS (ESI): Calcd for $C_{46}H_{41}N_3NaO_{11}$ [MNa^+] 834.2639, found 834.2638.

(2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-(*N*-phenoxycarbonyliminomethano)thymidine (4e)

Under an Ar atmosphere, *N*,*N*-diisopropylethylamine (0.12 mL, 0.72 mmol) and (*i*-Pr)$_2$NP(Cl)O(CH$_2$)$_2$CN (64 μL, 0.29 mmol) were added to a solution of **2e** (166 mg, 0.24 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. After quenching with sat. $NaHCO_3$ aq. and dilution with EtOAc, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (220 mg) was purified by column chromatography (silica gel 10 g, hexane:EtOAc = 2:1 to 2:3) to give **4e** as a white powder (188 mg, 88%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.99–1.17 (m, 12H), 1.53–1.57 (m, 3H), 2.39–2.40 (m, 1H), 2.51–2.57 (m, 1H), 3.45–3.67 (m, 8H), 3.80 (s, 3H), 3.80 (s, 3H), 4.46–4.53 (m, 1H), 4.92–5.03 (m, 1H), 5.76 (m, 1H), 6.85–7.46 (m, 18H), 7.69–7.73 (m, 1H), 8.50 (s, 0.3H), 8.68 (s, 0.7H). ^{31}P NMR (202 MHz, $CDCl_3$) δ: 149.4, 149.5, 149.6, 149.8. HRMS (ESI): Calcd for $C_{48}H_{54}N_5NaO_{10}P$ [MNa^+] 914.3506, found 914.3510.

(2′*R*)-2′,4′-(*N*-Benzyloxycarbonyliminomethano)-5′-*O*-(4,4′-dimethoxytrityl)thymidine (2f) and (2′*R*)-3′-*O*-benzyloxycarbonyl-2′,4′-(*N*-benzyloxycarbonyliminomethano)-5′-*O*-(4,4′-dimethoxytrityl)thymidine (3f)

Under an Ar atmosphere, Et_3N (98 μL, 0.70 mmol) and benzyl chloroformate (42 μL, 0.30 mmol) were added to a solution of **1**[11] (100 mg, 0.18 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 3 h. After quenching with sat. $NaHCO_3$ aq. and dilution with $CHCl_3$, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (159 mg) was purified by column chromatography (silica gel 5 g, hexane:EtOAc = 1:1 to 1:3) to give **2f** as a white powder (67 mg, 55%) and **3f** as a white powder (29 mg, 20%). Compound **2f**: IR (ATR) ν_{max}: 3390, 3203, 3066, 3034, 3009, 2953, 2933, 2911, 2836, 1708, 1682, 1607, 1583, 1508, 1458, 1444, 1427, 1361, 1349, 1297, 1274, 1249, 1214 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.61 (s, 3H), 2.30 (s, 0.5H), 3.09 (s, 0.5H), 3.34–3.64 (m, 4H), 3.78 (s, 6H), 4.27 (s, 0.5H), 4.32 (s, 0.5H), 4.68 (s, 0.5H), 4.76 (s, 0.5H), 5.10–5.18 (m, 2H), 5.56 (s, 0.5H), 5.61 (s, 0.5H), 6.84–6.85 (m, 4H), 7.24–7.45 (m, 14H), 7.64 (s, 1H), 8.46 (s, 0.5H), 8.80 (s, 0.5H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5. 51.5, 51.8, 55.2, 59.0, 59.1, 62.9, 67.4, 67.5, 69.4, 70.0, 86.8, 86.9, 87.0, 87.0, 88.0, 88.6, 110.3, 113.4, 127.2, 127.7, 127.9, 128.0, 128.1, 128.5, 130.0, 130.0, 134.7, 135.2, 136.1, 136.4, 144.3, 149.6, 155.3, 155.4, 158.7, 163.6, 163.8. HRMS (ESI): Calcd for $C_{40}H_{39}N_3NaO_9$ [MNa^+] 728.2584, found 728.2586. Compound **3f**: IR (ATR) ν_{max}: 3194, 3064, 3034, 3014, 2954, 2932, 2900, 2837, 1758, 1711, 1689, 1607, 1584, 1508, 1456, 1446, 1421, 1361, 1352, 1312, 1271, 1249, 1213 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.58 (s, 1.8H), 1.60 (s, 1.2H),

3.33–3.58 (m, 4H), 3.77–3.78 (m, 6H), 4.93 (s, 0.6H), 4.97 (s, 0.4H), 5.05–5.17 (m, 5H), 5.63 (s, 0.6H), 5.71 (s, 0.4H), 6.79–6.83 (m, 4H), 7.21–7.42 (m, 19H), 7.65 (s, 1H), 8.39 (s, 0.4H), 8.47 (s, 0.6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 12.5, 12.5, 51.9, 52.0, 55.2, 58.6, 58.6, 60.9, 61.2, 67.4, 70.6, 70.8, 73.0, 73.3, 86.9, 86.9, 87.0, 87.2, 87.3, 110.7, 110.8, 113.3, 113.4, 127.2, 127.7, 128.0, 128.1, 128.1, 128.4, 128.5, 128.5, 128.5, 128.7, 128.7, 128.9, 128.9, 130.0, 130.0, 134.1, 134.3, 134.9, 135.0, 136.0, 136.3, 143.9, 149.4, 149.5, 153.5, 153.6, 154.5, 154.7, 158.7, 163.4, 163.4. HRMS (ESI): Calcd for $C_{48}H_{45}N_3NaO_{11}$ [MNa^+] 862.2952, found 862.2950.

(2′*R*)-2′,4′-(*N*-Benzyloxycarbonyliminomethano)-3′-*O*-[2-cyanoethoxy(diisopropylamino)-phosphino]-5′-*O*-(4,4′-dimethoxytrityl)thymidine (4f)

Under an Ar atmosphere, *N*,*N*-diisopropylethylamine (0.28 mL, 1.6 mmol) and (*i*-Pr)$_2$NP(Cl)O(CH$_2$)$_2$CN (0.17 mL, 0.57 mmol) were added to a solution of **2f** (115 mg, 0.16 mmol) in anhydrous CH_2Cl_2 (3 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h. After quenching with sat. $NaHCO_3$ aq. and dilution with EtOAc, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (207 mg) was purified by column chromatography (silica gel 6 g, hexane:EtOAc = 1:2) to give **4f** as a white powder (114 mg, 77%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.93–1.32 (m, 12H), 1.51–1.55 (m, 3H), 2.28–2.57 (m, 2H), 3.35–3.66 (m, 8H), 3.79–3.80 (m, 6H), 4.35–4.45 (m, 1H), 4.84–4.87 (m, 1H), 5.09–5.27 (m, 2H), 5.60–5.67 (m, 1H), 6.81–6.86 (m, 4H), 7.23–7.45 (m, 14H), 7.68–7.70 (m, 1H), 8.13 (s, 1H). ^{31}P NMR (202 MHz, $CDCl_3$) δ: 149.1, 149.3, 149.4. HRMS (ESI): Calcd for $C_{49}H_{56}N_5NaO_{10}P$ [MNa^+] 928.3662, found 928.3665.

(2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-2′,4′-(*N*-propionyliminomethano)thymidine (2g)

Under an Ar atmosphere, Et_3N (64 μL, 0.46 mmol) and propionyl chloride (18 μL, 0.20 mmol) were added to a solution of **1**[11] (66 mg, 0.11 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 5 h. After quenching with 28% NH_3 aq. for 24 h and dilution with $CHCl_3$, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (65 mg) was purified by column chromatography (silica gel 3 g, hexane:EtOAc = 1:1 to 1:3) to give **2g** as a white powder (45 mg, 62%). IR (ATR) ν_{max}: 3345, 3195, 3061, 3006, 2936, 2878, 2836, 1684, 1654, 1634, 1608, 1584, 1561, 1508, 1462, 1445, 1392, 1383, 1347, 1301, 1273, 1249 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.05 (t, *J* = 7.5 Hz, 1.8H), 1.13 (t, *J* = 7.5 Hz, 1.2H), 1.61 (s, 1.2H), 1.62 (s, 1.8H), 2.19 (q, *J* = 7.5 Hz, 0.8H), 2.39 (dq, *J* = 7.5, 15.0 Hz, 0.6H), 2.54 (dq, *J* = 7.5, 15.0 Hz, 0.6H), 3.39–3.61 (m, 4.6H), 3.78 (s, 6H), 4.31 (s, 0.6H), 4.36 (s, 0.4H), 4.52 (s, 0.6H), 4.87 (s, 0.4H), 5.16 (s, 0.4H), 5.45 (s, 0.6H), 5.53 (s, 0.4H), 6.83–6.85 (m, 4H), 7.21–7.48 (m, 9H), 7.59 (s, 0.4H), 7.66 (s, 0.6H), 9.47 (s, 0.6H), 9.81 (s, 0.4H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 8.7, 9.1, 12.5, 12.5, 27.0, 27.4, 51.0, 52.3, 55.2, 59.1, 59.6, 61.7, 63.5, 69.0, 70.2, 86.7, 86.8, 86.9, 87.2, 88.0, 88.8, 110.0, 110.6, 113.3, 113.4, 113.4, 127.1, 127.1,

128.0, 128.1, 128.1, 130.0, 130.1, 130.1, 134.6, 134.9, 135.2, 135.3, 135.4, 144.3, 144.4, 149.8, 150.2, 158.7, 164.1, 164.5, 173.8, 174.0. HRMS (ESI): Calcd for $C_{35}H_{37}N_3NaO_8$ [MNa^+] 650.2478, found 650.2475.

(2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-2′,4′-(*N*-propionyliminomethano)thymidine (4g)

Under an Ar atmosphere, *N*,*N*-diisopropylethylamine (0.12 mL, 0.71 mmol) and (*i*-Pr)$_2$NP(Cl)O(CH$_2$)$_2$CN (55 μL, 0.25 mmol) were added to a solution of **2g** (45 mg, 0.071 mmol) in anhydrous CH_2Cl_2 (2 mL) at 0 °C. The reaction mixture was stirred at room temperature for 2.5 h. After quenching with sat. $NaHCO_3$ aq. and dilution with EtOAc, the mixture was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (96 mg) was purified by column chromatography (silica gel 3 g, hexane:EtOAc = 1:2) to give **4g** as a white powder (31 mg, 53%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.96–1.30 (m, 15H), 1.57–1.59 (m, 3H), 2.39–2.62 (m, 4H), 3.34–3.80 (m, 14H), 4.40–4.48 (m, 1H), 4.71–4.73 (m, 1H), 5.53 (s, 0.4H), 5.53 (s, 0.6H), 6.83–6.87 (m, 4H), 7.24–7.47 (m, 9H), 7.70 (s, 1H), 8.93 (s, 1H). ^{31}P NMR (202 MHz, $CDCl_3$) δ: 148.1, 149.1, 149.7. HRMS (ESI): Calcd for $C_{44}H_{54}N_5NaO_9P$ [MNa^+] 850.3557, found 850.3556.

Synthesis of oligonucleotides

Phosphoramidites **4a**–**4h**, dT-phosphoramidite (Sigma), Ac-dC-phosphoramidite (Glen Research), Pac-dA-phosphoramidite (Glen Research), iPr-Pac-dG-phosphoramidite (Glen Research), and Ac-d^{m}C-phosphoramidite (Sigma) were dissolved in anhydrous MeCN to a final concentration of 0.1 M. **ON1**–**18** was synthesized on a 0.2 μmol scale by using an automated DNA synthesizer (Gene Design nS-8II), with 0.25 M 5-(ethylthio)-1*H*-tetrazole in MeCN as an activator. The modified phosphoramidites **4a**–**4h** were incorporated into oligonucleotides at an extended coupling time of 10 min. The oligonucleotides, synthesized in trityl-on mode, were cleaved from the CPG resin by treatment with 50 mM K_2CO_3 in anhydrous MeOH at room temperature for 1.5 h. All the protecting groups of the oligonucleotides were removed by treatment with 50 mM K_2CO_3 in anhydrous MeOH at room temperature for 4 h. After neutralization with 2 M triethylammonium acetate buffer (pH 7.0), MeOH was removed *in vacuo*. The crude oligonucleotides were purified by Sep-Pak® Plus C18 cartridges (Waters), and the 5′-DMTr group was removed by treatment with 1% (v/v) aqueous trifluoroacetic acid in purification. The separated oligonucleotides were further purified by reversed-phase HPLC (Waters XBridge® MS C18 Column 5 μm, 10 × 50 mm). The compositions of the new oligonucleotides (**ON1**–**8** and **ON10**–**17**) were confirmed by ESI-TOF mass analysis. The deconvoluted ESI-TOF mass data [M] for **ON1**–**8** and **ON10**–**17** were as follows: **ON1**, found 3752.90 (calcd 3752.48); **ON2**, found 3776.90 (calcd 3776.51); **ON3**, found 3796.90 (calcd 3796.54); **ON4**, found 3780.90 (calcd 3780.54); **ON5**, found

3815.00 (calcd 3814.56); **ON6**, found 3829.00 (calcd 3828.58); **ON7**, found 3750.90 (calcd 3750.51); **ON8**, found 3694.80 (calcd 3694.45); **ON10**, found 4278.50 (calcd 4277.89); **ON11**, found 4302.40 (calcd 4301.91); **ON12**, found 4322.40 (calcd 4321.94); **ON13**, found 4306.20 (calcd 4305.94); **ON14**, found 4340.40 (calcd 4339.96); **ON15**, found 4354.40 (calcd 4353.99); **ON16**, found 4276.20 (calcd 4275.92); **ON17**, found 4220.40 (calcd 4219.85). All natural DNA, RNA, and hairpin dsDNA for the UV-melting experiments were purchased from GeneDesign, Inc.

UV-melting experiments

For UV-melting experiments using the duplexes formed by **ON1**–**9** and ssRNA (or ssDNA), the 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 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 accurate to within 1 °C.

For UV-melting experiments using the triplexes formed by **ON10**–**18** and dsDNA, the oligonucleotides were dissolved in 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM KCl and 10 mM $MgCl_2$ to give 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 obtain the T_m values and the final values were determined by averaging three independent measurements accurate to within 1 °C.

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