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Novel anthraquinone conjugate of 2,2-bis(hydroxymethyl)propionic acid incorporated to a TFO with phosphodiester linkage facilitates triplex formation with dsDNA bearing a pyrimidine-gapped polypurine sequence

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Abstract—An anthraquinone derivative conjugated with 2,2-bis(hydroxymethyl)propionic acid as a novel non-nucleosidic component was synthesized and successfully incorporated into an internal region of 14-mer triplex-forming oligonucleotide (TFO) via the phosphoramidite method. The resulted novel TFO exhibits remarkable enhancement effect on the thermal stability of a DNA triplex upon binding to a pyrimidine-gap containing polyprurine sequence.

For the past decades, 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 regulate the normal gene expression through the interference of the transcription and/or replication of genomic DNA.[1] Usually, a homopyrimidylate is used as a TFO which binds to a homopurine strand of dsDNA in the manner of parallel orientation. Therefore, a sufficiently long tract of the homopurine strand is required as the target strand for the triple helix formation. The thermal stability of the resulted triple-helical oligonucleotide is, however, generally much lower than that of the corresponding double helix. Furthermore, an interruption of the homopurine strand with even a single pyrimidine residue substantially lowers the stability of the triplex.[2] Several TFOs bearing a modified base, which is able to form hydrogen bonds with the inverted base pair in the homopurine strand, have been reported.[3] In the case that thymidine is placed as the interrupting pyrimidine residue, however, the approach using such modified bases has resulted in limited success.[3,4] Thus, the development of new TFO capable of forming a stable triplex upon binding to a pyrimidine interruption sequence possessing a T–A base pair would bring about a great improvement in the feasibility of TFO as a practical gene regulating agent.

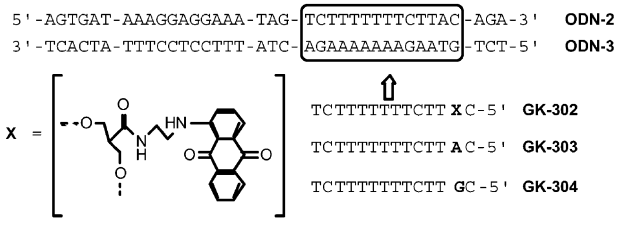

Figure 1. The structure and the sequence of TFO used in this study.

Attempting to achieve enhanced thermal stability of the triplex containing such inverted base pair, we have synthesized a novel non-nucleosidic phosphoramidite bearing an intercalative moiety. The derivative was incorporated into a modified TFO (**GK-302**, Fig. 1) at the position facing the T residue of the T-A inverted base pair in a polypurine sequence of dsDNA. UV-melting experiment revealed that the modified TFO brought about remarkable enhancement effect on the thermal stability of DNA triple helix upon binding to the target dsDNA.

The preparation of the key compound, bi-functional anthraquinone phosphoramidite reagent (**4**), was achieved by the following procedure (Scheme 1). In brief, anthraquinolylethylenediamine (**1**), which was

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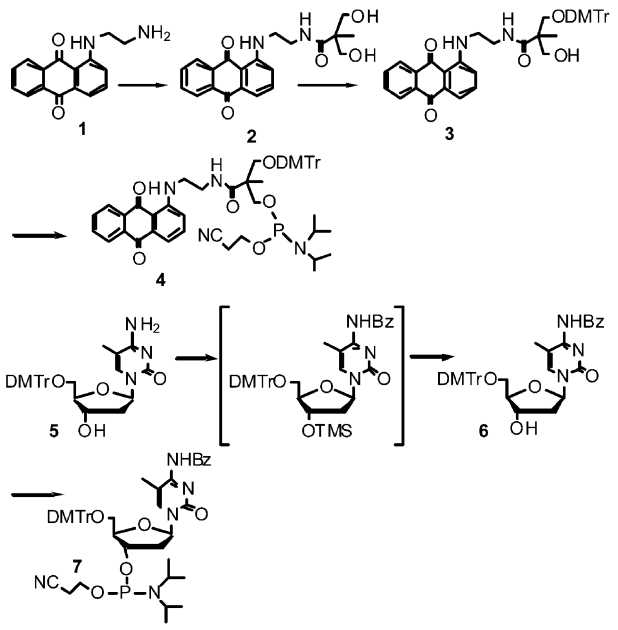

Scheme 1. Preparation of anthraquinone-bearing phosphoramidite reagent (**4**) and fully protected 5-methyl-2′deoxycytidine phosphoramidite reagent (**7**).

readily prepared from 1-chloroanthraquinone and ethylenediamine, was condensed with 2,2-bis(hydroxymethyl)-propionic acid by the action of dicyclohexylcarbodiimide (DCC) in dry pyridine.[5] The obtained conjugate (**2**) was reacted with slightly less equivalent (0.9 equiv) of dimethoxytrityl chloride to give the mono-tritylated desirable product (**3**) as the major product along with small amount of the di-tritylated by-product. The mono-tritylated compound **3** was further converted to the corresponding phosphoramidite derivative (**4**)[6] using chlorophosphoramidite reagent.[7] Meanwhile, 2′-deoxy-5-methylcytidine, which substitutes all normal deoxycytidine residue in **GK-302**, was prepared from 4-triazolylthymidine.[8] The nucleoside was readily converted to the 5′-dimethoxytritylated derivative (**5**). Further attempt to convert **5** to the corresponding N^4-benzoylated form (**6**)[9] with a standard transient-protection procedure, which has not been described in literature yet, using the TMS-group as the transient 3′-protecting group was not successful. This is due to the extraordinary vulnerability of the benzoyl group of the intermediate towards the alkaline hydrolysis used for the removal of the TMS-group. Even a brief treatment of the intermediate with 2 M ammonium hydroxide at room temperature caused the cleavage of the benzoyl group. After several experiments, we found that a brief treatment of the intermediate with a mixture of pyridine and aqueous methanol (pyridine:MeOH:H_2O = 1:8:1, 25 min) is effective for the selective and quantitative removal of the TMS-group. The protected nucleoside thus obtained (93.2% from **5**) was derived to the corresponding phosphoramidite derivative (**7**) as above.

GK-302 (14-mer) was synthesized using an automated DNA synthesizer (ABI-392) on a 1 μmol scale. The incorporation of **4** and **7** into the TFO was accomplished with the extended coupling period (360 s). After the usual treatment of DNA-bound CPG with concd ammonia, the oligomer was purified by reversed-phase HPLC, detritylation, ethanol precipitation and Sephadex G-25 gel filtration to give **GK-302** in a satisfactory yield (26.8%).[10]

The double-stranded DNA (**ODN-2** + **ODN-3**) containing a polypurine tract, in which a single pyrimidine interruption sequence possessing the T–A base pair is located near the end of the polypurine sequence as shown in Figure 1, was used as the target for triplex formation experiment. The stability of the triplex between the double-stranded DNA and **GK-302** was examined through the measurement of the UV-melting profile under a slightly acidic condition. The melting curves using **GK-303** and **-304**, in which the anthraquinone-bearing non-nucleosidic unit in **GK-302** was substituted with normal 2′-deoxyadenosine or 2′-deoxyguanosine unit, respectively, were also measured for comparison. As shown in Figure 2, UV-melting profiles of the mixtures gave a two-phase transition in all cases. The higher transitions are due to the dissociation of the duplex (**ODN-2** + **ODN-3**) into single strands. The lower transitions correspond to the dissociation of the triplex into the duplex and the third strand. The data indicates that the formation of the triplex actually occurred upon mixing all **GK**-series of oligonucleotide with the dsDNA under the conditions we used. The thermal stability of the triplex consisting of **GK-302** was compared to that of the triplexes consisting of **GK-303** and **-304** through the comparison of the *T*m values of the triplex estimated from the UV-melting profiles and those are listed in Table 1. As is clear from Table 1, **GK-302** greatly stabilizes the gap-containing triple helix since the T_m value for the triplex involving **GK-302** is remarkably higher (20 °C >) than those of the triplexes involving **GK-303** and-**304**. It would be noteworthy to point out that the *T*m values of **GK-303** and **-304** were considerably lower than that of the previously reported 11-mer TFO (**β-11mer**)[10] which covers only the polypurine region of the current target site (Fig. 1).

In an independent experiment monitoring the UV-absorption of the anthraquinone moiety of **GK-302**, we

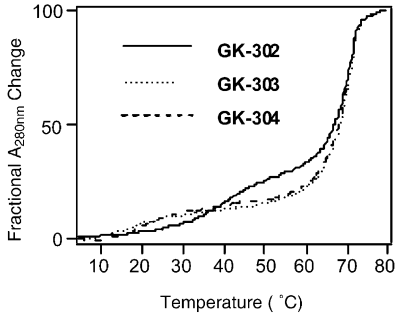

Figure 2. UV-melting profiles of the triple helices. The duplex consisting of **ODN-2** and **ODN-3** (1.5 μM) was mixed with an appropriate THO (1.5 μM) in sodium cacodylate buffer (10 mM, pH6.1) containing 100 mM NaCl, 0.5 mM spermine, and 10 mM $MgCl_2$. The temperature was raised at 0.1 °C/min and thermally induced transition of each mixture was monitored at 260 nm.

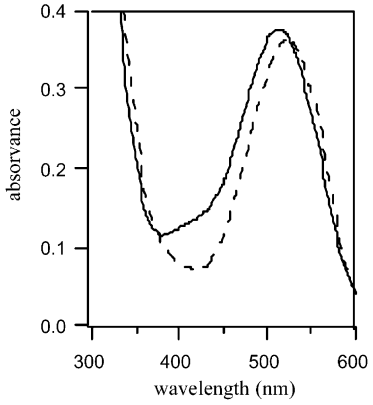

Figure 3. UV spectra of anthraquinone moiety incorporated into **GK-302**. The solid line represents **GK-302** (67 μM) alone. The broken line represent **GK-302** mixed with the double-stranded DNA (ODN-2 + ODN-3, 67 μM each) at 10 °C. The samples were measured in the same buffer system as it is indicated in Figure 2.

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

TFO	Triplex T_m[a]	Duplex T_m[a]
GK-302	40.7	71.0
GK-303	18.7	71.1
GK-304	18.5	70.9
β-11mer[b]	27.2	—

[a] T_m values (°C) were determined by computer fitting of the first derivative of the absorbance with respect to 1/T.
[b] The sequence of **β-11mer** is 5′-TCT TTT TTT CT-3′ which is complementary to the polypurine region of the target sequence.[11]

observed a characteristic red shift (518–522 nm) of the anthraquinone-based absorbance upon mixing of the TFO and the dsDNA as it is shown in Figure 3. These results suggest that the observed enhancement of the stability of the triplex containing **GK-302** is due to the intercalation of the anthraquinone moiety to the target dsDNA, to some extent. Interestingly, enhanced hyperchromicity was also clearly observed for the triplex-duplex transition of **GK-302** compared to **GK-303** and **-304**, as is also shown in Figure 2. This suggests that in the triplex form, stronger stacking effect exists in **GK-302** compared to other TFO, which would also contribute to the increment of the thermal stability of the triplex. Another interesting observation here is that the T_m values of the triplex containing either **GK-303** or-**304** are almost the same and no stabilizing effect of the triplex by the dG-residue in **GK-304** over the dA-residue in **GK-303** facing to the T–A pyrimidine gap position of the dsDNA was observed. The result is somewhat contrary to the previous findings that dG-residue in a TFO facing to the T–A pyrimidine gap stabilizes the triplex through the formation of a single hydrogen bond with T-residue.[2a,12] It should be pointed out that, however, the reported stabilizing effect of dG was observed when the T–A gap is located near the central region of the target polypurine strand which is not the case in this study. In addition, the 5′-neighboring triplet of G·TA in **GK-304** is C^+·GC which is known to destabilize the G·TA triplet to a considerable extent.[12a,12c] The observed total lack of the stabilizing effect of dG in **GK-304** would be attributed to these facts. The result also indicates that the current anthraquinone-bearing TFO would be a particularly useful TFO against a pyrimidine-gap containing polypurine sequence when such gap position is located near the end of the sequence.

The current anthraquinone-bearing phosphoramidite derivative unit might be more advantageous compared to the recently reported analogous amino acid derivative[13] because of the ease in preparation and straightforward handling in an automated DNA-synthesizer using phosphoramidite chemistry. The results presented here indicate that novel modified oligonucleotides containing an anthraquinone moiety as a non-nucleosidic component would expand the utility of TFO as an efficient and feasible gene-regulating agent. Optimization of the structural feature of the conjugate including the length of the linker portion is now underway and will be reported elsewhere.

Acknowledgements

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References and notes

1. (a) Reviews Hélène, C. *Anticancer Drug Design* **1991**, *6*, 569. (b) Cheng, Y. K.; Pettitt, B. M. *Prog. Biophys. Mol. Biol.* **1992**, *58*, 225. (c) Strobel, S. A.; Dervan, P. B. *Methods Enzymol.* **1992**, *216*, 309. (d) Thoung, N. T.; Hélène, C. *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 666. (e) Radhakrisnan, I. D.; Patel, D. J. *Biochemistry* **1994**, *33*, 11405. (f) Luyten, I.; Herdewijin, P. *Eur. J. Med. Chem.* **1998**, *33*, 515. (g) Praseuth, D.; Guieysse-Peugeot, A.-L.; Hélène, C. *Biochim. Biophys. Acta* **1999**, *1489*, 181.
2. (a) Grifiin, L. C.; Dervan, P. B. *Science* **1989**, *245*, 967. (b) Fossella, J. A.; Kim, Y.-J.; Shih, H.; Richard, E. G.; Fresco, J. R. *Nucleic Acids Res.* **1993**, *21*, 4511. (c) Mergny, J.-L.; Sun, J.-S.; Rougée, M.; Garestier, T.; Barcelo, F.; Chomilier, J.; Hélène, C. *Biochemistry* **1991**, *30*, 9791. (d) Best, G. C.; Dervan, P. B. *J. Am. Chem. Soc.* **1995**, *117*, 1187. (e) Greenberg, W. A.; Dervan, P. B. *J. Am. Chem. Soc.* **1995**, *117*, 5016. (f) Gowers, D. M.; Fox, K. R. *Nucleic Acids Res.* **1997**, *25*, 3787.
3. (a) Reviews Doronina, S. O.; Bear, J.-P. *Chem. Soc. Rev.* **1997**, 63. (b) Gowers, D. M.; Fox, K. R. *Nucleic Acids Res.* **1999**, *27*, 1569.
4. (a) Guianvarc'h, D.; Benhida, R.; Fourrey, J.-L.; Maurisse, R.; Sun, J.-L. *Chem. Commun.* **2001**, 1814. (b) Guianvarc'h, D.; Fourrey, J.-L.; Maurisse, R.; Sun, J.-L.; Benhida, R. *Org. Lett.* **2002**, *4*, 4209.
5. (a) Asanuma, H.; Ito, T.; Komiyama, M. *Tetrahedron Lett.* **1998**, *39*, 9015. (b) Liang, X.; Asanuma, H.; Komiyama, M. *Tetrahedron Lett.* **2001**, *42*, 6723.
6. ^{31}P NMR ($CDCl_3$, δ); 148.5, 148.6 (doublet).
7. Shinha, N. D.; Biernat, J.; Koster, H. *Tetrahedron Lett.* **1983**, *24*, 5843.
8. Sung, W. L. *Chem. Commun.* **1981**, 1089.
9. (a) Uesugi, S.; Miyashiro, H.; Tomita, K.; Ikehara, M. *Chem. Pharm. Bull.* **1986**, *34*, 51. (b) Ono, A.; Ueda, T. *Nucleic Acids Res.* **1987**, *15*, 219.

10. Following multiply chraged ion peaks were detected in ESI-masspectrometry; m/z 481.1 $[M]^{9-}$ (calcd 480.5), 540.9 $[M]^{8-}$ (calcd 540.7), 624.4 $[M+2Na]^{7-}$ (calcd 624.4), 727.8 $[M+2Na]^{-6}$ (calcd 728.6). The average molecular weight of **GK-302** calculated from these data is 4334.3 (calcd 4334.0).
11. Shinozuka, K.; Matsumoto, N.; Suzuki, H.; Moriguchi, T.; Sawai, H. *Chem. Commun.* **2002**, 2712.
12. (a) Belotserkovskii, B. P.; Veselkov, A. F.; Filippov, S. A.; Dobrynin, V. N.; Milikin, S. M.; Frank-Kamenetskii, M. D. *Nucleic Acids Res.* **1990**, *18*, 6621. (b) Radhakrisman, I.; Gao, X.; De los Suntos, C.; Live, D.; Patel, D. J. *Biochemistry* **1991**, *30*, 9022. (c) Kiessling, L. L.; Griffin, L. C.; Dervan, P. B. *Biochemistry* **1992**, *31*, 2829.
13. Mokhir, A. M.; Connors, W. H.; Richert, C. *Nucleic Acids Res.* **2001**, *29*, 3674.