Chemistry and Structure of Modified Uridines in the Anticodon, Wobble Position of Transfer RNA Are Determined by Thiolation

Hanna Sierzputowska-Gracz,*†,⊥ Elzbieta Sochacka,‡ Andrzej Malkiewicz,‡ Kenneth Kuo, Charles W. Gehrke, and Paul F. Agris⊥

Contribution from the Division of Biological Sciences and Experiment Station Chemical Laboratory, University of Missouri, Columbia, Missouri 65211, and Institute of Organic Chemistry Technical University, 90-924 Lodz, Poland. *Received January 27, 1987*

Abstract: Uridines found in the first or wobble position of transfer RNA anticodons are most often modified at base ring carbon-5 and many times also thiolated at carbon-2. It is important to understand the chemistry and structure of the modified uridines because they influence codon recognition by tRNA. Uridine and five biologically important 5-position derivatives and the six analogous 2-thiouridines were all investigated by high-performance liquid chromatography (HPLC) and ultraviolet (UV), infrared (IR), ^1H, and ^{13}C NMR spectroscopy under physiological conditions. The modified nucleosides were chemically synthesized, and purity was assessed by HPLC. Thiolation produced a more hydrophobic nucleoside independent of the chemical nature of the 5-position substituent as determined by HPLC. Thiolation also produced characteristic differences in IR and UV spectra. The six 2-thiouridines strictly conformed to predominating fractional populations in the C(3′) endo, gauche plus [C(4′)–C(5′)], anti structure as determined by NMR techniques. However, the six uridines were found to be much less restricted to their predominating C(2′) endo, gauche plus structure that was either syn or anti. The syn conformer was found for those uridines with bulky 5-position modifications. Thus, we postulate thiolation of tRNA wobble position uridines may produce the hydrophobic, restricted C(3′) endo, gauche plus, anti conformation best suited for anticodon base stacking, and thereby they may effect a selective codon recognition.

Uridines and 2-thiouridines modified at the 5-position of the base are often found in transfer RNA (tRNA) at nucleotide number 34, the first or "wobble" location within the anticodon. All modified uridines are synthesized post-transcriptionally in procaryotes, and in the nucleus and mitochondria of eucaryotes.[1,2] Modification at the 5-position of uridine-34 is as short as a methoxy group (mo^5U)[3] or as long as a ((carboxymethyl)-amino)methyl chain ($cmnm^5U$). Modification of the 2-position from carbonyl to thio does not always accompany derivatization of the 5-carbon. However, 2-thiouridine (s^2U) without some sort of modification at the 5-position has only been found in one of the anticodons of hundreds of mature tRNAs that have been sequenced.[1] Thiolation and the 5-position substituents have been shown in vitro, and more recently in vivo, to influence codon recognition[4-6] and aminoacylation[7,8] and thus are of chemical and structural importance to the tRNA function.

The first modified uridine to be described was 2-thio-5-((methylamino)methyl)uridine (s^2mnm^5U).[9] This was followed by the X-ray crystallographic study of the same nucleoside.[10] More than a half-dozen uridines and thiouridines have now been reported at tRNA position 34.[1,2] The most complete chemical and structural study of modified uridines (U) previously reported was conducted by Egert et al.[11] In a study of six naturally occurring (and thirteen unnatural) derivatives of U, the electronic effect of the substituent at position 5 influenced not only the base aromatic system but also the N(1)–C(1′) and C(1′)–O(4′) bond lengths in an opposing manner. The orientation of the base was correlated to sugar pucker. X-ray crystallographic studies showed that the N(1)–glycosidic bond angle was found to be $\chi < 35°$ for the C(3′) endo conformation and $\chi > 40°$ for the C(2′) endo conformation. These correlations seemed to hold, as well, for those uridines that were studied in D_2O solution by low-field ^1H NMR techniques.[11] In a more recent NMR study of six wobble position nucleosides (and their 5′-monophosphates) in D_2O, the 2-thiolated uridines (s^2U) were found predominantly in the C(3′) endo form, whereas non-analogous, 5-position derivatives of uridine were in a dynamic equilibrium between C(2′) and C(3′) endo forms.[12]

Here we report spectroscopic (NMR, UV, and IR) data for 12 natural and thus biologically important, chemically synthesized uridines. In contrast to previous studies,[11-16] the uridines chosen included six 5-position derivatives and their exact s^2U analogues (Figure 1), and studies were conducted in physiological saline solution. Therefore, we could compare the structures found within each U–s^2U pair and among the U's and the s^2U's with different 5-position modifications. Such comparisons should allow us to describe the chemical and physical parameters influencing native nucleoside structure and conformation that are relevant to tRNA studies under the same physiological conditions.

Experimental Section

Synthesis of Nucleosides. All of the nucleosides were chemically synthesized with the exception of uridine (U) which was purchased from

⊥ University of Missouri.
† H.S.-G. is on leave from the Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61-704 Poznan, Poland.
‡ Institute of Organic Chemistry Technical University.

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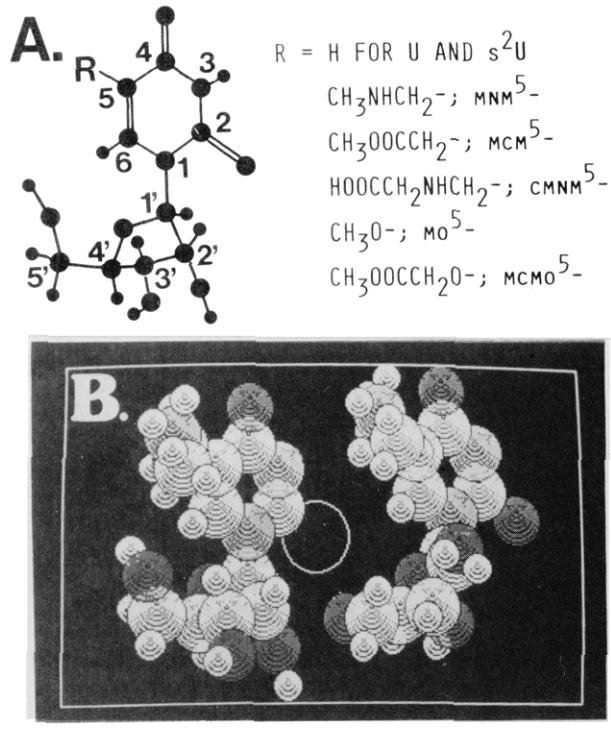

Figure 1. Molecular structures of investigated uridines and space-filling model. A computer drawn structural formula of a uridine derivative is shown in part A. The figure shows the numbering of the atoms. Uridine (U) and 2-thiouridine (s^2U, s = S) and five 5-position derivatives of each were investigated. The observations of the 5-position modifications are also shown. A computer-simulated, space-filling model of the X-ray crystallographic structure of s^2mnm^5U[10] is shown in part B. The structure of mnm^5U is an NMR-derived modification of the s^2mnm^5U crystal structure. Parts A and B were produced from the Cartesian coordinates of the respective X-ray crystallographic data by means of the molecular graphics computer program PC Model II (Academic Press Inc.).

Sigma Chemical (St. Louis). Syntheses of four of the nucleosides have not previously been published and are reported here in some detail.

2-Thiouridine (s^2U). The nucleoside was prepared according to the "silyl method" reported by Vorbruggen.[17] Instead of 1-chloro-2,3,4-tri-*O*-benzoyl-D-ribose, $AgClO_4$ as catalyst and benzene as solvent, 1-*O*-acetyl-2,3,5-*O*-benzene-D-ribose, $SnCl_4$ and CH_3CN were used. The yield of s^2U was 85%.

5-((Methylamino)methyl)uridine (mnm^5U). Treatment of s^2mnm^5U (see below) with H_2O_2 in 4 N NaOH produced mnm^5U in quantitative yield.[17] Crude product was passed through a Dowex H^+ column followed by elution with 10% NH_4OH. The eluate was evaporated, and the resulting residue was dissolved in ethanol and precipitated with ether.

2-Thio-5-((methylamino)methyl)uridine (s^5mnm^5U). The persilylated derivative of 5-(*N*-methyl-*N*-formylaminomethyl)-2-thiouracil was condensed with 1-*O*-acetyl-2,3,5-tribenzoyl-D-ribose. Benzoyl groups were removed with half-saturated NH_3/methanol solution. The nucleoside was isolated as the hydrochloride, and the free nucleoside was prepared by passage through Dowex H^+.[18,19]

5-((Methylcarboxy)methyl)uridine (mcm^5U). The nucleoside cm^5U was prepared as reported.[17] Esterification was accomplished by refluxing with 0.5 N H_2SO_4/methanol for 24 h. The reaction was neutralized with $BaCO_3$, the salt removed by filtration, and the filtrate evaporated to dryness. The product was purified by silica gel H column chromatography to give mcm^5U in 75% yield. The nucleoside, synthesized in another manner, has been described.[19]

2-Thio-5-((methylcarboxy)methyl)uridine (s^2mcm^5U). The synthesis was essentially that of Vorbruggen[17] except the catalyst and solvent were those reported here for s^2U.

5-(((Carboxymethyl)amino)methyl)uridine ($cmnm^5U$). Synthesis was according to our previously reported methods.[20,21]

2-Thio-5-(((carboxymethyl)amino)methyl)uridine (s^2cmnm^5U). The nucleoside was synthesized by transformation of 5-[*N*-((carbomethoxy)methyl)-*N*-(trifluoroacetyl)aminomethyl]-2′,3′-*O*-isopropylidene uridine[20] into the respective 2-*O*, 5′-anhydro nucleoside.[22] This was reacted with excess H_2S liquid/pyridine (1:1) to give 5-(*N*-(carboxymethyl)-*N*-(trifluoracetyl)aminomethyl)-2-thiouridine, which was then deprotected.[20]

5-Methoxyuridine (mo^5U). This derivative was prepared as reported.[23]

2-Thio-5-methoxyuridine (s^2mo^5U). The base 5-methoxy-2-thiouracil[24] was transformed to the 2-*S*, 6-*O*-bis(trimethylsilyl) derivative and condensed with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribose in the presence of $SnCl_4$ as catalyst in acetonitrile. The perbenzoylated derivative of s^2mo^5U (yield 62%) was easily separated from the N3 derivative (yield 15%) by silica gel column chromatography. Deprotection was accomplished with an 87% yield by treatment with 0.01 N methanolic sodium methoxide at room temperature for 24 h.

Uridine-5-oxyacetic Acid Methyl Ester ($mcmo^5U$). The monoanion of 2′,3′-*O*-isopropylidene-5-hydroxyuridine was condensed with ethyl iodoacetate to give the 2′,3′-*O*-propylidene derivative of uridine-5-oxyacetic acid ethyl ester (yield 52%). Simultaneous deprotection of the cis diol function and transesterification was achieved by action of 1 N HCl/methanol at room temperature for 24 h. The nucleoside was purified by silica gel column chromatography and was crystallized from methanol.

2-Thiouridine-5-oxyacetic Acid Methyl Ester (s^2mcmo^5U). The nucleoside was prepared by two methods. Synthesis from uridine-5-oxyacetic acid ethyl ester produced s^2mcmo^5U in 38% yield. Synthesis from 2-thiouracil-5-oxyacetic acid ethyl ester by condensation with blocked ribose according to the standard "silyl method" produced the perbenzoylated nucleoside in 57% yield. Deprotection and transesterification was achieved by action of 0.01 N sodium methoxide/methanol with 83% yield.

Purity of each of the nucleosides was determined by high-performance liquid chromatography (HPLC) and simultaneous UV spectral analysis (see below). All of the nucleosides were better than 95% pure compounds.

HPLC and UV Spectral Analysis of the Modified Uridines. The resolution, sensitivity, and speed of our previously reported chromatography methods for modified nucleoside analyses[25,26] have been improved further.[27,28] The new chromatography included the use of the following: a specially developed HPLC reversed-phase "nucleoside column" (Supelcosil LC-18S, 250 × 4.6 mm, Cat. No. 5-9825, Supelco Inc., Bellefonte, PA); a mobile phase consisting of a three-buffer, multiple-ramp elution gradient at 1.0 mL/min flow rate; and 26 °C isothermal column temperature. Buffer A was 2.5% CH_3OH in pH 5.30, 0.01 M $NH_4H_2PO_4$; buffer B was 20.0% CH_3OH in pH 5.10, 0.01 M $NH_4H_2PO_4$. The elution consisted of 100% buffer A isocratic from 0 to 12 min. Then, from 12 to 45 min a series of buffer A and B ramps were made from 100% A to 100% B. The gradient progressed from 100% B to 100% C from 45 to 80 min, followed by 80 to 87 min of 100% C, isocratic.[27,28] The column was washed with 70% CH_3OH in H_2O for 5 min and equilibrated with buffer A for 25 min before the next run. The RP–LC retention times of the different uridines are presented in Table I. The instrumentation consisted of a Hewlett-Packard Model 1090A liquid chromatography instrument equipped with DR-5 solvent delivery system, temperature-controlled column compartment, automatic injector, automatic sampler, diode-array detector, HP-80B controller, HP-7470A plotter, HP-9133 hard disc drive, HP Think jet printer, and DPU

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Table I. RP-LC Retention Times and Ultraviolet Absorption Maxima and Minima of Uridines

nucleoside[a]	retention time[b]	λ_{max}[c]	λ_{min}[c]
cmnm^{5}U	5.12	208	
		265	232
mnm^{5}U	5.43	208	
		265	232
U	8.39	207	
		262	232
s^{2}cmnm^{5}U	12.10	220	
		273	242
s^{2}mnm^{5}U	12.60	220	
		273	242
mo^{5}U	21.10	206	
		278	245
s^{2}U	21.60	219	
		275	244
mcm^{5}U	30.10	208	
		266	234
mcmo^{5}U	35.00	208	
		275	242
s^{2}mo^{5}U	35.80	235	205
		285	252
s^{2}mcm^{5}U	39.70	221	
		278	246
s^{2}mcmo^{5}U	47.30	227	
		285	250

[a] Nucleoside abbreviations: s = S, mnm = $-CH_2NHCH_3$, mcm = $-CH_2COOCH_3$, cmnm = $-CH_2NHCH_2COOH$, mo = $-OCH_3$, mcmo = $-OCH_2COOCH_3$. [b] Retention times are in minutes; dead volume time is 2.42 min. [c] Wavelengths (λ) are in nanometers. The maximum and minimum of each nucleosides were measured from the first derivative plot of the spectrum.

(data-process-unit) Multi-channel Integrator. Wavelengths at 254 + 2 nm and 280 + 2 nm were selected to quantify all the nucleosides.

NMR Spectroscopy. Solutions of mononucleosides were prepared in phosphate buffered saline in D_2O, PBS (10 mM $MgCl_2$, 15 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.2, 100 mM NaCl), and dried, deuteriated dimethyl sulfoxide (DMSO-d_6) from Merck Isotopes. All of the nucleosides were more easily dissolved in DMSO than in PBS. The low solubility of the thiolated and longer chain 5-position derivatives was particularly troublesome. Concentrations varied between 10 and 50 mM.

NMR spectra were obtained with a Nicolet 300-MHz (^{1}H) Fourier transform spectrometer with an Oxford Instrument wide bore magnet and Nicolet 1280 computer. The instrument was equipped with a Nicolet 5 mm single-frequency (75.5 MHz) ^{13}C probe and a Doty Scientific single-frequency ^{13}C preamplifier. Proton NMR spectra of all of the nucleosides were recorded at the frequency of 300 MHz with a 5-mm probe. Two-dimensional (2D) nuclear Overhauser effect (NOE) spectra were acquired with a 90° pulse width of 7.5 μs. Sample viscosity and temperature were kept constant for the 12 nucleosides. Spectra were recorded in 256 blocks of 32 acquisitions (512 points per block). The mixing time was τ = 0.7 s. Nucleoside ^{13}C signal assignments were made by comparison to already published data and by use of a wide range of available NMR techniques. Nucleoside proton resonance assignments were made by comparison of chemical shifts with that of their respective analogues, literature values, and spin–spin coulings for ribose protons in different nucleosides.

Infrared Spectroscopy. Infrared (IR) spectra were taken on a Nicolet FTIR spectrometer. All samples were prepared in KBr pellets.

Results

HPLC and UV Spectral Analyses. The purity of the 11 synthesized uridine derivatives (s^{2}U, mnm^{5}U, s^{2}mnm^{5}U, mcm^{5}U, s^{2}mcm^{5}U, cmnm^{5}U, s^{2}cmnm^{5}U, mo^{5}U, s^{2}mo^{5}U, mcmo^{5}U, s^{2}mcmo^{5}U) was found to be better than 95% by reverse-phase HPLC (RPLC) and UV spectral analyses. We have used this reverse-phase chromatography as a method of characterizing and comparing the chemistry of these nucleosides through their chromatographic retention times and UV spectra (Figure 2). Relative solubilities of the uridine derivatives were reflected in their retention times (Table I): the longer the retention times,

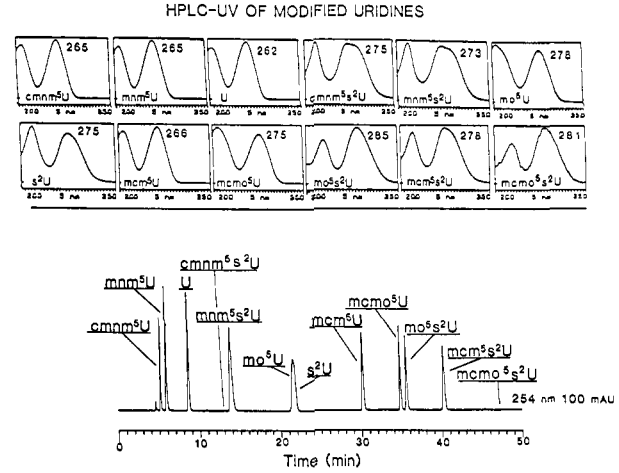

Figure 2. RP-LC retention time and HPLC-UV spectra of the 12 modified uridines. Twelve UV spectra are shown for the investigated uridines. Spectra are in the same sequence as the nucleosides eluted from the HPLC column. For this figure, we used a chromatogram where 10 of 12 uridines were run simultaneously. Two others run separately have elution positions as indicated in the figure. Details of the chromatographic procedures are found in the Experimental Section.

the lower the aqueous solubility. The order of RPLC retention time for the modified uridine nucleosides with different functional groups substituted at position C(5) was essentially the same as the order of decreasing polarity of the respective functional groups. This increasing order of retention is consistent with the partial positive and negative characteristics and hydrophobicity of the molecule. A substitution of oxygen by sulfur at position C(2) of uridine always substantially increased the retention time of the nucleoside, indicating less polarity. This is understandable because sulfur is considerably weaker in electron withdrawal than oxygen, thereby reducing the partial positive character of the base ring nitrogens. The thiocarbonyl (S=C⟨) group itself is also less polar than the carbonyl group (O=C⟨).

Substituting a hydrogen atom at the C(5) position of the uridine ring with a stronger electron donor group increases the π-electron density of the pyrimidine ring. This resulted in a shift of the UV absorption maximum (λ_{max}) to a longer wavelength (Figure 2 and Table I). The 2-thio substitution of uridines resulted in a larger shift of the λ_{max} to longer wavelengths than substitution at C(5). The effect on λ_{max} from these two substitutions is somewhat additive. Also, a skewing of the absorption band due to 2-thio substitution was observed. Under the chromatography conditions used the tautomeric thiol imine form of 2-thiouridines is more stable than the thionoamide form of 2-thiouridines. Two independent chromophore systems, –(R)N—C(SH)=N—C=O and –(R)N—C=C(R)—C=O, exist in the thiol imine tautometer. This caused the UV band skewing. The high stability of the thiol imine form is also observed in 4-thiouridine. The UV spectrum of 4-thiouridine has a very broad wavelength absorption maximum at 332 nm. This can result only when the thiol imine tautomer of 4-thiouridine is favored. It has the chromophore –(R)N—C=C—C(SH)=N—C=O, consisting of 3 pairs of π-electrons.

Infrared Spectroscopy. Infrared spectroscopy confirmed the purity of the nucleoside derivatives. In addition, the effects of 2-thiolation and structure of the 5-position substitutions on the carbonyl and thiol bands[29,30] were compared and assignments made (Table II). A band at 1680 cm^{-1} was present in the uridines and missing from the thio series of compounds. A more detailed look at the related functional groups of the uridines and thiouridines yielded additional band assignments. As seen in Table II for U and mo^{5}U there are three major bands correlated to functional groups: double bonded C(2)=O to the frequency 1680–1670

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Table II. Summary of Key Infrared Spectral Data[a] for Six Pairs of Uridines

5-position side chain	U[b]	s^2U[c]	5-position side chain	U[b]	s^2U[c]
H	1680		cmnm	1696	1696
	1660	1660		1684	
	1644	1644			1630
mnm	1699	1699		1620	1620
	1680		mo	1690	
	1670			1652	1660
	1648	1642		1630	1627
		1627	mcmo	1745	
mcm	1722	1722		1738	1740
	1688			1687	
	1668	1668		1665	1665
	1654			1638	1638

[a] IR frequencies are given in cm^{-1}. [b] U = uridine nucleoside derivatives. [c] s^2U = matching 2-thiouridines nucleoside derivatives.

cm^{-1}; double bonded C(4)=O to the frequency 1660–1652 cm^{-1}; and double bonded C=C to the frequency 1644–1630 cm^{-1}. Bands between 1660 and 1644 cm^{-1} are typical for this group. From the spectral results for s^2mnm^5U and mnm^5U, and s^2cmnm^5U and $cmnm^5U$, four main bands are assigned in the following manner. An absorption band at 1699 cm^{-1} is assigned to the C–N vibration; 1680 cm^{-1} is assigned to double-bonded C(2)=O; 1670 cm^{-1} is assigned to double-bonded C(4)=O; and 1648 cm^{-1} is assigned to the C=C double bond. The band at 1699 cm^{-1} seems typical for the CH_3NHCH_2 modification and is not present in uridine. The four uridines having the mcm and mcmo related functional groups exhibited four to five main IR bands. The 1745–1722-cm^{-1} frequencies are characteristic for the carboxylic ester moiety, while the band at 1688 cm^{-1} is again characteristic for double-bonded C(2)=O, that at 1665 cm^{-1} for C(4)=O, and that a 1638-cm^{-1} for C=C.

NMR Spectroscopy. Structural comparisons of the 12 uridines were accomplished by single and two-dimensional ^1H NMR and by ^{13}C NMR spectroscopy. The complete ^1H chemical shift comparisons of these nucleosides taken from spectra accumulated under physiological conditions are shown in Table III.

Some interesting ^1H chemical shift differences are seen and could be considered characteristic for each modified U in comparison to its analogous s^2U derivative, or for U and s^2U versus their respective 5-position analogues. Ribose H(1′) chemical shifts are different for the U versus s^2U series. The thio substitution at the 2-position of the base changes the electron distribution of the base ring substantially enough to influence the ribose anomeric proton chemical shift. The upfield change in chemical shift (Δ_{av}) averages −0.62 ppm (186 Hz). The H(1′) chemical shift is not effected by 5-position chain size or chemical character.

The H(5′) proton chemical shifts are found significantly different for U's vs. s^2U's with 5-position base substitutions. All H(5′) chemical shifts for s^2U derivatives averaged 0.11 ppm downfield of the U analogue. The effect on H(5″) is less evident but consistent and in the same direction (Δ_{av} = 0.05). Uridine derivatives had H(5′) chemical shifts consistently upfield of unmodified U (Δ_{av} = 0.05), whereas s^2U 5-position derivatives had 5′ proton chemical shifts consistently downfield of s^2U (Δ_{av} = 0.07). Both H(3′) and H(4′) chemical shifts for the uridine protons moved upfield slightly with 5-position substitution, whereas they moved downfield for the derivatives of s^2U. The difference in chemical shifts between the 3′ and 4′ protons seen in the uridine series disappears in the s^2U series.

The base proton H(6) chemical shift moved downfield with thiolation at position 2. The difference between U and s^2U (Δ = 0.09) is enhanced with 5-position substitution (Δ_{av} = 0.27). The chemical nature of the 5-position side chain effected the H(6) resonance for U and s^2U similarly.

Thiolation at the 2-position does not effect the carbon-bound proton chemical shifts of any side chain positions: CH_3; $C_\alpha H_2$; and $C_\beta H_2$. Chemical shift changes in CH_3 and $C_\alpha H_2$ protons due to differences in the side chain structures are consistent with the chemical nature of those structures.

Ribose coupling constants (J_{HH}) shown in Table IV were measured from spectra to determine and compare sugar conformation[31] for each of the 12 uridines. Simulation of each spectrum aided in the exact determination of coupling constants. Any one coupling constant, for instance $J_{1'2'}$, varied little between the 6 uridine derivatives. The $J_{3'4'}$ was found for both uridines and thiouridines to be the largest coupling other than 5′–5″.

The ratio of $J_{1'2}$ to $J_{3'4'}$ was used to calculate the relative fractional populations of C(2′) endo and C(3′) endo forms (K_{eq} = % S/% N = C(2′) endo/C(3′) endo = $J_{1'2'}/J_{3'4'}$).[32] Each uridine, as compared to the corresponding thiouridine, had a value of S/N indicative of a consistently strong difference in sugar pucker for the two groups. $J_{1'2'}$ was significantly small for the thiolated versus the corresponding non-thiolated U, whereas the $J_{3'4'}$ was consistently larger. Thus, S/N = $J_{1'2'}/J_{3'4'}$ was lower for the thiolated compound and indicative of the C(3′) endo conformation, ^3E, predominating (Table V). Two s^2U derivatives have been shown to be ^3E in the crystal form,[33,34] whereas U, which is predominantly C(2′) endo (^2E) in solution, is C(3′) endo in the crystal structure.[35] The length and chemical nature of the 5′ substituent did not seem to effect the couplings, at least not to the extent as to influence pucker.

Values of $J_{4'5'}$ and $J_{4'5''}$ were found to be lower for each of the s^2U's versus the corresponding U nucleoside (Table IV). Thus, about the C(4′)–C(5′) bond the conformation is strongly g+ for the s^2U's ($\gamma > 80\%$), but also g+ for the U series of nucleosides ($60\% < \gamma < 80\%$)[32] (Table V). Again, the length and nature of the 5-position side chain was inconsequential to this conformational element.

Results of two-dimensional nuclear Overhauser enhancement spectroscopy, or NOESY experiments, on each of the 12 uridines (Figure 3) confirmed the determination of sugar pucker (Table V) analyzed initially from the standpoint of coupling constants. The observed NOESY patterns for 10 of the 12 compounds are presented in Table VI and indicated either ^2E or ^3E conformations. The relative strengths of the NOE's between H(6) and H(2′), H(6) and H(3′), H(6) and H(1′), and H(1′) and H(2′) are indicative of either ^2E or ^3E conformations. In general, the presence of NOE's between H(6)–H(3′) and H(1′)–H(2′) and the absence of an H(6)–H(1′) NOE are characteristic of the C(3′) endo conformer, whereas the absence of an H(1′)–H(2′) NOE and presence of an H(6)–H(2′) NOE speak rather for C(2′) endo sugar pucker. Thus, NOE patterns observed for the thio compounds were characteristic for C(3′) endo sugar puckering. However, s^2mo^5U and s^2mcm^5U did exhibit small H(6)–H(1′) NOE's. Within the 3′-endo conformation an NOE would not be expected from the strictly anti, thiolated nucleoside.

Information on the syn/anti conformation about the N-glycosidic bond is found in proton-coupled, ^{13}C spectra and in the NOESY data. Proton-coupled, carbon-13 NMR spectroscopy reveals carbon–proton couplings that indicate the syn/anti conformations according to the following relationships: $J_{C(6)H(1')} > J_{(C)2H(1')}$ = anti; $J_{(C)2H(1')} > J_{C(6)H(1')}$ = syn.[36] For example, for mcm^5U, $J_{C(2)H(1')}$ = 3.5 Hz, which is larger than $J_{C(6)H(1')}$ = 2.5 Hz, and the nucleoside is considered in the syn conformation. However, for s^2cmnm^5U, $J_{C(6)H(1')}$ = 4.0 Hz which is larger than $J_{C(2)H(1')}$ = 2.5 Hz, indicating that the thio nucleoside is anti. The same conclusions were drawn from the results of NOESY experiments. For instance, for $cmnm^5U$ strong NOE's were observed

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Table III. Proton Chemical Shifts under Physiological Conditions[a,b]

	proton position										
nucleoside	H-5	H-6	CH_3	$CH_2(\alpha)$	$CH_2(\beta)$	H1′	H2′	H3′	H4′	H5′	H5″
U	5.9	7.90				5.85	4.32	4.21	4.11	3.90	3.76
s^2U	6.01	7.98				6.47	4.25	4.04	4.04	3.87	3.72
mnm^5U		8.07	2.61	3.87		5.87	4.24	4.12	4.03	3.83	3.72
s^2mnm^5U		8.33	2.61	3.88		6.40	4.29	4.08	4.08	3.94	3.76
mcm^5U		7.77	3.64	3.30		5.80	4.22	4.12	4.01	3.82	3.68
s^2mcm^5U		8.07	3.59	3.34		6.45	4.25	4.06	4.03	3.92	3.73
$cmnm^5U$		8.06		3.50	3.91	5.82	4.23	4.12	4.02	3.84	3.70
s^2cmnm^5U		8.3		3.47	3.85	6.44	4.27	4.07	4.04	3.94	3.76
mo^5U		7.49	3.62			5.82	4.22	4.16	4.02	3.85	3.72
s^2mo^5U		7.86	3.62			6.45	4.25	4.18	4.08	3.95	3.77
$mcmo^5U$		7.66	3.68	4.55		5.81	4.19	4.12	4.03	3.82	3.71
s^2mcmo^5U		7.94	3.71	4.59		6.44	4.25	4.11	4.08	3.94	3.76

[a] Phosphate buffered saline (PBS) of pH 7.2 in D_2O (10 mM $MgCl_2$, 15 mM KH_2PO_4, 10 mM Na_2HPO_4, 100 mM NaCl). [b] Chemical shifts are given in ppm from DSS as the internal reference at 0 ppm.

Table IV. Coupling Constants *J* (H–H) under Physiological Conditions[a]

	coupled protons					
nucleoside	1′2′	2′3′	3′4′	4′5′	4′5″	5′5″
U	4.6	5.2	5.2	2.8	4.3	12.5
s^2U	2.5	4.0	6.0	1.6	3.0	13.5
mnm^5U	4.2	5.4	5.7	2.5	3.9	12.8
s^2mnm^5U	2.3	4.0	7.0	1.6	3.2	13.0
mcm^5U	4.5	5.4	5.5	2.9	3.5	12.9
s^2mcm^5U	2.1	4.2	6.0	2.0	2.8	13.2
$cmnm^5U$	4.2	5.2	5.4	2.7	3.5	12.8
s^2cmnmU	1.8	4.0	6.0	1.7	2.5	13.0
mo^5U	4.0	5.1	5.4	2.7	2.8	12.8
s^2mo^5U	1.7	4.8	8.0	2.2	2.1	13.1
$mcmo^5U$	4.0	5.0	5.2	2.3	3.1	12.8
s^2mcmo^5U	1.7	4.0	6.0	1.7	2.1	13.0

[a] Coupling constants are given in Hz.

Table V. Conformational Percentages

nucleoside	P[a]	% S/% N[b]	γ[c]		γ[c]	χ[d]
U	^2E	0.88	g^+ 59%	g^- 28%		anti
s^2U	^3E	0.41	g^+ 94%	g^- 5%		anti
mnm^5U	^2E	0.73	g^+ 81%	g^- 24%		anti
s^2mnm^5U	^3E	0.30	g^+ 77%	g^- 17%		anti
mcm^5U	^2E	0.81	g^+ 66%	g^- 20%		syn
s^2mcm^5U	^3E	0.35	g^+ 82%	g^- 13%		anti
$cmnm^5U$	^2E	0.77	g^+ 68%	g^- 20%		syn
s^2cmnm^5U	^3E	0.31	g^+ 88%	g^- 21%		anti
mo^5U	^2E	0.74	g^+ 75%	g^- 13%		anti
s^2mo^5U	^3E	0.21	g^+ 87%	g^- 6%		anti
$mcmo^5U$	^2E	0.76	g^+ 76%	g^- 16%		syn
s^2mcmo^5U	^3E	0.28	g^+ 94%	g^- 2%		anti

[a] Ribose conformer derived from the pseudorotation phase angle, *p*. [b] S = 2′-endo conformer, N = 3′-endo conformer. [c] γ = conformation around the C(4′)–C(5′), γ = O(1′)–C(4′)–C(5′)–O(5′). [d] χ = conformation around the glycosidic bond given by dihedral angle χ = C(6)–N(1)–C(1′)–O(1′).

for H(6)–H(1′) and H(6)–H(2′). NOE's between the 5-position $C_\alpha H_2$, $C_\beta H_2$, and CH_3 protons and H(5′) or H(5″) were conspicuously absent. Thus, $cmnm^5U$ was syn (^2E). In contrast, s^2cmnm^5U exhibited strong NOE's for H(6)–H(3′) and H(6)–H(2′), and for CH_2–H(5″). Therefore, the thiouridine derivative was anti (^3E). In fact, all thiouridines were found to be anti (^3E) (Table V). Uridine, mnm^5U, and mo^5U were anti, whereas mcm^5U, $cmnm^5U$, and $mcmo^5U$ with bulkier side chains were syn.

The carbon chemical shifts for the U and s^2U series of nucleosides under physiological conditions are shown in Table VII. Significant differences in chemical shifts are noted in almost all of the carbons when comparing the thiolated compound to the non-thiolated uridine for each of the sets of nucleosides. For instance, the carbon chemical shift for the C(2) atom of the thiouridine is consistently upfield of that for the analogous uridine to the extent of Δ_{av} = −23 ppm (1736.5 Hz). The nature of the

Table VI. NOE Patterns for Each of the Twelve Uridines from NOESY Experiments[a]

nucleoside	carbonyl derivatives (O)	2-thio derivative (S)
mnm^5U	H(6)–H(1′)	H(6)–H(3′)
	H(6)–H(2′)	H(1′)–H(2′)
	H(6)–H(4′)	H(6)–H(2′)
mcm^5U	H(6)–H(1′)	H(6′)–H(3′)
	H(1′)–H(3′)	H(1′)–H(2′)
	H(1)–H(2′)	H(6)–H(2′)
$cmnm^5U$	H(6)–H(1′)	H(6)–H(3′)
	H(6)–H(2′)	H(1′)–H(2′)
	H(1′)–H(2′)	H(6)–H(2′)
		CH_2–H(5″)
mo^5U	H(6)–H(1′)	H(6)–H(3′)
	H(6)–H(2′)	H(1′)–H(2′)
	H(1′)–H(2′)	H(6)–H(2′)
	H(6)–H(3′)	CH_3–H(2′)
	CH_3–H(3′)	
$mcmo^5U$	H(6)–H(1′)	H(6)–H(3′)
	H(6)–H(2′)	H(1′)–H(2′)
	H(1′)–H(2′)	H(6)–H(2′)
	CH_2–H(2′)	CH_3–H(3′)
	CH_2–H(1′)	

[a] NOE pattern presents connectivities between protons of the base and ribose rings.

5-position substituent also effected the C(2) chemical shift. The directly bonded oxygen of mo^5U and $mcmo^5U$ shifted the C(2) resonance upfield for these uridine (Δ_{av} = −2.2 ppm) and thiouridine (Δ_{av} = −4.7 ppm) derivatives relative to the unsubstituted and other substituted uridines. Carbon atom C(4) exhibited a change in resonance for all the thiolated compounds in comparison to the analogous 2-carbonyls. Base carbon atom 5 experienced a change in chemical shift downfield for each of the 5-position derivatives relative to U and s^2U. The directly bound oxygen of mo^5 produced the largest changes in C(5) (Δ_{av} = 30.3 ppm). Interestingly, in comparing U to s^2U for each of the 6 sets of nucleosides the C(5) signal is shifted downfield an additional Δ_{av} = 4.2 ppm no matter what the character of the side chain. Carbon-6 demonstrated smaller chemical shift changes with thiolation at C(2). Those shifts were approximately 1 ppm and mostly upfield. As with C(5), C(6) chemical shifts dramatically demonstrated the effect of oxygen bound directly to the uridine aromatic ring at position 5. However, in contrast to C(5), the directly bound $mcmo^5$ substituents caused C(6) chemical shift changes upfield (Δ_{av} = −20.3 ppm) from that of U, s^2U, and the other 5-position derivatives. Carbon chemical shifts of the side chain are not substantially effected by thiolation at ring carbon 2 and show predictable changes with differences in side chain chemistry, i.e., chemical shifts of C. The almost 3 ppm upfield difference between C of $mcmo^5U$ and $mcmo^5s^2U$ is presently unexplainable.

In the ribose ring, the carbon that exhibited the largest chemical shift change was C(1′). With thiolation at C(2) of the base, a downfield shift was observed for C(1′) equal to 4.2 ppm. C(2′)

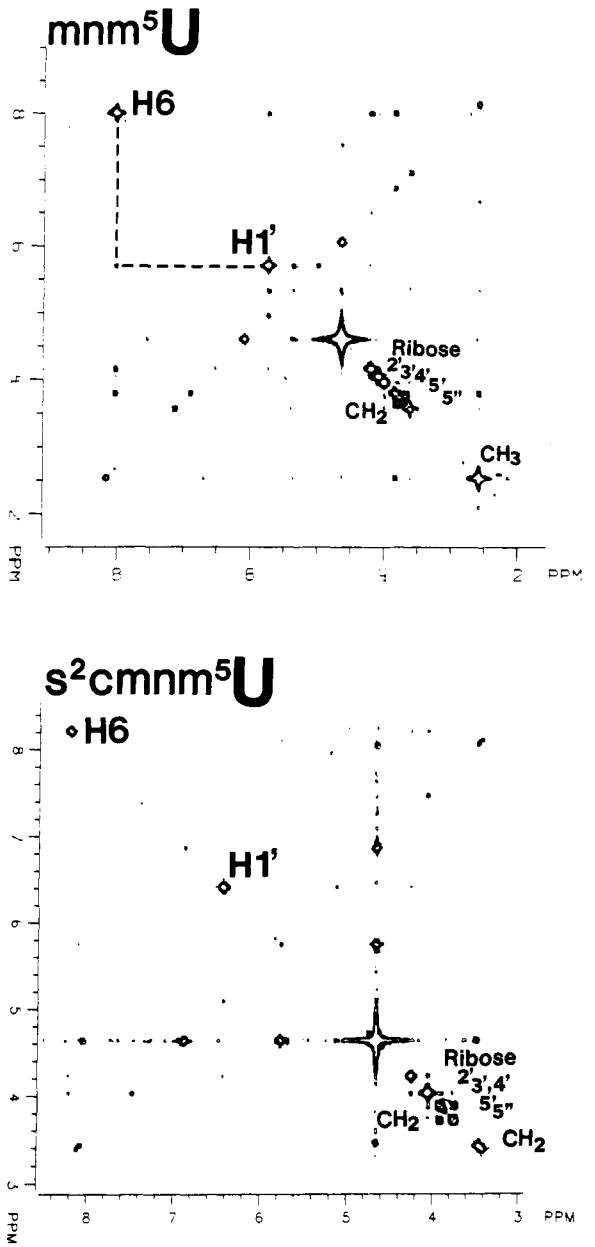

Figure 3. The 300-MHz two-dimensional NOE (NOESY) spectra of s^2cmnm^{5}U and mnm^{5}U. The single dimension proton spectrum is labeled on the diagonal of each of the contour plots. Mixing time was 0.7 s. Connectivity between H(6) and H(1′) is seen for mnm^{5}U, but not for s^2cmnm^{5}U. This demonstrates a consistent difference observed for the U and s^2U derivatives. Table VI contains all the connectivities found in the NOESY experiments.

had a small downfield chemical shift change (Δ_{av} = 0.9 ppm) with thiolation. Atoms C(3′) and C(5′) exhibited small upfield shifts, whereas C(4′) did not exhibit a resonance change in a comparison of the U's with the corresponding s^2U's. The chemical nature and length of the base 5-carbon substituent had virtually no effect on ribose carbon chemical shifts.

Discussion

We have been studying and comparing the chemistry and structure of five pairs of biologically important 5-position modified uridines and 2-thiouridines, as well as U and s^2U, themselves, under physiological conditions. All are found in the wobble position of tRNA anticodons. All of the nucleosides, except uridine, were chemically synthesized and were subsequently analyzed by HPLC and UV, IR, and NMR spectroscopy in our laboratories. These spectroscopic techniques, especially NMR, have been shown to be the methods of choice, along with circular dichroism, for the study of nucleic acids.[37] In order to understand the significance of these modifications to tRNA anticodon structure and function it was necessary to compare the various chemical and structural properties of each individual uridine and its analogous thiouridine, as well as the different 5-position modified uridines, or thiouridines.

Reverse-phase HPLC demonstrated quite dramatically the effect of the thio group at position 2 of the base on the chemistry of the nucleosides. Retention times and, thus, the hydrophobicity of the uridine derivative were increased with thiolation. The shorter the 5-position substituent was, the larger was the effect of thiolation. The various aqueous solubilities of the nucleosides were consistent with the HPLC results. Two 5-position substituents of U and s^2U, mnm^5 and cmnm^5, were more hydrophilic than U and s^2U, whereas all other 5-position derivatives were more hydrophobic than the parent compounds.

Ultraviolet spectral analyses revealed that both thiolation and 5-position derivatizations of uridine shifted the wavelength of maximum absorbance to longer wavelengths. Thiolation increased λ_{max} by 5–13 nm, whereas the increase affected by the 5-position substituents, 3–16 nm, was very dependent on their chemical structures (mnm^5 and mcm^5, only 3 nm; versus mo^5 and mcmo^5, 13–16 nm). Each of the 12 nucleosides studied exhibited unique, characteristic UV and IR spectra. For instance, infrared spectra revealed specific absorbance bands for C(2)=O and those characteristic for mnm^5 and mcm^5.

Two-dimensional NMR investigations under physiological saline conditions have been shown capable of detecting structural variations even in nucleic acid oligomers.[38] Our NMR investigations under physiological saline conditions revealed the predominating sugar puckers and the positioning of the base ring relative to the ribose (syn/anti conformation) for each of the 12 uridines. We have calculated the proportions of the major sugar conformers, as demonstrated by other investigators.[39] All 2-thiouridines were found to have major fractional populations in the ^{3}E form. The detailed comparison between different 5-substituted uridines showed that the positively charged (methylamino)methyl group or the more bulky (methoxycarbonyl)methyl group did not contribute to the high ^{3}E population. Thus, neither chemistry nor spacial size of the 5-position derivatives had much effect on this conformation of the s^2U's. In addition, s^2U's were predominantly gauche plus [C(4′)–C(5′)]. The conformation about the N(1)–glycosidic bond could not be deduced from vicinal (^{1}H) spin-coupling constants. The NOESY data together with $J(^1H–^{13}C)$ revealed the orientation around N(1)–glycosidic bonds. As seen from Table V, all thiolated compounds were anti.

In surprising contrast to the thiolated uridines, structures and conformations varied among uridines. Although all uridines were predominantly ^{2}E conformers, the gauche plus populations (average 70%) were significantly lower than that of the s^2U's. Also both syn and anti structures were exhibited by the uridines. Those uridines with bulkier 5-position derivatives (mcm^5, mcmo^5, and cmnm^5) exhibited syn structures. The chemical nature of the substituent did not seem to influence the N–glycoside bond angle hydrophilic mnm^{5}U is anti, and cmnm^{5}U is syn; the more hydrophobic mo^{5}U is anti, while mcm^{5}U and mcmo^{5}U are syn.

Conclusion

From the 12 uridines studied, there are three consistent observations of chemistry and structure. First, the 2-thio group is responsible for producing a more hydrophobic nucleoside, no matter what the nature of the 5-position substituent. The thio group produces unique and characteristic UV and IR spectra. Second, the 2-thio substitution is responsible for "fixing" a ^{3}E, gauche plus, anti conformation for all six s^2U's we investigated.

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Table VII. Carbon-13 Chemical Shifts[a,b]

	carbon position											
nucleoside	C=O	C-2	C-4	C-5	C-6	C-1′	C-2′	C-3′	C-4′	C-5′	CH_3	$CH_2(\alpha)$	$CH_2(\beta)$
U		152.34	167.3	103.4	143.0	90.6	74.8	70.6	85.5	62.0			
s^2U		176.84	163.88	107.29	142.47	94.17	75.43	69.27	84.62	60.69			
mnm^5U		152.14	165.38	105.63	143.94	90.47	74.70	69.84	85.00	61.17	33.03	45.97	
s^2mnm^5U		176.81	162.3	109.69	143.56	94.35	75.44	68.72	84.58	60.11	33.15	46.11	
mcm^5U	174.61	153.0	166.2	109.26	140.92	90.12	74.54	70.03	84.96	61.35	53.62	32.76	
s^2mcm^5U	174.04	175.95	163.13	113.74	141.02	94.22	75.44	68.86	84.51	60.25	53.66	32.98	
$cmnm^5U$	171.98	152.25	165.56	105.92	143.66	90.44	74.69	69.81	84.96	61.13		44.63	48.33
s^2cmnm^5U	171.38	176.33	164.11	110.55	142.83	94.38	75.65	68.87	84.53	60.32		45.33	49.64
mo^5U		150.84	162.33	137.48	120.14	90.15	74.77	69.94	84.83	60.92	57.85		
s^2mo^5U		171.23	159.8	141.68	120.64	94.82	75.74	68.58	84.34	59.56	57.58		
$mcmo^5U$	171.79	151.01	162.33	135.33	125.11	90.05	74.84	68.08	85.03	61.09	53.10	70.04	
s^2mcmo^5U	171.33	172.27	159.63	139.52	123.74	94.70	75.75	68.66	84.53	59.82	53.66	67.11	

[a] Chemical shifts are given in ppm relative to dioxane (67.4 ppm).

A computer-simulated, space-filling model of the X-ray crystallographic structure of s^2mnm^5U is shown in Figure 1. In the figure, comparison to the structure of mnm^5U, deduced from NMR data, demonstrate the spacial dominance of the thio over oxygen at position 2. Chemistry and spacial size of 5-position derivatives were inconsequential to this structure. Third, uridine and its derivatives in solution were all ^2E, but they had more varied C(4′)–C(5′) and syn/anti conformations. Here, bulk alone, not chemical nature of the 5-position derivative, seemed to affect the syn conformer in the nucleoside.

The strongly indicated fixed structure of the thiouridines and the more varied structures of the uridines are presently under further study to evaluate the actual transition energies between conformers for all 6 pairs of nucleosides under physiological conditions. The enthalpy difference between ^2E and ^3E forms has been reported to be only 0.37 kcal/mol for U and 0.58 kcal/mol for mo^5U, whereas it was 1.12 kcal/mol for s^2U and 1.32 kcal/mol for s^2mnm^5U in D_2O. We would postulate that the 2-thiouridine structure found here for six nucleosides is so substantially stable as to be the predominant conformer at the wobble position of the tRNA anticodon. Previous work has shown that in vitro, *E. coli* $tRNA^{Glu}$ with s^2mnm^5U at wobble position 34 prefers the GAA codon 3:1 over GAG, whereas the same tRNA lacking only the 2-thio group actually prefers the GAG codon 3:1 over GAA[4]. An *E. coli* $tRNA^{Tyr}$ suppressor with a non-thiolated, modified uridine in the wobble position also recognizes the G-containing codon, UAG terminator, rather than A-containing, UAA.[40] Even in DNA a base pair mismatch has been demonstrated in the non-thiolated dm^5U (dT):dG duplex.[41] In vivo studies of yeast $tRNA^{ser}$ suppressor recognition of the terminating UAA codon have demonstrated that thiolase deficient strains lack s^2mcm^5U-34. These strains are incapable of suppression.[6,42] Thus, at least for the cited tRNA cases, effective recognition of codons ending in A could be predicted upon the ^3E, gauche plus, anti conformation of the 2-thiolated uridines. Effective and perhaps stringent codon recognition seems dependent upon good anticodon base stacking.[10,43] Uridine is not the only thiolated nucleoside found in the anticodon loop, and for which there is possible evidence of translational control.[44] We would thus hypothesize that the 2-thio group in increasing the hydrophobic nature of the uridine-34 nucleosides enhances base stacking with nucleoside-35, and in fixing the ^3E, gauche plus, anti conformation effectively, it modulates the binding energy to the codon[45] for selective recognition of A in vivo.

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Registry No. $cmnm^5U$, 69181-26-6; mnm^5U, 72667-55-1; U, 58-96-8; s^2cmnm^5U, 78173-95-2; s^2mnm^5U, 32860-54-1; mo^5U, 35542-01-9; s^2U, 20235-78-3; mcm^5U, 29428-50-0; $mcmo^5U$, 66536-81-0; s^2mo^5U, 30771-43-8; s^2mcm^5U, 20299-15-4; s^2mcmo^5U, 110417-90-8; cm^5U, 20964-06-1; 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-D-ribose, 14215-97-5; 5-methoxy-2-thiouracil, 6939-11-3; 5-methoxy-2-thio-2′,3′,5′-tri-*O*-benzoyluridine, 34291-44-6; 5-methoxy-2-thio-3-(2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl)uracil, 110417-92-0; 2′,3′-*O*-isopropylidene-5-hydroxyuridine, 20406-82-0; ethyl iodoacetate, 623-48-3; 2′,3′-*O*-isopropylidene-5-(ethoxycarbonylmethoxy)uridine, 110417-93-1.

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