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Synthesis and Properties of 2′-*O*-Methyl-2-thiouridine and Oligoribonucleotides Containing 2′-*O*-Methyl-2-thiouridine

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Abstract—A new method for the synthesis of 2′-*O*-methyl-2-thiouridine (s^2Um) found in thermophilic bacterial tRNA was developed. Structural properties of s^2Um and s^2Um_pU were studied by using ^{1}H NMR spectroscopy. A modified nonaribonucleotide (RNA*: 5′-CGUUs^2UmUUGC-3′) was synthesized to study the base-recognition ability of s^2Um in formation of RNA–RNA and RNA–DNA duplexes. The UV melting experiments revealed that RNA*–RNA and RNA*–DNA duplexes having an s^2U-A base pair are more stable than those having a U-A base pair. On the contrary, the thermal stability of RNA*–RNA and RNA*–DNA duplexes having an s^2U-G wobble base pair was much lower than that of the unmodified duplexes having a natural U-G base pair. It is concluded that s^2Um has higher selectivity toward A over G than unmodified U.

Introduction

2′-*O*-Methyl-2-thiouridine is a doubly modified nucleoside discovered from tRNAs of extremely thermophilic archaebacteria (*Sulfolobus solfataricus*, *Thermoproteus neutrophilus*, and *Pyrodictium occultum*) by Edmonds et al.[1] Both the ribose residues of 2-thiouridine (s^2U)[2] and 2′-*O*-methyluridine (Um)[3] have a C3′-*endo* conformation, since the 2-thiocarbonyl and 2′-*O*-methyl groups are more sterically hindered than the 2-carbonyl and 2′-hydroxyl groups, respectively.[4] Therefore, the ribose puckering of s^2Um is predicted to have preferentially the C3′-*endo* form because of the additive effect of the 2-thiolation and 2′-*O*-methylation. The C3′-*endo* predominance of nucleosides is entropically favorable for stable A-type RNA duplex formation, because all the ribose conformations of A-type RNA duplexes are C3′-*endo*.[5] It was reported that RNA duplexes containing s^2U or Um were thermodynamically more stable than unmodified natural RNA duplexes.[6,7] Therefore, an oligoribonucleotide bearing s^2Um, which was predicted to have a rigid C3′-*endo* form, was expected to form more stable A-type RNA duplexes. Moreover, the hydrophobic nature of the 2-thiocarbonyl and 2′-*O*-methyl groups may enhance permeability to the cell membrane, and the 2′-*O*-methylated oligoribonucleotides acquire resistance against nucleases.[7b] These properties of s^2Um are expected to be significantly useful for the antisense strategy.[8]

In addition to this expectation, oligonucleotides containing s^2Um are thought to form double helixes more selectively than those containing uridines. The word "selectively" means that the 2-thiocarbonyl group destabilizes an s^2U-G wobble base pair because of the weaker hydrogen bonding nature of the thiocarbonyl group and the steric repulsion derived from the C=S bond that is longer than the C=O bond. Although the U-G wobble base pair is a well-known base mismatch, it does not decrease the *T*m value so much as other base mismatches like the C-A base pair. This is because the U-G pair can form two hydrogen bonds (N^3 of U$\cdots O^6$ of G, and O^2 of U$\cdots N^1$ of G).[9] Quite recently, the thermodynamic parameters (in 1 M NaCl) of RNA duplexes having an s^2U-G wobble base pair were reported by Testa et al.[10] They described that the melting temperatures of RNA duplexes having the s^2U-G wobble base pair were considerably lower than those obtained in the case of the matched s^2U-A base pair.

In order to examine the effects of the 2-thiocarbonyl group on formation of a base pair, we performed ab initio calculation of U-G wobble base pairs (Fig. 1).[11] The hydrogen bonding energy of the base pair formed between 1-methyl-2-thiouracil and 9-methylguanine was −10.4 kcal/mol, while that of the natural U-G base was calculated to be −14.1 kcal/mol.[12] These results apparently suggest the weakened hydrogen bonding property of the 2-thiocarbonyl group as expected above. In addition, the optimized geometry of the 1-methyl-2-thiouracil and 9-methylguanine base pair was simultaneously calculated.

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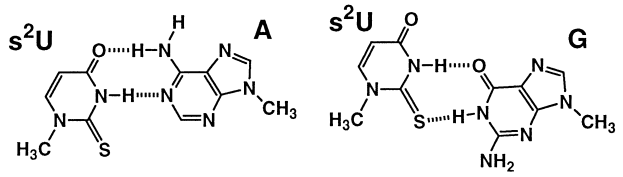


Figure 1. Match (left) and wobble (right) base pairs containing s^2U.

The distance between the two methyl groups of 1-methyl-2-thiouracil and 9-methylguanine was longer by ca. 1 Å than that of the corresponding U-G base pair. This implies that the steric repulsion also contributes to the duplex destabilization by s^2U.

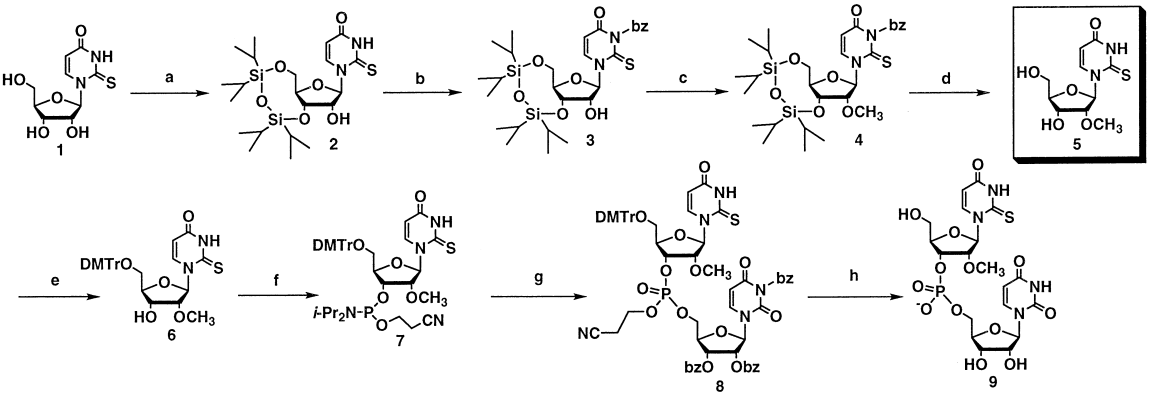
In this study, we developed a new route to s^2Um and a building block for incorporation of s^2Um into oligonucleotides. Furthermore, structural and thermodynamic properties of s^2Um and oligoribonucleotides having s^2Um were studied by means of ^1H NMR spectroscopy and *T*m experiments.

Results and Discussion

Edmonds et al. first reported that s^2Um was obtained by methylation of O^2,5′-anhydrouridine followed by a ring-opening reaction with H_2S.[1] However, no details of the 2′-*O*-regioselectivity of the methylation and the yield at each step were given. It seemed that it was difficult to separate the 2′-*O*-and 3′-*O*-methyl-O^2,5′-anhydrouridine derivatives unless HPLC was used. Therefore, we searched for a more practical route to s^2Um capable of large-scale synthesis of this material required for the antisense strategy. Consequently, all the reactive functional groups of s^2U were protected except for the 2′-hydroxyl group, and the successive 2′-*O*-methylation was performed, as depicted in Scheme 1.

First, the 3′- and 5′-hydroxyl groups of 2-thiouridine[13] (**1**) were protected by use of the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl (TIPDS) group.[14] Subsequently, the base moiety of the resulting product **2** was acylated via transient protection of the 2′-hydroxyl group with the trimethylsilyl group.[15] The 2′-*O*-methylation of the *N*-benzoylated uridine derivative **3** was examined by using CH_3I–NaH or CH_3I–Ag_2O.[16] When we used the former conditions, 2′-*O*-methylation of **3** was accomplished, but the latter caused complex side reactions because Ag_2O activated simultaneously the 2-thiocarbonyl group of **3**. Finally, both the TIPDS and benzoyl groups of the 2′-*O*-methylated product **4** were removed in the usual manner. Thus, the desired product **5** could be successfully synthesized in an overall yield of 49% from **1**.[17] The 5′-*O*-dimethoxytritylation of **5** followed by 3′-*O*-phosphitylation gave the building block **7** required for incorporation of s^2Um into oligonucleotides.[18] By using this building block, the fully protected dimer **8** bearing s^2Um as a 5′-upstream nucleoside was synthesized (Scheme 1). Since it was reported that the thiocarbonyl group of 2-thiothymidine reacted with I_2,[19] the phosphite intermediate was oxidized with *t*-butyl hydroperoxide in a manner similar to that described by Kumar et al.[6,20] The successive treatments of **8** with ammonium hydroxide and 80% AcOH gave the dinucleoside monophosphate **9** (s^2Um_pU) after reversed-phase HPLC purification. In order to investigate the conformational properties of s^2Um, the ^1H NMR spectra of **5** and **9** were measured in sodium phosphate buffer (pH 7.0). The ratio of the C3′-*endo* and C2′-*endo* conformers of s^2Um was calculated by using the coupling constant between the vicinal protons of **5** and **9**, and these results are listed in Table 1.[21]

The %N (C3′-*endo*) value of **5** was calculated to be 70%, while that of 2-thiouridine (s^2U) reported by Sierzputowska–Gracz was 78%.[2] In spite of the presence of the bulky 2′-*O*-methyl group, the predominance of the C3′-*endo* conformer of **5** was unexpectedly almost equal to that of s^2U. On the other hand, the ribose puckering of s^2Um in the dimer **9** was almost C3′-*endo* only ($^3J_{1'H\text{-}2'H} < 1$ Hz), although the predominance of the C3′-*endo* conformer of the 5′-upstream nucleoside in s^2UpU was at most 90%.[22] These results indicated that the steric repulsion between the 2-thiocarbonyl and 2′-*O*-methyl groups of s^2Um was more effective in the



Scheme 1. Synthesis of s^2Um and s^2Um_pU. Reagents and conditions: (a) $TIPDSCl_2$ in pyridine, 99%; (b) i. HMDS in CH_3CN, ii. BzCl, Bu_4NBr in CH_2Cl_2/Na_2CO_3 aq, iii. TFA in MeOH-CH_2Cl_2 (1:1, v/v), 76%; (c) NaH, CH_3I in DMF, 79%; (d) i. Bu_4NF, AcOH in THF, ii. Concd NH_3–pyridine (1:9, v/v), 83%; (e) DMTrCl in pyridine, 85%; (f) Chloro(2-cyanoethoxy)(*N*, *N*-diisopropylamino)phosphine, diisopropylethylamine in CH_2Cl_2, 81% (purity 89%); (g) i. N^3,2′,3′-*O*-Tribenzoyluridine, 1*H*-tetrazole in CH_3CN, ii) *t*-BuOOH, 38%; (h) i. Concd NH_3–pyridine (9:1, v/v), ii. 80% AcOH, 67%.

Table 1. Vicinal coupling constants ($^3J_{1'H\text{-}2'H}$) and C3′-*endo* preference of nucleosides

				s²UmpU		s²UpU		UpU	
	s²Um	s²U	U	5′-Nu	Nu-3′	5′-Nu	Nu-3′	5′-Nu	Nu-3′
$^3J_{1'H\text{-}2'H}$ (Hz)	3.1	2.5[a]	4.6[a]	⩽1.0	2.8	1.6[b]	3.5[b]	4.4[b]	3.7[b]
%N (C3′-*endo*)[c]	70	78	48	100	74	91	64	51	61

[a]These coupling constants were reported in ref. 2.
[b]These data were taken from ref. 22.
[c]These values were calculated according to the equation: %N (C3′-*endo*) = {(7.9−$^3J_{1'H\text{-}2'H}$ (Hz))/6.9}×100.[21]

presence of the 3′-phosphate group because of its steric hindrance. Therefore, it was expected that the ribose puckering of s²Um in oligonucleotides would also be C3′-*endo*. Interestingly, the C3′-*endo* preference of s²Um influenced the sugar conformation of the 3′-downstream nucleoside, which became C3′-*endo*. The %N (C3′-*endo*) value of the 3′-nucleoside in the s²Um$_p$U calculated was 74%, i.e., greater by 10% than that of s²U$_p$U which showed 64% C3′-*endo* predominance.

Since the C3′-*endo* preference of s²Um was clearly confirmed, the stability of duplexes containing s²Um was evaluated by the melting temperature (*T*m) measurement. An oligoribonucleotide nonamer (5′-CGUUs²UmUU GC-3′) was synthesized by the usual phosphoramidite method, except for the oxidation, similar to that described in the synthesis of dimer **8**. Subsequent deprotection was performed by the routine procedure, and the resulting mixture was separated by anion exchange HPLC.[23] For the control experiments, an oligoribonucleotide having s²U instead of s²Um was obtained by Kumar's method.[6]

UV melting curves of RNA–RNA and RNA–DNA duplexes were measured in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl, and these profiles and *T*m values are shown in Fig. 2 and Table 2, respectively. In the case of RNA–RNA duplexes, the *T*m value of the duplex containing either s²Um or s²U was higher than that of the wild-type matched duplex (ΔTm = +5.5 °C (s²Um), and +6.9 °C (s²U)). Such a tendency was also found in the case of an RNA–DNA matched duplex, but the ΔTm value became larger than those of the RNA–RNA series (ΔTm = +6.9 °C (s²Um), and +9.4 °C (s²U)). Although oligonucleotides modified with s²U or Um form thermodynamically stable A-type RNA duplexes,[6,7,10] the *T*m values of both RNA–RNA and RNA–DNA duplexes modified by s²Um were somewhat lower than those of the duplexes modified by s²U, and no additive effect of the double modification was observed.

The selectivity toward the target sequence is also an important property of antisense agents. Particularly, the discrimination of the U-G wobble base pair from the U-A Watson–Crick base pair is essential for a highly specific antisense method. Therefore, the thermal stability of RNA–RNA duplexes containing an s²Um-G wobble pair was examined.

In order to evaluate the selectivity toward the target sequence, difference in the *T*m value between the duplex having a s²Um-G and the duplex having a s²Um-A base pair (= ΔTm*, see Table 2) was calculated. These ΔTm* values were found to be −15.7 and −14.7 °C, for s²Um and s²U, respectively. However, the ΔTm* value between the unmodified duplexes was only −3.9 °C. Testa et al. also reported a similar trend for U-G and s²U-G, but the absolute values of ΔTm* are smaller than those in this study.[10] Similar experiments were performed in a series of RNA–DNA duplexes, and the ΔTm* values were determined to be −20.4, −18.5, and −11.2 °C for the duplexes having s²Um, s²U, and U, respectively. In both the RNA–RNA and RNA–DNA

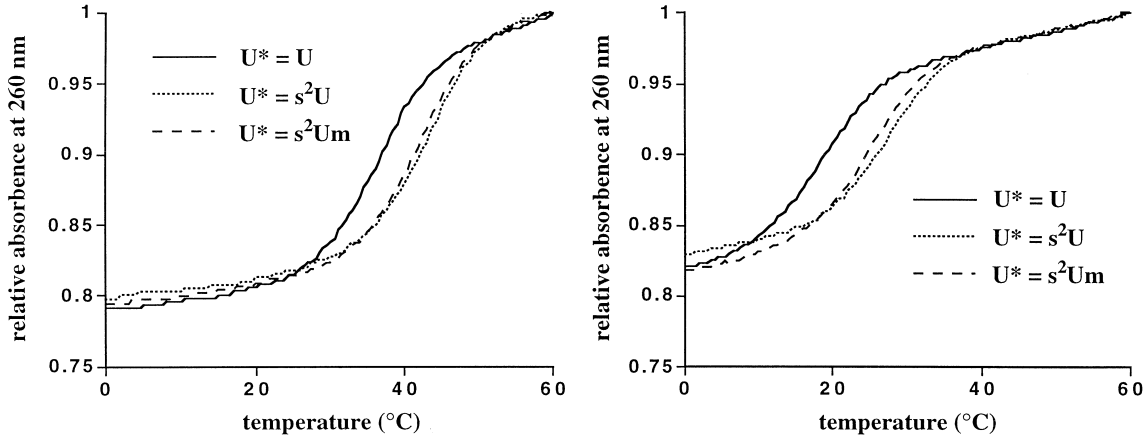


Figure 2. Melting curves of CGUUU*UUGC/GCAAAAACG (RNA–RNA series, left) and CGUUU*UUGC/d(GCAAAAACG) (RNA–DNA series, right).

Table 2. Melting temperature (*T*m Value, °C) of RNA–RNA and RNA–DNA duplexes containing an s^2Um-A (match) or s^2Um-G (wobble) base pair

	GCAAXAACG		d(GCAAXAACG)	
CGUUU*UUGC	X = A (match *T*m value (ΔTm*)[a]	X = G (wobble) *T*m value (ΔTm*)[b]	X = A (match) *T*m Value (ΔTm)[a]	X = G (wobble) *T*m value (ΔTm*)[b]
U* = U (control)	36.7	32.8 (−3.9)	18.6	7.4 (−11.2)
U* = s^2Um	42.2 (+5.5)	26.5 (−15.7)	25.5 (+6.9)	5.1 (−20.4)
U* = s^2U	43.6 (+6.9)	28.9 (−14.7)	28.0 (+9.4)	9.5 (−18.5)

[a]ΔTm = (*T*m value of a modified duplex) − (that of a wild-type duplex).
[b]ΔTm* = (*T*m value of a duplex having a U*-G wobble base pair) − (that of a duplex having a matched U*-A base pair in place of a U*-G base pair).

duplexes having s^2U the ΔTm* values of the ones modified by s^2U derivatives were always larger than those of the unmodified, and the ΔTm* value of s^2Um-containing duplexes was fortunately somewhat larger than those having s^2U.

The duplex destabilization by the s^2Um (or s^2U)-G base pair was a consequence of both the weaker hydrogen bonding nature and the steric hindrance of the 2-thiocarbonyl group, as expected by ab initio calculation. However, the reason why the ΔTm* values of the duplexes containing s^2Um were larger than those of the duplex containing s^2U is not clear. Since the effect of the uridine-2-thiolation on the stabilization and selectivity enhancement of RNA duplexes is excellent, we propose that several or all uracil residues of the antisense agents should be replaced by 2-thiouracil, if such replacement is chemically and economically possible.[24] Therefore, antisense agents having 2-thiouracil residues instead of uracil will be one of the second-generation antisense drugs. Further studies are under way in this direction.

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