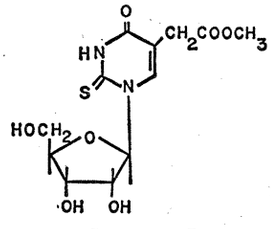
Sulfur-Containing Nucleoside from Yeast Transfer Ribonucleic Acid: 2-Thio-5(or 6)-uridine Acetic Acid Methyl Ester

Abstract. *A nucleoside, isolated from yeast transfer RNA, has been assigned the structure 2-thio-5-uridine acetic acid methyl ester on the basis of high-resolution mass spectrometry, chemical properties, and ultraviolet spectra. The alternate 6-substituted isomeric structure cannot yet be completely ruled out.*

A nucleoside isolated from the RNA (*1*) of yeast has been tentatively assigned the structure 2-thio-5-uridine acetic acid methyl ester (I). In comparison to nucleosides previously identified in yeast RNA, the most unusual features of this nucleoside are the presence of sulfur and an esterified carboxylic acid group.

For the isolation of the thionucleoside, 60 g of unfractionated yeast tRNA, prepared according to the method of Holley (*2*), was hydrolyzed enzymically to its constituent nucleosides. The hydrolyzate was fractionated by column partition chromatography (*3*). The eluate (1.5 liters), which came off the column immediately after the fraction containing the N^6-(Δ^2-isopentenyl)adenosine, was evaporated to dryness in a vacuum. The residue was dissolved in 5 ml of water, and the solution was streaked on Whatman No. 1 filter paper. After development in solvent system E (*4*), the paper contained six bands of ultraviolet-absorbing material. The fifth band (R_F=0.60) was eluted with water, and the product was chromatographed in solvent system D (*4*). The developed chromatograph contained four ultraviolet-absorbing bands; the third band (R_F=0.44) consisted of compound I. This sample was



Structure I

purified on a small partition column containing 50 g of Celite-545 (Johns-Manville) mixed with 28 ml of the lower phase of solvent system E [the column was prepared according to the general method of Hall (*5*)]. The column was washed with 150 ml of the upper phase of the solvent system before the sample was introduced in a solution of the upper phase (10 ml). The product was eluted in a bell-shaped peak between the 100- and 250-ml volume marks of the eluate (zero volume when sample introduced). The product yield was 65 O.D.$_{275\,nm}$ units. The sample was homogeneous on paper chromatography (*4*); solvent A, R_F=0.37; solvent B, R_F=0.62; solvent D, R_F=0.46; solvent E, R_F=0.62.

The solution containing the product was evaporated to dryness in a vacuum, and the residue was crystallized from a mixture of 200 μl of ethanol and 50 μl of water. After the solution was decolorized with a small amount of charcoal, the crystalline compound (I) was isolated (m.p. 198° to 200°C, uncorr.) and exhibited the ultraviolet absorption spectra shown in Fig. 1. The maxima (λ_{max}) are: at *p*H 1 to 7, 275 nm; at *p*H 12.5, 242 nm.

Because of the scarcity of the isolated material, mass spectrometry seemed the most promising method for obtaining an insight into the structure of this compound. A conventional mass spectrum indicated two very intense peaks at *m/e* 200 and 201. Beyond this point, however, the low intensity peaks at high mass were obscured by other peaks resulting from impurities which stemmed from the isolation procedure. Recourse was, therefore, taken to the use of high-resolution mass spectrometry, and the interpretation was based on the elemental composition of all the ions (*6*). This method facilitates the differentiation of the compound ions from the impurities, particularly in a case where one can expect the compound of interest to have a characteristic heteroatom content (large number of nitrogen and oxygen atoms, in the case of a nucleoside).

It became immediately apparent that nitrogen and oxygen were not the only heteroatoms present. The accurately measured mass of a number of ions did not correspond to a reasonable combination of C, H, N, and O. Examination of the accurate masses for the exact mass difference of light and heavy isotopes of Si, S, Cl, and Br revealed the presence of sulfur in a number of the ions, and the data were then interpreted in the light of this result.

The ions at mass 200 and 201 did, in fact, correspond to $C_7H_8N_2O_3S$ and $C_7H_9N_2O_3S$. It is well established (*7*) that nucleosides have the tendency to eliminate the base with rearrangement of one or two hydrogen atoms, and one can conclude that the structure of this new nucleoside is generally represented by structure II. The presence of a $C_5H_9O_4$ ion corresponding to the pentoside moiety further supports this formulation. The elemental composition data for the molecular ion at high mass did not reveal any species of the expected composition $C_{12}H_{16}N_2O_7S$; however, ions of the compositions $C_{11}H_{13}N_2O_6S$ and $C_{12}H_{14}N_2O_7$ were found. These fragments correspond to $(M-CH_2OH)^+$ and $(M-H_2S)^+$ ions, respectively, and indirectly confirm the postulated elemental composition of the compound. On the basis of the nitrogen content of the species, this structure must be a pyrimidine rather than a purine.

In an effort to decide whether the sulfur atom was part of the ring or the attached side chain, the elemental composition of the ions still containing both nitrogen atoms and sulfur were also examined. Ions with the lowest number of oxygen atoms correspond to $C_5H_5N_2OS$ and $C_5H_6N_2OS$. These ions were compatible with a substituted thiouracil rather than with a uracil. The minimum number of carbon atoms in the C,H,N_2,O_2,S group is 6, and in the C,H,N_2,O_3,S group it is 7. Such data are most compatible with structure III for the base (at this point, the position of the sulfur at C-2 and the side chain at C-5 is arbitrary). In principle, both isomeric methyl-5 or (6)-thiouracyl acetates would fit these data, but the ultraviolet spectra discussed below indicate a preference for C-5 substitution. The presence of an ester group was further

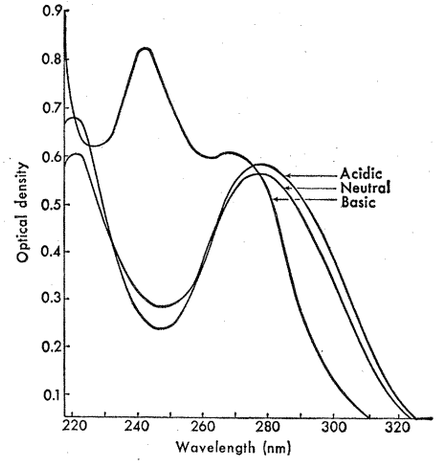
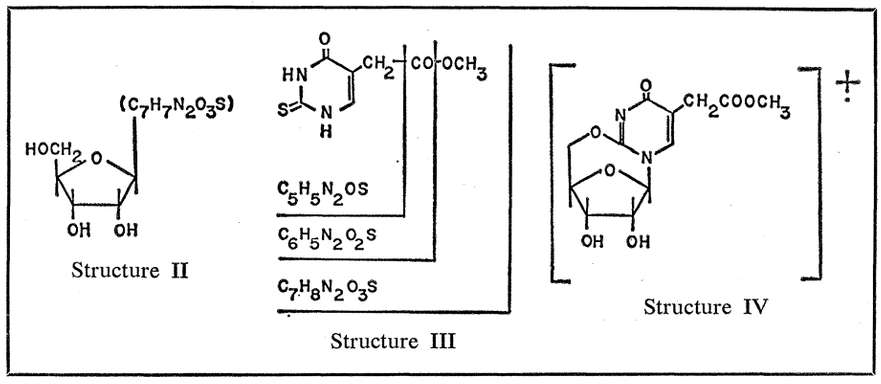


Fig. 1. Ultraviolet absorption spectra of the isolated thionucleoside I (*p*H 1.0; *p*H 7.0; *p*H 12.5).



supported by the change in chromatographic behavior of the compound upon mild alkaline hydrolysis.

While the mass spectral data do not reveal the exact position of the side chain, they do indicate the position of the sulfur atom at C-2 (rather than at C-4), since the loss of H_2S to produce the ion of composition $C_{12}H_{14}N_2O_7$ is best explained as the formation of a C-2, C-5′, ether bridge (as in IV) upon electron impact. This interpretation, in addition, represents presumptive evidence that the nucleoside exists in the β-D-ribofuranosyl configuration.

Independent support of structure I for the new nucleoside was then provided by comparison of its ultraviolet spectrum (Fig. 1) with that of 5-methyl-2-thiouridine (*8*, *9*) which it indeed resembles closely.

In order to confirm the presence of a saponifiable group, compound I was subjected to mild alkaline hydrolysis. A solution (600 μl) of compound I (10 O.D.$_{275\ nm}$ units) in 600 μl of 0.2*N* sodium hydroxide was heated for 45 minutes at 100°C. The digest was neutralized with hydrochloric acid, and the solution was evaporated at reduced pressure to dryness. The reaction product was purified by means of partition chromatography on a column (1 × 18 cm) containing 10 g of Celite-454, with solvent system C (*4*). Only one ultraviolet-absorbing product was eluted (between the 30- and the 85-ml volume marks of the eluate). This fraction was concentrated to a volume of 500 μl, and the material was chromatographed in solvent system A (*4*). A new ultraviolet-absorbing product was present and remained at the base line. None of the original sample of compound I was detected. The fact that the new compound did not migrate in a solvent containing ammonia indicates that, under these reaction conditions, the thionucleoside is converted to a product containing an acidic functional group.

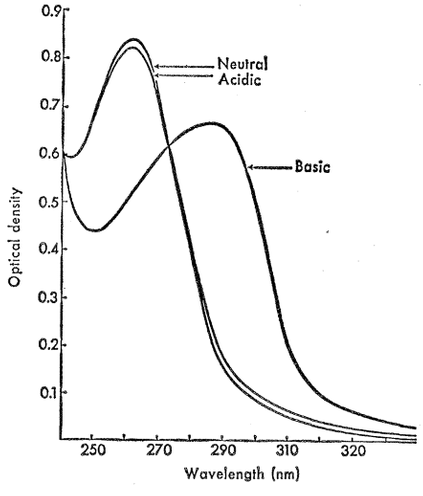


Fig. 2. Ultraviolet absorption spectra of the acid degradation product V, obtained from the thionucleoside (*p*H 1.0; *p*H 7.0; *p*H 12).

In order to compare the ultraviolet absorption spectra of the product with those of substituted uracil derivatives, compound I was converted to a uracil derivative by treatment with chloroacetic acid, which desulfurizes 2-thiouridine (*8*).

A solution of the thionucleoside, I, (1.0 O.D.$_{275\ nm}$ unit) in 50 μl of 20 percent aqueous monochloroacetic acid solution was placed in a sealed tube and heated for 18 hours at 100°C. The reaction mixture was chromatographed in solvent system B (*4*). The developed chromatogram contained two ultraviolet-absorbing spots, the starting material (R_F = 0.62), 63 percent, and a new compound designated V (R_F = 0.47), 37 percent. The ultraviolet-absorption spectra of this reaction product are shown in Fig. 2. Hydrolysis was also carried out with trichloroacetic acid under similar conditions. After 6 hours, a 45 percent yield of compound V was obtained.

The ultraviolet absorption spectra of compound V shown in Fig. 2 (λ_{max} at *p*H 1 to 7, 262 nm; at *p*H 12, 286 nm) correspond more closely to those of a 5-(alkyl substituted) uracil, than a 6-substituted derivative [ultraviolet absorption maxima of 5-methyluracil are at *p*H 4.7 to 7.2, 264.5 nm; at *p*H 12 to 13, 291 (*10*); and for 6-methyluracil at *p*H 4.6, 261 nm; at *p*H 13, 277 nm (*11*)].

Lipsett (*12*) has identified 4-thiouridine as a constituent of the tRNA of *Escherichia coli*. Carbon *et al.* (*9*) reported the presence of a 2-thiouridine derivative in the tRNA of *Escherichia coli*, but did not present a complete structure. The thionucleoside described in the present paper represents the first sulfur-containing nucleoside to be identified in yeast tRNA.

The esterified acetic acid side chain adds to the biological interest of this nucleoside. The esterification and deesterification of this group by an enzyme in the intact tRNA would constitute a mechanism by which the properties of a tRNA molecule could be changed. In this context the possibility exists that both the esterified and free carboxylic acid forms of compound I occur in the tRNA. Our isolation procedure would yield only the esterified form of the thionucleoside. Therefore, a significant portion of compound I as the free acid might not have been detected. Gray and Lane (*13*) report the presence of a nucleoside in yeast tRNA identified tentatively as uridine-5-acetic acid.

Our data point to the formulation of the isolated thionucleoside as 2-thio-5(or 6)-uridine acetic acid methyl ester. The similarity of the ultraviolet-absorption spectra of compounds I and V to the respective 5-(alkyl substituted)-2-thiouracil and uracil derivatives suggests that the natural product is the 5-substituted compound.

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References and Notes

1. Abbreviations used are as follows: tRNA, transfer ribonucleic acid; v/v, measurement by volume; *m/e*, mass-to-charge ratio; O.D. unit, optical density of 1 ml of solution at stated wavelength in nanometers.
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4. The solvent systems used were as follows: solvent A: 1-butanol, water, concentrated ammonium hydroxide (86 : 14 : 5); B: 1-butanol, glacial acetic acid, water (5 : 3 : 2);

C: ethyl acetate, 1-propanol, 0.1 percent aqueous formic acid (4 : 1 : 2); D: 2-propanol, water, concentrated amonium hydroxide (7 : 2 : 1); and E: ethyl acetate, 1-propanol, water (4 : 1 : 2).
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14. Supported by grants from NIH (CA-046401) and from the Mass Spectrometry Facility for Biomedical Research of M.I.T. (FR-00317). One of us (R.H.H.) thanks J. Mozeiko, R. Thedford, and L. Stasiuk for technical assistance.
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20 December 1967

Human Granulocyte Collagenase

Abstract. *A collagenase, operative at neutral and alkaline* p*H, has been extracted from the granule fraction of human granulocytic leukocytes. It digests reconstituted collagen fibrils and reduces the viscosity of collagen solutions. Cleavage of collagen in solution with purified enzyme produces the discrete products characteristic of other animal collagenases.*

The first animal enzyme known to lyse collagen at neutral *p*H was isolated by Gross, Lapiere, and their co-workers (*1*, *2*) from the culture fluid of tadpole skin. Collagenase has also been demonstrated in culture fluids of rat bone (*3*, *4*) and uterus (*5*). Extracts of rat bone have also been reported to contain collagenolytic activity (*6*). A similar activity of tissues in man was first demonstrated in culture fluids of gingivae by Fullmer and Gibson (*7*) and was later confirmed by other investigators (*8*, *9*). Recently collagenase has been found in culture fluids of human bone (*4*) and skin (*10–12*). Culture fluids of synovia obtained from patients with rheumatoid arthritis have also demonstrated collagenase activity (*13*, *14*). Most of these enzymes (*5*, *12–15*) have been shown to cleave the collagen molecule into two pieces representing three quarters and one quarter of the molecule (*15–17*). In view of this highly specific action, the finding of these products can be considered diagnostic of a true collagenase.

Digestion of collagen occurs on a large scale during inflammation and abscess formation at a time when large numbers of granulocytic leukocytes are present. However, the presence of collagenase has not been detected heretofore in these cells (*6*, *18*). This report describes investigations which demonstrate the presence of a collagenase in the granule fraction of human granulocytic leukocytes.

Preparations containing predominantly polymorphonuclear leukocytes were obtained by differential centrifugation and dextran sedimentation of the leukocyte-rich red blood cell interfaces pooled from six units of normal human blood. Red blood cell contamination was removed by hypotonic saline hemolysis (*19*). The granulocytes were washed three times with isotonic saline and collected by centrifugation at 400*g* for 10 minutes. After white blood cell counts and differential smears were made, a portion of each granulocyte preparation was frozen for use as a whole cell lysate. The remaining granulocytes were homogenized at a concentration of 1.8×10^8 cell/ml for 3 minutes in cold 0.34*M* sucrose solution in a ground glass homogenizer and were then fractionated into a nuclear, granule and post-granule supernatant fraction according to the method of Cohn and Hirsch (*20*). The granule fraction was examined by phase microscopy, and typical granules were seen.

Human lymphocytes were prepared by sedimenting the buffy coats from 4 units of fresh whole blood with dextran. The cells obtained were resuspended in heparinized fetal calf serum at 37°C. After 1 hour of incubation they were placed on a 45-cm glass bead column and the lymphocytes were eluted (*21*). Contaminating red blood cells were removed from the effluent by hypotonic saline hemolysis (*19*). The lymphocytes were then washed three times with isotonic saline and frozen after a white blood cell count and differential smear were obtained.

To prepare whole cell lysates, granulocyte and lymphocyte preparations were homogenized in a ground glass homogenizer for 3 minutes and then frozen and thawed ten times. The cell fractions from the granulocyte preparation were also disrupted by freezing and thawing ten times prior to their use. Immediately before assaying for collagenolytic activity all preparations were centrifuged at 25,000*g* for 15 minutes. The volume of the resultant supernatant was adjusted with Tyrode's solution to give a preparation derived from 1.8×10^8 cell/ml. Aliquots of the final adjusted supernatants were analyzed for protein according to the Miller modification of the Lowry technique (*22*).

Table 1. Collagenolytic activities of normal human white blood cells as determined by the reconstituted radioactive collagen fibril assay. The enzyme preparations were derived from 9×10^7 cells.

Preparation	Protein (mg)	Collagen gel lysed (%)
Human granulocyte homogenate*	2.7	21.0
Nuclear fraction	1.2	7.3
Granule fraction	0.5	21.0
Post-granule supernatant fraction	.9	5.0
Human lymphocyte homogenate†	2.4	5.5
Trypsin (25 μg)		5.0

* 81 percent granulocytes, 19 percent lymphocytes. †95 percent lymphocytes, 5 percent granulocytes.

Native acid-soluble rat skin collagen was prepared by a modification of the method of Kang *et al.* (*23*) after injecting the animals with glycine-^{14}C. The collagen prepared had a specific activity of 3750 disintegrations per minute per milligram, and when it was incubated with a number of different proteolytic enzymes it was found to be almost completely resistant to nonspecific digestion. Indeed, when a 1 : 2 weight ratio of trypsin to collagen was incubated in the C^{14}-labeled collagen fibril assay (described below) only 5 percent of the substrate was digested.

Reconstituted C^{14}-labeled collagen fibrils were prepared as a 0.2 percent collagen gel according to the method of Gross (*2*). To a 0.2-ml gel of fibrils (1500 disintegrations/min) in a 3-ml test tube were added 0.25 ml of 0.001*M* calcium chloride in 0.05*M* tris buffer, *p*H 7.6, and 0.5 ml of the sample to be assayed. The mixture was incubated for 18 hours at 37°C and then filtered through a 0.9-μ-pore-size, 13-mm-diameter Versapor filter (Gelman Instrument Company, Ann Arbor, Michigan) in a Swinney adapter. The filter retained undigested collagen fibrils but allowed the passage of soluble digestion products. A 0.5-ml aliquot of the filtered solution was added to 20 ml of Bray's solution (*24*) and counted in a liquid scintillation counter. All counts were corrected for quenching by the channels ratio method and adjusted to a 100 percent efficiency (*25*). In addition to buffer blanks, all experiments included trypsin controls (25 μg) in the incubation mixture in order to indicate the