Synthesis of 4-Thiopseudoisocytidine and 4-Thiopseudouridine as Components of Triplex-forming Oligonucleotides

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In this paper, we report convenient methods for the synthesis of 4-thiopseudoisocytidine ($s^4\psi iC$) and 4-thiopseudouridine ($s^4\Psi$). ^{1}H NMR spectral analysis of these modified nucleosides showed that both $s^4\Psi$ and $s^4\psi iC$ prefer C3′-*endo* ribose puckering. These conformational properties are favorable for the stabilization of triplex formation.

Using the antigene strategy, a large number of modified nucleosides have been synthesized to enhance the thermal stability of DNA triplexes formed by hybridization of the third DNA strands with DNA duplexes.[1–5] These studies showed that the use of homopyrimidine–oligodeoxynucleotides containing cytosine or 5-methylcytosine bases as triplex-forming oligodeoxynucleotides (TFOs) under weakly acidic conditions resulted in significant stabilization of the resulting parallel triplex structures. This was due to the formation of protonated cytosine or 5-methylcytosine bases that could bind to guanine bases at the Hoogsteen base-pairing site.[6–9] However, those acidic conditions limit the sequences of TFOs; therefore, antigene therapy using this strategy is not generally applicable. To overcome this limitation, several modified nucleosides have been developed to mimic the structure of the 3-N-protonated cytosine base.[10–15] 2′-*O*-Methylpseudoisocytidine (ψiCm) is known to form a triplet base pair with a G–C base pair under neutral conditions. However, TFOs containing ψiCm could not stabilize the triplex structure sufficiently at neutral pH.[10,11]

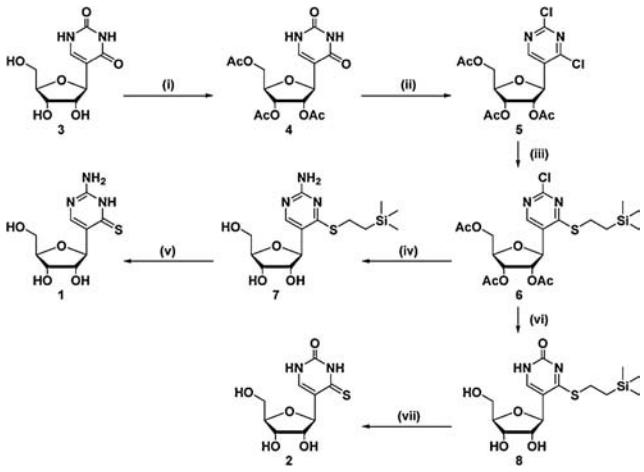
On the other hand, we have recently reported that TFOs containing 2′-*O*-methyl-2-thiouridine (s^2Um) or 2-thiothymidine (s^2T) formed quite stable parallel triplex structures.[16] Enhancement of the thermal stability of these parallel triplexes can be explained by means of the strong stacking interaction of the 2-thiocarbonyl group with the 5′-upstream or 3′-downstream bases. In particular, it was found that a consecutive alignment of s^2Um or s^2T in TFOs resulted in a more effective increase in the binding ability toward DNA duplexes.[16]

It was expected that a consecutive pile of 4-thiopseudoisocytidine (**1**: $s^4\psi iC$) in combination with s^2Um or s^2T might cause an increase in the thermal stability of the parallel triplex structures. In this paper, we report convenient methods for the synthesis of **1** and 4-thiopseudouridine (**2**: $s^4\Psi$), which can be derived from a synthetic intermediate of the former. Chemical structures of these modified nucleosides were shown in Figure 1.

Figure 1. Chemical structures of 4-thiopseudoisocytidine and 4-thiopseudouridine.

In the synthesis of 4-thiouridine (s^4U), it was reported that the thiolation of the pyrimidine ring at position 4 could be achieved by the reaction of 4-(2,4,6-triisopropylbenzenesulfonyl)pyrimidinone nucleoside derivatives with 3-sulfanylpropionitrile.[17–19] In addition, many reactions with pyrimidine rings substituted with leaving groups at position 4 were reported. Therefore, such types of substitution reactions might also produce 4-substituted Ψ derivatives. Townsend et al. reported 2,4-dichloro-5-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)pyrimidine as a Ψ derivative that has chloro groups at positions 2 and 4 on the pyrimidine ring.[20] Considering the reactivity of compound, we expected that the substitution reaction might occur predominantly at position 4.[21] According to Townsend's procedure (Scheme 1), pseudouridine **3** was converted to 2′,3′,5′-tri-*O*-acetylpseudouridine (**4**) in 92% yield. Compound **4** was further treated with excess $POCl_3$ to give 2,4-dichloropseudouridine derivative **5** in 91% yield.

After that, as expected, the reaction of compound **5** with 2-(trimethylsilyl)ethanethiol in *N*,*N*-dimethylacetamide in the presence of triethylamine formed only the 4-thiolated compound **6** in a high yield of 84%. The structure of this product was determined from the correlation between the ^{1}H signal of the 2-(trimethylsilyl)ethyl group and the ^{13}C signal of 4C on the pyrimidine ring, obtained by HMBC spectrum analysis. The chloro group of compound **6** was converted to an amino group by the reaction with concd NH_3 to form compound **7** in 44% yield. Treatment of **7** with Bu_4NF formed $s^4\psi iC$ (**1**) in 62% yield.[22]



Scheme 1. Reagents and conditions: (i) Ac_2O (10 equiv), pyridine, rt; (ii) *N*,*N*-diethylaniline hydrochloride (1.0 equiv), $POCl_3$ (20 equiv), reflux; (iii) 2-(trimethylsilyl)ethanethiol (1.2 equiv), triethylamine (1.2 equiv), DMA, rt; (iv) concd NH_3, dioxane, 100 °C; (v) TBAF (3.0 equiv), THF, 50 °C; (vi) $LiOH\cdot H_2O$ (5.0 equiv), DMA, 60 °C; and (vii) TBAF (1.5 equiv), THF, 60 °C.

Table 1. Conformational analysis of $s^4\Psi$ and $s^4\psi i$C in D_2O

	Ψ	$s^4\Psi$	$s^4\psi i$C
%N (C3′-*endo*)[a]	50%	78%	66%
$J_{1'H2'H}$	5.4 Hz	2.2 Hz	3.9 Hz
$J_{3'H4'H}$	5.4 Hz	7.8 Hz	7.1 Hz

[a]%N values of nucleosides were determined by following equation: $\%N\ (C3'\text{-}endo) = J_{3'H4'H}/(J_{1'H2'H} + J_{3'H4'H}) \times 100$.

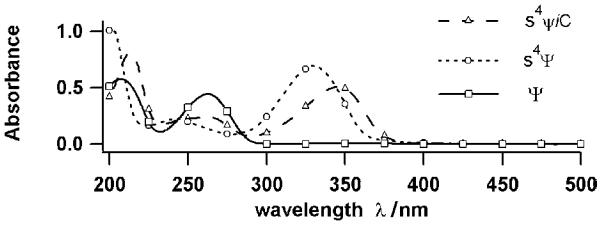


Figure 2. UV spectra of $s^4\psi i$C and $s^4\Psi$ in H_2O.

On the other hand, hydrolysis of compound **6** with LiOH afforded 4-(2-trimethylsilyl)ethyl-4-thiopseudouridine (**8**) in 36% yield. The low yields of the above two reactions of compound **6** forming compounds **7** and **8** were due to side reactions of position 4, since it is known that pyrimidine derivatives having alkylthio or sulfanyl groups at position 4 or 2 react easily with nucleophilic reagents.[23–26] The TBAF-mediated deprotection of compound **8** formed $s^4\Psi$ in 67% yield.[27]

To clarify the sugar conformations of $s^4\Psi$ and $s^4\psi i$C, ^{1}H NMR spectral analysis was performed. As shown in Table 1, it was found that $s^4\Psi$ and $s^4\psi i$C showed C3′-*endo* ribose puckering forms (%N; $s^4\Psi$: 78%; $s^4\psi i$C: 66%) more predominantly than Ψ. It was reported that s^2U derivatives prefer C3′-*endo* ribose puckering.[28–30] This conformational predominance is known to be caused by steric repulsion between the 2-thiocarbonyl group of s^2U and the 2′-hydroxy group.[28] The C3′-*endo* predominance observed could be explained by the same type of steric repulsion.

It is known that s^4U exhibited a unique UV absorption spectrum with maximum absorbance at 330 nm.[31] As shown in Figure 2, the UV absorption maxima of 4-thiopseudo-nucleosides $s^4\psi i$C and $s^4\Psi$ were shifted markedly from that of ψ (260 nm) to 345 and 331 nm, respectively. These spectral changes were very similar to those from U to s^4U. Since the structure of s^4U resembles that of $s^4\Psi$, these UV spectral changes also supported the view that the thiolation occurred at position 4 of the pyrimidine ring.

In conclusion, we synthesized $s^4\psi i$C and $s^4\Psi$ successfully. The ^{1}H NMR studies of these modified nucleosides showed that both $s^4\psi i$C and $s^4\Psi$ prefer C3′-*endo* ribose puckering. These conformational properties are favorable for the stabilization of both RNA-duplex and parallel triplex formation. Synthesis of oligonucleotides containing $s^4\psi i$C and study of their duplex- and triplex-forming abilities are now in progress.

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