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Remarkable stabilization of antiparallel DNA triplexes by strong stacking effects of consecutively modified nucleobases containing thiocarbonyl groups

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ABSTRACT

The consecutive arrangement of 2′-deoxy-6-thioguanosines (s^6Gs) and 4-thiothymidines (s^4Ts) in antiparallel triplex-forming oligonucleotides (TFOs) considerably stabilized the resulting antiparallel triplexes with high base recognition ability by the strong stacking effects of thiocarbonyl groups. This result was remarkable because chemical modifications of the sugar moieties and nucleobases of antiparallel TFOs generally destabilize triplex structures. Moreover, in comparison with unmodified TFOs, it was found that TFOs containing s^6Gs and s^4Ts could selectively bind to the complementary DNA duplex but not to mismatched DNA duplexes or single-stranded RNA.

The formation of naturally occurring DNA triplexes between DNA duplexes and external DNA single strands is well known, and for more than 2 decades, a large number of chemists have synthesized triplex-forming oligonucleotides (TFOs) that have been modified in various ways to increase the stabilities of the triplexes.[1] Because TFOs can strongly bind to the major grooves of complementary DNA duplexes, they can be very useful for the suppression of gene expression or for the creation of various artificial nanostructures. This is particularly important in gene therapy where the strong affinity of a TFO is one of the most important requirements because the length of the recognition sequence, which is a homopurine–homopyrimidine site, can be shortened in a gene.

There are 2 kinds of triplex formation: parallel- and antiparallel-oriented triplexes.[2] In a parallel-oriented triplex, 2 sets of planar triads that are composed of T–A–T and protonated C (C^+)–G–C and that involve the Hoogsteen hydrogen bonds contribute to the stabilization of the triplexes. By contrast, an antiparallel-oriented triplex contains 2 reverse-Hoogsteen base pairs [T (or A)–A–T and G–G–C], as shown in Figure 1. Many chemical modifications of nucleobases or sugar moieties have been developed to improve the hybridization affinity of parallel TFOs.[3] However, there have been few studies[4] on the chemical modifications of the nucleobases or the sugar moieties of antiparallel TFOs that increased their affinity, except for studies of the attachment of intercalators[5] or minor groove binders[6] to TFOs and the expansion of recognition sequences.[7] It is known that the introduction of chemical modifications into the sugar moieties and nucleobases of antiparallel TFOs generally destabilize triplex structures.[4b] Gee et al. synthesized modified antiparallel TFOs that contained 2′-deoxy-6-thioguanosine (s^6G) residues to avoid the self-aggregation of G-rich TFOs, such as the G-quadruplex, that occurs in the presence of physical concentrations of potassium ions.[4a] They found that the introduction of discontinuous s^6G residues into TFOs destabilized the triplexes while preventing the undesired self-aggregation that occurs in the presence of potassium ions.

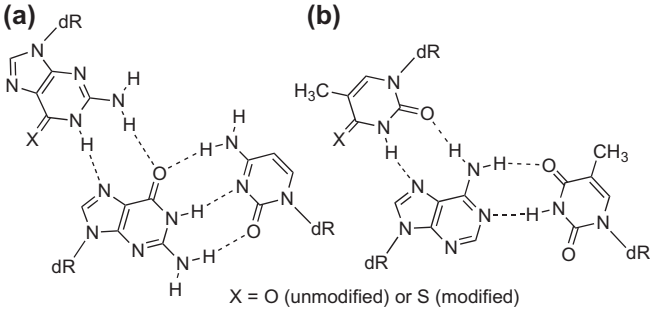

Figure 1. Base pairing of (a) G or s^6G with G–C and (b) T or s^4T with A–T.

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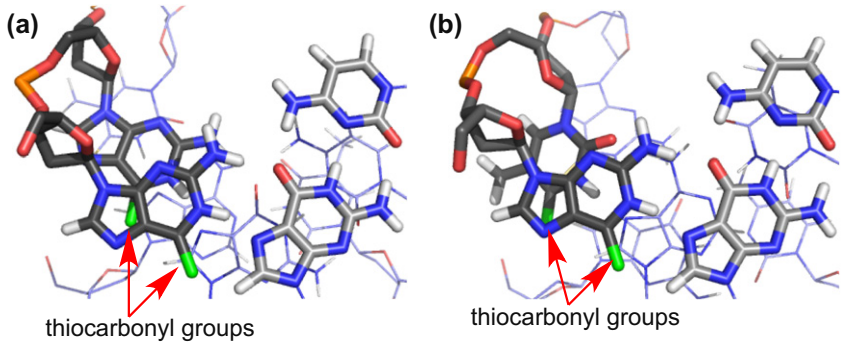


Figure 2. Computer model of a DNA triplex containing s^6G and s^4T residues. The sequences of the TFOs are (a) 5′ -s^6G s^6G T-3′ and (b) 5′ -s^6G s^4T G-3′.

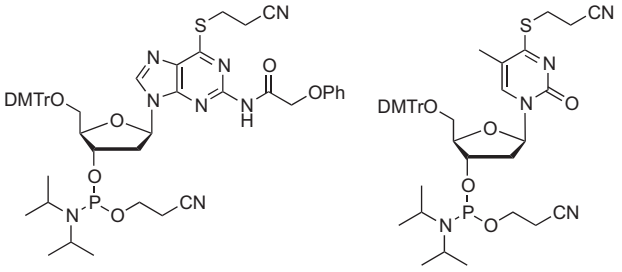


Figure 3. Chemical structures of the s6G and s4T phosphoramidite derivatives.

However, we recently reported that the consecutive arrangement of the thiocarbonyl groups, 2-thiothymidine (s^2T) and 2′-deoxy-5-methyl-2-thiocytidine (m^5s^2C), considerably stabilized the parallel-oriented triplex by the strong stacking effects of thiocarbonyl groups.[8] These results encouraged us to develop new antiparallel TFOs that contain consecutive thionucleobases. We expected that the thiocarbonyl groups of s^6G and 4-thiothymidine (s^4T) would interact with the 5′-upstream nucleobases in TFOs without interrupting the formation of the Hoogsteen base pairs, as shown in Figure 2. In this Letter, we focused on the triplex-forming and base recognition abilities of oligonucleotides containing s^6G and s^4T residues that were arranged consecutively.

In the synthesis of oligonucleotides containing consecutive s^6G and s^4T residues, the corresponding monomer building blocks were used in the general phosphoramidite approach, as shown in Figure 3. The cyanoethyl groups of the nucleobases and the phosphate linkages were deprotected by treatment with 1 M DBU in CH_3CN before the release of the oligonucleotides from the resins. First, we synthesized oligo DNA **1** d[T s^6G s^6G s^6G s^6G T] to examine the quadruplex formation of the oligomer containing consecutive s^6G residues because the self-aggregation of an oligonucleotide containing consecutive s^6G residues has not been previously reported; however, it was reported that the discontinuous introduction of s^6G destabilized the quadruplex.[4a] The G-quadruplex of the unmodified oligomer, d[TGGGGT] was observed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry, as shown in Figure S1. However, the oligo DNA **1** could not form the corresponding quadruplex. These results showed that the consecutive introduction of s^6G disturbed the formation of quadruplexes, which supported the results of the discontinuous introduction that were reported in the previous paper.[4a]

Subsequently, we examined the binding affinity of TFOs **1**, **2**, and **3** to the complementary HP DNA duplex **1**, which contained a sequence of the c-Myc P2 promoter,[9] at 37 °C, as shown in Table 1. Although the binding affinity of unmodified TFO **1** was very low (K_d >800 μM), the affinity of modified TFO **2** containing 2 sets of 3

Table 1
Binding assay[a] of TFOs **1**, **2**, and **3** to HP duplex DNA **1** containing the P2 promoter sequence

HP duplex DNA 1 (sequence of c-Myc P2 promoter)
3′- GCC CTTTTTCTTG C C T C C C T C C C T TG T
5′- FAM- CGGGAAAAAGAAC G G A G G G A G G G A AC T
T
TFO 1 3′- G G T G G G T G G G T
TFO 2 3′- G_sG $T_sG_sG_sG$ $T_sG_sG_sG$ T
TFO 3 3′- $G_sG_sT_sG_sG_sG_sT_sG_sG_sG_sT$ ($_sG = s^6G$, $_sT = s^4T$)

	Kd (μM)	ratio of binding affinity
TFO 1	> 800	—
TFO 2	7.3	> 1.1 x 10^2
TFO 3	0.67	> 1.2 x 10^3

[a] The conditions of binding assay: 50 mM HEPES buffer (pH 7.2) containing 10 mM $MgCl_2$, 100 mM NaCl, and 10% sucrose for 12 h at 37 °C.

Table 2
Binding assay[a] of TFOs **4** and **5** to HP duplex DNA **2** containing the sequence of the HIV2 nef gene at 37 °C

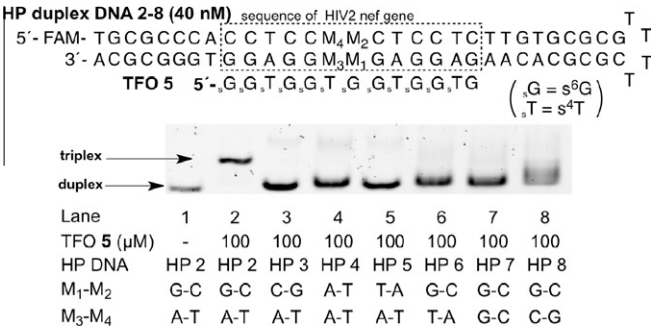
HP duplex DNA 2 (sequence of HIV2 nef gene)
5′- FAM- TGCGCCCA C C T C C T C C T C C TC T T GTGCGCG T
3′- ACGCGGGT G G A G G A G G A G G AG A A CACGCGC T
T
TFO 4 5′- G G T G G T G G T G G TG T
TFO 5 5′- $_sG_sG_sT_sG_sG_sT_sG_sG_sT_sG_sG_sT_sG$ ($_sG = s^6G$, $_sT = s^4T$)

	100 mM NaCl		100 mM KCl	
	Kd (μM)	ratio of binding affinity	Kd (μM)	ratio of binding affinity
TFO 4	17	—	33	—
TFO 5	0.4	43	0.2	1.7 x 10^2

[a] The conditions of binding assay: 50 mM HEPES buffer (pH 7.2) containing 10 mM $MgCl_2$, 100 mM NaCl, and 10% sucrose for 12 h at 37 °C.

consecutive s^6G residues was significantly higher. In addition, we found that the consecutive introduction of s^6G and s^4T into the antiparallel TFO surprisingly increased the triplex-forming ability by the strong stacking effects of thiocarbonyl groups. The binding affinity of TFO **3** was more than 1200 times higher than that of the unmodified TFO. This was the first promising observation suggesting that a chemical modification of a nucleoside moiety could enhance the triplex stability of an antiparallel TFO. Table 2 shows the binding affinity of the 13-nucleotide TFOs **4** and **5** to the HP DNA **2** containing a sequence of the HIV2 nef gene.[10] The binding affinity of TFO **5** containing consecutive s^6G and s^4T residues was 43 times higher than that of the unmodified TFO **4** in the presence of 100 mM NaCl. A similar result was also observed in the presence of 100 mM KCl. These results indicated that TFOs containing consecutive s^6G and s^4T residues could strongly bind to the complementary DNA duplexes without sequence dependency and the formation of G-quadruplexes.

Moreover, we examined the base recognition of s^6G and s^4T in the triplexes. Figure 4 shows the results of a gel mobility shift assay



Lane	1	2	3	4	5	6	7	8
TFO **5** (μM)	-	100	100	100	100	100	100	100
HP DNA	HP 2	HP 2	HP 3	HP 4	HP 5	HP 6	HP 7	HP 8
M_1-M_2	G-C	G-C	C-G	A-T	T-A	G-C	G-C	G-C
M_3-M_4	A-T	A-T	A-T	A-T	A-T	T-A	G-C	C-G

Figure 4. Electrophoretic mobility shift assay of the triplex formed between TFO 5 (100 μM) and fluorescently labeled HPs 2–8 (40 nM) on a 15% nondenaturing polyacrylamide gel after the incubation of the triplexes. The conditions of binding assay: 50 mM HEPES buffer (pH 7.2) containing 10 mM $MgCl_2$, 100 mM NaCl, and 10% sucrose for 12 h at 37 °C.

of the binding of TFO **5** (100 μM) to HP DNAs **2–8** (40 nM). Although triplex formation was observed in lane 2 with matched HP DNA **2**, TFO **5** could not form stable triplexes with mismatched HP DNAs **3–7**, even with the high concentrations of TFOs. Although we observed a smeared band in lane 8, the K_d value of the triplex formed between TFO **2** and HP DNA **8** was very low (K_d >100 μM). As a result, the base recognition ability of the TFO containing consecutive s^6G and s^4T residues was sufficiently high to distinguish the matched base pair from the other mismatched base pairs. There were more than 250-fold differences in the K_d values between matched (K_d = 0.4 μM) and mismatched triplexes.

Finally, the additional binding assay of TFO **4** or **5** to HP DNA **2** was conducted in the presence of the complementary RNA **1**, 5′-CACCACCACCACC-3′ (Fig. 5). Although the triplexes were observed with 40 μM of TFO **4** or **5**, as shown in lanes 2 and 5, the triplex that was formed between TFO **4** and HP DNA **2** disappeared, even in the presence of an equivalent RNA **1** (40 μM, lane 3). This might have resulted from duplex formation between TFO **4** and RNA **1**. However, a large amount of RNA **1** (40 equiv RNA) could not destabilize the triplex that was formed by TFO **5** and HP DNA **2**. These results suggested that TFO containing s^6Gs and s^4Ts could selectively bind to the complementary DNA duplex but not to the single-stranded RNA. This property of TFOs containing s^6Gs and s^4Ts is very useful for the direct regulation of gene expression without the interference of a huge number of mRNA copies containing similar sequences in the cytoplasm.

In summary, we found that the consecutive arrangement of the thiocarbonyl groups of s^6Gs and s^4Ts remarkably stabilized antiparallel triplexes by the strong stacking effects of thiocarbonyl groups. This is the first report of the enhancement of the structural stability of antiparallel triplexes by the introduction of chemical modifications into nucleoside moieties. Furthermore, it was observed that TFOs containing s^6Gs and s^4Ts could selectively bind to the complementary DNA duplex and not to the mismatched DNA duplexes or single-stranded RNA, compared with unmodified TFOs. These results indicated that TFOs containing s^6Gs and s^4Ts may be useful for the direct regulation of gene expression. The excellent properties of TFOs incorporating thionucleoside moieties could provide new insights into various fields of nanotechnology. Further studies on these issues are now in progress.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.11.079.

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