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Thermal stability of triple helical DNAs containing 2′-deoxyinosine and 2′-deoxyxanthosine

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Abstract—In this paper, we describe the synthesis and thermal stabilities of the triplexes containing either 2′-deoxyinosine (**1**) or 2′-deoxyxanthosine (**3**) in their second strands. It was found that the triplexes with the 2′-deoxy-5-methylcytidine(dM)•**1**:dC and dM•**1**:dA base triplets are thermally stable, but those containing the dM•**1**:T and dM•**1**:dG base triplets are unstable under both neutral and slightly acidic conditions. On the other hand, it was found that the oligonucleotide containing **3** could form thermally stable triplexes with the oligonucleotides that involve four natural bases opposite the sites of **3**. The rank of the thermal stabilities of the triplexes was as follows: the triplex containing the dM•**3**:dC base triplet > that containing the dM•**3**:dA base triplet > that containing the dM•**3**:T base triplet > that containing the dM•**3**:dG base triplet.

1. Introduction

Oligonucleotide-directed triple helix (triplex) formation offers means to target specific sequences in DNA and interfere with gene expression at the transcriptional level.[1] Triplex-forming oligonucleotides (TFOs) bind to the oligopurine:oligopyrimidine sequences, forming a stable, sequence-specific complex with duplex DNA. Pyrimidine-rich TFOs can bind parallel to the purine strand of the duplex and form the T•dA:T and dC•dG:dC base triplets by the Hoogsteen hydrogen bonds (in which '•' indicates Hoogsteen hydrogen bond and ':' indicates Watson–Crick hydrogen bond),[2,3] while purine-rich TFOs bind anti-parallel to the purine strand of the duplex and form the dA•dA:T (or T•dA:T) and dG•dG:dC base triplets by the reverse Hoogsteen hydrogen bonds.[4,5]

Recently, much attention is being given to the approach of targeting single-stranded DNA or RNA through the formation of triplexes using circular or branched oligonucleotides.[6–11] These oligonucleotides are more nuclease resistant than unmodified oligonucleotides, and have high sequence selectivities relative to simple Watson–Crick complements since the mismatches in the Watson–Crick base pairings simultaneously influence the Hoogsteen or reverse Hoogsteen hydrogen bonds between the second and third strands. Recently, we also synthesized the branched oligonucleotides that were linked with the pentaerithritol linker. We found that the branched oligonucleotides containing the 2′-*O*-methylribonucleosides, especially the oligonucleotide composed of the 2′-deoxyribonucleosides and 2′-*O*-methylribonucleosides, significantly stabilized the triplexes with single-stranded DNA or RNA.[11] These indicate that the branched and circular oligonucleotides have good properties as anti-sense molecules.

However, these branched and circular oligonucleotides cannot target the mixed sequences composed of the pyrimidine and purine bases because the first and second strands of the triplexes must be oligopyrimidines and oligopurines, respectively. It was reported that 2′-deoxyinosine (**1**) and 2′-deoxyxanthosine (**3**) could form base pairings with the four natural bases in DNA duplexes (Fig. 1).[12–15] These reports motivated us to investigate the properties of triplexes containing **1** and **3** in their second strands. We hypothesized that, when using the formations of the dC•**1**:dN or dC•**3**:dN types of base triplets (in which dN indicates dA, T, dG, or

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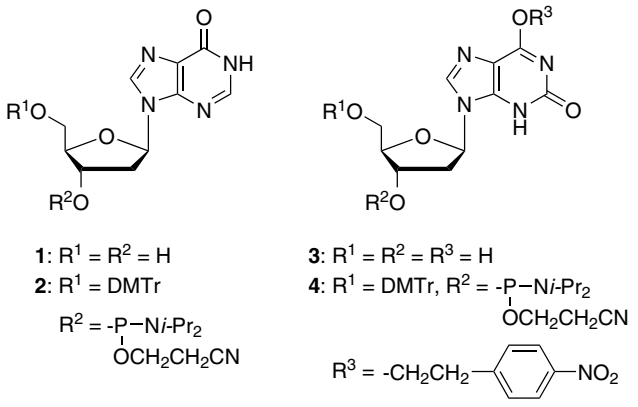

Figure 1. Structures of the nucleoside analogs and their corresponding phosphoramidites.

dC), the mixed sequences comprised of the pyrimidine and purine bases could be targeted.

In this paper, we report the synthesis and thermal stabilities of the triplexes containing either **1** or **3** in their second strands. The formation of these triplexes was also confirmed by circular dichroism (CD) measurement.

2. Results

2.1. Oligonucleotides

Sequences of the first, second, and third strands of the triplexes used in this study are represented in Figure 2. In the parallel triplex, the N-3 position of the cytosine residue of the third strand must be protonated in order to form two hydrogen bonds with the dC:dG base pair in the Watson–Crick duplex.[16,17] The incorporation of 5-methylcytosine into the third strand gives us a more stable triplex.[18,19] We thus introduced 2′-deoxy-5-methylcytidine (dM) into the third stand in place of dC. The oligonucleotides, **7** and **8**, which contained **1** or **3**, were synthesized using the properly protected 2′-deoxyinosine or 2′-deoxyxanthosine phosphoramidite units, **2** or **4**.[15,20] The fully protected oligonucleotides (1 μmol) linked to the solid support were treated with concentrated NH_4OH at 55 °C for 16 h. In the instance of the oligonucleotide containing **3**, it was treated with 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in pyridine for 60 min at room temperature, and then concentrated NH_4OH. The released oligonucleotides were purified by denaturing with 20% polyacrylamide gel electrophoresis (20% PAGE) to give the deprotected oligonucleotides, **7** and **8**, in 35 and 11 OD_{260} units, respectively. These oligonucleotides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). The observed molecular weights supported their structures.

```
5'-d(TTMMTTTTMTMMTTTMT)-3'     5
5'-d(AAGGAAAGGAGGAAAGA)-3'     6
5'-d(AAGGAAA1GAGGAAAGA)-3'     7
5'-d(AAGGAAA3GAGGAAAGA)-3'     8
5'-d(TCTTTCCTCATTTCCTT)-3'     9
5'-d(TCTTTCCTCGTTTCCTT)-3'    10
5'-d(TCTTTCCTCCTTTCCTT)-3'    11
5'-d(TCTTTCCTCTTTTCCTT)-3'    12

dM = 2'-deoxy-5-methylcytidine
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Figure 2. Sequences of the oligonucleotides used in this study.

2.2. Study of triplex formation by thermal denaturation

The formation of a duplex from two single-stranded oligonucleotides generates a hypochromic effect at the UV absorption of the nucleotides in the wavelength range of 240–290 nm. Thus, the melting of the duplex can be detected by measuring the absorption of the sample at a fixed wavelength within this range as a function of the temperature. A wavelength of 260 nm, which is close to the maximum of the absorption band of the nucleosides, is commonly used for the detection of the melting of the Watson–Crick duplex. Likewise, the formation of a triplex from a duplex and a pyrimidine-rich single-stranded oligonucleotide also yields a hypochromic effect at 260 nm, so that this process can be followed in the same way as for the duplex. In addition, a hyperchromic effect at around 300 nm occurs along with a triplex formation due to the protonation of the cytosine residues of the third strand.[21–24] This effect is absent in the melting of the duplexes. Therefore, the simultaneous measurement of the absorption at 260 and 300 nm allows distinguishing between and characterizing these two transitions.

Thermal denaturation of the complexes was performed in a buffer of 10 mM PIPES (pH 7.0) containing 100 mM NaCl and 10 mM $MgCl_2$. Melting transitions were monitored at 260 and 300 nm. Figure 3a shows the melting profiles of the complexes among **5**, **6**, and **9** (an open circle), **5**, **6**, and **10** (an open square), **5**, **6**, and **11** (a closed circle), and **5**, **6**, and **12** (a closed square) at 260 nm. Two distinct transitions were observed around 29 and 58 °C in the melting profile of the complex of **5**, **6**, and **11**, while one large transition was observed around 57 °C in other profiles of other complexes. Figure 3d represents the melting profiles of the complexes in the same buffer at 300 nm. A transition based on the hyperchromic effect due to the deprotonation of the dM residues of the third strand was observed for the complex of **5**, **6**, and **11** (a closed circle) around 29 °C, whereas no transition was observed for the other complexes. The T_m values are shown in Table 1. It was found that the triplexes containing the dM•dG:dA, dM•dG:T or dM•dG:dG base triplets are thermally very unstable under the given conditions.

Figure 3b shows the melting profiles of the complexes involving the oligonucleotide **7** containing **1** at 260 nm.

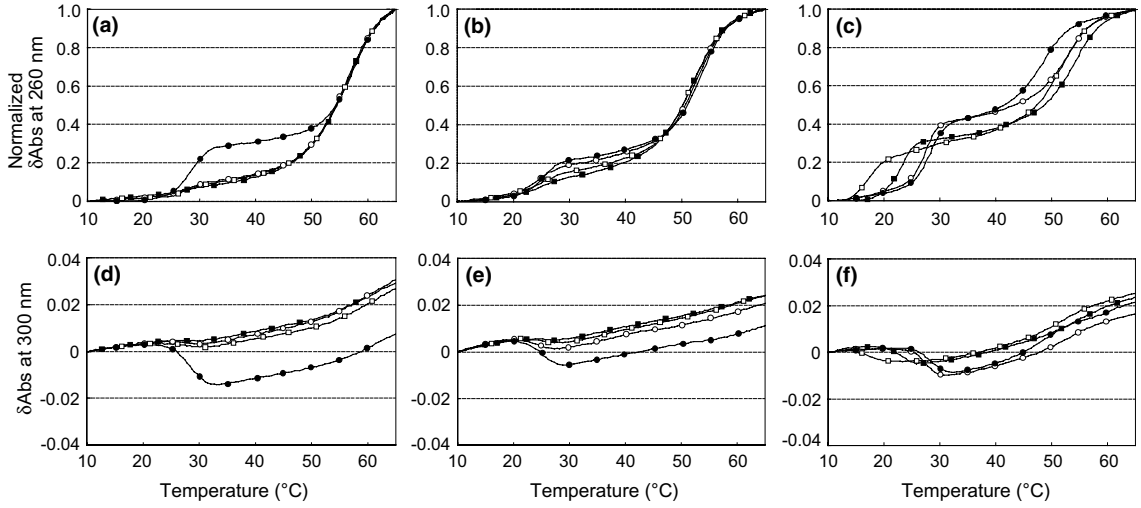

Figure 3. Melting profiles at a pH of 7.0. (a, d) the complex between **5**, **6**, and **9** (○); the complex between **5**, **6**, and **10** (□); the complex between **5**, **6**, and **11** (●); the complex between **5**, **6**, and **12** (■). (b, e) The complex between **5**, **7**, and **9** (○); the complex between **5**, **7**, and **10** (□); the complex between **5**, **7**, and **11** (●); the complex between **5**, **7**, and **12** (■). (c, f) The complex between **5**, **8**, and **10** (○); the complex between **5**, **8**, and **10** (□); the complex between **5**, **8**, and **11** (●); and the complex between **5**, **8**, and **12** (■). Experimental conditions are described in Section 4.

Table 1. Hybridization data

Complex (base triplet)	T_m (°C) at pH 7.0	T_m (°C) at pH 6.5
(*a*)		
5•6:9 (dM•dG:dA)	ND (56.4)	ND (60.8)
5•6:10 (dM•dG:dG)	ND (56.4)	ND (60.8)
5•6:11 (dM•dG:dC)	29.1 (58.4)	42.8 (62.2)
5•6:12 (dM•dG:T)	ND (56.4)	ND (61.3)
(*b*)		
5•7:9 (dM•**1**:dA)	24.7 (52.8)	34.9 (53.3)
5•7:10 (dM•**1**:dG)	ND (51.8)	ND (52.4)
5•7:11 (dM•**1**:dC)	25.5 (53.3)	37.4 (52.8)
5•7:12 (dM•**1**:T)	ND (52.3)	ND (51.7)
(*c*)		
5•8:9 (dM•**3**:dA)	25.7 (47.9)	35.5 (48.4)
5•8:10 (dM•**3**:dG)	15.3 (45.9)	24.9 (47.4)
5•8:11 (dM•**3**:dC)	26.1 (43.4)	37.8 (44.4)
5•8:12 (dM•**3**:T)	21.1 (48.5)	30.5 (51.7)

The T_m values of the duplexes are indicated in parentheses.

Two transitions were observed around 25 and 53 °C in the melting profiles of the complexes between **5**, **7**, and **9** (an open circle), and **5**, **7**, and **11** (a closed circle), while one large transition around 52 °C and an obscure transition around 25 °C were observed in the profiles of the complexes between **5**, **7**, and **10** (an open square), and **5**, **7**, and **12** (a closed square). Figure 3e represents the melting profiles of the complexes in the same buffer at 300 nm. A transition based on the hyperchromic effect was observed for the complex of **5**, **7**, and **11** around 25 °C, while an obscure transition was observed in each profile of the complexes between **5**, **7**, and **9**, **5**, **7**, and **10**, and **5**, **7**, and **12**. The T_m values are listed in Table 1. It was revealed that the triplex containing the dM•**1**:dC base triplet is thermally stable, but those containing the dM•**1**:T and dM•**1**:dG base triplets are unstable under the given conditions.

Figure 3c shows the melting profiles of the complexes involving the oligonucleotide **8** containing **3** at 260 nm. Two distinct transitions were observed in the profiles of all of the complexes, although the T_ms of each complex were deferent. Figure 3f represents the melting profiles of the complexes in the same buffer at 300 nm. Transitions based on the hyperchromic effect were also observed for all of the complexes. The T_m values are summarized in Table 1. It turned out that the triplexes consisting of the dM•**3**:dA, dM•**3**:T, and dM•**3**:dG base triplets as well as that consisting of the dM•**3**:dC base triplet are thermally stable under the given conditions.

The parallel triplex containing cytosines in the third strand is known to be thermally stabilized in slightly acidic solutions when compared to neutral or basic solutions because the N-3 positions of the cytosine residues of the third strand must be protonated to form two hydrogen bonds with the dC:dG base pairs in the Watson–Crick duplex.[16,17] Thermal denaturation was thus carried out in a buffer with a pH of 6.5. The T_m values are also summarized in Table 1. Although all of the triplexes containing **3** in their second strands were stabilized in the acidic solutions, the complexes between **5**, **7**, and **10**, and **5**, **7**, and **12** did not form any triplexes in the acidic solutions. Consequently, the order of the thermal stabilities of the triplexes containing **3** at pH levels of 6.5 and 7.0 was as follows: the triplex containing the dM•**3**:dC base triplet > that containing the dM•**3**:dA base triplet > that containing the dM•**3**:T base triplet > that containing the dM•**3**:dG base triplet.

2.3. Circular dichroism

It was reported that a negative cotton effect was observed at ~218 nm in the circular dichroism (CD) spectra of the parallel triplex.[21,25] In order to confirm the triplex formation, we next measured the CD spectrum

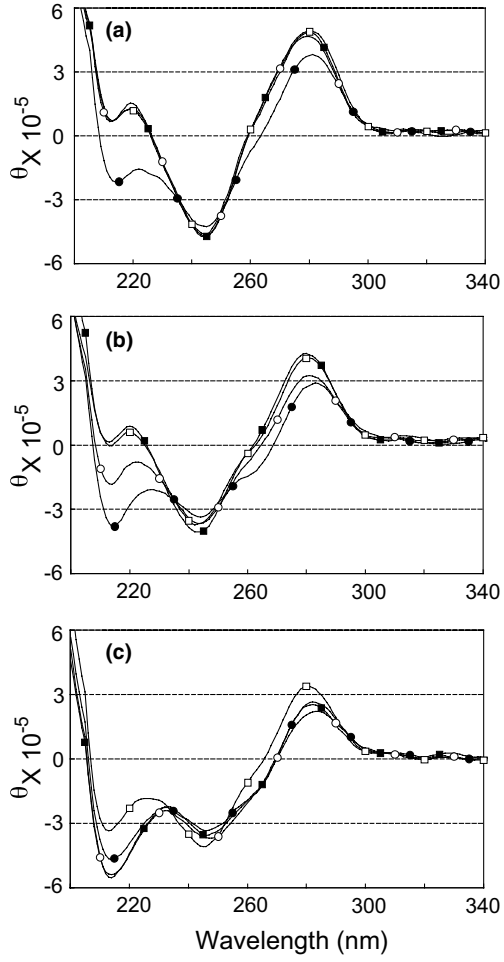

Figure 4. CD spectra at a pH of 7.0. (a): the complex between **5**, **6**, and **9** (○); the complex between **5**, **6**, and **10** (□); the complex between **5**, **6**, and **11** (●); the complex between **5**, **6**, and **12** (■). (b) The complex between **5**, **7**, and **9** (○); the complex between **5**, **7**, and **10** (□); the complex between **5**, **7**, and **11** (●); the complex between **5**, **7**, and **12** (■). (c) The complex between **5**, **8**, and **10** (○); the complex between **5**, **8**, and **10** (□); the complex between **5**, **8**, and **11** (●); the complex between **5**, **8**, and **12** (■). Experimental conditions are described in Section 4.

of each complex. Figure 4 shows the CD spectra of the mixtures of a 1:1:1 molar ratio of the first, second, and third strands in a buffer of 10 mM PIPES (pH 7.0) containing 100 mM NaCl and 10 mM $MgCl_2$. As shown in Figure 4a and b, the CD spectra of the **5**:**6**:**11** (Fig. 4a, ●) and **5**:**7**:**11** (Fig. 4b, ●) solutions had negative CD bands around 218 nm, which would be based on the binding of the third strand **5** into the major grooves of the **6**:**11** and **7**:**11** duplexes. The CD spectrum of the **5**:**7**:**9** (Fig. 4b, ○) solution also exhibited a negative CD band around 218 nm, but its intensity was clearly less than that of the **5**:**7**:**11** solution. On the other hand, the CD spectra of the **5**:**7**:**10** (Fig. 4b, □) and **5**:**7**:**12** (Fig. 4b, ■) solutions did not show the negative CD bands around 218 nm. The CD spectra of the mixtures involving the oligonucleotide **8**, that contains the nucleoside **3**, are also shown in Figure 4c. The CD spectra of all of the solutions exhibited negative CD bands around 218 nm, although the CD band of the **5**:**8**:**10** (□) solution was less intense relative to the others.

3. Discussion

2′-Deoxyinosine (**1**) and 2′-deoxyxanthosine (**3**), which are called the universal bases, can form base pairings with the four natural bases in the DNA duplexes. Based on the base pair structures of **1** and **3** in the duplexes previously reported,[13–15] the possible base triplet structures of **1** and **3** with the four natural bases in the triplexes are, respectively, shown in Figures 5 and 6.

The nucleoside **3** can form base pairs with T in two different manners (Fig. 6, V and V′), while **1** can form a base pair with T only in one manner (Fig. 5, I). The duplex containing **1** opposite the site of T did not form a triplex, whereas the duplex containing **3** opposite the site of T formed the thermally stable triplex at both pH levels of 6.5 and 7.0 (T_m of the triplex at pH 6.5 = 30.5 °C, that at pH 7.0 = 21.1 °C). The thymine base of the **1**:T base pair in the duplex is presumed to project into the major groove of the duplex when compared to the pyrimidine bases in the canonical Watson–Crick duplex. This projecting thymine base may prevent the third strand from binding to the duplex. The geometry of the V-type structure of the dM:**3**:T base triplet is similar to that of the dM:**1**:T base triplet. However, **3** can take the V′-type structure of dM:**3**:T base triplet. Thus, in contrast to the duplex containing **1**, the duplex containing **3** opposite the site of T would form the thermally stable triplex with the third strand by taking the V′-type base triplet.

Although the T_m values of the triplexes containing the dM:**1**:dA and dM:**3**:dA base triplets are almost the same under both neutral and acidic conditions (Table 1), the transitions of the triplexes containing the dM:**1**:dA base triplet in their melting profiles were less clear when compared to those of the triplexes containing the dM:**3**:dA base triplet. Furthermore, the CD band of the triplex containing the dM:**1**:dA base triplet around 218 nm was less intense relative to the triplex containing the dM:**3**:dA base triplet. The geometries of the dM:**1**:dA (Fig. 5, III) and dM:**3**:dA (Fig. 6, VII) base triplet structures in the triplexes are surmised to be similar to each other. Although the precise reason for these differences is unclear, the existence of the enolate anion oxygen at the 2-position of **3** might alter the electronic structure of the purine base, which might influence on the thermal stability of the triplexes.

The duplex containing the **1**:G base pair did not form the triplex in either the neutral or acidic solutions, whereas the T_m values of the triplexes containing the dM:**3**:dG base triplet were 15.3 and 24.9 °C in the neutral and acidic solutions, respectively. The formation of the triplex containing the dM:**3**:dG base triplet was also supported by the CD spectrum. It was reported that **1** forms the base pair with dG by adopting the *syn*-conformation, as shown in Figure 5. Thus, this conformational change around the glycosyl bond of **1** would

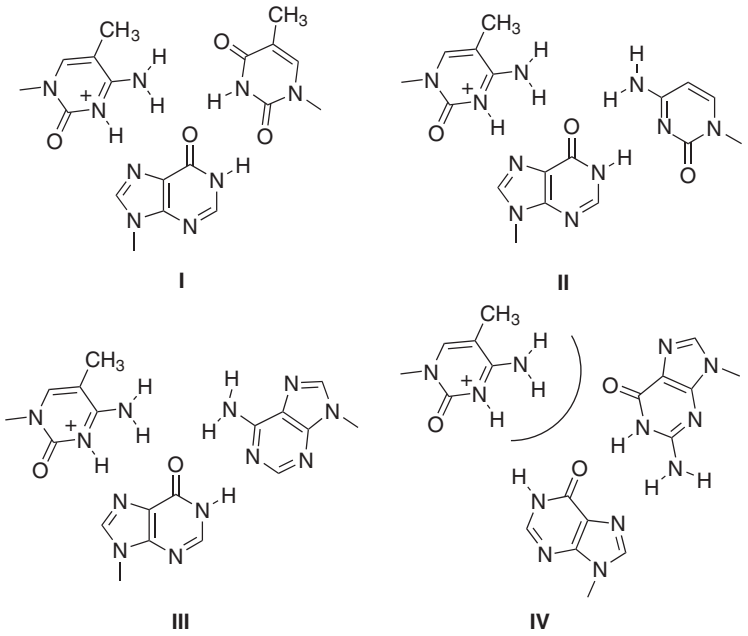

Figure 5. Postulated base triplet structures involving 2′-deoxyinosine.

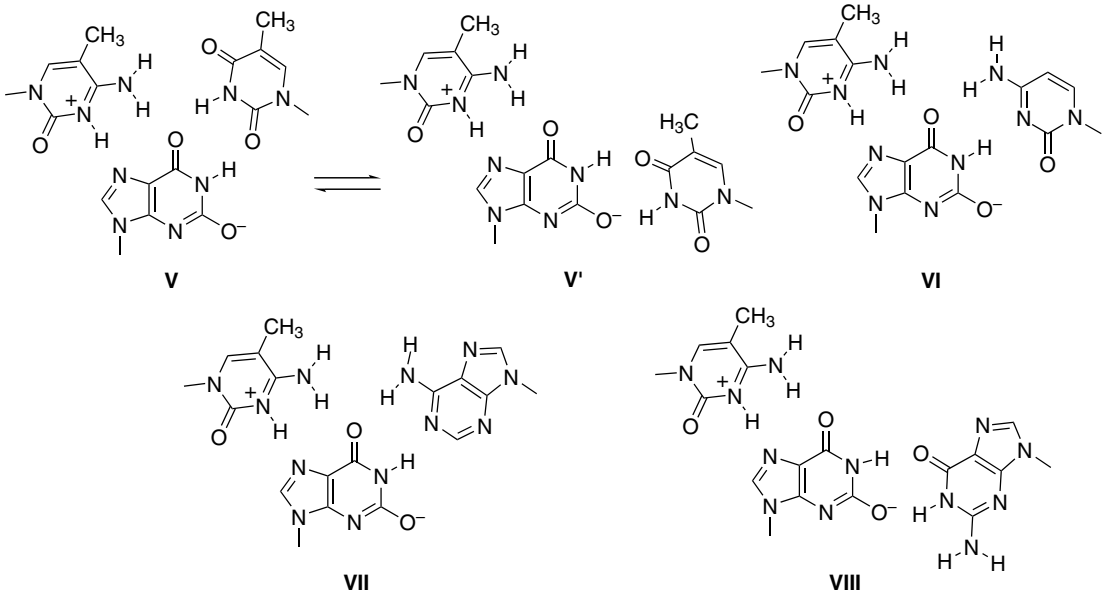

Figure 6. Postulated base triplet structures involving 2′-deoxyxanthosine.

prevent the third strand from binding to the duplex containing the **1**:dG base pair. On the other hand, **3** can form the base pair with dG by adopting the *anti*-conformation, as shown in Figure 6, since **3** has the enolate anion oxygen at the 2-position. Therefore, the duplex containing the **3**:dG base pair would be able to form the triplex by taking the VIII-type base triplet.

So far, only a few research groups have reported the properties of the triplexes containing a xanthine base.[20,26,27] However, to the best of our knowledge, this is the first report that has showed the oligonucleotide containing the xanthine base being able to form the thermally stable triplexes in all cases with four natural bases opposite the sites of the xanthine base.

In conclusion, we have demonstrated the synthesis and thermal stabilities of the triplexes containing **1** or **3** in their second strands. The formation of these triplexes was also confirmed by CD measurement. It was found that the oligonucleotide containing **3** could form the thermally stable triplexes with the oligonucleotides, which involve four natural bases opposite the sites of **3**. Thus, the mixed sequences comprised of the pyrimidine and purine bases could be targeted by the formation of the dC•**3**:dN base triplets. These novel base

triplet motifs presented here could be applicable for the anti-sense method.

4. Experimental

4.1. Synthesis of oligonucleotides

Oligonucleotides were synthesized on a DNA/RNA synthesizer (Applied Biosystems Expedite Model 8909) by the phosphoramidite method. The inosine phosphoramidite **2** was purchased from GLEN Research. The xanthosine phosphoramidite **4** was synthesized according to the reported method.[15,20] The oligonucleotides linked to the resins were treated with concentrated NH_4OH at 55 °C for 16 h, and the released oligonucleotides were purified by denaturing 20% polyacrylamide gel electrophoresis (20% PAGE) run at 600 V for 8 h. The oligonucleotides were visualized by UV shadowing and eluted from crushed gel slices by incubation at room temperature in 0.1 M triethylammonium acetate (TEAA, pH 7.0) and 1 mM EDTA for 15 h. The oligonucleotides were further purified by a Sep-Pak C18 cartridge to give the deprotected oligonucleotides **7** (35) and **8** (11). The yields are indicated in parentheses as OD units at 260 nm starting from 1 μmol scale.

4.2. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Spectra were obtained on a Voyager Elite reflection time-of-flight mass spectrometry (PerSeptive Biosystems, Inc., Framingham, MA) equipped with a nitrogen laser (337 nm, 3 ns pulse). 3-Hydroxypicolinic acid (HPA), dissolved in H_2O to give a saturated solution at room temperature, was used as the matrix. Time-to-mass conversion was achieved by calibration by using the peak representing the C^+ cation of the charged derivative to be analyzed. Oligonucleotide **7**: calculated mass, 5359.5; observed mass, 5356.3. Oligonucleotide **8**: calculated mass, 5375.5; observed mass, 5374.4.

4.3. Thermal denaturation and CD spectroscopy

The solution that contained the oligonucleotide **5** (2 μM) and each duplex (2 μM) in a buffer of 10 mM PIPES (pH 6.5 or 7.0), 100 mM NaCl, and 10 mM $MgCl_2$ was heated at 50 °C for 3 min, then cooled gradually to an appropriate temperature, and used for the thermal denaturation study. Thermal-induced transitions of each mixture were monitored at 260 nm on a Beckman DU 650 spectrophotometer. Sample temperature was increased 0.5 °C/min. Extinction coefficients of the branched oligonucleotides were calculated from those of mononucleotides and dinucleotides according to the nearest-neighbor approximation.[28] CD spectra were measured by a JASCO J-820 spectropolarimeter. Samples for CD spectroscopy were prepared by the same procedure used in the thermal denaturation study, and spectra were measured at 10 °C. The ellipticities of triplexes were recorded from 200 to 350 nm in a cuvette with a path length 1 mm. CD data were converted into mdeg mol of residues^{-1} cm^{-1}.

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