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α–β Chimeric oligo-DNA bearing intercalator-conjugated nucleobase inside the linker sequence remarkably improves thermal stability of an alternate-stranded triple helix

A. T. M. Zafrul Azam, Minoru Hasegawa, Tomohisa Moriguchi and Kazuo Shinozuka*

Department of Chemistry, Faculty of Engineering, Gunma University, 1-5-1 Tenjin-cho, Kiryu 376-8515, Japan

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Abstract—Novel α–β chimeric oligodeoxynucleotides bearing an intercalator-conjugated nucleobase located at the internal 4-nt linker region were synthesized, and their triplex-stabilizing property was examined. The triple helical DNA formed between the modified chimera DNA and double-stranded DNA exhibited remarkable thermal stability; however, the position of the intercalator-conjugated nucleobase had little influence on the stability. Among the examined, modified chimera DNA bearing the two intercalator-conjugated nucleobases at adjacent positions exhibited the highest stability.

Oligonucleotides capable of forming a triple helix with double-stranded DNA (dsDNA) have been extensively studied, because an appropriately designed triplex-forming oligonucleotide (TFO) would inhibit the normal gene expression through the sequence-specific binding to the genomic DNA.[1] Homopyrimidylate is commonly used as a TFO, which binds to a homopurine strand of dsDNA by the Hoogsteen hydrogen-bonding in the manner of parallel orientation. The thermal stability of the triplex is, however, lower than that of the complementary double helix, in general. Thus, the formation of a stable triplex requires a sufficiently long tract of the homopurine strand as the target strand. Unfortunately, this could limit the feasibility of TFO as the gene-regulating agent since it would be not so easy to find sufficiently long tracts of homopurine in one strand of a certain gene. One way to extend the range of the target is to utilize a specifically designed TFO capable of forming a triplex with adjacent and alternating homopurine strand. For example, several 3′–3′ or 5′–5′ linked oligopyrimidylates were synthesized and were proved to form an alternate-stranded triplex with a double-stranded DNA having adjacent and alternating homopurine strand.[2] Meanwhile, we found that a chimeric oligo-DNA (**GK-300**, previously designated as **ODN-1**) composed of a tandem β-anomeric polypyrimidine strand and an α-anomeric polypyrimidine strand could also form an alternate-stranded triplex.[3] The resulting alternate-stranded triplex was thermally more stable than the triplexes formed with the parental α-DNA, β-DNA or a mixture of both.[3] The melting point of the alternate-stranded triplex was, however, just above the physiological temperature and further improvement of the thermal stability of the triplex is highly desired.

These facts prompted us to synthesize a modified chimeric DNA bearing an intercalator-conjugated nucleobase at the internal linker region. We found that the novel modified chimeric DNA exhibited remarkable stabilizing ability of the alternate-stranded triplex upon binding to the target duplex. Here, we would like to report the synthesis of the modified chimeric DNA (**GK-329** to **GK-332**, Fig. 1) and the result of UV-melting experiment of the triplex formed between the chimeric DNA and the target dsDNA.

The synthesis of the modified chimeric DNA bearing an intercalator-conjugated nucleobase was carried out by the known procedure utilizing the 4-triazolyl derivative of 5-methyl-2′-deoxycitidine as summarized in Scheme 1, with a slight modification. In brief, 1-(6-aminohexyl-1-amino)anthraquinone (**1**), which was readily prepared from 1-chloroanthraquinone and 1,6-diaminohexane,[4] was reacted in dimethylacetamide with 2′-deoxy-3′-*O*-acetyl-5′-*O*-dimethoxytrityl-4-triazolyl-5-methylcytidine

Keywords: Alternate-stranded triplex; Chimeric DNA; Intercalator.
* Corresponding author. Tel.: +81 277 30 1320; fax: +81 277 30 1321; e-mail: sinozuka@chem.gunma-u.ac.jp

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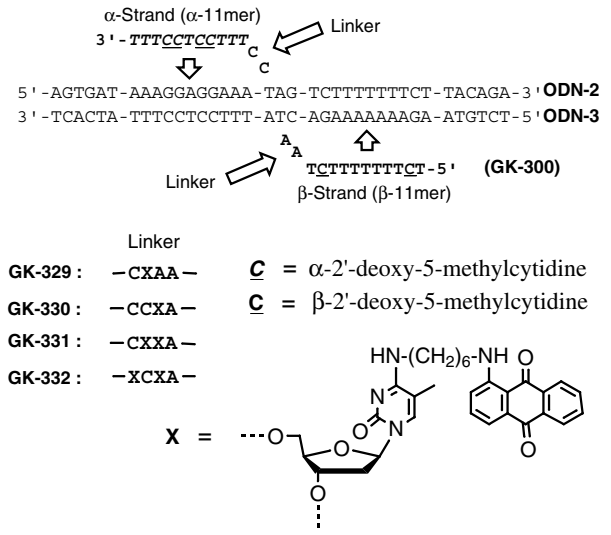
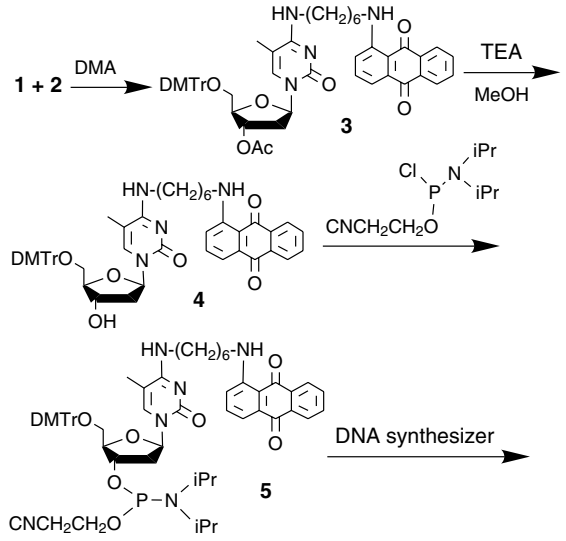


Figure 1. The structure and the sequence of α–β chimeric DNA (**GK-300** and **GK-329** to **GK-332**). In the sequence, italic letters represent α-anomeric polypyrimidylate portion and roman letters represent β-anomeric polypyrimidylate portion. The linker portion of the chimeric DNA consists of β-anomeric 4-nt sequence.



Scheme 1. Preparation of fully protected anthraquinone-bearing nucleoside phosphoramidite reagent (**5**).

(**2**), which was obtained from the corresponding protected thymidine and triazole.[5] Treatment of the resulting conjugate (**3**) with a mixture of triethylamine and MeOH (1:1) afforded the 5′-protected nucleoside (**4**), quantitatively. The nucleoside **4** was further derived to the corresponding phosphoramidite derivative (**5**).[6] The introduction of the phosphoramidite (**5**) into the chimeric DNA was accomplished with automated DNA synthesizer (ABI 392) on a 0.2 μmol scale starting from CPG-bound α-deoxynucleoside as reported previously.[3] It should be noted that the coupling period for the amidite **5** was extended to 360 s. Under the conditions, the coupling yield estimated from the conventional trityl assay for **5** was about 90–92%. After the usual treatment of DNA-bound CPG with concd ammonia (55 °C for 8 h), the oligomer was purified by reversed-phase HPLC, detritylation, ethanol precipitation, and Sephadex G-25 gel filtration to give 26-mer modified TFOs (**GK-329**, **-330**, **-331**, **-332**). The structures of the oligomers were confirmed by ESI-mass spectrometry.[7]

The formation and thermal stability of the complex obtained by the mixing of the modified chimera DNA and the dsDNA consisting of **ODN-2** and **-3** was monitored by UV-melting experiments under near physiological conditions and the results are shown in Figure 2. The all complexes exhibited a two-phase transition upon increasing the temperature as shown in Figure 2. The higher transitions are due to the dissociation of the duplex (**ODN-2** + **ODN-3**) into single strands. Meanwhile, the lower transitions are presumed to correspond to the dissociation of the triple helices into the duplex and the third strand.

It is clearly seen from Figure 2 that the lower transitions of all triplexes formed with the modified chimera DNA and the dsDNA markedly shifted towards higher temperature range compared to that of the unmodified parental chimera DNA (**GK-300**, blue line). The data indicates that the triplexes containing the modified TFO has higher stability compared to the triplex containing **GK-300**. The T_m values of the triplex estimated from the first derivative of the UV-melting profiles are listed in Table 1. As shown in Table 1, the modified TFO greatly stabilizes the triple helix since the T_m values of the triplexes containing the modified TFO are remarkably higher (10 °C>) than that of the triplexes containing unmodified **GK-300**. Also, the stabilizing effect is influenced by the number of the intercalator-conjugated nucleobases incorporated to the TFO. Thus, the T_m values for **GK-331** and **-332** bearing two of the modified bases were higher than those of **GK-329** and **-330** bearing a single modified base. Meanwhile, the position of the modified base in the TFO seems to have little effect since the T_m values of **GK-329** and **-330** were almost the same and as that of **GK-331** and **-332**. Among the

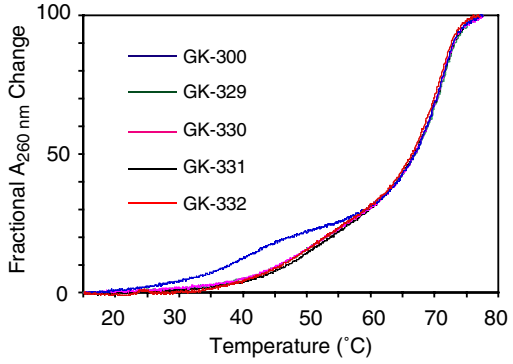


Figure 2. UV-melting profiles of the triple helices. The duplex consisting of **ODN-2** and **ODN-3** (1.5 μM) was mixed with an appropriate TFO (1.5 μM) in sodium cacodylate buffer (10 mM, pH 6.1) containing 100 mM NaCl, 0.5 mM spermine, and 10 mM $MgCl_2$. The temperature was raised at 0.1 °C/min and thermally induced transition of each mixture was monitored at 260 nm.

Table 1. Melting temperature (T_m) of the triplexes and the duplex

TFO	Triplex T_m[a]	Duplex T_m[a]	ΔT_m[b]
GK-300	38.6	71.1	—
GK-329	49.4	71.3	10.8
GK-330	49.0	71.1	10.4
GK-331	52.1	71.1	13.5
GK-332	51.2	70.9	12.6

[a] T_m values (°C) were determined by computer fitting of the first derivative of the absorbance with respect to $1/T$.
[b] ΔT_m indicates the deviation from the T_m value of the corresponding triplex composed of **GK-300** and the duplex.

modified TFO, **GK-331** having the adjacent modified bases exhibited the highest stabilizing effect, although the difference in the T_m between **GK-331** and **332** was only about 1 °C. Presumably, the observed T_m increment effect of the modified TFO is due to the interaction of the anthraquinone moiety and the double-stranded portion of the target dsDNA.

To confirm such interaction, we carried out a set of experiments monitoring the change in UV-absorption of the anthraquinone moiety. Thus, the complex formed between **GK-332** and the dsDNA was heated at elevated temperature and the UV-absorbance at the range of 400–700 nm was monitored. As shown in Figure 3a, the anthraquinone-based absorbance exhibited a marked shift to the shorter wave length (blue shift) according to the elevation of the temperature. At the same time, the absorption maximum was also increased (hyperchromic effect). A combination of these characteristic behaviors in UV-spectroscopy strongly suggests that the anthraquinone moiety actually intercalates to the target dsDNA when the modified TFO binds it to form the triplex.

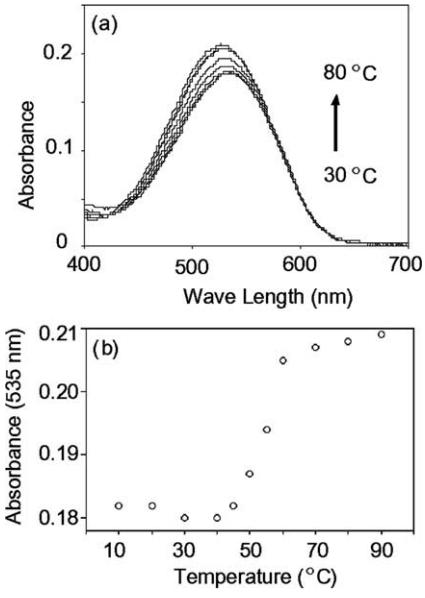


Figure 3. Temperature dependent UV-change of anthraquinone moiety in **GK-332**. The experiment was carried out in the same condition as the UV-melting experiment, although the concentration of the triplex was 18.6 μM.

Next, the UV-absorbance at 535 nm in Figure 3a was plotted against temperature and the result is shown in Figure 3b. As is clearly seen from Figure 3b, the plot shows prominent increment of the absorbance between 50 and 60 °C. The essentially same results were obtained from the corresponding experiment for other modified TFOs. The obtained plots reflect the association and dissociation of the intercalator moiety from the target dsDNA, presumably. The results strongly suggest that the intercalator moiety in the modified TFOs works as a molecular anchor to fasten the TFO to the target.

In conclusion, we have successfully demonstrated the preparation and the remarkable triplex-stabilizing ability of novel α–β chimeric oligo-DNA bearing an intercalator-conjugated nucleobase located at the linker region as a unique alternate-stranded triplex forming oligonucleotide. The triplex-stabilizing ability of the modified chimera DNA arises from the intercalation of the intercalative moiety.

The ease in preparation and the substantial triplex-stabilizing effect of the current modified chimera DNA would make it more advantageous compared to the previously reported modified chimera bearing a dinucleoside phosphotriester unit conjugated with the same intercalative moiety having hexamethylene tether group at its internucleotide linkage in a stereospecific manner.[8] Also, the results presented here indicate that the C-4 position of the pyrimidine base in the linker portion of the chimera is a very suitable position to incorporate a certain molecule capable of interacting with dsDNA, such as DNA cleaving molecule.

Optimization of the structural feature of the conjugate including the length of the linker portion is now under way and will be reported elsewhere.

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7. The average molecular weight of the modified chimera DNA calculated from the multiply charged ion peaks were as follows; **GK-329**: calcd mass, 8144.40; obsd mass, 8145.49. **GK-330**: calcd mass, 8120.39; obsd mass, 8120.89. **GK-331**: calcd mass, 8439.55; obsd mass, 8441.05. **GK-332**: calcd mass, 8439.55; obsd mass, 8439.66.
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