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Double-stranded oligonucleotides containing 5-aminomethyl-2′-deoxyuridine form thermostable anti-parallel triplexes with single-stranded DNA or RNA

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ABSTRACT

This Letter describes the synthesis and properties of double-stranded antisense oligonucleotides connected with a pentaerythritol linker. We found that double-stranded antisense oligonucleotides with aminomethyl residues have high affinity for single-stranded DNA or RNA in buffer solutions with and without $MgCl_2$. Thus, these oligonucleotides would be useful as antisense oligonucleotides for targeting single-stranded RNA through triplex formation.

Thanks to the discovery of a large number of non-coding RNAs, gene suppression by using antisense oligonucleotides has once again attracted much attention.[1–3] An antisense oligonucleotide binds to the target mRNA via Watson–Crick hydrogen bonds, whereas an antigene oligonucleotide binds to the major groove of double-stranded DNA by Hoogsteen or reverse Hoogsteen hydrogen bonds and forms a local triple helix (triplex).[4] Recently, we reported an approach whereby single-stranded DNA or RNA is targeted through triplex formation by using a branched oligonucleotide.[5–7]

Approaches using branched oligonucleotides can be classified into 2 categories based on the orientation of the triplexes: one method utilizes parallel triplexes, in which the third strand binds to the second strand via Hoogsteen hydrogen bonds (Fig. 1a), whereas the other method utilizes antiparallel triplexes, where the third strand binds to the second strand via reverse Hoogsteen hydrogen bonds (Fig. 1b). In a previous report, we described the synthesis of double-stranded antisense oligonucleotides connected with a pentaerythritol linker that could target single-stranded DNA or RNA by forming parallel triplexes.[5–7] It was found that the double-stranded oligonucleotides formed more thermally stable triplexes than the corresponding Watson–Crick duplex with a single-stranded RNA.[6,7] This suggests that the double-stranded oligonucleotides are useful as an antisense oligonucleotide. In this Letter, we describe the synthesis and properties of double-stranded antisense oligonucleotides that can target single-stranded DNA or RNA by forming antiparallel triplexes.

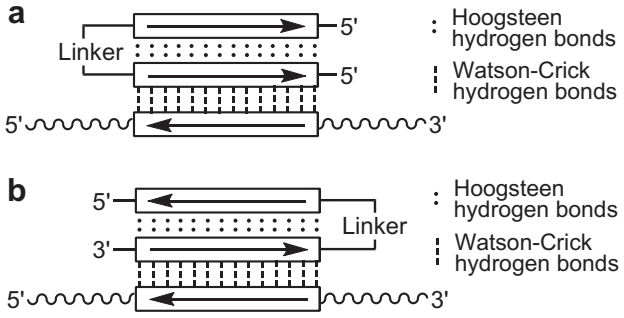

Figure 1. Schematic representation of branched oligonucleotides binding to single-stranded nucleic acids.

Thus far, oligonucleotide analogs carrying various polyamines have been synthesized,[8–14] and some of them have been shown to increase the thermal stability of duplexes and parallel triplexes. However, to the best of our knowledge, the stabilization effects of aminoalkyl modifications on antiparallel triplex formation have not been examined. X-ray crystal structure analysis of antiparallel triplexes showed that the 5-position of the thymidine residues of the third strand is positioned very close to the phosphate backbone

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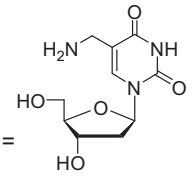
target duplex

II 3'-d(GAGGAGGGAGGA)-5'

I 5'-d(CTCCTCCCTCCT)-3'



	3rd strand	K_d (nM)
III	5'-d(GTGGTGGGTGGT)-3'	115 ± 9
IV	5'-d(GTGGTGGG**Z**GGT)-3'	128 ± 19
V	5'-d(GTGG**Z**GGG**Z**GGT)-3'	82 ± 4
VI	5'-d(G**Z**GG**Z**GGG**Z**GGT)-3'	48 ± 6
VII	5'-d(G**Z**GG**Z**GGG**Z**GG**Z**)-3'	68 ± 4

Figure 2. Binding abilities of the oligonucleotides for the target duplex.

of the second strand.[15] Thus, we hypothesized that introduction of an aminomethyl residue into the 5-position of the 2′-deoxyuridine in the third strand would increase the thermal stability of the antiparallel triplex because the ammonium cations of the aminomethyl residues would reduce the anionic electrostatic repulsion between the phosphate moieties.

Oligonucleotides containing 5-aminomethyl-2′-deoxyuridine (**Z**) were synthesized using the phosphoramidite method (Fig. 2). The synthetic route of a **Z** phosphoramidite is shown in Figure S1. All **Z**-containing oligonucleotides were synthesized using a DNA/RNA synthesizer. The obtained oligonucleotides were analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), and the observed molecular weights were in agreement with their structures.

First, binding of the aminomethyl-modified oligonucleotides to target DNA was examined using an electrophoretic mobility shift assay (EMSA). Figure 2 summarizes the dissociation constants (K_d) of the third strands bound to the duplex DNA target. The K_d of the unmodified third strand, **III**, to the target DNA was 115 ± 9 nM, whereas those of the third strands **V**, **VI**, and **VII** containing 2, 3, and 4 **Z** residues were 82 ± 4, 48 ± 6, and 68 ± 4 nM, respectively. Thus, this proved that the aminomethyl modification at the 5-position of the 2′-deoxyuridine in the third strand increases the stability of the antiparallel triplex. The K_d (128 ± 19 nM) of the third strand, **IV**, containing 1 **Z** residue was slightly lower than that of the unmodified third strand, **III**. Thus, plural **Z** residues seemed to be required for the triplex stabilization.

Branched oligonucleotides **IX** and **X** were synthesized using an appropriately protected pentaerythritol linker (Fig. 3). The stability of triplexes formed with these oligonucleotides was studied by thermal denaturation in Tris–HCl buffer solution (15 mM, pH 7.0) containing 25 mM NaCl and 5 mM $MgCl_2$ (Figs. 3 and S2). One transition was observed in the melting profile when branched oligonucleotide **IX** and single-stranded RNA **VIII** were used, and the melting temperature (T_m) was 67.6 °C. In contrast, the T_m of the duplex between single-stranded DNA **II** and RNA **VIII** was 54.5 °C. The increment in the absorbance melting curve of the **VIII**:**IX** complex was greater than that of the **VIII**:**II** duplex. This means that thermal denaturation from triplex to complete random coil for the **VIII**:**IX** complex occurred cooperatively in a single transition. On the other hand, the **VIII**:**II**:**III** triplex did not show a clear T_m transition, that might be due to aggregation of the oligonucleotide strands containing many guanine residues. Thus, it turned out that the oligonucleotide **IX** connected with the pentaerythritol linker forms a more thermostable triplex with the single-stranded RNA **VIII** than the corresponding **VIII**:**II** duplex. We also performed a thermal denaturation study with target RNA that contained one base mismatch (Fig. S3). The ΔT_m (T_m [a complex between an oligonucleotide and a complementary target] − T_m [a complex between an oligonucleotide and a target containing a mismatched base]) ranged from −7.2 to −12.4 °C. Thus, it was found that the branched oligonucleotide **IX** has sufficient base discrimination ability as an antisense oligonucleotide. Incorporation of 3 aminomethyl residues into the branched oligonucleotide slightly stabilized the triplex in the buffer solution containing $MgCl_2$. The ΔT_m between the triplexes **VIII**:**X** and **VIII**:**IX** was +2.7 °C. Similar phenomena were observed when single-stranded DNA **I** was used as the target (Fig. 3, top graph).

I 5'-d(CTCCTCCCTCCT)-3'

VIII 5'-r(CUCCUCCCUCCU)-3'

II 3'-d(GAGGAGGGAGGA)-5'

IX 5'-d(GTGGTGGGTGGT)—OH
3'-d(GAGGAGGGAGGA)—OH

X 5'-d(G**Z**GG**Z**GGG**Z**GGT)—OH
3'-d(GAGGAGGGAGGA)—OH

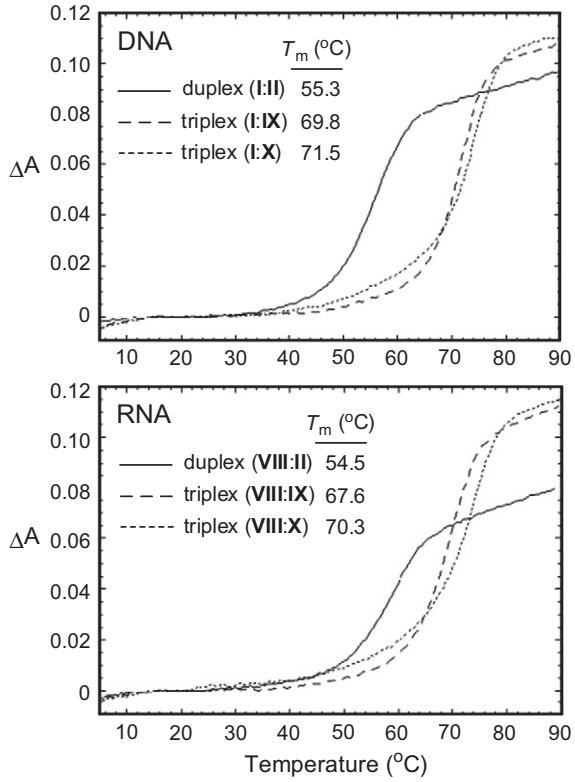


Figure 3. UV melting profiles. Melting experiments were performed in Tris–HCl buffer solution (15 mM, pH 7.0) containing 25 mM NaCl and 5.0 mM $MgCl_2$. The concentrations of the complexes were 2.0 μM.

It was reported that divalent cations, such as Mg^{2+}, are necessary for the formation of an antiparallel triplex.[16,17] Next, we demonstrated thermal denaturation in a buffer solution that did not contain $MgCl_2$. As shown in Fig. S2, the T_m of the complexes **I**:**IX**, **I**:**X**, **VIII**:**IX**, and **VIII**:**X** were significantly decreased. The T_m of the complexes were almost equal to those of the corresponding **I**:**II** and **VIII**:**II** duplexes. However, intriguingly, the increment in the absorbance melting curves of the **VIII**:**X** complex was greater than that of the **VIII**:**II** duplex. This suggested that the aminomethyl residue-containing branched oligonucleotide **X** formed an antiparallel triplex with the single-stranded RNA **VIII** in the absence of Mg^{2+}. The circular dichroism spectrum demonstrated not only triplex formation between the branched oligonucleotide **X** and the target RNA **VIII** but also triplex formation between the

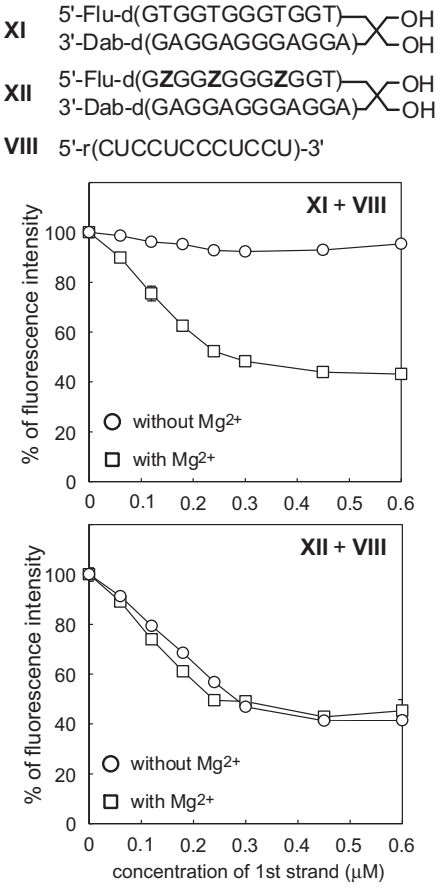


Figure 4. Profiles of the fluorescence intensities versus the first strand concentrations. Experiments were performed in Tris–HCl buffer solution (15 mM, pH 7.0) containing 25 mM NaCl in the presence or absence of 5.0 mM $MgCl_2$. The concentrations of the labeled oligonucleotides (**XI** and **XII**) were 0.30 μM. Excitation wavelength: 485 nm. Emission wavelength: 535 nm.

branched oligonucleotide **X** and the target DNA **I** in the absence of Mg^{2+} (Fig. S4).

In order to confirm the formation of the triplex, we next performed fluorescence resonance energy transfer (FRET) analysis. For this, we synthesized the branched oligonucleotides **XI** and **XII**, which were modified with fluorescein (Flu) at the 5′-end and with dabcyl (Dab) at the 3′-end as a quencher (Fig. 4). When the branched oligonucleotide **XI** was mixed with the target RNA **VIII** in the absence of Mg^{2+}, the fluorescence intensities of the solutions were almost the same irrespective of the concentrations of the first strand **VIII** (Fig. 4, top graph). On the other hand, the fluorescence intensities of the solutions decreased as the concentrations of the first strand **VIII** increased, and plateaued at a 1:1 branched oligonucleotide **XI** and first strand **VIII** ratio when the branched oligonucleotide **XI** was mixed with the target RNA **VIII** in the presence of Mg^{2+}. This indicates that the branched oligonucleotide **XI** forms a triplex with the target RNA **VIII** in the presence of Mg^{2+} but not in the absence of Mg^{2+}.

When the branched oligonucleotide **XII** containing the aminomethyl residues was used, the fluorescence intensities of the solutions decreased as the concentrations of the first strand **VIII** increased even in the absence of Mg^{2+}, and plateaued near a 1:1 branched oligonucleotide **XII** and first strand **VIII** ratio (Fig. 4, bottom graph). This proved that the branched oligonucleotide **XII**, which is modified with the aminomethyl residues, could form an antiparallel triplex even in the absence of a divalent cation such as Mg^{2+}.

In conclusion, we have demonstrated the synthesis of branched oligonucleotides containing aminomethyl residues, and found that these oligonucleotides have a high affinity for single-stranded DNA or RNA in buffer solutions with and without $MgCl_2$. Thus, these oligonucleotides would be useful as antisense oligonucleotides for targeting single-stranded RNA through triplex formation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.024.

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