Modified α–β chimeric oligoDNA bearing a multi-conjugate of 2,2-bis(hydroxymethyl)propionic acid–anthraquinone–polyamine exhibited improved and stereo-nonspecific triplex-forming ability†

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Novel α–β chimeric oligonucleotides bearing a propionic acid derivative of an anthraquinone–polyamine conjugate in the "linker" region sequence-specifically formed a substantially stable alternate-stranded triplex with dsDNA almost regardless of the stereochemistry of the derivative.

Triplex-forming oligonucleotide (TFO) has been attracting great interest as a possible new therapeutic agent since it would regulate gene expression through direct interaction with genomic dsDNA. Indeed, oligonucleotides forming a triplex with certain gene promoters can modulate the level of transcription of that gene (anti-gene strategy).[1] One major drawback of the strategy is that the thermal stability of the resulting triplex under physiological conditions is not very high compared to that of the duplex having an analogous sequence. Furthermore, sufficiently long polypurine tracts in one strand of genomic DNA, which serves as the target for TFO, are not always found in a biologically important sequence. Considerable effort has been devoted to overcoming these limitations of the anti-gene strategy. Accordingly, a unique type of TFO consisting of 3′-3′ conjugated polythymidylate was reported by Horne and Dervan.[2] The TFO forms a so-called alternate-stranded triplex with an adjacent and alternating homopurine strand in the manner of parallel orientation. As a result, the TFO could expand its target and form a sufficiently long triplex with improved thermal stability. Since then, a number of 3′-3′ or 5′-5′ conjugated oligopyrimidylates have been synthesized having higher affinity to the target dsDNA through forming an alternate-stranded triple helix.[3]

Meanwhile, we have reported the synthesis and alternate-stranded triplex formation of a chimeric oligoDNA composed of a contiguous β-anomeric polypyrimidine strand and an α-anomeric polypyrimidine strand.[4] Since the thermal stability of the resulting triplex was just above the physiological temperature even at pH 6.1, we subsequently developed several modified α–β chimera TFOs possessing either a novel dinucleoside phosphotriester unit conjugated with an anthraquinone moiety at the internucleotidic linkage in a stereospecific manner[5] or an anthraquinone-attached nucleobase, both in the linker region of the chimera DNA.[6]

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† Electronic supplementary information (ESI) available: UV-melting curves of triplex at pH 6.5 and 7.0 (Figs. S1–S4). See DOI: 10.1039/b514325j

In our continuing studies to bring about further improvement in the affinity of chimera TFO to its target sequence, we have designed a new type of modified α–β chimera TFO, **GK-354** and **GK-358**. In these TFOs, a normal nucleoside unit in the "linker" region is substituted with a multi-conjugate of a non-nucleosidic unit, 2,2-bis(hydroxymethyl)propionic acid, and an intercalator–polyamine complex (Fig. 1). Although the TFOs become a set of diastereomers due to the presence of a stereogenic carbon atom in the non-nucleosidic unit, a UV-melting experiment revealed that the TFOs have almost equal and remarkable triplex-stabilizing ability. Here, we would like to report the synthesis of the multi-conjugate of 2,2-bis(hydroxymethyl)propionic acid–anthraquinone–polyamine and its incorporation into the chimera TFO, as well as the thermal stability of the alternate-stranded triplex formed with dsDNA and the modified chimera TFO.

The synthetic procedure of the key compounds, bi-functional 5-tris(aminoethyl)anthraquinone phosphoramidite reagents (**7**) is

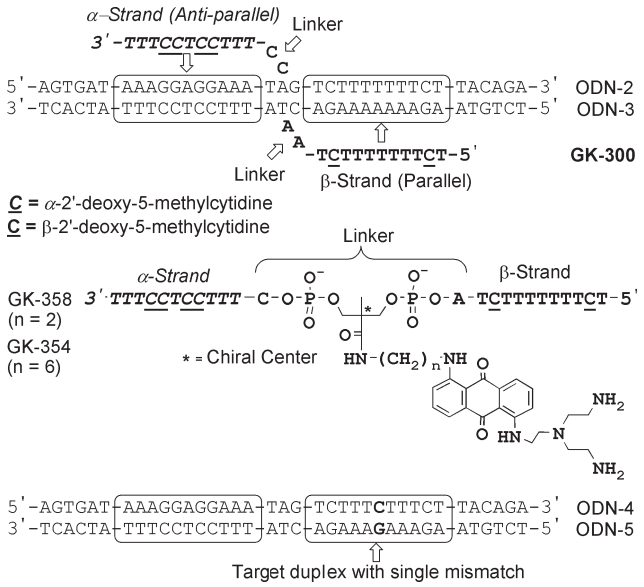
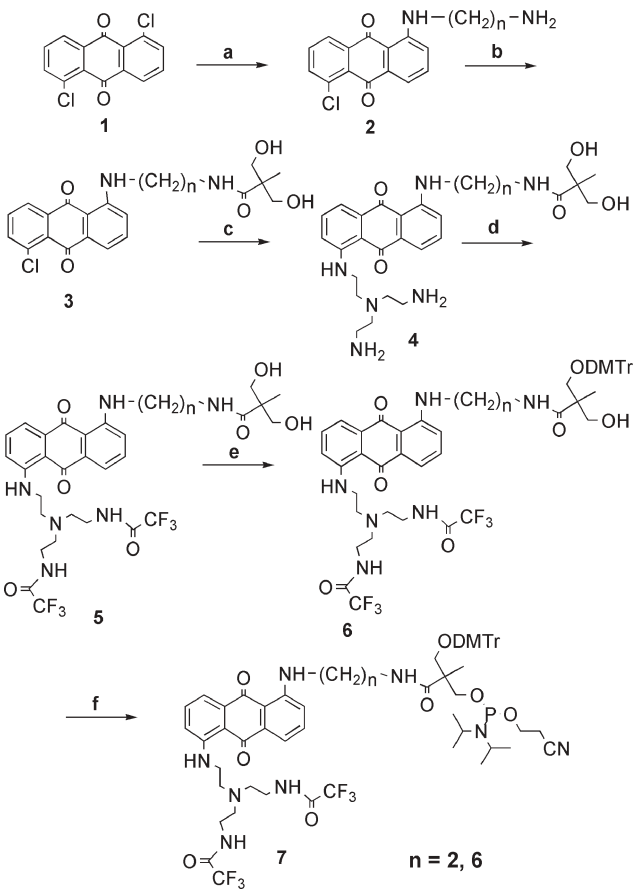


Fig. 1 The sequence and the structure of α–β chimeric TFOs (**GK-300**, **GK-354** and **GK-358**). In the sequence, italic letters represent the α-anomeric polypyrimidylate component and roman letters represent the β-anomeric polypyrimidylate component, respectively. The linker portion of the chimera TFO consists of a β-anomeric 5′-AACC-3′ sequence in **GK-300** whereas AC of the portion is replaced with a non-nucleotidic anthraquinone bearing unit in **GK-354** and **GK-358**. Duplex containing **ODN-2** and **ODN-3** represent a full-matched target whereas **ODN-4** and **ODN-5** represent a single mismatched target of TFO.

summarized in Scheme 1. In brief, 5-chloroanthraquinolyl diamine derivative (**2**), which was readily prepared from 1,5-dichloroanthraquinone (**1**) and *n*-alkyl diamine, was condensed with 2,2-bis(hydroxymethyl)propionic acid by the action of dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole in dry pyridine.[7] The obtained conjugate (**3**) was reacted under reflux with tris(2-aminoethyl)amine in toluene to give compound (**4**). The trifluoroacetyl-protected compound (**5**) was prepared by the treatment of (**4**) with trifluoroacetic acid ethyl ester in the presence of triethylamine in dry dichloromethane. The obtained conjugate (**5**) was reacted with dimethoxytrityl chloride to achieve the monotritylated desired compound (**6**) as the major product along with a small amount of the ditritylated by-product. Compound (**6**) was further converted to the corresponding phosphoramidite derivative‡ (**7**) by a standard procedure[8] for the automated solid-phase DNA synthesis.



Scheme 1 Preparation of 5-tris(aminoethyl)anthraquinone bearing phosphoramidite reagents for the automated solid-phase oligonucleotide synthesis. a) 3 equiv. of NH_2-$(CH_2)_n$-NH_2, reflux in xylene, 30 min (56.5%, n = 2; 56.3%, n = 6); b) 1.3 equiv. of 2,2-bis(hydroxymethyl)propionic acid, 2.0 equiv. of DCC and 1.5 equiv. of HOBt in dry pyridine, r.t., 4 h (60.4%, n = 2; 81.3%, n = 6); c) 4.0 equiv. of tris(2-aminoethyl)amine, reflux in toluene, 6 h (51.6%, n = 2; 65.0%, n = 6); d) 5.0 equiv. of $CF_3COOC_2H_5$, 10 equiv. of TEA in dry CH_2Cl_2, r.t., 3 h (60.7%, n = 2; 85.4%, n = 6); e) 1.2 equiv. of DMTr-Cl, 1.5 equiv. of DIPEA and 0.05 equiv. of DMAP in dry pyridine, r.t., 3 h (66.1%, n = 2; 74.0%, n = 6); f) 2.5 equiv. of (iPr_2N)($NCCH_2CH_2O$)-PCl, 3.0 equiv. of DIPEA in dry CH_2Cl_2, r.t., 30 min (70.1%, n = 2; 81.0%, n = 6).

Meanwhile, unnatural α-2′-deoxythymidine was prepared from natural β-2′-deoxythymidine by an epimerization reaction.[9] α-2′-Deoxy-5-methylcytidine was prepared from α-4-triazolyl-2′-deoxythymidine as described previously.[10] The nucleosides were converted to the corresponding phosphoramidite derivatives in the same manner as above.

The incorporation of the phosphoramidite derivative bearing conjugated anthraquinone (**7**) into the "linker" region of the chimera TFO was accomplished with an automated DNA synthesizer (ABI-392) on a 0.2 μmol scale starting from CPG-bound α-deoxynucleoside which was prepared according to the reported procedure.[11] The coupling reaction of the phosphoramidite (0.3 M) was carried out for 360 s with a double coupling procedure. The coupling yield estimated from the conventional trityl assay was about 95% for both **GK-354** and **GK-358**. The incorporation of other unnatural nucleoside units into the chimera TFO was also accomplished with an extended coupling period (360 s). The obtained CPG-bound oligomer was treated with concentrated ammonia at 55 °C for 12 h. Then the oligomer was purified by reversed-phase HPLC.

Fig. 2 shows the HPLC profile of **GK-354**. In the DMTr-ON form (Fig. 2a), **GK-354** gave a single product peak at retention time 15.7 min. After isolation and detritylation, the sample was again analyzed by HPLC. This time, the sample gave two peaks at retention time 30.9 min and 33.0 min with almost equal intensity. Because the introduced anthraquinone derivative has a stereogenic carbon atom, these could be diastereomers of the desired chimera DNA. Therefore, we isolated these two peaks separately and designated the faster eluate as **GK-354A** and the slower eluate as **GK-354B**. The **GK-358A** and **GK-358B** were isolated similarly and the oligomers were further purified by ethanol precipitation and Sephadex G-25 gel filtration.§

The formation and thermal stability of the complex formed with the isolated modified TFO and the dsDNA consisting of **ODN-2** and **ODN-3** were examined by UV-melting experiments under near physiological conditions (pH 6.5 and 7.0). In the experiments, all complexes exhibited a two-phase transition upon increasing the temperature (Figs. S1–S4[12]). The lower transitions of all triplexes formed with the modified TFO, however, shifted towards a higher

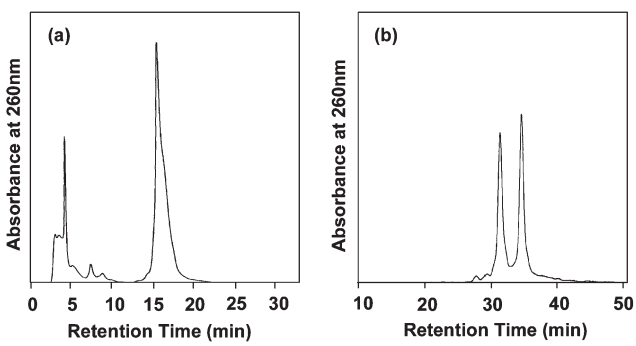


Fig. 2 The HPLC profile of **GK-354**. (a) DMTr-ON form; conditions: A buffer = 100 mM TEAA and B buffer = 100% acetonitrile; gradient: time in min (B buffer%), 0 (20), 10 (30), 30 (30), 35 (20). (b) DMTr-OFF form; conditions: A buffer = 50 mM TEAA and B buffer = 70% acetonitrile in 50 mM TEAA; gradient: time in min (B buffer%), 0 (18), 35 (25), 45 (25), 50 (18). For both cases the flow rate was 1 ml/min and the column was Wakosil 5C18 (ø 4.6 mm × 250 mm).

Table 1 Melting temperature (T_m) of the triplexes and the duplex at pH 6.5

TFO	T_m[a] (Full-match)			T_m[a] (Mismatch)		
	Triplex[d]	Duplex[d]	ΔT_m[b,d]	Triplex[d]	Duplex[d]	ΔT_m[c,d]
GK-300	35.8 (23.6)	72.2 (71.5)	—	26.2 (16.3)	72.2 (71.7)	−9.6 (−7.3)
GK-354A	50.3 (40.4)	71.8 (71.5)	14.5 (16.8)	42.4 (33.3)	72.1 (71.5)	−7.9 (−7.1)
GK-354B	51.2 (40.2)	71.7 (71.3)	15.4 (16.6)	42.2 (33.6)	71.9 (71.4)	−9.0 (−6.6)
GK-358A	46.4 (35.5)	71.7 (71.2)	10.6 (11.9)	39.1 (28.8)	71.9 (71.4)	−7.3 (−6.7)
GK-358B	46.6 (36.4)	71.6 (71.2)	10.8 (12.8)	39.4 (30.1)	71.8 (71.5)	−7.2 (−6.3)

[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 consisting of **GK-300** and the duplex. [c] ΔT_m indicates the deviation from the T_m value of the corresponding full-match triplex. [d] Values in parentheses indicate the T_m at pH 7.0. Each T_m value is the average of three separate experiments.

temperature range compared to that of the triplex formed with an unmodified parental chimera (**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 effectively stabilizes the triplex since the T_m values of the triplexes consisting of the modified TFO and full-matched target (**ODN-2** : **ODN-3**) are considerably higher (> 10 °C) than that of the triplex containing unmodified **GK-300**. The triplex-stabilizing effect is, however, dependent on the length of the alkyl linker connecting the anthraquinone moiety and the propionic acid moiety in the incorporated multi-conjugate. Thus, the TFOs bearing a longer alkyl linker (**GK-354A** and **-354B**) exhibited higher T_m values compared to the TFOs bearing a shorter alkyl linker (**GK-358A** and **-358B**). It is noteworthy that the T_m values of **GK-354A** and **-354B** are higher than the physiological temperature even at pH 7.0.

Interestingly, the data in Table 1 also revealed that the difference in the T_m values between the two diastereomers was quite small in all cases (< 1 °C). This small difference in the T_m values between the diastereomers indicates that the environment around the linker portion of the modified TFOs is very flexible. It also suggests that it is highly versatile for the modification at the linker portion of the chimera TFO with this type of non-nucleosidic unit to functionalize it without considering the stereochemistry in the modification. As a result, both **GK-354A** and **-354B** stabilized an alternate-stranded triplex at more than 15 °C with even a single modification of the parental chimera TFO.

In addition to these results, the T_m values of the triplexes consisting of the modified TFO and the mismatched target (**ODN-4** : **ODN-5**) are significantly reduced in Table 1. The magnitude of the T_m decrement is nearly the same as that of the parental unmodified **GK-300** at both pH 6.5 and 7.0. The results clearly indicate that the modified TFOs retain almost the same degree of sequence discrimination ability as the unmodified TFO and the introduction of the intercalator–polyamine conjugate to the chimeric TFO would not abolish the sequence discrimination ability despite its apparent enhancement effect on the thermal stability with a full-matched target.

In conclusion, we were able to successfully synthesize a multi-conjugate of 2,2-bis(hydroxymethyl)propionic acid–anthraquinone–polyamine and incorporate it into the α–β chimera TFO. The modification was quite effective at enhancing the triplex-forming ability of the TFO without abolishing sequence specificity. The enhancement effect depends on the length of the alkyl linker, however, it was almost independent of the stereochemistry of the incorporated multi-conjugate. Work to optimize the length of the alkyl linker as well as to create novel functional chimera TFOs utilizing the current modification methodology is now underway in our laboratory and will be reported elsewhere.

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Notes and references

‡ ^{31}P NMR ($CDCl_3$, δ); 148.6 ppm (singlet) (n = 2); 148.4 ppm (singlet) (n = 6).

§ The average molecular weights of the modified chimera TFOs calculated from the multiply charged ion peaks were as follows: **GK-300**, 7824.91 (calcd 7825.25); **GK-354A**, 7868.55; **GK-354B**, 7868.32 (calcd 7867.46) and **GK-358A**, 7813.19; **GK-358B**, 7813.29 (calcd 7811.40).

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