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Synthesis and Triplex Formation of Oligonucleotides Containing 8-Thioxodeoxyadenosine

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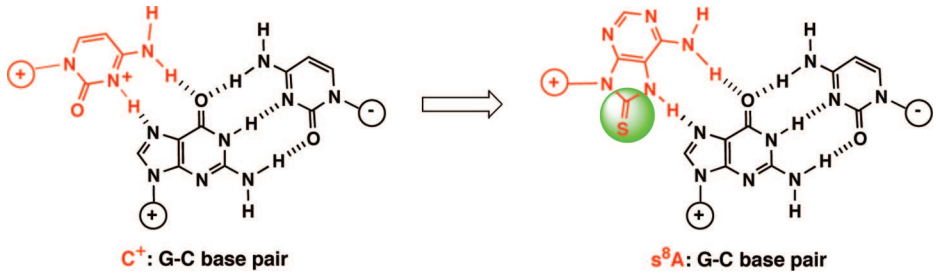
ABSTRACT



For more effective DNA triplex formation under neutral conditions, we synthesized triplex-forming oligonucleotides containing 8-thioxodeoxyadenosine (s^8dA) residues in place of the protonated deoxycytidines required for the third base pairing with DNA duplexes. Consequently, it was found that s^8dA exhibited much stronger hybridization ability than dC under neutral conditions when four s^8dA bases were arranged in a consecutive sequence.

Triplex formation between DNA duplexes and external DNA single strands has been extensively studied during the past two decades.[1] In naturally occurring triplexes, it is well-known that two sets of planar triads composed of T-A-T and C^+:G-C, in which Hoogsteen types of hydrogen bonds are involved, contribute to stabilization of the triplexes in a parallel manner as shown in Figure 1.[2]

To date, a large number of studies on increasing the hybridization affinity of triplex-forming oligonucleotides (TFOs) with the complementary duplex under neutral conditions, using artificially designed nucleobases in place of the cytosine base, have been reported.[3,4] Among them, 5-methylcytosine (m^5C) can bind more strongly to a G-C pair compared with cytosine in triplexes having discontinuous C^+:G-C sequenes under neutral conditions because the pK_a value of m^5C is higher than that of C.[5−7] However, a series of C^+:G-C sequences arranged in a straight manner resulted in a significant decrease in the thermal stability of the resulting triplexes because of the internal cation repulsion arising from the protonated cytosine bases.[8,9] Although 6-oxocytosine,[10−13] pseudoisocytosine,[14,15] and 1-deaza-6-

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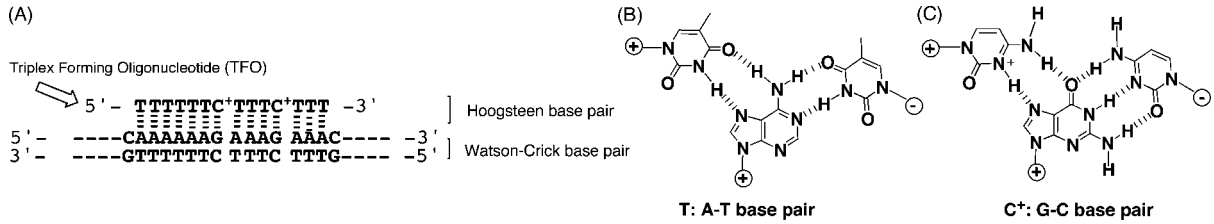


Figure 1. Parallel stranded triplex formation by naturally occurring nucleobases.

azacytosine[16] derivatives have been proposed as neutral modified bases capable of triplex formation independent of pH, a synthesis of these modified bases requires multistep reactions, and some C-nucleoside derivatives tend to epimerize at the anomeric center.

On the other hand, Miller et al. reported that 8-oxoadenine (o^8A) can be used as a neutral species that can bind to G of the G-C pair using two N−H···N hydrogen bonds.[17] As a result, the binding ability of oligodeoxyribonucleotides having o^8A toward G of the G-C pair of DNA duplexes at physiological pH was similar to that of oligodeoxyribonucleotides having cytosines. Davison et al. also synthesized oligodeoxyribonucleotides incorporating a cytosine, 8-oxoadenine, or adenine base and showed their effects on the thermal stability of the triplexes formed with a DNA duplex.[18] These results also showed that 8-oxoadenine base could bind to the G-C base pair, whereas the unmodified A could not. The orientation of the glycosyl bond of 8-oxoadenosine is known to be *syn*.[19] It is also suggested that this modified base has a *syn* conformation in its deoxyribonucleoside counterpart.[20] Only the *syn* conformation, which is in equilibrium with the *anti* conformation, allows the formation of a third base pair between o^8A and G-C. Therefore, if it is possible to make this equilibrium *syn* conformation, formation of the triad would be entropically favorable.

Because the sulfur atom is larger than the oxygen atom, it was expected that 8-thioxoadenine (s^8A) should favor the *syn* orientation compared with o^8A. In addition to this advantage, the use of the thio-carbonyl group could enhance the stacking interaction with the downstream bases in TFO because the thiouracil base showed such an effect upon incorporation into oligonucleotides.[21,22] On the basis of these results, we thought that s^8A would be the choice of modified adenine for increasing the hybridization ability of TFO having a sequence of consecutive cytosine bases as shown in Figure 2.

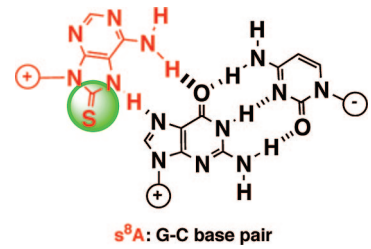
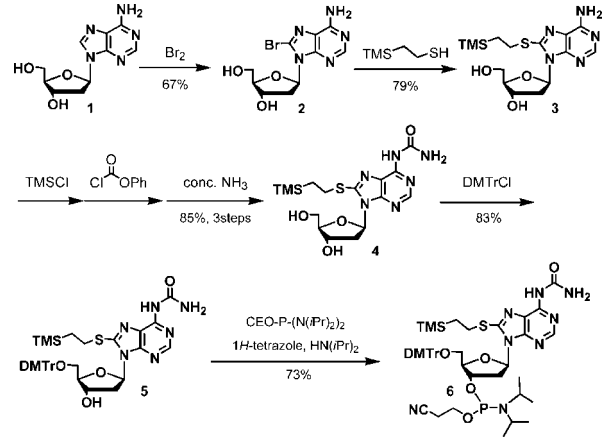


Figure 2. Structure of 8-thioxodeoxyadenosine (s^8A) and triplex formation with a G-C base pair.

To incorporate this modified deoxyribonucleoside into DNA oligomers, the phosphoramidite building block **6** was synthesized, as shown in Scheme 1. The trimethylsilylethyl

Scheme 1. Synthesis of 6-*N*-Carbamoyl-8-thioxodeoxyadenosine 3′-Phosphoramidite **6**



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group was chosen as the protecting group for a thiol group, which is tautomerized with the 8-thioxo group of s^8A, because 8-[2-(trimethylsilyl)ethylthio]deoxyadenosine **3** has been synthesized from deoxyadenosine **1** via 8-bromodeoxyadenosine **2**, reported by Chambert et al.,[23] as shown in Scheme 1. Additionally, we also chose the unsubstitued carbamoyl group as a protecting group of an amino group at the 6-position of adenine, which could be removed by treatment with a 1 M tetrabutylammonium fluoride (TBAF) solution in THF to give the desired oligomers (see Figure S1 in Supporting Information).

The carbamoyl group could be easily introduced into the 6-amino group by treatment of **3** with phenyl chloroformate in the presence of trimethylsilyl chloride and triethylamine followed by ammonolysis to produce the 2-*N*-carbamoyl derivative **4** in 85% yield. The usual dimethoxytritylation of **4** followed by 3′-phosphitylation afforded the phosphoramidite building block **6** via the 5′-tritylated species **5**.

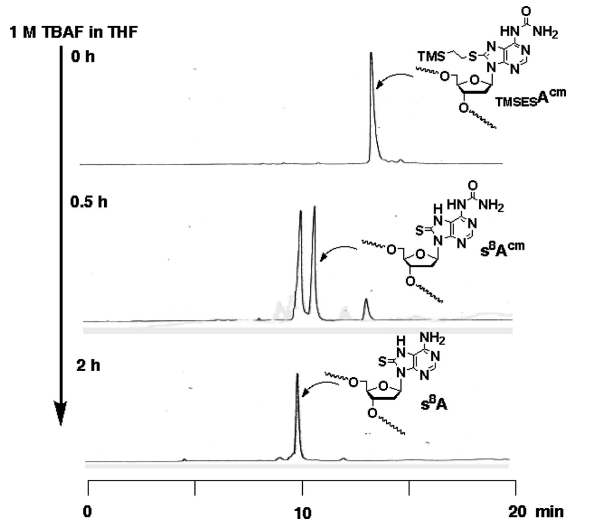

Figure 3. Time course of deprotection of the TMSE and carbamoyl groups by treatment with a 1 M TBAF silution in THF. Each peak was identified by MALDI-TOF mass spectroscopy.

The synthesis of oligodeoxyribonucleotides (TFOs 2, 3, and 7, as shown in Tables 1 and 2) containing s^8A was carried out using an automated synthesizer. After the synthetic cycles were finished, they were isolated in the usual way. The oligodeoxyribonucleotides having a 5′-terminal DMTr group were absorbed on a C_{18} cartridge. The eluate was treated with a 1 M TABF solution in THF. Figure 3 shows a reverse-phase HPLC analysis of the synthesis of TFO 2. A somewhat stable but degradable intermediate with a peak at 10.4 min was observed after 30 min. A mass spectrographic analysis suggested that this early product was an oligonucleotide containing an s^8A^{cm} residue. Upon prolonged treatment, this peak was completely converted to a new peak at 9.96 min. This final product was identified with the target DNA 14mer containing a s^8A residue by MALDI-TOF mass spectrometry. TFOs 3 and 7 containing s^8A residues were also synthesized by the same treatment as described above.

To examine the triplex-forming ability of TFOs 2 and 3, T_m experiments of the triplexes formed between these oligomers and the DNA duplex HP 1 having a hairpin structure described in Table 1 were carried out. In addition, we synthesized an unmodified DNA oligomer (TFO 1) and two oligomers (TFOs 4 and 5) containing one or two o^8As to compare the effect of the thioxo group on the thermal

Table 1. T_m Values for DNA Triplexes Containing C, s^8A, and o^8A

5′-TTTTTTT **X** TTT **X′**TTT-3′ — TFOs 1-5
5′-CAAAAAA **Y** AAA **G** AAAC $^{T}_{T}$ — hairpin duplexes (HPs 1-2)
3′-GTTTTTT **Z** TTT **C** TTTG $_{T}^{T}$

entry	triplex	X	X′	Y	Z	T_m (°C)	ΔT_m (°C)
1	TFO 1 - HP 1	C	C	G	C	30.5[a]	
2	TFO 2 - HP 1	s^8A	C	G	C	29.0[a]	−1.5[c]
3	TFO 3 - HP 1	s^8A	s^8A	G	C	27.4[a]	−3.1[c]
4	TFO 4 - HP 1	o^8A	C	G	C	28.2[a]	−2.3[c]
5	TFO 5 - HP 1	o^8A	o^8A	G	C	22.4[a]	−8.4[c]
6	TFO 1 - HP 1	C	C	G	C	41.9[b]	
7	TFO 1 - HP 2	C	C	C	G	16.3[b]	−25.6[d]
8	TFO 2 - HP 1	s^8A	C	G	C	39.7[b]	
9	TFO 2 - HP 2	s^8A	C	C	G	8.9[b]	−30.8[d]
10	TFO 3 - HP 1	o^8A	C	G	C	33.8[b]	
11	TFO 3 - HP 2	o^8A	C	C	G	15.3[b]	−18.5[d]

[a] T_m values are accurate within ±0.5 °C. The T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate buffer (pH 7.0), 500 mM NaCl, 10 mM $MgCl_2$, and 2 μM triplex. [b] T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate buffer (pH 6.0), 500 mM NaCl, 10 mM $MgCl_2$, and 2 μM triplex. [c] ΔT_m is the difference in the T_m value between the unmodified triplex (entry 1) and the modified triplexes having s^8A or o^8A (entries 2−5). [d] ΔT_m is the difference in the T_m value between the matched triplex (entries 6, 8, 10) and mismatched triplexes (entries 7, 9, 11).

Table 2. T_m Values for DNA Triplexes Containing Consecutive Cs, s^8As, and o^8As.

5′-TTTTTT **XXXX** TTTT-3′ — TFOs 6-8
5′-CAAAAAA **GGGG** AAAAC $^{T}_{T}$ — hairpin duplex (HP 3)
3′-GTTTTTT **CCCC** TTTTG $_{T}^{T}$

entry	triplex	X	T_m (°C)[a]	ΔT_m (°C)[b]
1	TFO 6 - HP 3	C	9.7	
2	TFO 7 - HP 3	s^8A	36.8	+27.1
3	TFO 8 - HP 3	o^8A	17.4	+ 7.7

[a] T_m values are accurate within ±0.5 °C. The T_m measurements were carried out in a buffer containing 10 mM sodium cacodylate buffer (pH 7.0), 500 mM NaCl, 10 mM $MgCl_2$, and 2 μM triplex. [b] ΔT_m is the difference in the T_m values between the unmodified triplex (entry 1) and the modified triplexes having s^8A or o^8A (entries 2−3).

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stability of triplexes. The results of the T_m experiments are also shown in Table 1.

The T_m value of TFO 2 containing one s^8A in entry 2 was lower than that of unmodified TFO 1 in entry 1 (29.0 vs 30.5 °C), although the T_m value of TFO 2 containing one s^8A was higher than that of TFO 3 containing one o^8A in entry 3 (29.0 vs 28.2 °C). It was also found that the T_m value of the TFO decreased further by the addition of a discontinuous s^8A as shown in entry 3 (27.4 vs 30.5 °C) though the T_m values of TFO 3 obtained at various pHs showed the pH-independence of TFO 3 in Figure S2 in Supporting Information. In entry 5, the T_m value of TFO 5 containing two discontinuous o^8As was significantly lower (by 8.4 °C) than that of unmodified TFO 1. Because the backbone structure was somewhat disturbed by the presence of a bigger s^8A base in place of the protonated C, the distance between the C1′ atoms in the neighboring mononucleotide units became longer only at the modified bases so that the constant structure could not be preserved around the modified sites.

Subsequently, we examined the selectivity of modified DNA oligomers to study whether the s^8A base can actually form a third Hoogsteen-type base pair with the guanine base. In the hairpin duplex (HP 2), we replaced the central C-G base pair with a base pair of G-C that cannot form hydrogen bonds with s^8A or o^8A, as shown in entries 6−11 of Table 1. We carried out the T_m experiments at pH 6.0 since the triplexes formed between TFO 1 or 2 and HP 2 were very unstable at pH 7.0. It should be noted that the selectivity of s^8A with ΔT_m of 30.8 °C (difference in the T_m values between entry 8 and entry 9) was superior to that of the unmodified cytosine with ΔT_m of 25.6 °C (difference in the T_m values between entry 6 and entry 7). On the other hand, the o^8A base has a poorer selectivity with ΔT_m of 18.5 °C (difference in the T_m values between entry 10 and entry 11). Because the s^8A base has a higher selectivity than the protonated C base, it is likely that the former can form two hydrogen bonds with G of G-C. The predominance of s^8A over o^8A could be explained in terms of the higher contribution of the *syn* form around the glycosyl bond of the former.

Next, we synthesized oligodeoxyribonucleotides having four s^8A or o^8A bases in a consecutive sequence and studied their hybridization property to examine if our proposal is correct. It was expected that when a consecutive sequence of four s^8A bases is incorporated into the DNA oligomer TFO 7, the stacking effect of two s^8A bases was enhanced, as reported in the case of poly-2-thiouridylates.[21] The T_m values of the oligomers having four consecutive unmodified and modified (o^8A and s^8A) bases are summarized in Table 2. The T_m value of TFO 6 with a consecutive sequence of four cytosines to HP 3 was very low ($T_m = 9.7$ °C) compared with the T_m value (24.4 °C) of TFO 9 having four discontinuous cytosine bases, 5′-TTTCTTCTTCTTCT, to the complementary HP 4, 5′-CAAAGAAGAAGAAGACTTT-TGTCTTCTTCTTCTTTG. The significant decrease in the T_m value could be explained in terms of the increasing electronic repulsion between the protonated cytosine bases. Similar phenomena have been observed in earlier studies by Roberts[8] and recent studies by James.[9] In contrast, the T_m values of TFOs 7 and 8 having s^8A and o^8A residues, respectively, as the protonated cytosine base analogs increased compared with the unmodified TFOs (entries 2 and 3). In particular, the T_m value of TFO 7 having four consecutive s^8A bases was significantly higher (by 27.1 °C) than that of TFO 6. These results strongly support our proposal that the hybridization ability of TFO by incorporating a sequence of consecutive s^8A bases would be increased.

In summary, we synthesized the s^8A phosphoramidite unit **6** and TFOs having s^8A residues for the first time. The selectivity of a TFO having an s^8A residue was higher than that of the unmodified TFO, whereas the hybridization ability of a TFO having an s^8A was lower than that of the unmodified TFO. Moreover, it was also found that the hybridization ability of TFO 7 having four consecutive s^8A bases was significantly higher than those of the corresponding unmodified TFO 6 or TFO 8 containing four consecutive o^8A as a well-known modified base of a protonated cytosine. These results indicate that it might be necessary to use the cytosine analogs properly in sequences of discontinuous and consecutive cytosine bases. In the case of TFOs containing discontinuous cytosine bases, the use of 5-methylcytosine accessible to the protonation[5,6] of the cytosine ring in a TFO under neutral conditions might be better because of its stronger hydrogen bonding ability. On the other hand, s^8A should be used in place of cytosine to avoid electronic repulsion and enhance the stacking interaction when a series of cytosine bases are arranged in a consecutive sequence. Further studies are now under way in this direction.

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Supporting Information Available: Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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