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Synthesis and structural characterization of diastereomeric isomers of RNA trimer adenylyl(3′-5′)adenylyl(3′-5′)adenosine

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Abstract—We have synthesized the diastereomeric isomers of adenylyl(3′-5′)adenylyl(3′-5′)adenosine (ApApA), and investigated their helical structures and hybridization properties with D-poly(U) by circular dichroism (CD) and UV melting experiments. The configuration of the 5′-end residue of ApApA has little effect on the helical structure. The configuration of the 3′-end residue has little effect on the stability of the triplex, and the chiral modifications cause complicated effects on the helical structure of ApApA and its triplex-forming ability with D-poly(U), depending on the site of the modification.

1. Introduction

Biological molecules such as sugars and amino acids are chiral, and hence enantiomers exist. However, organisms strictly discriminate between enantiomers, and usually synthesize and use only one of them. In particular, organisms strictly discriminate both enantiomers of ribose, the chiral component of nucleic acids. Nucleic acids consist of only D-ribose as the sugar unit, and the L-isomer has not yet been found in nature. It was thus considered that the strict chiral selection during the early stage of chemical evolution would have been essential for the emergence of functional RNA molecules. Since the chiral homogeneity is thought to be essential for the formation of higher order structures of biopolymers and their interaction with specific ligands, it would be useful to understand the structures and properties of chirally modified RNA molecules for considering the establishing process and the significance of the homochirality of RNA. Several groups have reported the structure and properties of mirror image DNAs[1,2] and RNAs.[3–8] However, there is little information about the structure and properties of heterochiral nucleic acids,[9–16] especially heterochiral RNAs.[17,18] In this context, we have investigated the helical structure of the diastereomeric isomers of adenylyl(3′-5′)adenosine (ApA).[17] Ts'o et al. reported the properties of unnatural homochiral L-(ApA), which adopts a left-handed helical structure and forms a right-handed triplex with D-poly(U), and the triplex is less stable than that with natural D-(ApA).[19] We found that heterochiral ALpAD and ADpAL form the right- and left-handed helical structure, respectively. ALpAD formed the triplex with D-poly(U) while its thermal stability was quite similar to the D-(ApA)·D-poly(U) triplex, whereas the triplex of ADpAL with D-poly(U) showed a quite similar stability to that of L-(ApA), whose stabilities were significantly lower than those of D-(ApA) and ALpAD.[17] This means that the configuration of the 3′-end residue of ApA primarily determines the helical sense of ApA and its triplex-forming ability with poly(U). Here, we report the synthesis of the eight diastereomeric isomers of ApApA and the structural characterization, in particular, the effects of configuration of each residue on the helical structure of ApApA.

2. Results and discussion

2.1. Synthesis of trimers

The diastereomeric isomers of ApApA were synthesized by a phosphotriester method[20] as shown in Schemes 1–5. D- and L-N^6-Benzoyladenosines were converted to 2′,3′-dibenzoates **3** and **10**, as well as 2′-*O*-tetrahydrofuranyl-protected 3′-nucleotides **7** and **14**. Compounds **3** and **10** were allowed to condense with either **7** or **14** in the presence of MSNT [1-(2-mesitylenesulfonyl)-3-nitro-1,2,4-triazolide] followed by deprotection of the

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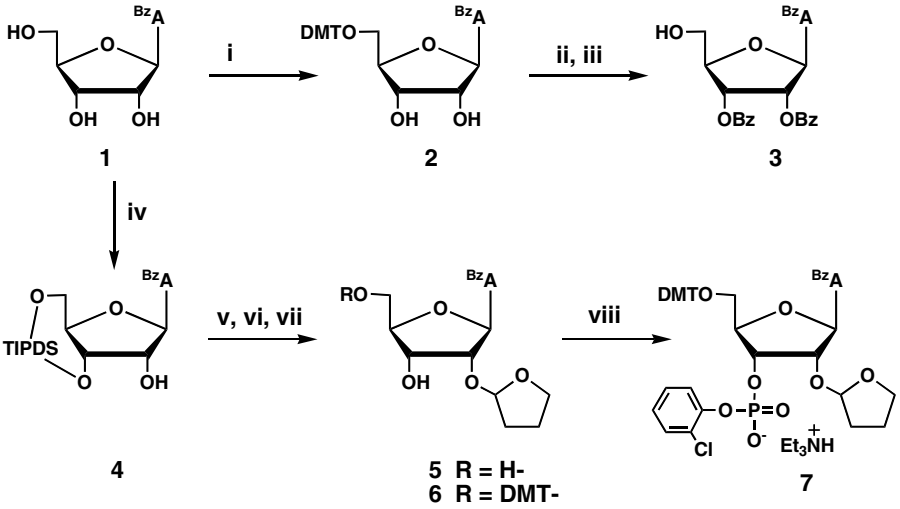
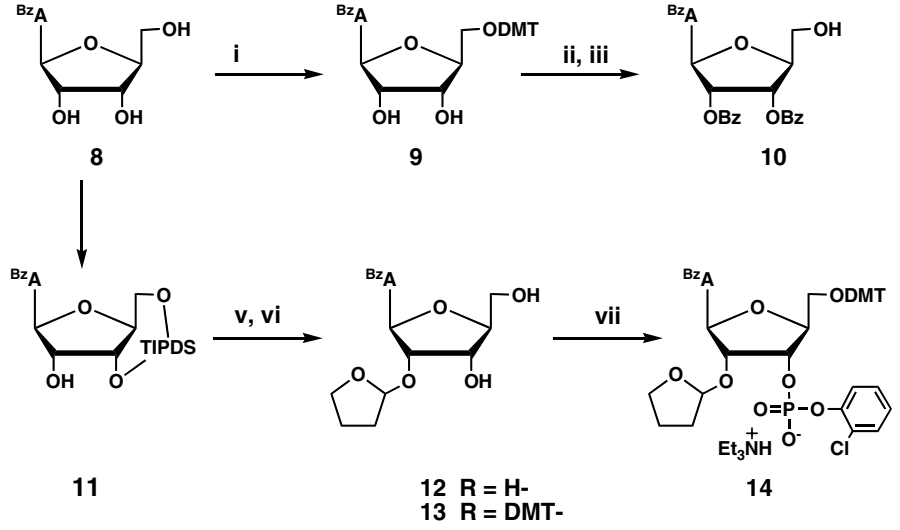


Scheme 1. Reagents and conditions: (i) DMT–Cl, pyridine; (ii) Bz_2O, DMAP, CH_2Cl_2; (iii) 2% benzenesulfonic acid, MeOH/CH_2Cl_2; (iv) 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, pyridine; (v) 2,3-dihydrofuran, PPTS, CH_2Cl_2; (vi) 1 M TBAF/THF; (vii) DMT–Cl, pyridine; (viii) *o*-chlorophenylphosphorditriazolide, pyridine, dioxane.



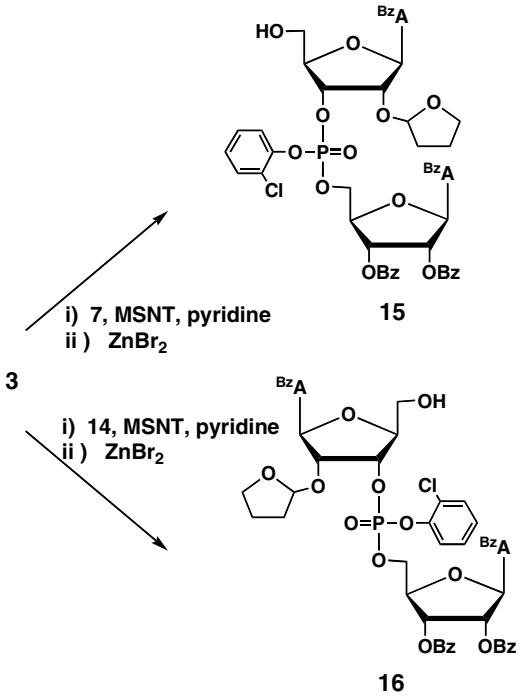
Scheme 2. Reagents and conditions: (i) DMT–Cl, pyridine; (ii) Bz_2O, DMAP, CH_2Cl_2; (iii) 2% benzenesulfonic acid, MeOH/CH_2Cl_2; (iv) 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane, pyridine; (v) 2,3-dihydrofuran, PPTS, CH_2Cl_2; (vi) 1 M TBAF/THF; (vii) DMT–Cl, pyridine; (viii) *o*-chlorophenylphosphorditriazolide, pyridine, dioxane.

DMT group to give 5′-OH free dimers **15**–**18**. Subsequently, these dimers were allowed to condense with either **7** or **14** in the presence of MSNT to give fully protected trimers **19**–**26**. After deprotection of these trimers with concentrated ammonium hydroxide at 55 °C for 8 h, followed by 80% acetic acid treatment, the purification was conducted by using reversed phase HPLC. The purities of the trimers were more than 95%.

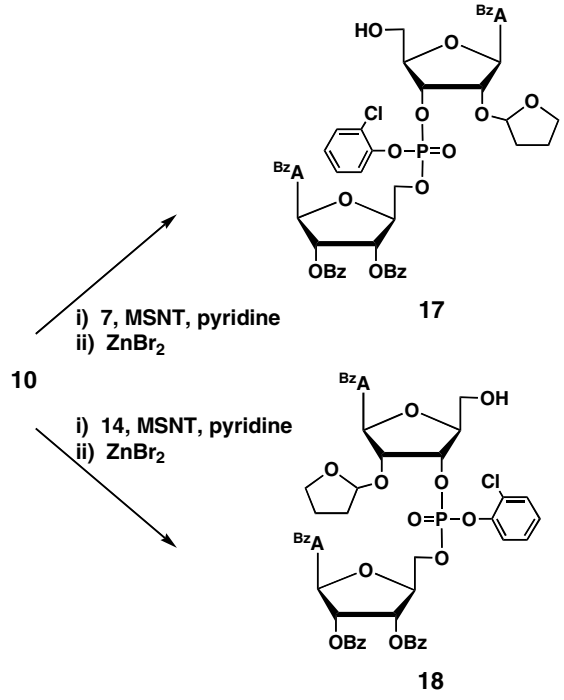
2.2. Characterization of trimers

The reactivity of nuclease P1 and snake venom phosphodiesterase (SVPD) towards each isomer of diastereomeric ApAs has been well characterized.[17] Nuclease P1 cleaves the phosphodiester bonds at the P–O(3′) bond of AdpAd and AdpAl, whereas SVPD cleaves the phosphodiester bonds at the P–O(3′) bond of AdpAd and AlpAd. The trimers other than AlpAlpAd, AdpAlpAl and l-(ApApA) could be completely hydrolyzed to adenosine and 5′-AMP by these enzymes, and their molar extinction coefficients were determined by comparing absorbances before and after the hydrolysis (Table 1).

We tried to characterize the isomers of ApApA by analyzing the digestion products obtained by the reaction with these enzymes. However, SVPD, which is a 3′-exonuclease, showed the unexpected reactivity for trimers that contain the l-adenosine residue at the 3′-end. An example is shown in Figure 1. AdpAdpAl was expected to be resistant to digestion by SVPD, however, the reaction afforded degradation products in which both of the phosphodiester bonds were cleaved (Fig. 1). Damha et al. reported that SVPD cleaves l-oligomers very

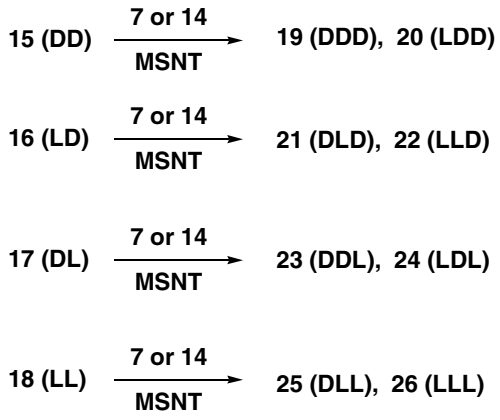


Scheme 3.



Scheme 4.

slowly compared to the corresponding D-oligomers and also *endonucleolytically* cleaves the heterochiral oligomers that contain the L-nucleotide residue at the 3′-end, such as d(TTTTTC$_L$).[13] Our findings seem to be similar to those reported by Damha et al. We tested several enzymes (nuclease S1, micrococcal nuclease, RNase T_2) from commercial sources, however, we could not find another enzyme that hydrolyzed the phosphodiester bonds of ALpAD as well as SVPD does.



Scheme 5.

Table 1. Hypochromicity and molar extinction coefficients of the trimers

Trimer	Hypochromicity (%)	ε at 260 nm (L/mol cm)
D-(ApApA)	26.9	35,400
ALpADpAD	22.0	36,800
ADpALpAD	21.9	36,800
ALpALpAD	—	36,300[a]
ADpADpAL	23.6	36,300
ALpADpAL	23.2	36,500
ADpALpAL	—	36,800[a]
L-(ApApA)	—	35,400[a]

[a] Not determined. The values were assumed to be the same as the corresponding enantiomers.

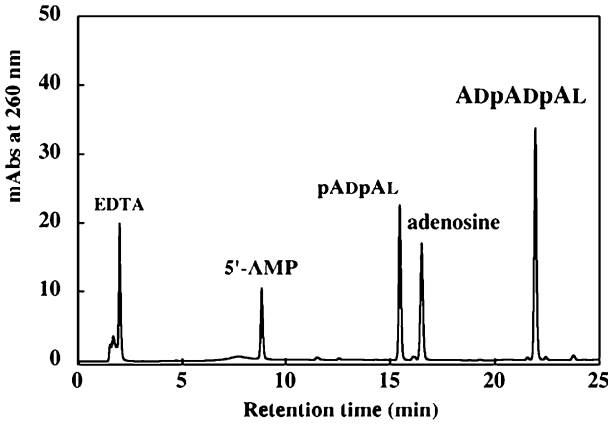


Figure 1. Reversed phase HPLC chromatogram of the reaction of ADpADpAL with SVPD.

We thus changed the strategy to characterize the trimers. Each trimer was hydrolyzed with aqueous potassium hydroxide solution to give adenosine, 2′- and 3′-AMPs, and the resulting mixture treated with calf intestine alkaline phosphatase (CIAP) to give adenosine. The enantiomers of adenosine were then separated by using chiral HPLC to evaluate the molar ratios of D- and L-adenosines (Fig. 2), and the trimers classified by the

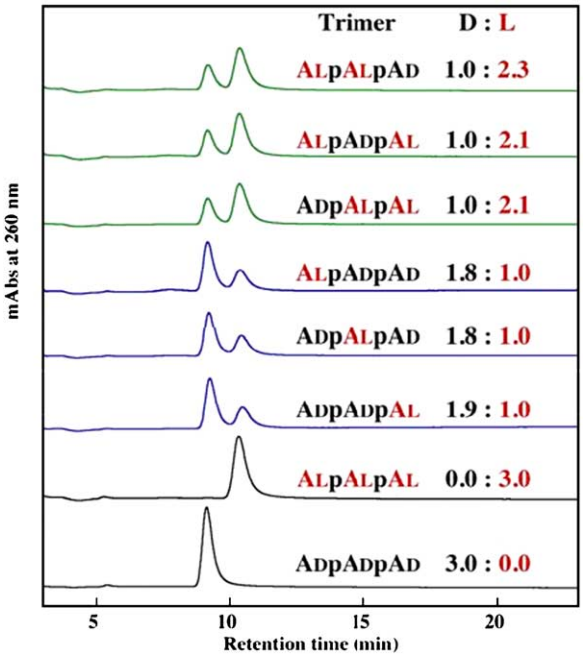


Figure 2. HPLC chromatograms for the chiral resolution of D- and/or L-adenosine obtained by the alkaline hydrolysis of the trimers followed by treatment with APH.

D/L ratios of their constituent adenosine as shown in Table 2, in which the expected degradation products by nuclease P1 for each trimer are also shown. Although some trimers afford the same degradation products as each other by treatment with nuclease P1, for example, both ADpADpAD and ADpADpAL afford 5′-AMP and adenosine, the classification of the trimers by the D/L ratios made it possible to distinguish between them by reversed phase HPLC. Figure 3 shows the reversed phase HPLC chromatograms of the trimers after nuclease P1 digestion. All the trimers gave the expected degradation products and were successfully characterized by a combination of the classification by the D/L ratios and nuclease P1 treatment.

2.3. CD spectra of trimers

In order to understand the effects of chiral modification on the helical structure of ApApA, CD experiments were conducted with the results shown in Figure 4. The natural trimer D-(ApApA) shows a typical conservative CD spectrum by exciton interaction, with positive and negative bands at 267 and 250 nm, respectively. The positive sign of the long-wavelength component of the conservative bands indicates a right-handed helical twist of the transition moment of the bases when the bases are nearly perpendicular to the helix axis.[21] The spectrum of L-(ApApA) shows the opposite sign but the same intensity as that of D-(ApApA). These results suggest that D-(ApApA) has a right-handed helical sense and L-(ApApA) is its mirror image, as expected. In the case of the

Table 2. Classification of the trimers by the D/L ratios of constituent adenosine and expected products after nuclease P1 digestion[a]

D/L Ratio	Trimer	Expected products after nuclease P1 digestion
D:L = 3:0	D-(ApApA)	5′-AMP + adenosine
D:L = 0:3	L-(ApApA)	L-(ApApA)
D:L = 2:1	ALpADpAD	5′-AMP + ALpAD
	ADpALpAD	Adenosine + pALpAD
	ADpADpAL	5′-AMP + adenosine
D:L = 1:2	ALpALpAD	ALpALpAD
	ALpADpAL	5′-AMP + ALpAD
	ADpALpAL	Adenosine + pALpAL

[a] The configuration of the monomers in the expected degradation products are not indicated because of the inability of the reversed phase HPLC in separating enantiomers.

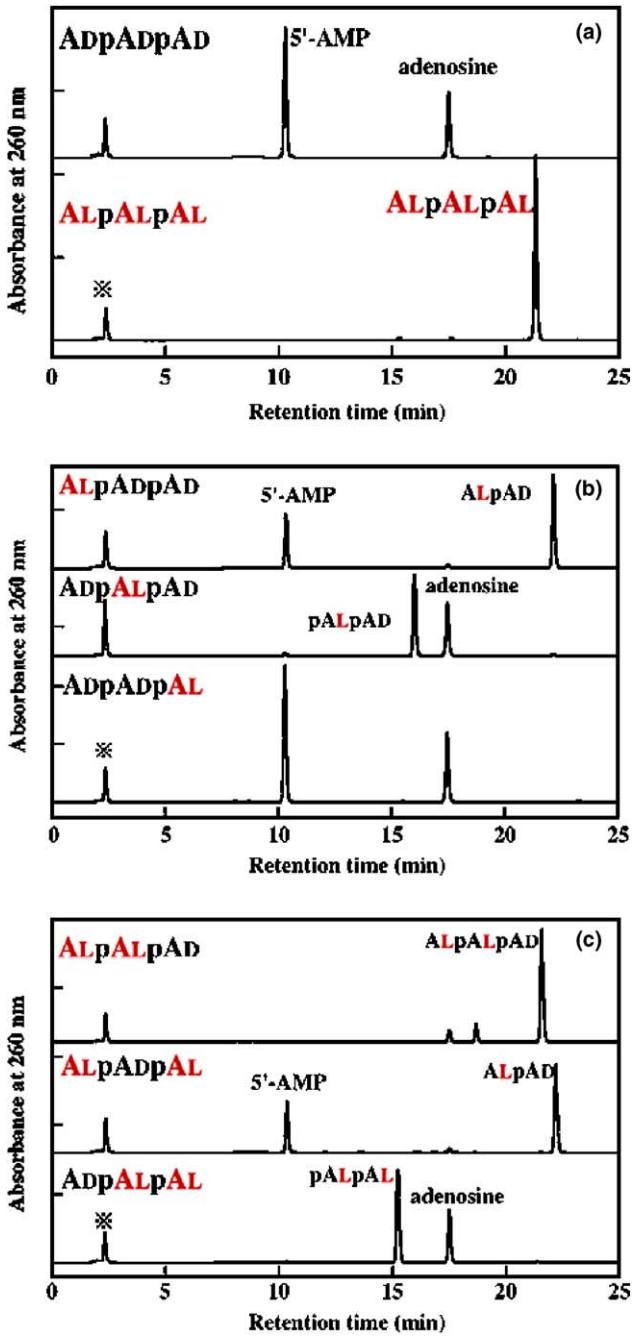


Figure 3. Reversed phase HPLC chromatograms for the reactions of the trimers with nuclease P1. D/L ratios are (a) 3:0 or 0:3, (b) 2:1 and (c) 1:2.

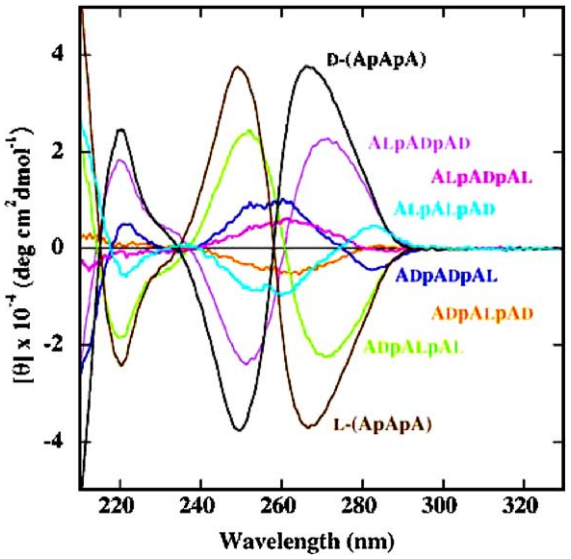


Figure 4. CD spectra of the trimers at 0 °C. Samples contained 40 μM trimer in 0.1 M NaCl, 10 mM sodium phosphate, pH 7.0.

heterochiral trimers, ALpADpAD and ADpALpAL also exhibit spectra, which are symmetrical to each other. Although these trimers show somewhat decreased CD intensity when compared to D- and L-(ApApA), the spectra are still considered to be conservative. Thus, it is very likely that ALpADpAD has the right-handed helical sense, whereas ADpALpAL has the left-handed helical sense. Conversely, the other heterochiral ApApAs exhibit the non-conservative CD spectra in extremely weakened strength. It is thus unlikely that these trimers form a definite helical structure. These results mean that the trimers possessing the same chirality at the internal and 3′-end residues form the definite helical structure, in other words, the chirality of the 5′-end residue of ApApA hardly affects the helical structure, whereas that of the other residues is essential for the formation of the helical structure of ApApA.

2.4. Triplex stability of trimers with D-poly(U)

Figure 5 shows UV mixing curves of the ApApA isomers with D-poly(U). All the cases showed an absorption minimum at A:U = 1:2, and it is thus very likely that all the ApApA isomers form a triple helix with D-poly(U), as seen in the case of the diastereomeric isomers of ApA.[17] Figure 6 shows the CD spectra of these triplexes, which indicate that all the triplexes adopt a right-handed helical structure. To evaluate the thermal stability of each ApApA-poly(U) triplex, UV melting experiments were conducted with the results shown in Figure 7a. The triplexes other than ADpALpAD·2poly(U) and ADpALpAL·2poly(U) show a cooperative sigmoidal curve, suggesting a two-state transition from the triplex to the single strand. However, ADpALpAD·2poly(U) and ADpALpAL·2poly(U) reveal a non-cooperative curve, which means a complex melting process. Figure 7b shows the first derivative plots of the melting curves of Figure 7a. ADpALpAD·2poly(U) exhibits two maxima (7 and 18 °C) as well as ADpALpAL·2poly(U) (7 and 12 °C). The presence of these two maxima means that two kinds of complex formations between the trimers and D-poly(U) exist. The maximum at 7 °C suggests a very weak complex formation between the trimers and D-poly(U).

Table 3 shows the T_m values of the triplexes and their relationship with the configuration of each residue of the trimers. With respect to the configuration of the internal residue, the trimers possessing the D-configuration at the internal residue form the more stable triplexes with D-poly(U) than those possessing L-configuration at the same site. Among the trimers possessing the internal D-configuration, those possessing D-configuration at the 5′-end residue (DDD and DDL) form the more stable

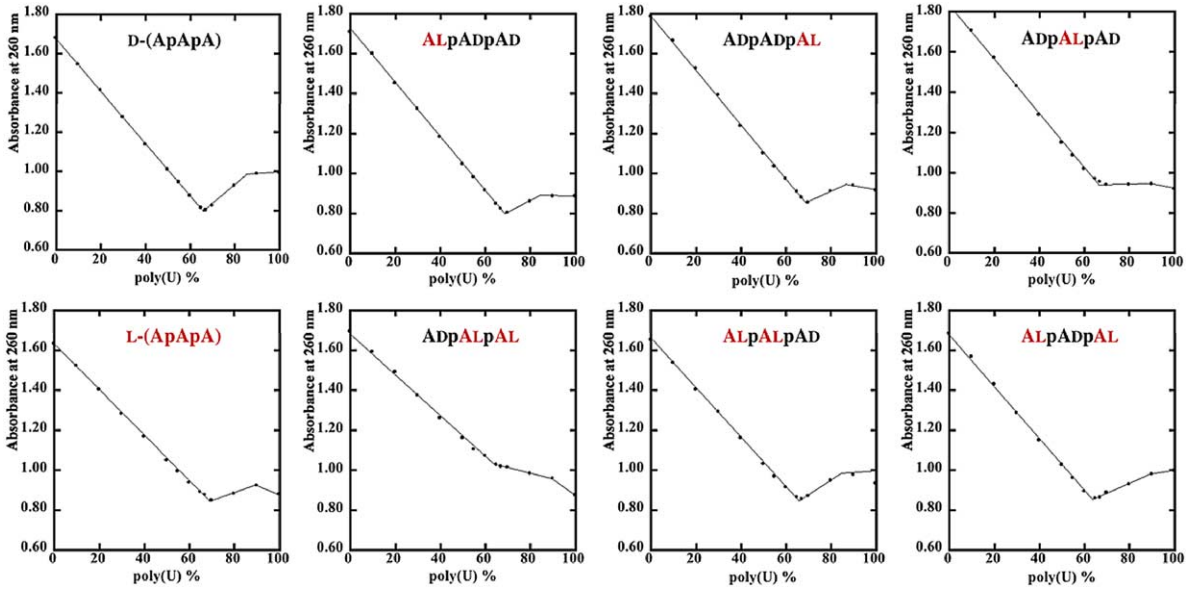


Figure 5. UV mixing curves of the trimers with D-poly(U) in 10 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5 at 0 °C. Total base concentration is 150 μM.

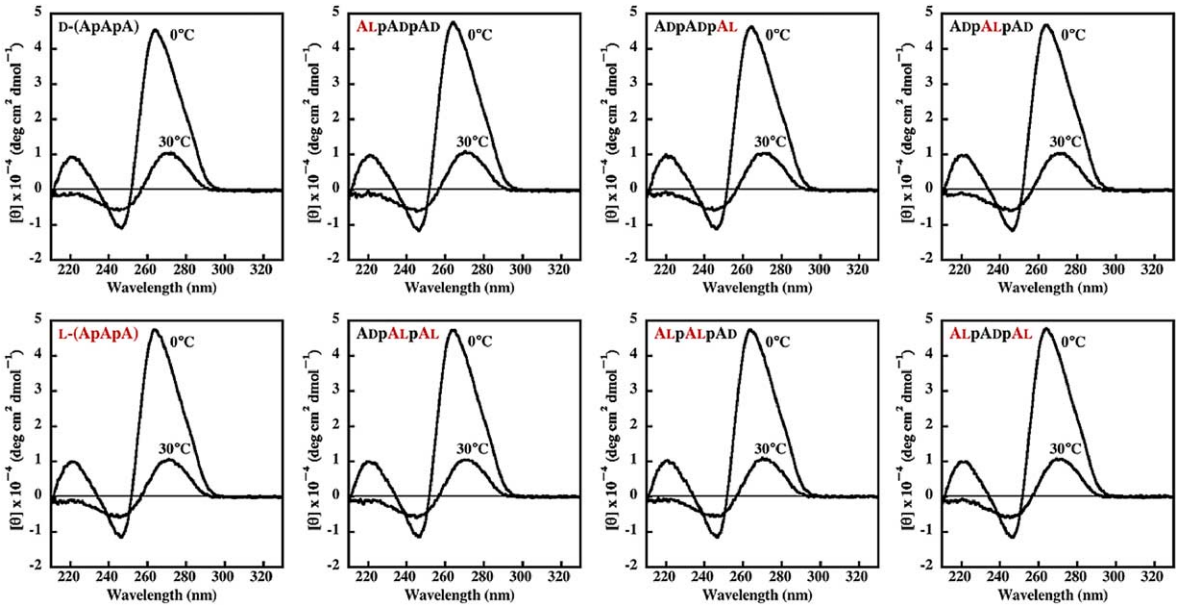


Figure 6. CD spectra of the triplexes of the trimers with D-poly(U) in 10 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5 at 0 °C. Total base concentration is 90 μM.

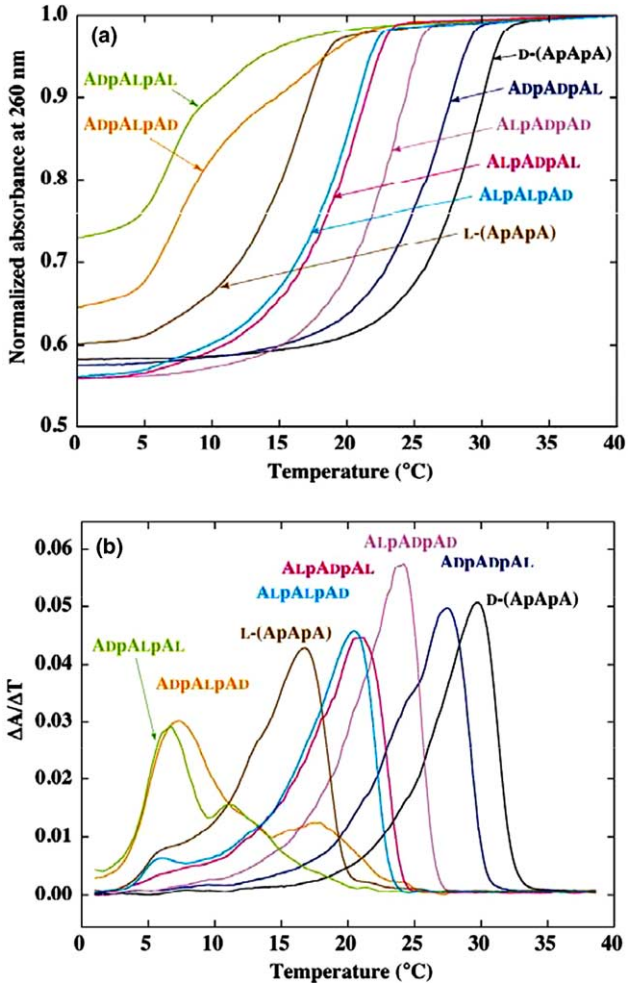


Figure 7. (a) UV melting profiles of the triplexes of the trimers with D-poly(U). Base concentrations for trimer and D-poly(U) are 30 and 60 μM, respectively, in 10 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5. (b) First derivative plots of the UV melting curves of the triplexes of the trimers with D-poly(U).

Table 3. Relationship between the configuration of each residue of the trimers and their triplex stability with D-poly(U)

Trimer	Chirality	T_m (°C)
ADpADpAD	DDD	29.8
ADpADpAL	DDL	27.4
ALpADpAD	LDD	24.2
ALpADpAL	LDL	21.1
ALpALpAD	LLD	20.5
ALpALpAL	LLL	16.7
ADpALpAD	DLD	7.3
ADpALpAL	DLL	6.6

triplexes with D-poly(U) than those possessing L-configuration at the same site (LDD, LDL). However, among the trimers possessing the internal L-configuration, those possessing L-configuration at the 5′-end residue (LLD, LLL) form the more stable triplexes with D-poly(U) than those possessing D-configuration at the same site (DLD, DLL). Therefore, the same configuration at the 5′-end and internal residues is somewhat more advantageous for the triple helix formation of ApApA with D-poly(U) than the different configuration at the same sites.

In the case of the ApA diastereomeric isomers, D-(ApA) and ALpAD were considered to have the right-handed helical sense, whereas L-(ApA) and ADpAL were considered to have the left-handed helical sense from their CD spectra. Furthermore, the triplexes of D-(ApA) and ALpAD with D-poly(U) showed similar melting behaviors (T_m value; 14.7 and 13.7 °C, respectively), while their stability was remarkably higher than that of the triplexes of L-(ApA) and ADpAL with D-poly(U), which showed a similar thermal stability (T_m value; 5.7 and 6.6 °C, respectively).[17] It was thus thought that the configuration of the 3′-end residue of the dimers primarily determines the helical sense of ApA and its triplex stability

with D-poly(U). The present results indicate that the chiral modifications cause complicated effects on the helical structure of ApApA and its ability for the triplex formation with D-poly(U). Compared with homochiral D-(ApApA) and L-(ApApA), the CD strengths of ALpAD-pAD and ADpALpAL are significantly decreased, although the spectra show the conservative Cotton band as D-(ApApA) and L-(ApApA) (Fig. 4), respectively. Thus, the chiral modification at the 5′-end residue does not induce such a dramatic structural alteration for ApApA. However, the chiral modifications at the internal and 3′-end residues induce dramatic distortions on the helical structure. ADpADpAL, ADpALpAD and their enantiomers show the CD spectra in extremely weakened strength, which are no longer conservative, and the trimers are not considered to form a definite helical structure. Therefore, the configuration of the internal and 3′-end residues is a crucial factor for maintaining the helical structure of ApApA. From the viewpoint of the triplex formation with D-poly(U), the configuration of the internal and 5′-end residues of ApApA plays important roles for the stability, while the configuration of the 3′-end residue had little effect on the triplex stability.

In the template-directed oligomerization of activated mononucleotides, oligomerization of the monomers proceeded in the 5′→3′ direction, and incorporation of an L-nucleotide at the 3′-end of oligomers prevents further elongation by incorporation of either monomer.[22] The trimers without stable triplex-forming ability and the stable triplex-forming trimers with an L-nucleotide at the 3′-end are thought to be inactive for further elongation. Furthermore, the stable triplex-forming trimers with an L-nucleotide at the 3′-end would compete with the trimers proper for the elongation on the template. These characteristics of heterochiral oligomers may lead to an extremely reduced efficiency of template-directed oligomerization of racemic mononulceotides.[22]

3. Conclusion

In conclusion, the results reported herein indicate that the homochirality of the internal and 3′-end residues is the primary factor for maintaining the helical structure of ApApA, whereas the configuration of the 3′-end residue has little effect on the triplex formation. Thus, the chiral modifications result in the different influences on the helical structure of ApApA and on its triplex-forming ability with D-poly(U). These findings would provide useful information for the application of L-RNA to the development of novel functional molecules and for also considering the process of the chiral selection during the chemical evolution of RNA.

4. Experimental

4.1. General

TLC analyses were carried out on Merk silica gel $60F_{254}$ plates, which were visualized by UV illumination at 254 nm and spraying of diluted aqueous sulfuric acid solution, followed by heating. For silica gel column chromatography, either Wakogel FC-40 or C-400HG (Wako Pure Chemical Industries) was used. The elution was carried out by increasing the amounts of methanol in chloroform unless otherwise noted. ^{1}H NMR spectra were measured on either a Varian Gemini 200 or Mercury 300 spectrometer with tetramethylsilane as the internal standard. Nuclease P1, snake venom phosphodiesterase (SVPD) and calf intestine alkaline phosphatase (CIAP) were purchased from Yamasa Co. (Chiba, Japan), Roche Diagnostics (Mannheim, Germany) and Takara Bio Inc. (Kyoto, Japan), respectively. Reversed phase HPLC was performed on a column of μBondasphere C18 5 μm 100 Å column (3.9 × 150 mm, Waters), and the elution was performed with a linear gradient of acetonitrile (0–10%) in 50 mM potassium phosphate (pH 4.0) on a Shimadzu LC-10A HPLC system. The synthesis of L-ribose was reported previously.[23] L-Ribose was converted to 1-*O*-acetyl-2,3,5-*O*-tribenzoyl-L-ribofuranose according to the literature procedure for the corresponding D-isomer.[24] N^6-Benzoyl-L-adenosine was synthesized by the trimethylsilyl trifluoromethanesulfonate-catalyzed coupling of silylated N^6-benzoyladenine with 1-*O*-acetyl-2,3,5-*O*-tribenzoyl-L-ribofuranose.[25]

4.2. Synthesis of trimers

4.2.1. 5′-*O*-(4,4′-Dimethoxytrityl)-N^6-benzoyl-D-adenosine 2. A solution of **1** (2.97 g, 8.0 mmol) and 4,4′-dimethoxytritylchloride (3.25 g, 9.6 mmol) in dry pyridine (40 mL) was stirred at room temperature for 1 h. After the addition of EtOH (4 mL), the solvent was evaporated under reduced pressure. The residue was extracted with chloroform and washed with satd $NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated. The residue was chromatographed on a silica gel (100 g) to give **2** (4.74 g, 87.9%).

4.2.2. N^6,2′,3′-*O*-Tribenzoyl-D-adenosine 3. A solution of **2** (5.34 g, 7.92 mmol), benzoic anhydride (5.34 g, 23.6 mmol), triethylamine (5.21 mL, 23.6 mmol) and 4-dimethylaminopyridine (575 mg, 4.72 mmol) in dry dichloromethane (47 mL) was stirred at room temperature for 1 h. After the addition of EtOH (4 mL), the solution was diluted with chloroform and washed with satd $NaHCO_3$ and 0.5 M KH_2PO_4 aqueous solutions. The organic layer was dried over Na_2SO_4 and concentrated. The residue was dissolved in chloroform (140 mL) and the solution cooled with an ice bath and treated with benzenesulfonic acid solution in MeOH (6.67%, 60 mL). After stirring at 0 °C for 15 min, the reaction was quenched by adding satd $NaHCO_3$ aqueous solution. The solution was diluted with chloroform and washed with satd $NaHCO_3$ aqueous solution. The organic layer was dried over Na_2SO_4 and concentrated. The residue was chromatographed on a silica gel (100 g) to give **3** (3.96 g, 86.3%).

4.2.3. 3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxanylidene)-N^6-benzoyl-D-adenosine 4. A solution of **1** (0.37 g, 1.0 mmol) and 1,1,3,3-tetraisopropyl-1,3-dichlorodisiloxane (0.38 mL, 1.20 mmol) in dry pyridine (40 mL) was

stirred at room temperature overnight. After the addition of H_2O (2 mL), the solvent was evaporated under reduced pressure. The residue was extracted with chloroform and washed with satd $NaHCO_3$ aqueous solution. The organic layer was dried over Na_2SO_4 and concentrated. The residue was chromatographed on a silica gel (20 g) to give **4** (0.61 g, 99.3%).

4.2.4. 2′-*O*-Tetrahydrofuranyl-*N*^{6}-benzoyl-D-adenosine 5. A solution of **4** (1.01 g, 1.65 mmol), 2,3-dihydrofuran (0.25 mL, 3.36 mmol) and pyridinium *p*-toluenesulfonate (0.084 g, 0.34 mmol) in dry dichloromethane (10 mL) was stirred at room temperature overnight. After addition of satd $NaHCO_3$ aqueous solution, the solution was diluted with chloroform. The mixture was washed with satd $NaHCO_3$ aqueous solution. The organic layer was dried over Na_2SO_4 and concentrated. The residue was treated with 1 M tetrabutylammonium fluoride in THF solution (3.3 mL) for 10 min. The solvent was evaporated under reduced pressure and the residue chromatographed on a silica gel (30 g) to give **5** (0.70 g, 95.6%).

4.2.5. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-tetrahydrofuranyl-*N*^{6}-benzoyl-D-adenosine 6. A solution of **5** (0.21 g, 0.48 mmol) and 4,4′-dimethoxytritylchloride (0.195 g, 0.58 mmol) in dry pyridine (3 mL) was stirred at room temperature for 2 h. After the addition of EtOH (0.5 mL), the solvent was evaporated under reduced pressure. The residue was extracted with chloroform and washed with satd $NaHCO_3$ aqueous solution. The organic layer was dried over Na_2SO_4 and was concentrated. The residue was chromatographed on a silica gel (10 g) to give **6** (0.33 g, 92.3%).

4.2.6. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-tetrahydrofuranyl-*N*^{6}-benzoyl-D-adenosine 3′-*O*-(2-chlorophenyl)phosphate 7. Compound **6** (0.10 g, 0.13 mmol) was coevaporated with dry pyridine three times, and treated with 0.33 M 2-chlorophenylphosphoroditriazolide solution in dioxane (0.83 mL, 0.27 mmol). After 2 h, 50 mM triethylammonium bicarbonate buffer (TEAB, pH 7.5, 2 mL) was added, and the mixture extracted with chloroform–pyridine (3:1) and washed with 50 mM TEAB. The organic layer was evaporated under reduced pressure and the residue was dissolved in small amount of chloroform. The solution was added dropwise to 0.1% triethylamine in *n*-hexane (50 mL) to give **7** (0.158 g, quant.).

4.2.7. 5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{6}-benzoyl-L-adenosine 9. Yield: 0.29 g, 85.0%.

4.2.8. *N*^{6},2′,3′-*O*-Tribenzoyl-L-adenosine 10. Yield: 0.23 g, 92.3%.

4.2.9. 3′,5′-*O*-(1,1,3,3-Tetraisopropyldisiloxanylidene)-*N*^{6}-benzoyl-L-adenosine 11. Yield: 0.84 g, 91.2%.

4.2.10. 2′-*O*-Tetrahydrofuranyl-*N*^{6}-benzoyl-L-adenosine 12. Yield: 0.455 g, 75.3%.

4.2.11. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*- tetrahydrofuranyl-*N*^{6}-benzoyl-L-adenosine 13. Yield: 0.733 g, 95.7%.

4.2.12. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-tetrahydrofuranyl-*N*^{6}-benzoyl-L-adenosine 3′-*O*-(2-chlorophenyl)phosphate 14. Yield: 1.24 g, quant.

4.2.13. 5′-Free dimer 15. A mixture of **3** (0.107 g, 0.185 mmol) and **7** (0.23 g, 0.222 mmol) was coevaporated with dry pyridine and dissolved in dry pyridine (1.5 mL). After 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT, 0.132 g, 0.444 mmol) was added, the solution was kept at room temperature for 40 min. The mixture was diluted with chloroform and washed with 50 mM TEAB. The organic layer was evaporated under reduced pressure and the residue chromatographed on a silica gel (10 g) to give fully protected dimer (0.233 g, 84.2%).

The fully protected dimer (0.134 g, 0.09 mmol) was coevaporated with dry pyridine and dry toluene, and treated with 1 M $ZnBr_2$ solution in dichloromethane–*i*-PrOH (85:15) (5 mL, 5 mmol) at room temperature for 30 min. The reaction was quenched by adding 1 M aqueous ammonium acetate and diluted with chloroform. The organic layer was separated and washed with 1 M aqueous ammonium acetate. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was chromatographed on a silica gel (10 g) to give **15** (0.104 g, 97.2%).

4.2.14. 5′-Free dimer 16. Yield: 0.195 g, 90.4%.

4.2.15. 5′-Free dimer 17. Yield: 0.113 g, 86.0%.

4.2.16. 5′-Free dimer 18. Yield: 0.257 g, 90.5%.

4.2.17. Fully protected trimer 19. A mixture of **15** (0.11 g, 0.088 mmol) and **7** (0.138 g, 0.133 mmol) was coevaporated with dry pyridine and dissolved in dry pyridine (1.5 mL). After MSNT (0.079 g, 0.266 mmol) was added, the solution was kept at room temperature for 40 min. The mixture was diluted with chloroform and washed with 50 mM TEAB. The organic layer was evaporated under reduced pressure and the residue chromatographed on a silica gel (10 g) to give **19** (0.151 g, 80.6%).

4.2.18. Fully protected trimer 20. Yield: 0.205 g, 64.7%.

4.2.19. Fully protected trimer 21. Yield: 0.190 g, 77.8%.

4.2.20. Fully protected trimer 22. Yield: 0.176 g, 73.1%.

4.2.21. Fully protected trimer 23. Yield: 0.317 g, 77.0%.

4.2.22. Fully protected trimer 24. Yield: 0.259 g, 86.2%.

4.2.23. Fully protected trimer 25. Yield: 0.199 g, 83.8%.

4.2.24. Fully protected trimer 26. Yield: 0.233 g, 92.2%.

4.2.25. Deprotection and purification of trimers. The trimer (10–20 μmol) was suspended in pyridine (2 mL) and

concentrated ammonium hydroxide (10 mL). The reaction vessel was sealed and heated at 55 °C for 8 h. After cooling, the solvents were evaporated under reduced pressure and the residue treated with 80% aqueous acetic acid for 2 h. The mixture was concentrated and coevaporated with H_2O several times, and the residue dissolved in 20 mM TEAB and washed with diethyl ether. After the aqueous layer was lyophilized, the residue was purified on a C18 silica gel column (7×150 mm) with a linear gradient of acetonitrile (0–15%) in 50 mM triethylammonium acetate (TEAA, pH 7.0). Fractions containing the pure timer were collected and evaporated under reduced pressure, and the residue was then lyophilized several times to remove TEAA. The purity of the trimers tested by the above HPLC system was more than 95%.

4.3. Enzymatic digestion

For nuclease P1 digestion, solutions of the trimers (1 OD unit) in 50 mM ammonium acetate (pH 5.0, 18 μL) were treated with nuclease P1 (1 mg/mL, 2 μL). For SVPD digestion, solutions of the trimers (1 OD unit) in 10 mM $MgCl_2$ and 50 mM Tris–HCl (pH 8.0, 18 μL) were treated with SVPD (0.5 mg/mL, 2 μL). After the reactions were incubated at 37 °C for 3 h, 30 mM of EDTA (4 μL) was added. The mixtures were filtered through Ultrafree-MC 5000NMWL (Millipore) and analyzed by a reversed phase HPLC. Molar extinction coefficients of the trimers were determined by comparison of the absorbances of the trimer solutions before and after the complete enzymatic digestion into adenosine and 5′-AMP. The coefficients of the trimers (ALpApAD, ADpALpAL and ALpALpAL) that could not be cleaved completely by any of nuclease P1, SVPD or their combination were assumed as the same as those of the enantiomers.

4.4. Degradation of trimers into adenosine and its optical resolution

The trimers (1 OD unit) were treated with 0.1 M potassium hydroxide (200 μL) at 80 °C for 2.5 h. After the pH was adjusted to about 9.0 with 1 M HCl, a solution of 10 mM $MgCl_2$, 500 mM Tris–HCl (pH 9.0, 20 μL) and CIAP (0.5 units/μL, 2 μL) was added. After 3 h at 37 °C, the mixtures were desalted with a Sep-Pak Plus C18 cartridge (Waters), and analyzed by chiral HPLC. The chiral HPLC analyses were performed on a column of SUMICHIRAL OA-6000 (4.6×150 mm, Sumika Chemical Analysis Service, Japan) with an isocratic elution of 8% acetonitrile in 2 mM $CuSO_4$.

4.5. Measurements of CD spectra

For measurements of CD spectra of the trimers, a solution (30 μM) of each trimer containing 0.1 M NaCl and 10 mM sodium phosphate (pH 7.0) was placed in a 1 cm path-length quartz cell. For the measurements of the spectra of the ApApA·D-poly(U) triplexes, each trimer and poly(U) was mixed at a total base concentration of 90 μM with the molar ratio A:U = 1:2, and dissolved in a buffer containing 10 mM $MgCl_2$ and 10 mM Tris–HCl (pH 7.5, 4 mL). The solution was placed in a 1 cm path-length quartz cell. Spectra were measured on a JASCO J-820 spectropolarimeter equipped with a temperature control unit.

4.6. UV mixing curves

Each trimer and D-poly(U) was mixed with various molar ratios of the bases at the total base concentration of 150 μM. The samples were dissolved in 10 mM $MgCl_2$ and 10 mM Tris–HCl (pH 7.5, 3 mL), and the absorbance at 260 nm of the solutions was measured at 0 °C.

4.7. Measurements of melting curves

Each trimer and D-poly(U) was mixed at a total base concentration of 90 μM with the molar ratio A:U = 1:2, and dissolved in a buffer containing 10 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.5, 4 mL). The solutions containing triplexes were heated at 80 °C and cooled gradually to room temperature. Melting curves were obtained at least twice by measuring the absorbance at 260 nm every 0.2 °C on a JASCO Ubest-55 spectrophotometer. The temperature was raised at a rate of 0.5 °C/min, and the T_m values were obtained by the first-derivative plots of the melting curves.

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