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Novel dinucleoside phosphotriester unit conjugated with an intercalative moiety in a stereospecific manner enhances thermal stability of an alternate-stranded triple helix

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Abstract—A novel phosphoramidite synthon of a dinucleoside phosphotriester unit bearing an intercalative moiety at its internucleotide linkage in a stereospecific manner was prepared and successfully incorporated into the middle portion of α-β chimeric oligoDNA. One of the resulting stereoisomeric chimera DNAs strongly enhances the thermal stability of an alternate-stranded triplex formed between the chimera and a double-stranded DNA.

Use of synthetic oligonucleotides to form triple helices with double-stranded (ds) genomic DNA has been attracting great interest as a possible new therapeutic method, because it would specifically modulate the expression of a certain gene at the level of transcription (anti-gene strategy).[1–4] Usually, a homopyrimidylate is used as the triplex-forming third strand (TFO) which binds to a homopurine strand of dsDNA in the manner of parallel orientation. The thermal stability of the resulted triple-helical complex is, however, generally much lower than that of the complementary double helix. The stability of the triplex could be increased, for example, by extending the range of recognition on the dsDNA which, at the same time, requires a long tract of the homopurine target. Unfortunately, it would be not so easy to find sufficiently long tracts of homopurine in one strand of a certain gene providing a suitable triplex formation site, and this could limit the applicability of TFO as a feasible gene-regulating agent. Nevertheless, two adjacent short homopurine tracts locating in an alternate-stranded matter would be more conceivable.

Recently, we have reported a chimeric oligoDNA (**GK-300**, previously reported as **ODN-1**) composed of a tandem β-anomeric polypyrimidine strand and an α-anomeric polypyrimidine strand as a new TFO which is capable of binding to the two adjacent short homopurine tracts locating in the alternate-stranded matter,[5] as is depicted in Figure 1.[6] The thermal stability of the resulted alternate-stranded triplex was higher than that of the triplexes formed with the parental α-DNA, β-DNA or a mixture of both. This may be due to the fact that the chimera has an extended range of recognition, therefore, an extended number of hydrogen bonding, compared to the parental oligomers.

In the course of our study to establish further improvement of the thermal stability of the alternate-stranded triplex, we have developed a novel dinucleoside phosphotriester intermediate unit (**4**) bearing an intercalative molecule, namely, an anthraquinone moiety, at the internucleotide linkage in a stereo-specific manner. The intermediate was derived to the corresponding phosphoramidite synthon (**5**) and successfully incorporated into the middle of the linker portion of the chimeric DNA (Fig. 1). Here, we would like to report the synthesis of the synthon and its incorporation into the chimeric DNA, as well as the thermal stability of the alternate-stranded triplex formed with the dsDNA and the modified chimeric DNA.

The synthetic procedure of the synthon is summarized in Scheme 1. The starting material **1** was prepared by the coupling of 2′-deoxy-3′-*O*-levulinyl-N^4-benzoylcytidine[7] and the commercially available 3′-phosphoramidite derivative of 2′-deoxy-5′-*O*-dimethoxy-

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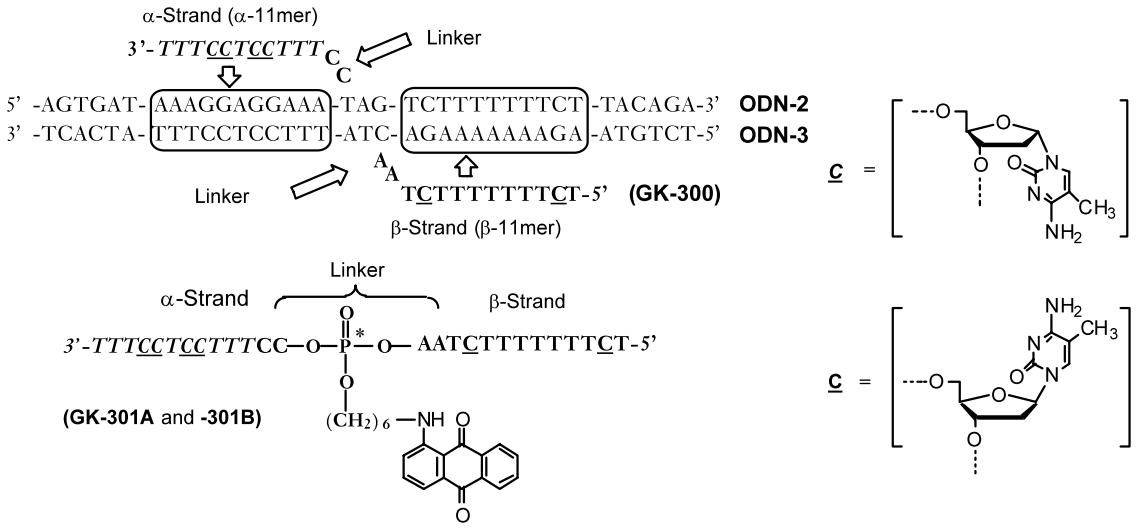
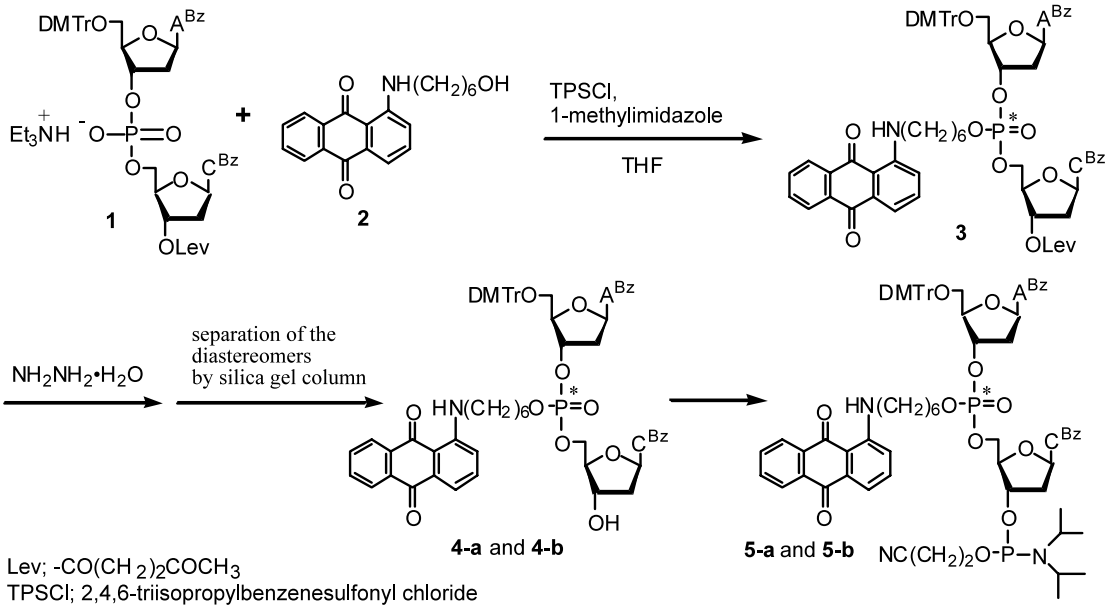


Figure 1. The sequence and the structure of α-β chimeric DNA (**GK-300** and **GK-301**). In the sequence, italic letters represent the α-anomeric polypyrimidylate component and roman letters represent the β-anomeric polypyrimidylate component, respectively. The linker portion of the chimeric DNA consists of β-anomeric 5′-AACC-3′ sequence.



Scheme 1. Preparation of anthraquinone bearing phosphoramidite synthon (**5-a** and **5-b**).

trityl-N^6-benzoyladenosine followed by oxidation of the resulting phosphite intermediate with I_2/H_2O[8] and removal of the 2-cyanoethyl group with triethyl amine. Compound **1** was condensed with 1-(6-hydroxyhexyl-1-amino)anthraquinone (**2**), which was obtained from 1-chloroanthraquinone and 6-aminohexanol, using 2,4,6-triisopropylbenzenesulfonyl chloride under the presence of 1-methylimidazale[9] to give the anthraquinone bearing fully protected dinucleoside phosphotriester compound (**3**). Removal of the levulinyl group from **3** was accomplished by treatment with hydrazine hydrate[10,11] to afford the diastereomeric intermediate compounds (**4-a** and **4-b**). The separation of the diastereoisomers was successfully achieved by silica gel column chromatography using a mixture of chloroform and ethanol (10:1) as the eluent. Through the chromatography, compound **4-a** (yield 36% from **1**, ^{31}P NMR; –0.96 ppm, mp; 98–100°C) eluted faster than **4-b** (yield 29% from **1**, ^{31}P NMR; –0.70 ppm, mp; 96–98°C). Each separated isomer was converted to the corresponding 3′-phosphoramidte derivative **5-a** and **5-b** by reaction with 2-cyanoethyl tetraisopropylphos-phordiamidite and 1*H*-tetrazole.[12,13] It would be worth noting that 2-cyanethyl *N*,*N*-diisopropylchlorophos-phoramidite,[14] a commonly used reagent for the phosphoramidation of protected nucleosides, gave considerable amounts of by-products in the above reaction.

Next, we examined the stability of the phosphotriester linkage in **4-a** and **4-b** by heating (55°C) the compounds

with concentrated ammonium hydroxide in a sealed tube for a substantial period (ca. 8 h). The condition is long enough to cleave the acyl protecting groups used at the exocyclic amino groups of dA and dC residues in an oligoDNA. Under the condition, no sign of the cleavage of the anthraquinolyl moiety was detected. Partial degradation of the linkage was, however, observed under prolonged treatment (ca. 16 h).

The incorporation of the synthon into the chimeric DNA was accomplished with automated DNA synthesizer (ABI 392) on 0.2 μmol scale starting from CPG-bound α-deoxynucleoside which was prepared according to the reported procedure.[15] The solubility of the phosphoramidite compounds **5-a** and **5-b** in CH_3CN was somewhat lower than that of the usual deoxynucleoside phosphoramidite reagents. Therefore, a mixture of DMF-CH_3CN (9:1) was required to obtain a clear solution with the concentration of 0.1 M. The coupling reaction of the synthon to the growing DNA chain was carried out for 360 sec. Also, an additional washing cycle of the lines using the DMF–CH_3CN mixture was set after the delivery of the synthon to the column to prevent possible clogging. Otherwise, the manufacturer-supplied standard procedure was applied for the oligomer synthesis. Under the conditions, the coupling yield estimated from the conventional trytyl assay was more than 95% for both **5-a** and **5-b**. The introduction of 5-methyl deoxycytidine residues to the oligomer was achieved by using the 4-triazolyl derivative of thymidine-3′-phosphoramidite as described previously.[16] The CPG-bound oligomer, thus obtained, was treated with concentrated ammonia at 55°C for 6 h. After the reversed-phase HPLC purification, detritylation with 20% AcOH, ethanol precipitation and gel filtration (Sephadex G-25), the desired **GK-301A** (from **5-a**) and **GK-301B** (from **5-b**) were obtained in satisfactory yields (29 and 19%, respectively). The structures of the oligomers were confirmed by ESI-mass spectrometry.[17]

We further examined the thermal stability of the complex formed with either **GK-301A** or **-301B** and the dsDNA formed by **ODN-2** and **-3** by UV-melting experiments, and the results are summarized in Figure 2. As shown in Figure 2, by-phasic sigmoidal curves were obtained for both complexes. The higher transitions are due to the dissociation of the duplex (**ODN-2**+**ODN-3**) into single strands while the lower transitions correspond to the dissociation of the triple helices into the duplex and the third strand. The estimated *Tm* values of the triplex containing **GK-301A** or **-301B** are listed in Table 1 along with the *Tm* of the triplex containing unmodified **GK-300** as the third strand.

As shown in Table 1, the triplex containing **GK-301B** exhibited marked enhancement of thermal stability compared to the triplex containing the parental **GK-300** and diastereomeric counterpart **GK-301A**. The enhanced stability is presumably due to the interaction of the intercalative moiety in the modified oligomer to the double-stranded region of the target duplex. Since the intercalative moiety in the modified oligomers is

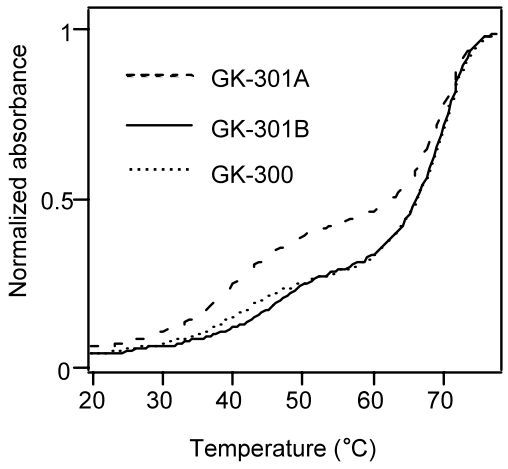


Figure 2. UV melting profiles of the triplexes. ODN-2+ODN-3 duplex (1.7 μM) was mixed with an appropriate third-strand (1.7 μM) in sodium cacodylate (pH 6.4) containing 100 mM NaCl, 0.5 mM spermine and 10 mM $MgCl_2$. The temperature was increased at the rate of 0.1°C per minute and thermally induced transition of each mixture was monitored at 280 nm.

Table 1. Melting temperature (*Tm*) of the triplexes and the duplexes.

TFO	Triplex *Tm*[a]	Duplex *Tm*[a]
GK-300	40.7	71.0
GK-301A	41.1	70.4
GK-301B	46.4	70.6

[a] *Tm* values (°C) were determined by computer fit of the first derivative of the absorbance with respect to $1/T$.

connected to the phosphorous atom in a stereospecific manner, the observed difference in the *Tm* increment effect between **GK-301A** and **-301B** could be due to the difference in the ease of the interaction of the intercalative moiety towards the double strand. It should be noted that in the different sets of experiment using the modified DNA possessing the same type of dimer block and the complementary single-stranded DNA, the duplexes composed of these oligomers failed to show any *Tm* increment (stabilization) effect regardless of the stereochemistry of the phosphorous atom bearing the intercalative moiety (data not shown). Thus, the stabilizing effect of the current dinucleoside phosphotriester unit conjugated with an intercalative moiety seems to be specific to the DNA triplex, and, at the same time, it depends on the stereochemistry of the phosphorous atom bearing the intercalative moiety. These findings would allow us to speculate that the overall structure around the intercalative moiety of the dimer is rather rigid and only the steroisomer having preferable spatial conformation could interact with the target oligonucleotides.

In conclusion, we were able to successfully develop a feasible TFO capable of forming a thermally stable alternate-stranded in the matter of thermal stability. The novel TFO would be a useful gene-regulating agent applicable to the anti-gene strategy.

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