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Stable triplex formation using the strong stacking effect of consecutive thionucleoside moieties†

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In this study, it was found that the arrangement of consecutive thiocarbonyl groups of s^2T and m^5s^2C remarkably stabilized the pre-protonated form of the triplex, and that the stabilization of the pre-protonated form increased the p*K*a value of a cytosine derivative in the triplex.

Over the last two decades, researchers have reported a large number of synthetic DNA and RNA oligomers[1] having various functional groups as useful molecules for gene therapies[2] such as RNAi and antisense strategies, the exhaustive analysis of gene expression,[3] the detection of single nucleotide polymorphisms,[4] and DNA- (or RNA-) nanostructure.[5] Among them, triplex-forming oligonucleotides (TFOs)[6] have long been recognized as powerful tools for straightforward regulation of gene expression. This antigene strategy is apparently rational because it allows a single target of nuclear DNA, but the other methods require targeting a large number of mRNA copies in the cytoplasm. Although this advantage is often considered, there is a disadvantage that the binding affinity of an unmodified TFO to the corresponding DNA duplex is very weak under neutral conditions compared with that of an antisense molecule with the target RNA. To overcome this serious problem, a large number of research groups have reported various chemical modifications of TFOs. In particular, it is well known that the introduction of amino moieties, such as aminopropargyl, guanidium ethyl, and 2-aminoethyl groups, into the 5-position of pyrimidine bases and the 2′-position of the ribose moiety can considerably increase the stability of triplexes even under neutral conditions because of the charge–charge interactions between cationic amino and anionic phosphate groups of DNA duplexes.[6b–d] In addition, Imanishi *et al.* reported that 2′,4′-BNA with *N*-form sugar puckering could strongly enhance the stability of the triplex structure.[6e]

On the other hand, we previously reported that the replacement of the thymidine of TFOs with 2-thiothymidine (s^2T)[7] in a TAT triad increased their affinity for DNA duplexes because of the strong stacking effect of the thiocarbonyl group at the 2-position of the pyrimidine base. In this study, we focused our interest on this strong stacking effect of the thiocarbonyl groups of 2-thiothymidine (s^2T), 5-methyl-2-thiocytidine (m^5s^2C), 8-thioxoadenosine (s^8A), and 8-thioxoinosine (s^8I) (Fig. 1) and tried to increase the triplex-forming abilities of TFOs by using these thionucleosides arranged in a consecutive manner.

To incorporate the thionucleosides into DNA- and RNA-type TFOs 1–10, we first synthesized their corresponding phosphoramidite units 1–5 (Schemes S1–S4, ESI†). The elongation and deprotection of TFOs containing consecutive thionucleoside residues was performed using these phosphoramidite units in a DNA synthesizer by the general procedure. Interestingly, during deprotection of the 2-(trimethylsilyl)ethylthio (TMSES) group from the protected s^8A and s^8I species in 2′-OMe RNAs, we found that the desulfurization of the thiocarbonyl group occurred when TFOs 11 and 12 were treated with tetrabutylammonium fluoride (TBAF) for more than 1 h, although such a thiocarbonyl group in DNA was stable under the same conditions for 2 h.[8] Therefore, the TMSES group was deprotected by treatment with 1 M TBAF in THF for a short time of 30 min in the synthesis of TFOs 11 and 12. After TFOs were released from resins, they were isolated by HPLC and characterized by MALDI-TOF mass spectrometry.

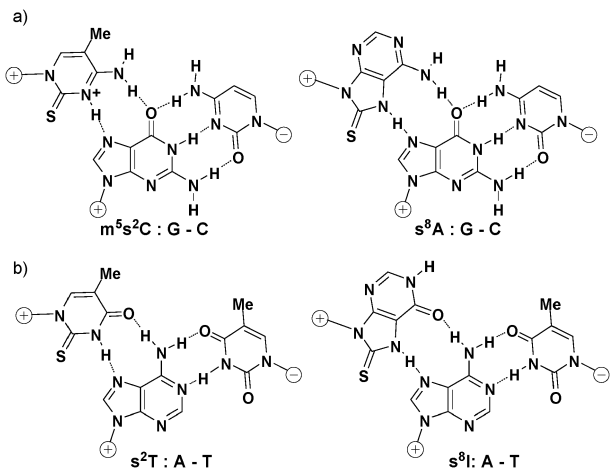

Fig. 1 Base pairs of (a) m^5s^2C or s^8A with G–C and (b) s^2T or s^8I with A–T.

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Table 1 T_m values of triplexes and duplexes containing C, m^5s^2C and s^8A at pH 7.0

TFO 1	5′-d[T T T C T T C T T C T T]-3′
TFO 2	5′-d[T T T C T T m^5s^2C T T C T T]-3′
TFO 3	5′-d[T T T C s^2T s^2T C s^2T s^2T C T T]-3′
TFO 4	5′-d[T T T C s^2T s^2T m^5s^2C s^2T s^2T C T T]-3′
TFO 5	5′-d[T T T m^5s^2C s^2T s^2T m^5s^2C s^2T s^2T m^5s^2C T T]-3′
HP 1	5′-d[G A A A G A A G A A G A A A C^T_T 3′-[C T T T C T T C T T C T T T G$_T$T
TFO 6	5′-d[T T T s^2T s^2T C C C C s^2T s^2T T T]
TFO 7	5′-d[T T T s^2T s^2T m^5s^2C m^5s^2C m^5s^2C m^5s^2C s^2T s^2T T T]
TFO 8	5′-d[T T T s^2T s^2T s^8A s^8A s^8A s^8A s^2T s^2T T T]
HP 2	5′-d[G A A A A A A G G G G A A A A C^T_T 3′-[C T T T T T C C C C T T T T G$_T$T
TFO 9	5′-d[s^2T s^2T s^2T s^2T s^2T m^5s^2C s^2T m^5s^2C s^2T m^5s^2C s^2T m^5s^2C s^2T m^5s^2C T]
TFO 10	5′-$_{Me0}$[s^2T s^2T s^2T s^2T s^2T m^5s^2C s^2T m^5s^2C s^2T m^5s^2C s^2T m^5s^2C s^2T m^5s^2C] dT
TFO 11	5′-$_{Me0}$[s^2T s^2T s^2T s^2T s^2T s^8A s^2T s^8A s^2T s^8A s^2T s^8A s^2T s^8A] dT
TFO 12	5′-$_{Me0}$[s^8I s^8I s^8I s^8I s^8I s^8A s^8I s^8A s^8I s^8A s^8I s^8A s^8I s^8A] dT
HP 3	5′-d[A A A A A G A G A G A G A C^T_T 3′-[T T T T T C T C T C T C T G$_T$T
RNA 1	3′-r[A A A A A G A G A G A G A]

Entry	Oligonucleotides	T_m/°C	ΔT_m/°C
1	TFO 1–HP 1	16[a]	—
2	TFO 2–HP 1	17[a]	1
3	TFO 3–HP 1	37[a]	—
4	TFO 4–HP 1	44[a]	7
5	TFO 5–HP 1	52[a]	15
6	TFO 6–HP 2	14[a]	—
7	TFO 7–HP 2	13[a]	−1
8	TFO 8–HP 2	55[a]	41
9	TFO 9–HP 3	29[b]	—
10	TFO 10–HP 3	55[b]	26
11	TFO 11–HP 3	57[b]	28
12	TFO 12–HP 3	19[b]	−10
13	TFO 10–RNA1	77[c]	22
14	TFO 11–RNA1	28[c]	−29

[a] The T_m values are accurate within ±0.5 °C. The T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate (pH 7.0), 500 mM NaCl, 10 mM $MgCl_2$, and 2 μM triplex. The T_m values of HP 1–2 themselves were 79 °C and 82 °C, respectively. [b] T_m conditions; 10 mM sodium phosphate (pH 7.0), 100 mM NaCl, and 2 μM triplex. The T_m value of HP 3 itself was 78 °C. [c] T_m conditions; 10 mM sodium phosphate (pH 7.0), 100 mM NaCl, and 2 μM duplex.

Table 1 shows the T_m values of triplexes formed between TFOs 1–12 and three hairpin-type duplexes (HPs) 1–3, under neutral conditions (pH 7.0). When a m^5s^2C was incorporated into an unmodified oligonucleotide (TFO 1) in place of C at the middle position, the hybridization affinity of TFO 2 containing a m^5s^2C for HP 1 was almost the same as that of the unmodified TFO 1 [16 °C (entry1) *vs.* 17 °C (entry 2)]. However, the hybridization affinity of TFO 4 containing a m^5s^2C was greater by 7 °C than TFO 3 containing a C [44 °C (entry 4) *vs.* 37 °C (entry 3)] when four s^2T residues were incorporated into TFOs 1–2 to stabilize the triplex structures by the stacking effect of the thiocarbonyl group. In addition, the hybridization affinity of TFO 5 containing three discontinuous m^5s^2C residues surprisingly increased to 52 °C, as shown in entry 5. These results indicated that the presence of the consecutive 2-thiocarbamoyl groups could remarkably result in the stabilization of the triplex structure by three-dimensionally fixing the positions of m^5s^2Cs because of the stacking effect (Fig. 2), so that the hybridization affinities of TFOs 4 and 5 having m^5s^2Cs were greater than

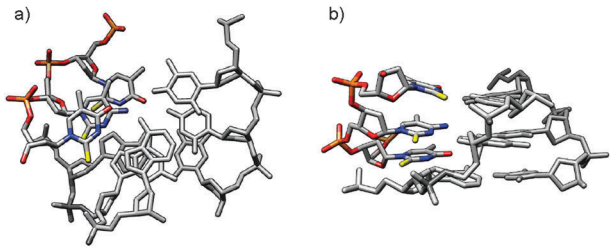

Fig. 2 Computer modelling of a DNA triplex containing s^2T and m^5s^2C residues. (a) Top view and (b) side view. The sequence of TFO is 5′-s^2Tm^5s^2Cs^2T-3′.

those of TFO 3 having Cs. Moreover, we also found that the base-pair recognition ability of a m^5s^2C (ΔT_m = 28 °C), which refers to the difference (ΔT_m) in the T_m values between the matched and most stable mismatched triplexes, was greater than those of C (ΔT_m = 22 °C) (Table S1, ESI†).

Next, we measured the T_m values of a triplex formed between TFO 16 containing a m^5s^2C residue and HP 8 at various pHs (pH 6.0–7.2) to examine if protonation could occur by the consecutive 2-thiocarbonyl groups under mild conditions near pH 7.0 (Fig. S1, ESI†). As a result, we found that the p*K*a value of a m^5s^2C in the triplex surprisingly increased to 6.7. This value near 7.0 should be noteworthy because the p*K*a value of the protonated C moiety in an unmodified DNA triplex was reported to be 5.5 according to Dervan and Singleton.[9] We also found that the p*K*a value of the protonated C moiety in a triplex formed between TFO 17 and HP 5 increased to 6.3 (Fig. S2, ESI†). The p*K*a value of a protonated m^5s^2C monomer (p*K*a = 4.24) was almost equal to that of the protonated C monomer (p*K*a = 4.3); these results strongly suggested that the protonation of a cytosine derivative in the triplexes could be considerably promoted by the pre-protonated form stabilized by the stacking effect of the thiocarbonyl groups.

Entries 6–8 (Table 1) show the T_m values of triplexes formed between TFOs 6–8 containing four consecutive C derivatives and HP 2 under neutral conditions. The hybridization affinity of TFO 7 containing four m^5s^2Cs was slightly lower than that of TFO 6 containing Cs. These results demonstrated that the destabilizing effect of the internal cation repulsion arising from the consecutive protonated cytosine bases was much stronger than the stabilizing effect of the consecutive thiocarbonyl groups of m^5s^2Cs. Therefore, we changed the sequence of four m^5s^2Cs to an alternate sequence of four s^8As as neutral thionucleobase analogs having two donor protons (see Fig. 1–a) to avoid internal cation repulsion. As a result, the hybridization ability dramatically increased to 55 °C (ΔT_m = 41 °C). It should be emphasized that the alternate arrangement of two modified bases, s^8A and s^2T, keeping eight thionucleoside residues in a consecutive sequence, such as s^2T-s^2T-s^8A-s^8A-s^8A-s^8A-s^2T-s^2T, was highly effective in enhancing the binding affinities of TFOs for double-stranded DNAs.

Subsequently, we changed the sugar moieties of TFOs incorporating consecutive thionucleobases from 2′-deoxyribose to 2′-OMe-ribose to increase their triplex-forming abilities, because it is well known that RNA-type TFO can strongly bind to the target DNA duplex compared with the corresponding DNA-type TFO. The triplex-forming ability of RNA-type TFO 10 having s^2Ts and

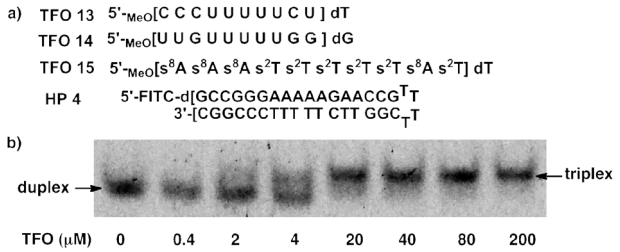

Fig. 3 Triplex formation of TFOs 13–15 with HP 4 containing the sequence of *c-myc* P2 promoter. (a) Sequences of TFOs 13–15 and HP 4. (b) Electrophoretic mobility shift assay of the triplex formed between TFO 15 and HP 4 (40 nM) on a 10% nondenaturing polyacrylamide gel at pH 7.0. At various concentrations, TFO 15 was incubated with HP 4 for 2 h at 37 °C.

discontinuous m^5s^2Cs was expectedly much greater than the corresponding DNA-type TFO 9 (entries 9 and 10, Table 1). In addition, we found that the use of s^8As in place of m^5s^2Cs in entry 11 slightly enhanced its binding affinity even in the sequence of TFOs containing discontinuous C derivatives.

We previously reported that the backbone structure of triplexes was somewhat disturbed by the presence of a bigger s^8A base in place of the protonated C because the distance between the C1′ atoms in the neighboring mononucleotide units increased only at the position of s^8A (Fig. S3, ESI†).[8] To avoid distortion of the triplex structure and reinforce the structure, we introduced s^8I into TFO 11 in place of s^2T. The distortion of the triplex structure might not have occurred because the size of s^8I was very similar to that of s^8A. However, we unexpectedly found that the T_m value of TFO 12 was much lower than that of TFO 11 (19 °C *vs.* 57 °C).

Moreover, we examined the hybridization affinity of TFO 10 containing m^5s^2Cs and TFO 11 containing s^8As toward a single-stranded RNA (entries 13 and 14, Table 1). The T_m value of TFO 10 toward the complementary RNA was higher than that toward the complementary DNA duplex (77 °C *vs.* 55 °C). These results indicated that TFO containing m^5s^2Cs could strongly bind to the complementary single-stranded RNA compared to the complementary DNA duplex, although the m^5s^2C residue of TFO 10 would be expected to interfere with the duplex formation due to the steric hindrance resulting from the thiocarbonyl group.[7a] On the other hand, the T_m value of TFO 11 toward the complementary RNA was much lower than that toward the complementary DNA duplex (28 °C *vs.* 57 °C). These results suggested that TFO containing s^8As and s^2Ts could selectively bind to the complementary DNA duplex but not to the single-stranded RNA. This property of TFO containing s^8As and s^2Ts is very useful for the direct regulation of gene expression without interference by a huge number of mRNA copies in the cytoplasm.

Finally, we examined the triplex formation of TFOs targeting the model sequence of the *c-myc* P2 promoter[10] by gel mobility shift experiments at 37 °C and pH 7.0 (Fig. 3). We found that the triplexes formed by the unmodified parallel- and antiparallel-type TFOs 13–14 were unstable under neutral conditions and could not be detected even at a concentration 25 000-fold that of TFO (Fig. S4 and S5, ESI†). However, the triplex formation using 2′-OMe TFO 15 having s^2T and s^8A residues was complete at a concentration 500-fold that of TFO under neutral conditions. These results also suggested that the triplex forming ability of TFOs incorporating consecutive modified nucleobases having the thiocarbonyl group was surprisingly greater (more than 500-fold) than that of unmodified oligonucleotides.

In summary, we found that the arrangement of consecutive thiocarbonyl groups of s^2T and m^5s^2C remarkably stabilized the pre-protonated form of the triplex, and that the stabilization of the pre-protonated form increased the p*K*a value of a cytosine derivative in the triplex. In addition, we could avoid cation repulsion in a triplex having a series of C^+-G-C sequences arranged in a continuous manner by converting the sequence of four m^5s^2Cs into the alternate arrangement of four s^8As. Furthermore, we also observed that 2′-OMe TFOs containing s^8As and s^2Ts could strongly and selectively bind to the complementary DNA duplex (but not to the single-stranded RNA) compared with the unmodified TFOs and DNA-type modified TFOs. These results indicated that TFOs having s^2Ts and s^8As might be useful for the direct regulation of gene expressions. The excellent properties of TFOs incorporating thionucleoside moieties could provide new insight into various fields of nanotechnology. Further studies in this direction are now in progress.

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