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Synthesis and triplex-forming properties of oligonucleotides containing thio-substituted *C*-nucleoside 4-thiopseudoisocytidine

Shi-Qi Cao, Itaru Okamoto, Hirosuke Tsunoda, Akihiro Ohkubo, Kohji Seio, Mitsuo Sekine *

Department of Life Science, Tokyo Institute of Technology, 4295 Nagatsuta, Midoriku, Yokohama 226-8501, Japan

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ABSTRACT

We synthesized the 3′-phosphoramidite building block of 4-thiopseudoisocytidine ($s^4\Psi iC$) and incorporated it into triplex-forming oligonucleotides (TFOs). The results of thermal denaturation of triplexes incorporating $s^4\Psi iC$ showed that $s^4\Psi iC$ could be used as a nucleoside component of TFOs to increase the thermal stability of triplexes at pH 7.

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In recent years, triplex-forming oligonucleotides (TFOs) have been extensively studied because they can be used in the antigene strategy targeting cellular double helical DNAs.[1–4] Researchers have shown that, under suitable conditions, TFOs can bind to the major groove of DNA duplexes and form triplexes in parallel and antiparallel orientations.[1–4] In the parallel orientation, triple helixes can be constructed by the T·A–T and C^+·G–C triad formations of thymine (T) and protonated cytosine (C^+) in TFOs and A–T and G–C base pairs in DNA duplexes, respectively, through Hoogsteen hydrogen bonds. However, there is a major drawback in the C^+·G–C motif.[5,6] Protonation of the N^3-position of C in TFOs is necessary for the formation of its hydrogen bond with N^7 of G in DNA duplexes, requiring an optimized range of pH 5–6, which does not meet the physiological conditions required for the antigene therapy. To overcome this restriction, several modified nucleosides have been developed to mimic the structure of the N^3-protonated C.[7–11] For example, 2′-O-methylated pseudoisocytidine with 2-amino-4-oxopyrimidin-5-yl moiety (ΨiC) as an aglycone was used to form a triad with a G–C base pair under neutral conditions when incorporated into TFOs.[10,11] As indicated in the base pair motif shown in the right side of Figure 1, there is a proton at the N^3 position of ΨiC (X = O) which is available for a Hoogsteen hydrogen bond with the N^7 position of G. TFOs containing ΨiC could stabilize the triplex structure more than those containing unmodified C or 5-methylcytosine at neutral pH.

On the other hand, TFOs containing thionucleosides such as 2′-*O*-methyl-2-thiouridine or 2-thiothymidine formed relatively stable parallel triplexes when they were bound to DNA duplexes.[12] The enhancement of the thermal stability of these triplexes can be explained by the strong stacking interaction of the thiocarbonyl group.

Accordingly, we designed 4-thiopseudoisocytidine ($s^4\Psi iC$) in which the thiocarbonyl group at position 4 was replaced with a carbonyl group (see Fig. 1, left). Previously, we synthesized $s^4\Psi iC$ successfully.[13] From ^{1}H NMR spectral analysis, we determined that the preferred sugar conformation of this nucleoside was a C3′-*endo* pucker. As it is well known that RNA-type TFOs form more stable triplexes than their deoxy counterparts,[14] we expected that $s^4\Psi iC$ would be favorable for the stabilization of triplexes when we used it as the nucleoside component of TFOs. However, to the best of our knowledge, no group has reported the synthesis of the

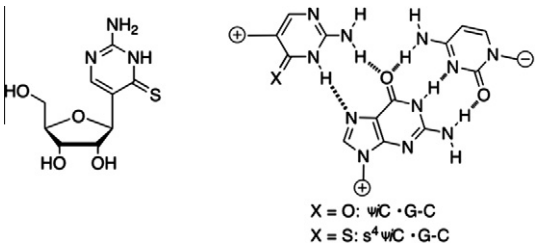

Figure 1. Structure of 4-thiopseudoisocytidine (left); proposed hydrogen bonding schemes of ΨiC·G–C and $s^4\Psi iC$·G–C base triads (right).

* Corresponding author. Tel.: +81 45 924 5706; fax: +81 45 924 5772.
E-mail address: msekine@bio.titech.ac.jp (M. Sekine).

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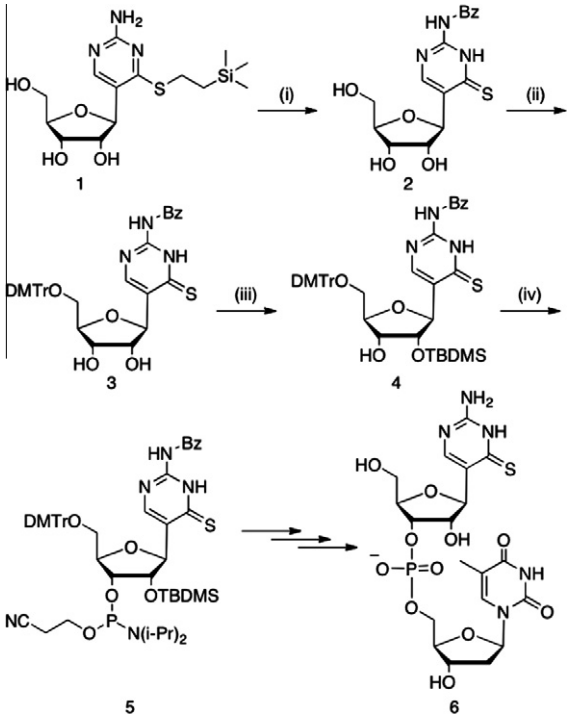

Scheme 1. Reagents and conditions: (i) (a) TMSCl (10 equiv), pyridine, rt, 1 h; (b) PhC(O)Cl (2.0 equiv), pyridine, rt, 2 h; (c) 1.0 M TBAF/THF (10 equiv), rt, overnight, 53% (three steps); (ii) DMTrCl (1.2 equiv), pyridine, rt, 5 h, 61%; (iii) TBDMSCl (1.2 equiv), imidazole (3 equiv), rt, 4 h, 26%; (iv) $NCCH_2CH_2OPCl(i\text{-}Pr)_2$ (2.5 equiv), 2,4,6-collidine (7.5 equiv), *N*-methylimidazole (0.5 equiv), THF, rt, 40 min, 80%.

corresponding thio-modified *C*-ribonucleoside phosphoramidite and oligonucleotides incorporating this modified nucleoside.

In order to incorporate $s^4\Psi iC$ into TFOs and study their triplex formation, we developed a practical approach for the synthesis of the 3′-phosphoramidite derivative (**5**) of $s^4\Psi iC$ as shown in Scheme 1. We began the synthesis of **5** from compound **1**, which we synthesized via a four-step reaction from pseudouridine.[13] According to the procedure described by Jones and co-workers,[15] compound **1** was first treated with chlorotrimethylsilane in pyridine for transient protection of the hydroxyl groups and then with benzoyl chloride to protect the exocyclic amino group.

Treatment with 1.0 M tetrabutylammonium fluroride (TBAF) in THF removed the trimethylsilylethyl (TMSE) group and the TMS groups to give **2** in 53% yield. Separately, we also tried to use the TMSE group as the protecting group of the thiocarbonyl group during chain elongation required for the synthesis of oligonucleotides incorporating compound **1**. However, the TMSE group was too stable to easily remove after the incorporation to the oligonucleotides without damaging the phosphodiester backbones, and the 4-thiocarbonyl group was rather labile (date not shown).

Subsequently, we selectively protected the 5′-OH group of **2** with the DMTr group to give **3** in 61% yield. For the protection of the 2′-OH group, we protected it using TBDMSCl, and the 2′-protected derivative **4** could be isolated in 26% yield from a mixture with the 3′-protected and 2′,3′-diprotected products. Finally, the reaction of **4** with $NCCH_2CH_2OPCl(i\text{-}Pr)_2$ in the presence of 2,4,6-collidine and *N*-methylimidazole[16] gave the phosphoramidite **5** in 80% yield. We should note that the unprotected thiocarbonyl group did not react with $NCCH_2CH_2OPCl(i\text{-}Pr)_2$ under the conditions used in this procedure.

In order to examine the stability of $s^4\Psi iC$ during the solid phase DNA synthesis, we first synthesized the dimer ($s^4\Psi iC$)pT (**6**) on the solid supports, using the phosphoramidite **5**. According to the usual protocol of DNA and RNA synthesis, we used 1*H*-tetrazole as the activator for the coupling step.[17] Because $s^4\Psi iC$ is a ribonucleoside, this step was performed for 20 min, according to the usual RNA synthetic protocol.[18] We used the 0.02 M I_2 in THF/pyridine/H_2O as the oxidation reagent.[19] Capping occurred with Ac_2O in 0.1 M DMAP–pyridine (1:9, v/v). From the DMTr cation assay, we calculated the overall coupling yield as 35%. According to the method reported by Ogilvie and co-workers,[20] we attempted to cleave the dimer intermediates from the CPG supports via treatment with 3:1 ammonium hydroxide–ethanol (3:1, v/v) for 2 h at room temperature to minimize the removal of the TBDMS group at this step. Before removal of the TBDMS group, the crude mixture was analyzed via reversed-phase HPLC–MS.

However, we observed several unexpected products. As shown in Figure 2, in addition to the desired intermediate **6′** at 21.5 min and the unreacted deoxythymidine (dT) at 6.7 min, we observed compounds containing a disulfide bond and one or two Bz groups at longer retention times. For example, compound **7** at 23.4 min was assigned as the dimer of **6′** with a disulfide bond. Compound **8** at 25.1 min was the mono-benzoylated product of **7**. Compound **9** at 26.0 min was the di-benzoylated product of **7**. In addition, we found a small amount of mono-benzoylated compound **10** at 29.3 min.

Therefore, in order to remove the residual Bz group, we treated the mixture again with 3:1 ammonium hydroxide–ethanol at 55 °C for 2 h. In addition, NaSH was added to the reaction mixture to convert the disulfide bond to two thiocarbonyl groups.[21] After

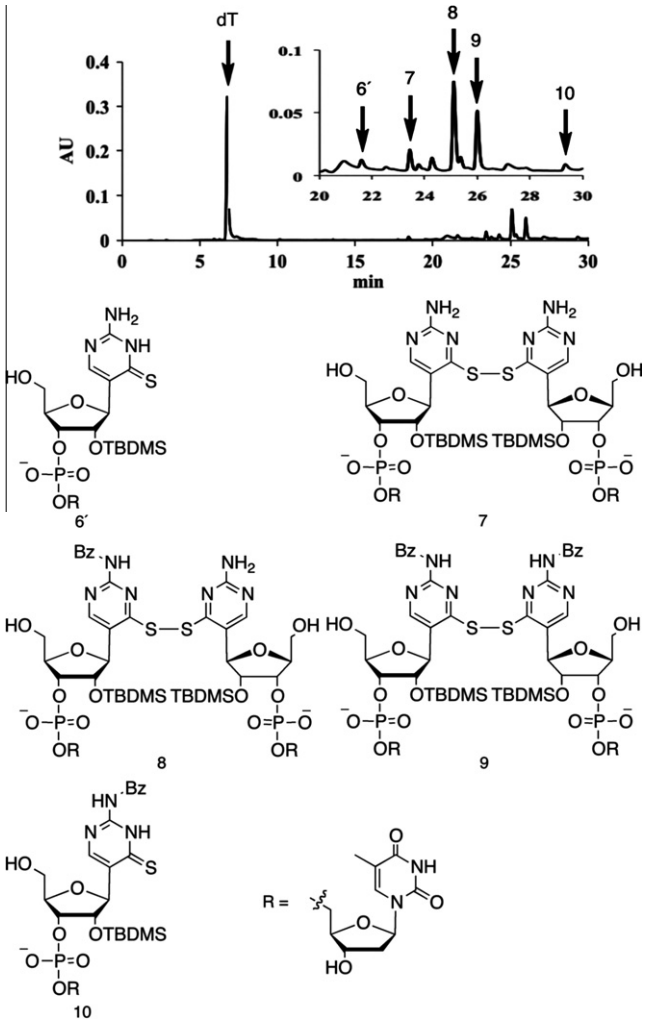

Figure 2. RP-HPLC profile and products of the synthesis of ($s^4\Psi iC$)pT dimers.

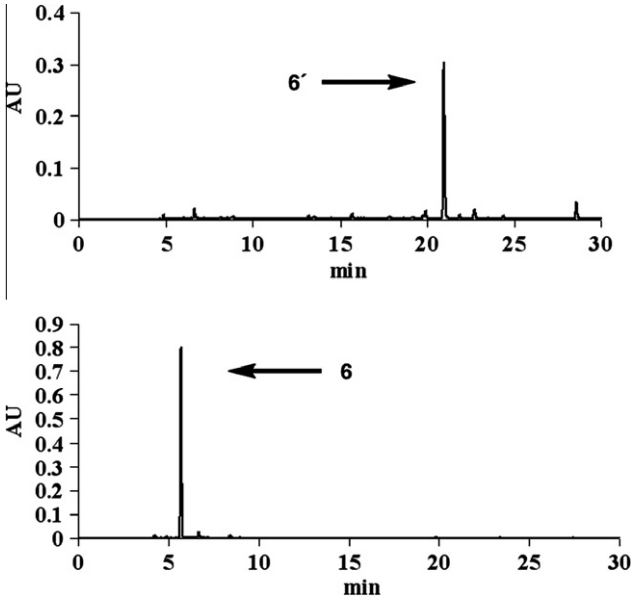

Figure 3. RP-HPLC profiles of compound **6′** (top) and compound **6** (bottom).

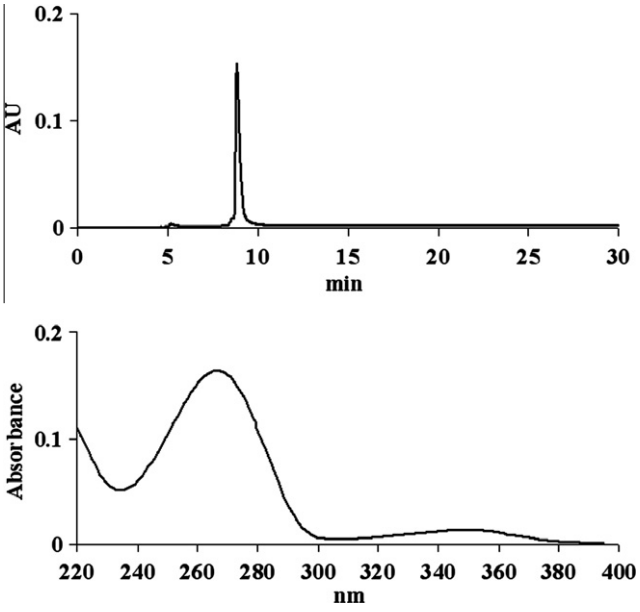

Figure 4. RP-HPLC profile of **TFO-1** (top) and UV spectra of **TFO-1** (bottom).

these reactions, we purified the mixture with a C-18 cartridge column. As expected, the Bz group was removed, and the disulfide bonds were also successfully reduced to give the 2′-*O*-TBDMS protected compound **6′** (see Fig. 3, upper panel).

After removal of ammonia and the solvents, according to the method reported by Sekine and co-workers,[22] we added 20% AcOH in H_2O to compound **6′** to remove the TBDMS group. The use of these acidic conditions was essential for the removal of this TBDMS group because the thiocarbonyl group was converted to the carbonyl group if usual desilylating agents such as TBAF were used (data not shown). The reaction mixture was kept at 30 °C for 2 h, and then NH_4OAc buffer was added to quench the reaction. After lyophilization, we obtained the target molecule ($s^4\Psi i$C)pT (**6**) quantitatively (see Fig. 3, lower panel).

Based on the results of the dimer synthesis, we successfully synthesized 14mer **TFO-1**, 5′TTTTTTs$^4\Psi i$CTTTCTTT 3′containing $s^4\Psi i$C using the above mentioned ammonia treatment in the presence of NaSH and acid treatment for removal of the TBDMS group. After purification, **TFO-1** was analyzed via reversed-phase HPLC as shown in Figure 4, upper panel. From the UV absorption spectrum of **TFO-1** at 8.9 min, we observed an absorbance at 350 nm (see Fig. 4, lower panel). This result suggested that a thiocarbonyl group existed in this oligonucleotide. The structure was also confirmed by MALDI-TOF-MS analysis. The observed molecular weight (4197.96) was consistent with the calculated mass (4197.67).

Finally, to assess the triplex-forming ability of **TFO-1**, we conducted melting temperature (T_m) experiments using a hairpin DNA molecule, 5′CAAAAAAGAAAGAAACTTTTGTTTCTTTCTTTTT-TG3′, which contains a stem region shown by the underlined portions and a four base internal loop region. For comparison, control TFO, 5′TTTTTTXTTTCTTT 3′ with deoxycytidine (**TFO-2**: X = dC; **TFO-3**: X = C) in place of $s^4\Psi i$C was also hybridized with the hairpin oligonucleotide.

Table 1 lists the results of the T_m experiments using **TFO-1** and **TFO-2** under deferent conditions (pH 7.0, 5.7, and 5.0). At pH 7.0, the **TFO-2** with unmodified dC showed a T_m value of 29 °C. On the other hand, the T_m value (45 °C) of a one-point modified triplex that resulted from **TFO-1** having $s^4\Psi i$C was much greater (ΔT_m 16 °C) than those (29 °C and 26 °C) of the triplexes derived from **TFO-2** and **TFO-3**. We measured the pH dependency of T_m in the triplexes containing **TFO-1** and **TFO-2**. With a decrease of pH, the T_m values of the former significantly increased. On the other hand, the T_m values of the latter moderately increased up to 55 °C. At pH 5.0 where the two dCs in **TFO-2** could be completely protonated, the triplex containing **TFO-1** showed a somewhat higher T_m value than that containing **TFO-2**. At pH 5.7, the triplex containing **TFO-1** showed a significantly higher T_m value than that containing **TFO-2**.

These results indicated that **TFO-1** containing one $s^4\Psi i$C residue could form a more stable triplex with the hairpin duplex than the unmodified one under the neutral conditions because the Hoogsteen base pair of $s^4\Psi i$C and guanine does not require protonation at position 3, as shown in Figure 1.

The stabilization effects of the triplex derived from **TFO-2** under acidic conditions are explained in terms of protonation at the N^3-position capable of formation of Hoogsteen hydrogen bonds and neutralization of the neighboring anionic phosphate residues by generation of the positive cation on the cytosine moiety. It is likely that the triplex containing **TFO-1** that might be protonated at pH

Table 1
T_m analysis of DNA triplexes

TFOs	X	pH 7.0		pH 5.7		pH 5.0	
		T_m[a] (°C)	ΔT_m[b] (°C)	T_m[a] (°C)	ΔT_m[b] (°C)	T_m[a] (°C)	ΔT_m[b] (°C)
TFO-1	$s^4\Psi i$C	45	+16	52	+8	55	+3
TFO-2	dC	29		44		52	

[a] T_m values are accurate within ±0.5 °C. The T_m measurements were conducted with 2 μM triplex in a buffer containing 10 mM sodium cacodylate buffer, 500 mM NaCl, 10 mM $MgCl_2$ adjusted at pH 7.0, 5.7, or 5.0.

[b] ΔT_m is the difference in the value between the modified triplex containing **TFO-1** and the unmodified triplex containing **TFO-2**.

5.0 exhibited an additional stacking effect of the thiocarbonyl group toward the nearby bases and/or a stronger effect of the modified base on the Hoogsteen hydrogen bonds.

In conclusion, we synthesized $s^4\Psi i$C 3′-phosphoramite **5** and succeeded in incorporating this thio-substituted *C*-nucleoside into TFOs. In the synthesis of the modified oligonucleotides, we found that the deprotection and cleavages by ammonia should be performed in the presence of NaSH and that the removal of the TBDMS groups should be performed under acidic conditions to suppress the side reactions at the thiocarbonyl group. Furthermore, under neutral conditions, the TFO containing $s^4\Psi i$C exhibited a significant increase in triplex stability as compared to the unmodified TFOs. More detailed studies of the mechanism associated with the stabilization of triplexes with $s^4\Psi i$C are now in progress.

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