BIOORGANIC CHEMISTRY 6, 103–116 (1977)

Cysteine Adduct of Pseudouridine: A Model for Aminoacyl-tRNA Binding to Ribosomes[1]

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Received August 16, 1976

Pseudouridine and the analogous 5-(1-hydroxyethyl)uracil form adducts with cysteine which have been isolated and characterized. A model is proposed for the process of binding of the aminoacyl-tRNAs to the ribosomes which includes formation of a covalent adduct of the type characterized, i.e. 5-[1-(*S*-cysteine)-2,3,4,5-tetrahydroxypentane]uracil, and which accommodates many previous observations. The proposal involves two sequential molecular events: recognition through H-bonding between complementary oligonucleotides of the tRNA and the ribosome surface, followed by formation of a reversible covalent bond between ribosomal protein and tRNA.

INTRODUCTION

Recent studies on the mode of binding of aminoacyl-tRNAs to ribosomes during protein synthesis have provided evidence suggesting that the pseudouridine loop of tRNAs interacts with ribosomal components. The presence of pseudouridine in the sequence G–rT–ψ–C–G(A)p,[2] first reported by Zamir et al. (*1*) and thus far found in every tRNA that is active in the elongation step of protein biosynthesis (*2*), indicates a specific role for that oligonucleotide. Those tRNAs which are either initiators (*3–5*) or participate in peptidoglycan synthesis (*6,7*) lack that sequence. The above sequence probably represents part of the normal ribosome binding site in tRNAs (*8–10*). There has been some evidence that ribosomal proteins are involved in the binding of aminoacyl-tRNAs (*11,12*), but the exact nature of this event at the molecular level has not been elucidated.

The presence of ψ instead of U in the region of tRNA under discussion is intriguing for several reasons. The general pathway leading to modified nucleosides, including ψ, in tRNA is due to enzymatic action at the polynucleotide level (*13,14*). Moreover, the enzymatic activity responsible for the conversion Up to ψp in the G–rT–ψ–C–G(A)p loop (loop IV) is quite specific for that position only and differs from that affecting the same transformation near the anticodon loop (*15,16*). Because of the conformational similarities of ψ to U (*17*), it appears likely that either one of these nucleotides would interact equally well through hydrogen bonding, via N_3–H, C_2=O or N_3–H, C_4=O, respectively, with the corresponding adenosine component of the complementary oligonucleotide C–G–A–A–C(U)p, which is found in 5 S ribosomal RNAs (*18–20*).

[1] A preliminary report was presented at the 67th Annual Meeting of the American Society of Biological Chemists, San Francisco, California [*Fed. Proc.* **35**, 1466 (1976)].

[2] Abbreviations used: G, guanosine; rT, ribosylthymine; U, uridine; ψ, pseudouridine; C, cytidine; A, adenosine; Pu, purine nucleoside.

ISSN 0045-2068

Unless the presence of ψ is accidental,[3] that modified nucleoside may provide for another, or an additional, unique mode of interaction, related to the unusual lability inherent in the allylic oxygen bridge in its "glycosyl" moiety. A reversible formation of heteroadducts of ψ with nucleophilic groups of ribosomal protein might be one molecular event in the functioning of the pentanucleotide G–rT–ψ–C–G(A)p. In order to investigate the feasibility of such reactions, we have studied the reaction of ψ with cysteine. In this report we present the results of such experiments and speculate on their biological implication with regard to the role of ψ in tRNAs.

The formation of adducts of various kinds of substances with nucleic acids has been studied extensively. Detailed studies have already been done on photo-cross-linked complexes of aminoacyl-tRNA synthetases with tRNA (*22–24*). Photoinduced cross-linkage of ribosomal proteins to tRNA in intact 50S ribosomal subunits (*25*) as well as the addition, induced by ionizing radiation, of amino acids (*26*) and α-chymotrypsin (*27*) to DNA have also been demonstrated.

The chemistry of pseudouridine has been reviewed, and most of its unique properties have been satisfactorily explained by the lability of the allylic ether function of the "glycosyl" moiety. This function is subject to reversible cleavage in either acid or base solution, giving rise to transient electrophilic intermediates (*28*). Formation of substitution products from 5-hydroxymethyluracil and derivatives has been well documented (*29*), and the relevance of such reactions to the mode of reaction of thymine synthetase has been demonstrated (*30*). For pseudouridine only solvolytic cleavage reactions leading either to intramolecular additions with isomerization of the 5-"glycosyl" moiety or to adducts with water formed *in situ* and subjected to further oxidation have been reported (*28*). A related reaction is the addition of ammonia to 5-(D-altro-pentaacetoxypentyl)uracil to give 5-(D-altro-1-amino-2,3,4,5-tetrahydroxypentyl)uracil (*31*).

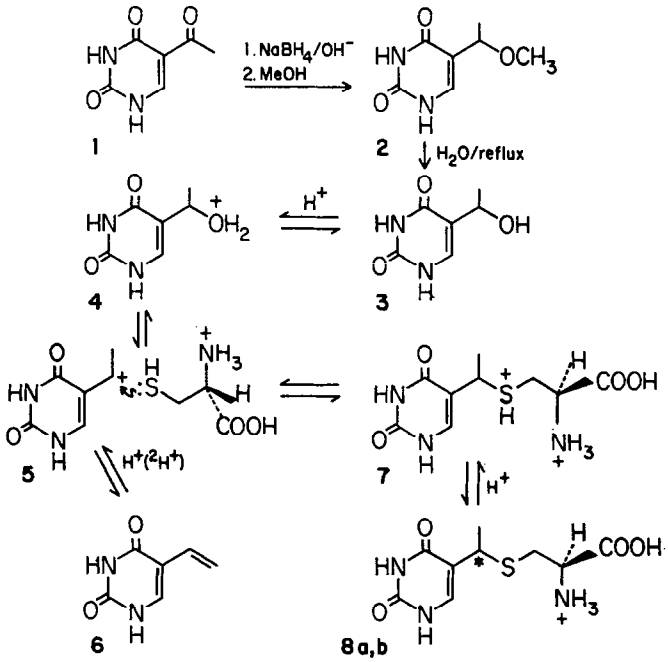
RESULTS AND DISCUSSION

S-Cysteine derivatives of pseudouridine and 5-(1-hydroxyethyl)uracil have been prepared in reactions that offer a simple model for a possible binding of tRNA to ribosomal protein. This model takes advantage of the highly reactive vinylogous carbinolamine (*30*) functionality present in β-pseudouridine as a consequence of its 5-"glycosyl" structure. This high degree of reactivity has been amply demonstrated in the case of 5-hydroxymethyluracil, a model compound containing the same vinylogous carbinolamine system, for which acid-catalyzed nucleophilic substitution has been reported for O (*29,32,33*), S (*29,32*), N (*30*), and C (*34*). Although the derivatives reported herein were prepared under acidic conditions not present *in vivo*, such extremes are unlikely to be necessary with appropriate enzymatic catalysis. For example, in liver monoamine oxidase (*35*), the linking *in vivo* of a cysteine residue of the protein to FAD

[3] However, that would seem unlikely because of the demonstrated topological and enzymic specificity of ψ biosynthesis and the conservation of the entire sequence G–rT–ψ–C–G(A)p through evolution. These events are presumably the result of mutations which must have produced a greater *reproductive potential* (*21*) in order to have been retained.

through a similarly activated sulfur–carbon bond presumably occurs readily by enzymatic assistance, although, for the chemical synthesis of the 8-α-cysteinylriboflavin, the more reactive 8-α-bromotetraacetylriboflavin and an aprotic solvent (dimethylformamide) were employed (*36*).

The reaction of cysteine with 5-(1-hydroxyethyl)uracil (**3**) (Scheme 1) proceeds rapidly and quantitatively at pH 2, and the two expected diastereomeric products **8a** and **8b** were readily separated on a Dowex 50 column. The two isomers could be isolated by lyophilization of the respective fractions. Each gave rise to nmr spectra consistent with



SCHEME 1.

structure **8**, with the chemical shifts for each proton of one isomer slightly offset from those found for the other. With a mixture of **8a** and **8b** all of the spectral lines were doubled.

The ABX spectrum of the cysteine fragment is essentially the same as that found in other *S*-cysteine derivatives in D_2O solution at acid pH, as illustrated in Table 1. The lack of substantial change of J_{AX} and J_{BX} from these models and within the two diastereomers themselves suggests that approximately the same relative population of the three CH_2–CH bond rotamers occurs in each case.

The downfield shift of $H_{1'}$ from –O– to –S– substitution is in the direction expected for the less electronegative heteroatom (*37*). Moreover, the vicinal coupling $J_{CH_3,\,CH(1')}$ increases significantly from 6.4 to 7.1 Hz, as expected for the less electronegative heteroatom (*37*).

Isomer **8a** could be readily crystallized from an alcoholic solution containing a small volume of HCl. This same method applied to a homogeneous sample of isomer **8b** (as evidenced by nmr and chromatographic data) failed to yield a crystalline product,

TABLE 1

NUCLEAR MAGNETIC RESONANCE DATA FOR *S*-CYSTEINE DERIVATIVES

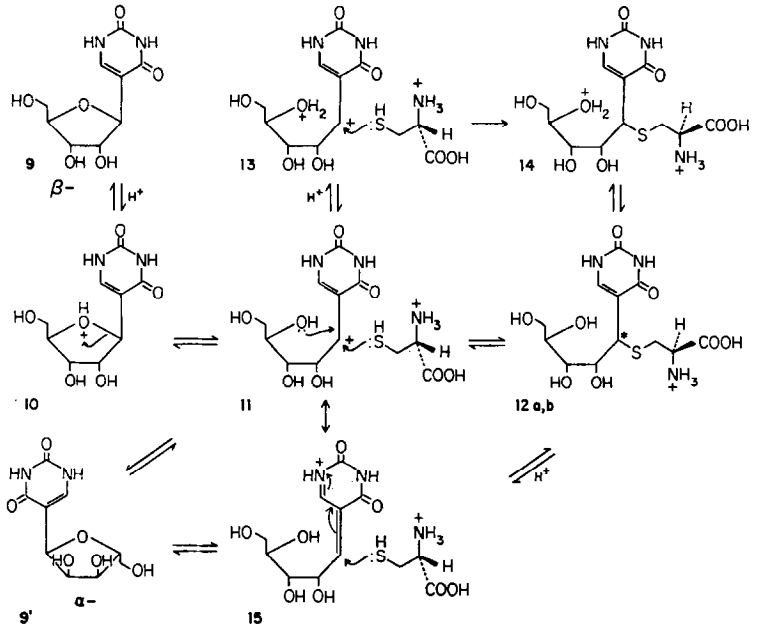
Compound	H_A[a]	H_B[a]	H_X[a]	J_{AB}[b]	J_{AX}[b]	J_{BX}[b]
S-Benzylcysteine[c,d]	3.08	3.18	4.29	−15.1	7.9	4.4
S-Allylcysteine[c,d]	3.07	3.19	4.32	−15.0	7.9	4.4
S-Methylcysteine[c,d]	3.11	3.20	4.32	−15.0	7.6	4.6
8a[e]	2.97	3.20	4.27	−14.6	7.2	4.6
8b[e]	3.07	3.28	4.38	−15.0	6.6	4.6
12a[f]	3.14	3.26	4.38	−15.2	7.0	4.3
12b[f]	3.15	3.27	4.41	−14.9	6.1	4.5

[a] In parts per million downfield from internal DSS.
[b] In hertz.
[c] Solution in 10% (w/w) CF_3CO_2D in D_2O.
[d] Bartle et al. (*63*).
[e] Solution of the hydrochloride in D_2O: pD 2.3.
[f] Solution in ~2 *N* DCl/D_2O.

except after an extended period of time. However, the crystals thus obtained proved (by nmr) to be those of a 1:1 mixture of **8a** and **8b**, a fact which is consistent with an acid-catalyzed epimerization at the benzylic carbon. This interconversion was confirmed in a separate set of experiments by following the appearance of a second set of nmr signals, starting either with **8a** or **8b** in D_2O/DCl solution. This isomerization proceeds to a common equilibrium mixture and is accompanied by a concomitant deuterium exchange of the protons of the side chain methyl group. This exchange could be accounted for by the reversible formation of the olefinic compound **6** from the key carbonium ion intermediate **5** as shown in Scheme 1.

The corresponding reaction with β-pseudouridine takes place under the same conditions but proceeds only partially to completion. Maximum yields could be obtained using a larger excess of cysteine, slightly more acidic conditions, and longer reflux time. It is likely that the initially formed carbonium ion (Scheme 2) is subject to competitive internal attack by the 4′-hydroxyl, so that only a small fraction of ring openings leads ultimately to thioether product. Strongly acidic conditions may repress this effect by protonation of this 4′-hydroxyl (**13**, Scheme 2).

A similar separation of the pseudouridine reaction mixture on a Dowex 50 column produced only one product peak, which on lyophilization gave a satisfactory elemental analysis for the hydrochloride salt of the *S*-cysteine substitution product **12** (Scheme 2). This product gives a positive violet ninhydrin reaction, and the uv spectrum is shifted bathochromically as in the case of **8** and some other model compounds (Table 2). Furthermore another isomer, presumably α-pseudouridine (**9′**), formed via the common intermediate **11**, is isolated along with unreacted β-pseudouridine. These facts are consistent with **12** being a 1:1 adduct of cysteine hydrochloride and pseudouridine, with attack and binding of the nucleophilic sulfhydryl group of the former to the C_1' position of the latter. The 220-MHz nmr spectrum (Fig. 1) is also consistent with this structure, chemical shifts and coupling falling within the ranges expected from a series of model compounds (Tables 1 and 2). In addition, the spectrum reveals a doubling of all clearly



SCHEME 2.

resolved signals. The two diastereomers **12a** and **12b** corresponding to these two sets of signals are in approximately a 2:1 ratio as judged by the relative intensities of these sets of doubled signals.

Incubation of a 0.5 *N* HCl solution of a pure sample of **12** overnight at 100°C produced an equilibrium mixture, the chromatographic profile of which was qualitatively the same as that of the original reaction mixture. This suggests the reversibility of this reaction.

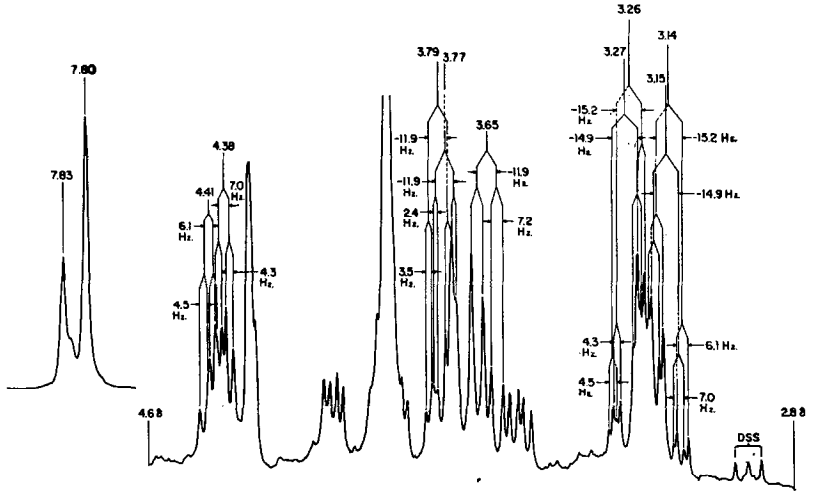
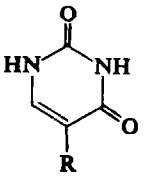
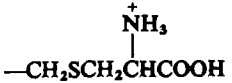
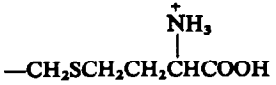
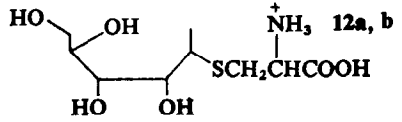


FIG. 1. The 220-MHz (FT) nmr spectrum of **12** with assignments as shown, obtained at a 500-Hz sweep width. The H_6 signal inset at the left was obtained at a 250-Hz sweep width.

TABLE 2

ULTRAVIOLET PROPERTIES OF



Compound	Notes	pH	Charge (pyrimidine)	λ_{max} [nm ($\varepsilon \times 10^{-3}$)]	λ_{min} [nm ($\varepsilon \times 10^{-3}$)]
	*a*	2	0	265.5 (7.6)	237 (2.9)
		12	−1	291.5 (6.8)	252 (2.4)
	*a*	2	0	266 (6.4)	237 (2.6)
		12	−1	291.5 (6.1)	252 (2.2)
 **8a**		6	0	266 (7.2)	237.5 (3.4)
		13	−1	288 (6.5)	249 (2.8)
8b		7	0	265.5 (6.2)	237.5 (2.9)
		12	−1	290 (5.4)	250 (2.3)
 **12a, b**		7	0	267.5 (5.5)	238.5 (2.9)
		12	−1	292 (5.1)	254

CH_3 | —CHOH	*b*	1	0	263 (7.6)	
		13	−1	287 (9.2)	
CH_3 | —$CHOCH_3$		6	0	263 (7.3)	232 (2.2)
		12	−1	288.5 (6.8)	245 (2.3)
β-Pseudouridine	*c*	7	0	207 (10.8), 263 (8.1)	232
		12	−1	218 (10.9), 287 (8.4)	245
	d	7	0	262 (7.9)	
		12	−1	287 (7.8)	
α-Pseudouridine	*d*	7	0	263 (7.9)	
		12	−1	287 (6.3)	

[a] Cline et al. (*29*).
[b] Evans et al. (*62*).
[c] Dlugajczyk (*64*).
[d] Brown et al. (*65*).

A Model

While the outlines of protein biosynthesis have been elucidated many individual details, such as the precise functioning of tRNA in the ribosome, are still unknown. Experimental evidence suggests no significant conformational change in the secondary structure of tRNA induced by aminoacylation, at least in the case of yeast $tRNA^{Phe}$ (*38*), but the functioning of the ψ loop, which is otherwise unavailable (*40–42*), has been explained by the induction of allosteric conformational changes following codon–anticodon interaction (*39,43,44a,b*). Alternatively, the precise alignment of the ψ loop was explained by a rotation of the L-shaped tRNA molecule around the axis defined roughly by the anticodon loop and the amino acid acceptor end (*45*). The evidence for a four-point interaction occurring exclusively through hydrogen bonding among complementary tetranucleotides commonly used in tRNA "binding" experiments (*12,44b*) has been critically reviewed (*46*), and the availability of ribosomal components implicated in such "binding" has been studied (*47,48*). This mode of interaction is plausible but not limiting. Conclusions regarding the "binding" of acyl-tRNAs to ribosomes, derived from elegant experiments using soluble oligonucleotide fragments, should be approached with caution, because the observed associations might involve only some individual components of the complementary fragments considered or might occur between the fragments used and allosteric sites otherwise unavailable.

The involvement of ribosomal sulfhydryl groups in the "binding" of aminoacyl-tRNAs (*49–53*) and in the process of translocation (*54*) has been demonstrated. Interestingly, it has been suggested that the structural basis for the action of sparsomycin, which stimulates the binding of initiator- or peptidyl-tRNA to ribosomes but blocks the peptidyl transferase reaction, is due to the pseudouridine-like moiety of that antibiotic which is attached to a peptide-like grouping (*55*). For these reasons the results purporting to prove the "binding" of the tetranucleotide rT–ψ–C–Gp to the complementary segment C–G–A–Ap cannot be accepted as conclusive when extrapolated to the complete acyl-tRNA–ribosome system.[4]

It is likely that the phenomena of tRNA binding during the various phases of protein synthesis are more complex than can be inferred when only hydrogen bonding interactions are taken into account. We therefore suggest that an interaction between a ribosomal protein and aminoacyl-tRNA through a reversible covalent bond between ψ and a nucleophile, probably a cysteine sulfhydryl group, should be considered as an additional or alternative factor relevant to the translocation and peptide transfer reaction. This model accommodates many previous observations regarding the participation of the ψ loop of tRNAs and SH groups of ribosomal proteins in such binding. We have

[4] In an apparent contradiction to the specific role suggested for the tetranucleotide rT–ψ–C–Gp in the elongation factor EF-T-dependent GTP hydrolysis (*56*) and in the stringent factor-directed synthesis of tetra- and pentaphosphates of guanosine (*57*), a tRNA, in which the uridine, pseudouridine, ribothymidine, and dihydrouridine were replaced to the extent of 87% or better by 5-fluorouridine, was fully active in *in vitro* protein synthesis (*58*) and in stimulating the synthesis of pppGpp (*59*). It is conceivable that in the latter cases a reversible addition of a sulfhydryl group to the 5,6-double-bond of the fluorouridine, occupying the locus of ψ in loop IV, can occur in a manner analogous to that observed in the reaction of FdUMP with thymidylate synthetase (*60*) and of UMP with cysteine (*61*).

demonstrated that the formation of such adducts is chemically possible, as shown by the model reactions reported here.

EXPERIMENTAL

Materials and Methods

Pseudouridine (Grade I, natural β-isomer) was purchased from Sigma Chemical Co. The ^{1}H nmr spectra were obtained with a Jeol PFT-100 and a Varian HR-220 spectrometer. Routine uv (H_2O) and infrared spectra were determined with a Unicam SP800 and a Perkin–Elmer Infracord spectrophotometer. Accurate ε values in the uv were measured with a Beckman DU spectrophotometer. All solvents were removed in a Buchler flash evaporator under reduced pressure, unless otherwise indicated. All solids were dried under reduced pressure over P_2O_5 at suitable temperature. An Eastman Chromagram silica gel sheet was used for thin-layer chromatography and developed as indicated.

5-(1-Methoxyethyl)uracil (**2**)[5]

A 2.00-g (13.0 mmol) sample of 5-acetyluracil (**1**) (Nutritional Biochemicals, Corp.) was dissolved, with slight warming, in 170 ml of 0.1 *N* sodium hydroxide solution, and 1.90 g (50.2 mmol) of $NaBH_4$ (Alfa-Ventron) was added slowly with stirring. The mixture was reacted for 3 hr at room temperature and quenched with the careful addition of 100 ml of "wet" AG 50W-X8, H^+ form (200–400 mesh, Bio-Rad), resin. The resin was collected on a sintered-glass funnel, and the clear, colorless solution was evaporated to dryness on a rotary evaporator. The residue was taken up in absolute methanol and evaporated to dryness. This process was repeated several times to give a constant-weight, borate-free residue. Prior to the final co-evaporation, the solution was decolorized with Norit and filtered. The white crystalline solid was dried *in vacuo*; yield, 2.16 g (100%). A portion of **2** was recrystallized from methanol; mp 182–184°C; nmr: (DMSO-d_6) δ 1.24 (*d*, 3, $J = 6.4$ Hz, CH_3), 3.17 (*s*, 3, $-OCH_3$), 4.18 (*q*, 1, $J = 6.5$ Hz, CH–OCH_3), 7.20 (*d*, 1, $J = 6.0$ Hz, H_6), 10.81 (*br. d*, 1, NH(1)), 11.08 (*br. s*, 1, NH(3)); ir (KBr, cm^{-1}): 3450 (*sh*), 3150 (*s*), 3000 (*br. s*), 2840 (*sh*), 1740 (*s*), 1650 (*br. s*), 1505 (*m*), 1450 (*s*), 1425 (*m*), 1370 (*w*), 1322 (*w*), 1310 (*w*), 1222 (*s*), 1204 (*sh*), 1150 (*w*), 1116 (*s*), 1082 (*m*), 1060 (*m*), 1010 (*w*), 995 (*w*), 925 (*w*), 868 (*w*), 820 (*br. m*), 790 (*sh*), and 780 (*m*). *Anal.* Calcd for $C_7H_{10}N_2O_3$: C, 49.41; H, 5.92; N, 16.46. Found: C, 49.41; H, 5.91; N, 16.47.

5-(1-Hydroxyethyl)uracil (**3**)

A 1.07-g (6.30 mmol) sample of 5-(1-methoxyethyl)uracil (**2**) was dissolved in 150 ml of water and refluxed for 2 hr. Monitoring by thin-layer chromatography (4:1 Bz/MeOH) showed complete conversion of starting material to a single product. The pale yellow solution was decolorized with Norit and evaporated to dryness to give 0.98 g (100%) of a white crystalline product. A small quantity of **3** was recrystallized from water. nmr (DMSO-d_6): δ 1.22 (*d*, 3, $J = 6.3$ Hz, CH_3), 4.56 (apparent quintet, 1, $H_{1'}$),

[5] This procedure is essentially that reported by Evans et al. (*62*), except that these authors isolated the alcohol, **3**, rather than the methyl ether, **2**, from the methanol co-evaporation.

4.95 (*d*, 1, $J = 4.7$ Hz, –OH), 7.19 (*br. s*, 1, H_6), 10.70 (*br. s*, 1, NH(1) or NH(3)), 11.01 (*br. s*, 1, NH(1) or NH(3)); ir (KBr, cm^{-1}): 3450 (*sh*), 3240 (*s*), 3100 (*s*), 2850 (*sh*), 1750 (*s*), 1680 (*s*), 1480 (*sh*), 1440 (*s*), 1365 (*m*), 1320 (*w*), 1290 (*w*), 1240 (*m*), 1200 (*m*), 1160 (*m*), 1080 (*m*), 1028 (*sh*), 1016 (*m*), 950 (*w*), 904 (*w*), 844 (*br. m*), 794 (*m*), 764 (*m*).

5-[1-(S-Cysteine)ethyl]uracil Hydrochloride (**8**)

A 0.42-g (2.7 mmol) sample of 5-(1-hydroxyethyl)uracil (**3**) and 0.47 g (2.7 mmol) of cysteine hydrochloride monohydrate (Fisher Reagent) were dissolved in 35 ml of water (pH 2) and heated to reflux. The reaction was monitored by thin-layer chromatography (9:1 EtOH/NH_4OH; ninhydrin/uv) and stopped after 6 hr. The solution was concentrated *in vacuo* and applied to a 2.5 × 90-cm AG 50W-X8, H^+ form (200–400 mesh, Bio-Rad), column and eluted at 1.0 ml/min with 1 *N* HCl. The eluents consisted of a small amount of unreacted starting material (6 hr) and two broad, but well-resolved product peaks (**8a**, 39–45 hr; and **8b**, 48–54 hr). The combined eluents for each product were frozen and lyophilized at high vacuum. Each was taken up in water, decolorized with Norit, and relyophilized. Each yielded a white amorphous foam; **8a**, 0.37 g (93%);[6] **8b**, 0.29 g (73%). Each isomeric product gave a positive violet ninhydrin test; the nmr spectra were nearly identical, and the uv spectra were completely indistinguishable. For **8a**: nmr[7] (D_2O) (downfield from DSS), δ 1.53 (*d*, 3, $J = 7.3$ Hz, CH_3), 2.97 (*q*, 1, $J_{AB} = -14.6$ Hz, $J_{AX} = 7.2$ Hz, H_A); 3.20 (*q*, 1, $J_{AB} = -14.6$ Hz, $J_{BX} = 4.6$ H_Z, H_B), 4.05 (*q*, 1, $J = 7.0$ Hz, C*H*-CH_3), 4.27 (double doublet, 1, $J_{AX} = 7.2$ Hz, $J_{BX} = 4.6$ Hz, H_X), 7.62 (*s*, 1, H_6); $[\alpha]_D^{23°C} = -37.9°$. For **8b**: nmr ($D_2O$), δ 1.55 (*d*, 3, $J = 7.0$ Hz, CH_3), 3.07 (*q*, 1, $J_{AB} = -15.0$ Hz, $J_{AX} = 6.6$ Hz, H_A), 3.28 (*q*, 1, $J_{AB} = -15.0$ Hz, $J_{BX} = 4.6$ Hz, H_B), 3.98 (*q*, 1, $J = 7.2$ Hz, C*H*-CH_3), 4.38 (double doublet, 1, $J_{AX} = 6.6$ Hz, $J_{BX} = 4.6$ Hz, H_X), and 7.58 (*s*, 1, H_6); $[\alpha]_D^{23°C} = -42.8°$.

Crystallization of **8a** from EtOH/1 *N* HCl (5:1) yielded white needles, mp 227–229°C. *Anal.* Calcd for $C_9H_{14}N_3O_4SCl$: C, 36.55; H, 4.77; N, 14.21; S, 10.84; Cl, 11.98. Found: C, 36.36; H, 4.85; N, 14.05; S, 10.97; Cl, 12.05.

Attempted crystallization of **8b** from EtOH/1 *N* HCl (5:1) produced crystals (mp ~225°C) which were proven by nmr to be a 1:1 mixture of **8a** and **8b**. *Anal.* Calcd for $(C_9H_{13}N_3O_4S)_2 \cdot HCl$: C, 38.95; H, 4.90; N, 15.14; Cl, 6.39; S, 11.55. Found: C, 39.05; H, 5.02; N, 15.16; Cl, 6.45; S, 11.50.

Cysteine–ψ Adduct: 5-[1-(S-Cysteine)-2,3,4,5-tetrahydroxypentane]uracil (**12**)

A 100-mg (0.41 mmol) sample of β-pseudouridine[8] (Sigma Chemical Co.) and 723 mg (4.1 mmol) of cysteine hydrochloride monohydrate were dissolved in 5 ml of water. The solution was strongly acidified by the addition of 0.5 ml of 12 *N* HCl and refluxed overnight. The subsequent mixture was concentrated to a small volume on a rotary evaporator, applied to a 2.5 × 90-cm, AG 50W-X8, H^+ form (200–400 mesh,

[6] The yield has been calculated on the basis of the hydrochlorides, each isomer taken as 50% of the total product.

[7] The nmr data for the two diastereomers was determined in D_2O from an approximately equimolar synthetic mixture of the two. Line assignments for each diastereomer were made by comparing successive spectra to which increasing amounts of one diastereomer had been added.

[8] In subsequent experiments a mixture of recycled α- and β-pseudouridine from the previous reaction was added to the fresh β-pseudouridine.

TABLE 3

COMPARISON OF SOME NMR PARAMETERS OF **6a**, **6b**, **4a**, AND **4b** WITH LITERATURE VALUES[a]

Compound	H_6[b]	$H_{5'B}$[b]	$H_{5'C}$[b]	$J_{4',5'B}$[c]	$J_{4',5'C}$[c]	$J_{5'B,5'C}$[c]
6a	7.80	3.77	3.65	2.4	7.2	−11.9
6b	7.83	3.79	(3.65)	3.5	(6.1)	−11.9
4a	7.62	—	—	—	—	—
4b	7.58	—	—	—	—	—
β-Pseudouridine[d,e]	7.65	3.83	3.72	3.2	4.6	−12.7
α-Pseudouridine[d,e]	7.57	3.87	3.70	2.4	5.7	−12.4
1-(β-D-Ribofuranosyl) cyanuric acid[d,e]	—	3.85	3.71	3.2	6.2	−12.3
Dihydrouridine[f]	—	3.80	3.73	3.6	4.8	−12.6
D-Ribose diphenyl dithioacetal[g,h,i]	—	—	—	2.6	7.2	−10.3
Tetra-*O*-acetyl-D-ribose diphenyl dithioacetal[i,j,k]	—	—	—	3.8	7.3	−12.2
Tetra-*O*-acetyl-D-ribose diethyl dithioacetal[i,j,k]	—	—	—	3.1	7.7	−12.0

[a] Values determined in D_2O unless otherwise indicated.
[b] Chemical shifts downfield from internal DSS.
[c] In hertz.
[d] Deslauriers and Smith (*66*).
[e] Values at pD 1.1; chemical shifts vary only 0.01 ppm up to pD 6.7; these coupling constants exhibit no apparent change over this pD change.
[f] Deslauriers et al. (*67*).
[g] Horton and Wander (*68*).
[h] Spectrum determined in 1:1 methanol-d_4–pyridine-d_5.
[i] Chemical shifts not indicated as they cannot be compared directly to the D_2O values.
[j] Horton and Wander (*69*).
[k] Spectrum determined in chloroform-*d*.

Bio-Rad), column and eluted with 1.0 *N* HCl at a 1.1 ml/min flow rate. Three major peaks were collected: *A*, 3.75 hr; *B*, 5 hr; and *C*, 7.75 hr. The solutions were lyophilized, redissolved in water, decolorized with Norit, and relyophilized. Fractions *A* (20.5 mg) and *B* (9.0 mg) were identified as β-pseudouridine and its α-anomer by comparison of their chromatographic properties and characteristic uv spectra with those of authentic samples. Fraction *C* (41.2 mg) exhibits a bathochromic shift (Table 2) expected for replacement of O by S at the C_1' allylic carbon, and gives a positive violet ninhydrin test consistent with **12**.

Nmr (220 MHz, ~2 *N* DCl[9] in D_2O/DSS): δ 7.83 (*s*, 1, $(H_6)_b$),[10] 7.80 (*s*, 1, $(H_6)_a$), 4.41 (*q*, 1, $J_{AX} = 6.1$ Hz, $J_{BX} = 4.5$ Hz, $(H_X)_b$), 4.38 (*q*, 1, $J_{AX} = 7.0$ Hz, $J_{BX} = 4.3$ Hz,

[9] The addition of DCl causes an increased resolution of the signals due to the two diastereomers.
[10] The subscripts *a* and *b* refer to diastereomers *a* and *b*, which occur in approximately a 2:1 ratio, respectively; spectral lines were readily assigned to each diastereomer on the basis of their relative intensities.

$(H_X)_a$), 4.31 (narrow envelope),[9] 4.08 (q),[9] 3.93 (narrow envelope),[11] 3.79 (q, 1, $J_{4',5'B}$ = 3.5 Hz,[12] $J_{5'B,5'C} = -11.9$ Hz, $(H_{5'B})_b$), 3.77 (q, 1, $J_{4',5'B} = 2.4$ Hz, $J_{5'B,5'C} = -11.9$ Hz, $(H_{5'B})_a$), 3.65 (q, 1, $J_{4',5'C} = 7.2$ Hz, $J_{5'B,5'C} = -11.9$ Hz, $(H_{5'C})_a$),[13] 3.56 (q),[9] 3.27 (q, 1, $J_{BX} = 4.5$ Hz, $J_{AB} = -14.9$ Hz, $(H_B)_b$), 3.26 (q, 1, $J_{BX} = 4.3$ Hz, $J_{AB} = -15.2$ Hz, $(H_B)_a$), 3.15 (q, 1, $J_{AX} = 6.1$ Hz, $J_{AB} = -14.9$ Hz, $(H_A)_b$), 3.14 (q, 1, $J_{AX} = 7.0$ Hz, $J_{AB} = -15.2$ Hz $(H_A)_a$). *Anal.* Calcd for $C_{12}H_{20}N_3O_8SCl$: C, 35.87; H, 5.02; N, 10.46; S, 7.98; Cl, 8.82. Found: C, 35.99; H, 5.06; N, 10.33; S, 8.04; Cl, 8.81.

ACKNOWLEDGMENTS

We are indebted to Dr. George Bosworth Brown for his advice and continued interest, and we express our appreciation to James Lehnberg for excellent technical assistance and Marvin Olsen for recording the nmr spectra (100 MHz). We wish to thank Drs. Edwin D. Becker and Cherie L. Fisk of the Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, for providing us with some of the 220-MHz nmr spectra.

This investigation was supported in part by Grants No. CA 16191 and CA 08748, awarded by the National Cancer Institute, DHEW, and the pmr experiments performed at our nmr laboratory (100 MHz) were supported in part by Grant No. CA 17085, awarded by the National Cancer Institute. The use of the nmr laboratory at Rockefeller University (220 MHz) was supported in part by the National Science Foundation, Grant No. GB 299946, and grants from the Research Corporation and the Sloan Foundation.

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[11] These bands are unassigned; their total integration is satisfactory for $H_{1'}$, $H_{2'}$, $H_{3'}$, and $H_{4'}$ for both diastereomers, which represent the remaining unassigned protons.

[12] This value is not precisely determined as the transitions involve overlap with those of the more intense *a* diastereomer.

[13] $(H_{5'C})_b$ is probably close to degenerate and hidden under this quartet.

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