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Identification and Synthesis of a Naturally Occurring Selenonucleoside in Bacterial tRNAs: 5-[(Methylamino)methyl]-2-selenouridine†

Arthur J. Wittwer,* Lin Tsai, Wei-Mei Ching, and Thressa C. Stadtman

ABSTRACT: *Escherichia coli*, *Clostridium sticklandii*, and *Methanococcus vannielii* synthesize ^{75}Se-labeled amino acid transfer ribonucleic acids ([^{75}Se]tRNAs) when grown with low levels (≈1 μM) of $^{75}SeO_3^{2-}$. When *E. coli* [^{75}Se]tRNA was digested to nucleosides and analyzed by reversed-phase high-performance liquid chromatography, a single selenonucleoside accounted for 70–90% of the ^{75}Se label in the bulk tRNA. This nucleoside was shown to be indistinguishable in a number of its properties from authentic 5-[(methylamino)methyl]-2-selenouridine. Preparation of the authentic selenonucleoside was accomplished and the synthetic compound characterized by its UV and ^{1}H NMR spectral properties. The new selenonucleoside also accounted for 40–60% of the ^{75}Se found in [^{75}Se]tRNA from *C. sticklandii* or *M. vannielii*. Each of these anaerobic bacteria contains one additional selenonucleoside in their tRNA populations distinct from 5-[(methylamino)methyl]-2-selenouridine. Pure seleno-tRNA^{Glu} isolated from *C. sticklandii* contains one 5-[(methylamino)methyl]-2-selenouridine and one 4-thiouridine per tRNA molecule.

It is well established that selenium is an essential component of several bacterial enzymes and of mammalian glutathione peroxidase (Stadtman, 1980). Selenium also has been shown (Chen & Stadtman, 1980) to be a specific constituent of another class of macromolecules, the amino acid transfer ribonucleic acids (tRNAs). A selenium-containing tRNA^{Glu} from *Clostridium sticklandii* has been purified (Ching & Stadtman, 1982), lysine- and glutamate-accepting tRNAs of *Escherichia coli* have been shown to contain selenium (Wittwer, 1983), and recently, we have found extensive incorporation of selenium in several tRNA species from *Methanococcus vannielii* (Ching et al., 1984). Lability of the incorporated selenium in these tRNAs to treatment with CNBr, KBH_4, or iodoacetate and its relative stability at pH 9 (a condition used to deesterify aminoacylated tRNAs) suggested the selenium was present in a modified nucleoside. We now report the isolation of a prominent selenium-containing nucleoside from several bacterial seleno-tRNAs and its identification as 5-[(methylamino)methyl]-2-selenouridine. The synthesis of this new, naturally occurring nucleoside also is described.

Experimental Procedures

Growth of Bacteria and Isolation of ^{75}Se-Labeled tRNA. Wild-type *E. coli* strain WG1 was obtained, grown in minimal glucose/salts medium, and labeled with $^{75}SeO_3^{2-}$ or $^{75}SeO_4^{2-}$, and ^{75}Se-labeled tRNA was isolated as described by Wittwer (1983). Selenium-enriched [^{75}Se]tRNA was prepared from bulk tRNA (isolated from cells incubated with 0.85 μM $^{75}SeO_3^{2-}$ and 8 mM SO_4^{2-}) by successive chromatography on Sepharose 4B (Holmes et al., 1975) and the RPC-5 reversed-phase system of Pearson et al. (1971) at pH 4.5 and then at pH 7.5 using conditions described previously (Wittwer, 1983). *C. sticklandii* was cultured in $^{75}SeO_3^{2-}$-supplemented media, and ^{75}Se-labeled tRNAs were isolated from the radioactive cells (Chen & Stadtman, 1980; Ching & Stadtman, 1982). In some experiments, the *C. sticklandii* tRNA population was labeled with ^{75}Se in cultures prevented from further growth by the addition of chloramphenicol. Pure ^{75}Se-labeled tRNA^{Glu} from *C. sticklandii* was isolated as described by Ching & Stadtman (1982). Preparations of ^{75}Se-labeled *M. vannielii* cells and purification of ^{75}Se-labeled tRNAs from this organism were as described by Ching et al. (1984).

Reversed-Phase High-Performance Liquid Chromatography (HPLC) Analysis of Nucleosides. Up to 3 A_{260} units of tRNA was rapidly hydrolyzed with nuclease P1 and bacterial alkaline phosphatase as described by Gehrke et al. (1982).

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Digestions were performed under argon in the presence of 2 mM dithiothreitol. The entire hydrolysate was then analyzed by HPLC with reversed-phase μBondapak C_{18} columns from Waters Assoc. In the experiments described in Figure 1, two 0.4 × 30 cm columns connected in series were used at 35 °C and a flow rate of 1 mL/min. At sample injection (0 min), the mobile phase was 97% 10 mM ammonium acetate, pH 5.3, and 3% methanol. This composition was held for 25 min, followed by a linear gradient from 3 to 20% methanol from 25 to 60 min. In other cases, a single reversed-phase column was used at room temperature and 1 mL/min flow rate. The same mobile phase at sample injection was used, but linear gradients of 3 to 5% methanol from 0 to 12.5 min and 5 to 20% methanol from 12.5 to 27.5 min were applied. Following each HPLC analysis the columns were washed with 100% methanol for 5–10 min before reequilibrating with the initial mobile phase. A Hewlett-Packard 1084B liquid chromatograph system was used for the experiments of Figure 1. Later experiments employed a Spectra-Physics SP8700 solvent delivery system with a Hewlett-Packard 1040A spectrophotometric detector. Radioactivity in fractions collected from the column was determined by liquid scintillation counting in aqueous counting scintillant (ACS) from Amersham Corp. or by using a Beckman 5500 gamma counter. Spectra of HPLC fractions were measured on a Hewlett-Packard 8450A spectrophotometer.

Preparation of 5-Methyl-2-selenouridine and 4-Selenouridine. These compounds were prepared from 5-methyl-2-thiouridine (Sigma) and 4-thiouridyl residues in intact *E. coli* tRNA (Sigma) by treatment with cyanogen bromide and sodium hydrogen selenide as described by Pal & Schmidt (1977) for the preparation of 1-methyl-4-selenouracil and selenated tRNA. The products were purified by HPLC (intact tRNA was first hydrolyzed to nucleosides) as described above.

Synthesis of 5-[(Methylamino)methyl]-2-selenouridine. (*i*) *Analytical Methods and Materials.* Melting points were taken on a Kofler hot stage and are uncorrected. UV spectra were measured in a Perkin-Elmer Model 559 spectrophotometer. ^{1}H NMR spectra were determined in a Varian HR220 spectrophotometer or a Nicolet NT 360 NMR spectrometer. Chemical shifts are reported in δ-units relative to internal tetramethylsilane. Mass spectra were recorded on a LKB-9000 spectrometer using a direct inlet system at 70 eV. Analytical thin-layer chromatography (TLC) was carried out on Eastman silica gel sheets and preparative TLC on Merck silica gel plates of 2-mm thickness, in solvent systems of (A) $CHCl_3/CH_3OH$ (9:1) and (B) $CHCl_3/CH_3OH$/concentrated NH_4OH (25:20:1). Microanalyses were performed by Gailbraith Laboratory, Inc., Knoxville, TN. Ethyl 3-(methylamino)-propionate was prepared from *N*-methyl-β-alaninenitrile by a procedure reported by Vorbrüggen & Krolikiewiez (1980). Selenourea was purchased from Sigma; *N*-methyl-β-alaninenitrile and β-D-ribofuranose 1-acetate 2,3,5-tribenzoate were from Aldrich Chemical Co.; *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Pierce Chemical Co.

(*ii*) *5-[(N-Methylformamido)methyl]-2-selenouracil* (**1**). A mixture of ethyl 3-(methylamino)propionate, 4.3 g (33 mmol), and ethyl formate, 6.1 g (83 mmol), was added dropwise to a stirred mixture of sodium hydride oil dispersion, 3.3 g (68 mmol), in 40 mL of dry 1,2-dimethoxyethane under an atmosphere of argon and cooling in an ice–water bath. The mixture was stirred in the cold for 1 h and then at room temperature for 0.5 h. After addition of 25 mL of water, the mixture was adjusted to pH 8.5 by addition of a few drops of glacial acetic acid. To this mixture, selenourea, 3.69 g (30 mmol), was added, and the mixture was heated in a 95 °C bath under argon atmosphere and stirring for 8 h. The cooled dark red mixture was extracted with 20 mL of cyclohexane, and the aqueous phase was filtered through celite. The clear aqueous filtrate was adjusted to pH 5 by careful addition of glacial acetic acid under ice cooling. Upon being allowed to stand, pink crystals of **1** were collected, washed with methanol, and dried in vacuo (0.5 mmHg) at room temperature overnight: yield 2.8 g (34.5%) of **1**; mp 185 °C dec; UV λ_{max} (CH_3OH) 315 nm (ε 1.10 × 10^4), λ_{max} (pH 1) 310 nm (ε 1.44 × 10^4), λ_{max} (0.1 N KOH) 279 nm (ε 9.73 × 10^3); ^{1}H NMR (220 MHz) (D_2O/Na_2CO_3) δ 2.74, 2.95 (s, 3 H, N–CH_3), 4.20 (s, 2 H, N–CH_2), 7.51, 7.62 (s, 1 H, H6), 8.03, 8.16 (s, 1 H, CHO) (the double signals for N–CH_3, H6, and CHO are due to the presence of rotamers of the N–CHO function); mass spectrum, *m/e* (relative intensity) 249 (19),[1] 248 (10), 247 (100),[1] 246 (5), 245 (53),[1] 244 (18),[1] 243 (19).[1] Anal. Calcd for $C_7H_9N_3O_2Se$: C, 34.16; H, 3.69; N, 17.07. Found: C, 34.32; H, 3.80; N, 16.92.

(*iii*) *5-[(Methylamino)methyl]-2-selenouracil Hydrochloride* (**2**). One gram of **1** in 32 mL of 2 N HCl was heated in a 95 °C bath under argon atmosphere and stirring for 1.75 h. The mixture was filtered through Celite while hot. The clear yellow filtrate upon being cooled in a −10 °C bath gave bright yellow crystals of **2**: 0.84 g (81%); mp 265 °C dec; UV λ_{max} (CH_3OH) 324 nm (ε 1.35 × 10^4), λ_{max} (pH 5) 313 nm (ε 1.35 × 10^4), λ_{max} (0.1 N KOH) 290 nm (sh) (ε 1.11 × 10^4) and 268 nm (sh) (ε 1.23 × 10^4); ^{1}H NMR (220 MHz) (D_2O) δ 2.73 (s, 3 H, N–CH_3), 3.72 (s, 2 H, N–CH_2), 7.71 (s, 1 H, H6); mass spectrum, *m/e* (relative intensity) 221 (14),[1] 220 (9), 219 (100),[1] 218 (9), 217 (40),[1] 216 (18),[1] 215 (18).[1] Anal. Calcd for $C_6H_9N_3OSe$·HCl: C, 28.31; H, 3.56; N, 16.51. Found: C, 28.17; H, 3.71; N, 16.32.

(*iv*) *2′,3′,5′-Tribenzoyl-5-[(methylamino)methyl]-2-selenouridine* (**4**). To a suspension of **2**, 110 mg (0.5 mmol), in 2.5 mL of dry acetonitrile, 1 mL of BSTFA was added under argon atmosphere and stirring. The mixture was stirred in a 90 °C bath for 3 h. The excess reagent and solvent were removed by vacuum distillation at 60 °C, yielding a clear yellow oil. To this was added β-D-ribofuranose 1-acetate 2,3,5-tribenzoate (**3**), 250 mg (0.5 mmol), in 2.5 mL of 1,2-dichloroethane. The mixture was cooled to −5 °C, and under argon atmosphere and stirring, a solution of 0.12 mL (ca. 1 mmol) of stannic chloride in 1 mL of 1,2-dichloroethane was added dropwise over a period of 10 min. The mixture was stirred at room temperature for 3 h. The brown reaction mixture was diluted with 40 mL of methylene chloride and 25 mL of methanol and shaken up with 15 mL of saturated sodium bicarbonate solution. The aqueous phase was extracted 3 times with 25 mL of methylene chloride each. The organic phase and methylene chloride extracts were combined and washed with 20 mL of saturated sodium chloride solution, dried over magnesium sulfate, and evaporated under reduced pressure, giving 187 mg of crude product as a light orange resin. Purification by preparative TLC (solvent A) furnished **4** as light yellow amorphous solid: 64 mg (20%); mp 65–67 °C; UV λ_{max} (MeOH) 311 nm (ε 1.40 × 10^4) and 228 nm (ε 5.00 × 10^4). Anal. Calcd for $C_{32}H_{29}N_3O_8Se·H_2O$: C, 56.47; H, 4.59; N, 6.17. Found: C, 56.54; H, 4.50; N, 6.11.

(*v*) *5-[(Methylamino)methyl]-2-selenouridine* (**5**). The benzoate (**4**), 37 mg, was suspended in 10 mL of 0.01 M sodium methoxide in methanol. The mixture was stirred under

[1] The relative intensities of these peaks correspond well with the natural abundance of the selenium isotopes.

argon atmosphere at room temperature. A clear yellow solution was obtained after a few minutes; stirring was continued for 4 h. To this solution 5 μL of glacial acetic acid was added, and the solvent was removed at 40 °C under reduced pressure. The residue was taken up in 10 mL of methanol and purified by preparative TLC (solvent B). 5-[(Methylamino)-methyl]-2-selenouridine (**5**) was obtained as light straw color microcrystals: 12 mg (61%); mp 155–160 °C dec; UV λ_{max} (MeOH) 312 nm (ϵ 1.83 × 10^4) and 223 nm (ϵ 1.97 × 10^4), λ_{max} (H_2O) 313 nm (ϵ 1.73 × 10^4), λ_{max} (0.1 N KOH) 290 nm (ϵ 1.43 × 10^4) and 241 nm (ϵ 2.76 × 10^4); ^{1}H NMR (360 MHz) (D_2O) δ 2.67 (s, 3 H, N–CH_3), 3.90 (s, 2 H, N–CH_2), 3.85 (d, J = 14 Hz, 1 H, H5′), 4.01 (d, J = 14 Hz, 1 H, H5′), 4.17 (m, 2 H, H3′ and H4′), 4.37 (m, 1 H, H2′), 6.75 (m, 1 H, H1′), 8.32 (s, 1 H, H6). Anal. Calcd for $C_{11}H_{17}N_3O_5Se{\cdot}H_2CO_3$: C, 34.96; H, 4.65; N, 10.19. Found: C, 34.88 and 34.69; H, 4.52 and 4.61; N, 10.03 and 10.15.

Results and Discussion

Preliminary analyses of *C. sticklandii* ^{75}Se-labeled tRNA preparations that had been digested to nucleotides by prolonged incubation in 0.1 M KOH indicated that this procedure was unsuitable because of alkali lability of the selenium-containing nucleotide. Digestion to nucleotides by successive treatment with ribonuclease T_1 at pH 7.6 followed by ribonucleases T_2 and A at pH 5 resulted in 60–70% conversion to low molecular weight products as determined by molecular sieve chromatography on P-2 polyacrylamide. Ion-exchange chromatography of the ^{75}Se-labeled fraction on DEAE-cellulose, with a 0.1–0.15 M LiCl gradient for elution, partially separated a ^{75}Se-labeled nucleotide that emerged between guanylic acid and 4-thiouridylic acid. This elution pattern suggested that the selenium-containing nucleotide was not 4-selenouridylic acid since the more acidic selenium analogue would be expected to follow rather than precede the corresponding sulfur nucleotide. This observation was of interest in view of earlier reports that a selenium-labeled nucleoside from *E. coli* tRNAs cochromatographed with carrier 4-selenouridine (Hoffman & McConnell, 1974; Prasado Rao & Cherayil, 1974). However, other analytical and separation methods (e.g., thin-layer chromatography or electrophoresis) available then and at earlier stages of this work were generally unsuitable since they had limited resolving power and usually led to significant decomposition.

With the advent of high-performance liquid chromatographic systems, separations of closely related modified nucleosides could be achieved rapidly under mild conditions and in deoxygenated solvents. Additionally, a rapid hydrolysis procedure developed by Gehrke et al. (1982), employing nuclease P1 and bacterial alkaline phosphatase, minimized exposure of the nucleosides to basic pH. By use of these methods, the selenonucleoside in digests of ^{75}Se-labeled tRNA from *E. coli* was isolated in amounts sufficient for characterization.

Isolation of Selenonucleoside from E. coli tRNA. Separation of the *E. coli* nucleosides by HPLC revealed one ^{75}Se-labeled peak (solid line of Figure 1). In this experiment, the tRNA preparation was derived from midlog phase *E. coli* cultures that had been incubated with $^{75}SeO_3^{2-}$ in the presence of chloramphenicol. Similar results were obtained with tRNA preparations from cells that had been allowed to multiply in the presence of $^{75}SeO_3^{2-}$ or $^{75}SeO_4^{2-}$. In each instance, a single labeled peak with the retention time shown in Figure 1 represented 70–90% of the radioactivity applied to the HPLC column. When bacterial alkaline phosphatase was omitted during the hydrolysis of the ^{75}Se-labeled tRNA preparation, the radioactive peak eluted earlier from the column (dashed line of Figure 1), presumably due to the influence of the phosphoryl group. This result served to confirm the nucleoside nature of the ^{75}Se-labeled material detected in the phosphatase-treated samples.

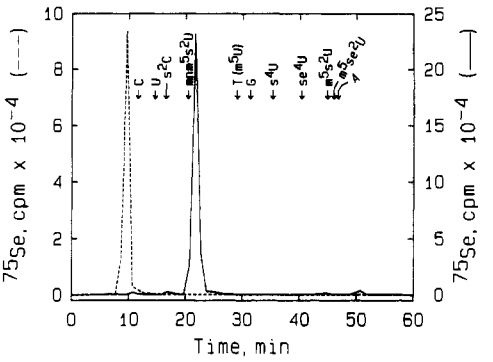


FIGURE 1: HPLC analysis of enzymatically digested ^{75}Se-labeled tRNA. Labeled tRNA isolated from *E. coli* cells incubated with 0.1 μM $^{75}SeO_3^{2-}$ and 8 mM SO_4^{2-} was digested to nucleosides and chromatographed as described under Experimental Procedures. The solid line shows the radioactivity profile of the fractions collected from the column. A separate ^{75}Se-labeled tRNA preparation (the selenium-enriched tRNA described in the text) was similarly treated, but bacterial alkaline phosphatase was omitted during the digestion. The dashed line shows the radioactivity profile of this chromatogram. The elution positions of several standard nucleosides under these same conditions are indicated by arrows: C, cytidine; U, uridine; s^2C, 2-thiocytidine; mnm^5s^2U, 5-[(methylamino)methyl]-2-thiouridine; T (m^5U), ribothymidine (5-methyluridine); G, guanosine; s^4U, 4-thiouridine; se^4U, 4-selenouridine; m^5s^2U, 5-methyl-2-thiouridine; m^5se^2U, 5-methyl-2-selenouridine; A, adenosine.

The elution positions from the HPLC μBondapak C_{18} column of the major nucleosides that occur in tRNA and a few known sulfur- and selenium-containing nucleosides are indicated in Figure 1. Sulfur-containing nucleosides eluted from the column after the analogous oxygen-containing compounds, and the seleno analogues eluted even later (cf. the elution position of C and s^2C; U, s^4U, and se^4U; and T (m^5U), m^5s^2U, and m^5se^2U). This illustrates the general property of many types of selenium compounds to exhibit tighter binding than their corresponding sulfur analogues to various chromatographic matrixes. Thus, the elution position of the ^{75}Se-labeled nucleoside (Figure 1) immediately after a sulfur-containing nucleoside, 5-[(methylamino)methyl]-2-thiouridine, furnished a clue as to its identity. It should be pointed out, however, that in the case of seleno- and thionucleosides this generalized behavior depends on the pH at which the compounds are compared. For example, when chromatographed in pH 7 rather than pH 5.3 buffer, 5-methyl-2-selenouridine precedes 5-methyl-2-thiouridine, and the ^{75}Se-labeled nucleoside elutes before 5-[(methylamino)methyl]-2-thiouridine in the HPLC profile (data not shown). This is presumably due to the difference in pK_a values of the seleno and thio groups; e.g., the pK_a values of 2-selenouracil and 2-thiouracil are 7.18 and 7.75, respectively (Mautner, 1956).

To obtain amounts of the ^{75}Se-labeled nucleoside suitable for further characterization, a selenium-enriched [^{75}Se]tRNA preparation (about 1 mol of Se/4 mol of tRNA, 2.9 total A_{260} units) was digested to nucleosides and subjected to HPLC analysis. A new peak of UV-absorbing material that eluted immediately after 5-[(methylamino)methyl]-2-thiouridine was evident and was coincident with 83% of the ^{75}Se applied to the column (data not shown). The absorbance maximum of the material in this radioactive peak was 313 nm at pH 5 (Figure 2a). The pH-dependent shifts in the absorbance maxima of this compound (Figure 2a) resembled those of 2-selenouridine but not those of 4-selenouridine, 6-seleno-

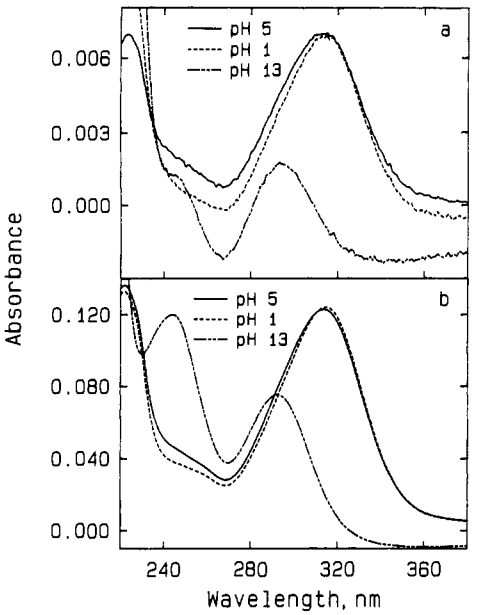


FIGURE 2: UV absorbance spectra of the natural ^{75}Se-labeled nucleoside (a) and synthetic 5-[(methylamino)methyl]-2-selenouridine (b). The natural ^{75}Se-labeled nucleoside was isolated by HPLC as described in the text. A 0.5-mL fraction from the tailing side of the selenonucleoside peak (to minimize any contamination with earlier eluting 5-[(methylamino)methyl]-2-thiouridine) was used to obtain the spectra in panel a. Synthetic 5-[(methylamino)methyl]-2-selenouridine was purified by HPLC prior to the recording of the spectra in panel b. The solution containing the nucleoside was first adjusted to pH 1 and then to pH 13 by the addition of 6 N HCl and 6 N KOH, respectively. Identical acid spectra were regenerated by further addition of 6 N HCl (not shown), indicating that the brief exposure to alkaline pH did not degrade the selenium-containing compounds. Spectra are uncorrected for volume increases during pH adjustment.

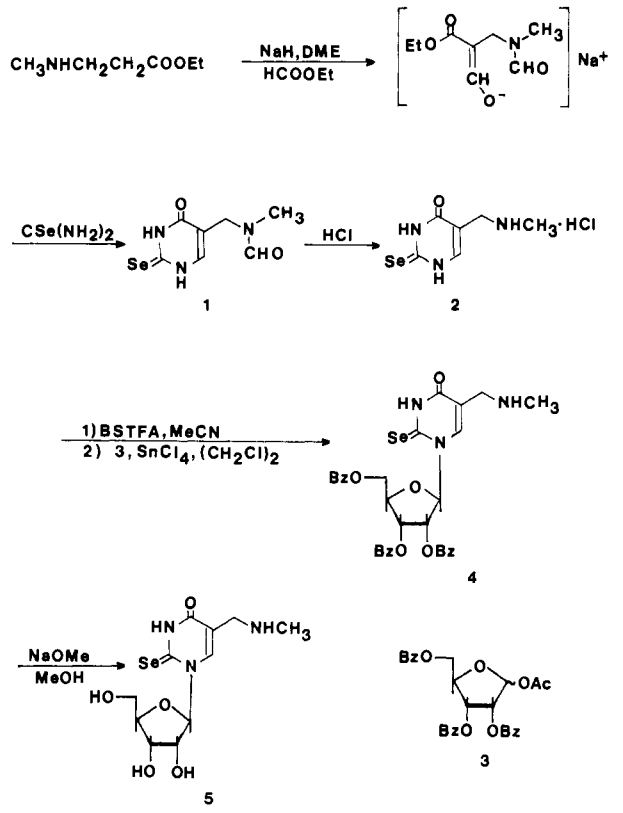
Table I: Absorbance Maxima of Selenonucleosides

compd	pH 1	MeOH	pH 11	ref
2-selenouridine	308	312	293, 237	*a*
4-selenouridine	366, 259	372, 265	338, 283, 236	*a*
6-selenoguanosine	366, 260	359 (water)	330, 256	*b*
2-selenocytidine	235	306, 252	299, 251	*c*
bacterial seleno-nucleoside (Figure 3a)	314	313 (pH 5)	294, 244[d] (pH 13)	

[a] Wise & Townsend, 1972. [b] Milne & Townsend, 1971. [c] Wise & Townsend, 1979. [d] Shoulder.

guanosine, or 2-selenocytidine (Table I). Thus, of the possible selenium analogues of uridine, guanosine, or cytidine (involving a single substitution of Se for O), only 2-selenouridine has spectral properties similar to those of the isolated selenonucleoside. Assuming 1 mol of Se/mol of nucleoside, an absorbance of 2×10^4 M^{-1} at 313 nm was calculated for the isolated, radioactive nucleoside. This is within the range of values [$(1-2) \times 10^4$ M^{-1} cm^{-1}] reported for various selenonucleosides or pyrimidine bases at their absorbance maxima (Wise & Townsend, 1979; Milne & Townsend, 1971; Mautner, 1956). These observations further suggested that the ^{75}Se-labeled nucleoside might be a 2-selenouridine. In addition, the finding that the selenium-containing *E. coli* tRNAs primarily have lysine- or glutamate-accepting activity (Wittwer, 1983) correlated with the known occurrence of 5-[(methylamino)methyl]-2-thiouridine in *E. coli* $tRNA^{Lys}$ (Chakraburtty et al., 1975) and $tRNA^{Glu}$ (Ohashi et al., 1970). Therefore, on the basis of the implications of amino acid acceptance activity, HPLC elution position, and UV spectral properties the new selenonucleoside was tentatively assigned the structure 5-[(methylamino)methyl]-2-selenouridine.

Scheme I



Synthesis and Characterization of 5-[(Methylamino)-methyl]-2-selenouridine. To provide an authentic sample for comparison, a synthesis of 5-[(methylamino)methyl]-2-selenouridine was undertaken. The synthesis (Scheme I) is designed after that reported by Vorbrüggen & Krolikiewicz (1980) for the synthesis of the corresponding 2-thiouridine derivative. However, the following particulars are to be noted. (a) Since many seleno compounds are more sensitive to light and oxygen than the corresponding thio compounds, as a precautionary measure, all the reactions were conducted in dim light under argon. (b) The *N*-formyl protecting group was introduced in the same step for α-C-formylation by using 2 equiv of sodium hydride and excess ethyl formate. (c) The conditions for the condensation of this α-formyl derivative with selenourea were critical due to the instability of selenourea in strong base; for instance, the presence of a slight excess of sodium hydride led to rapid decomposition of selenourea. The condensation was moderately successful when the reaction mixture was adjusted to pH 8.5–9.5. (d) Wise & Townsend (1972) reported successful conversion of 2-selenouracil to 2-selenouridine under conditions similar to those for the sulfur compounds. However, under these conditions, much decomposition of **2** was observed. With the use of a more effective silylating reagent, BSTFA, persilylated **2** was obtained at a lower temperature and shorter reaction time. The coupling of persilylated **2** with the ribofuranose derivative (**3**) in the presence of stannic chloride furnished the benzoylated nucleoside (**4**) in only 20% yield. Attempts to improve this reaction by using other Lewis acid catalysts (Vorbrüggen et al., 1981) were unsuccessful. (e) The conversion of **4** to **5** was accomplished by sodium methoxide in methanol. An alternative procedure employing methanolic ammonia yielded a much contaminated product. Both the UV spectra (Figure 2b) and the ^{1}H NMR spectrum (see Experimental Procedures) of the synthetic product are consistent with the structure 5-[(methylamino)methyl]-2-selenouridine (**5**).

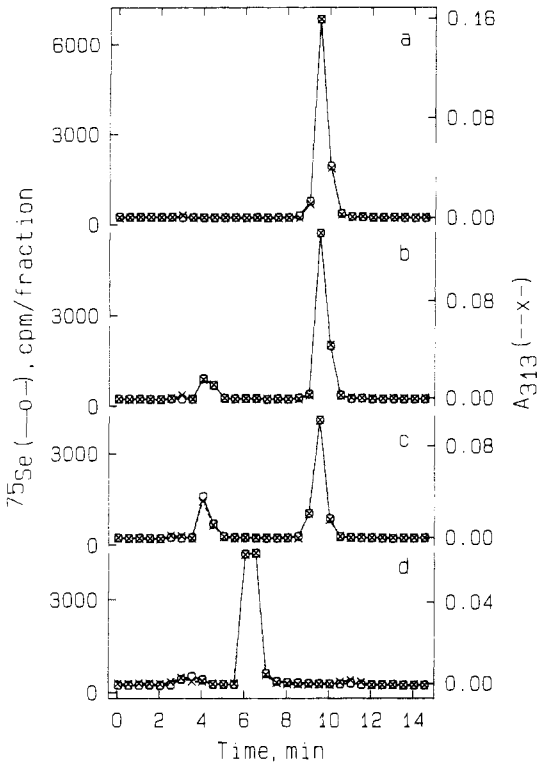


FIGURE 3: HPLC of the natural ^{75}Se-labeled nucleoside and synthetic 5-[(methylamino)methyl]-2-selenouridine following various treatments. About 0.1 A_{313} unit of synthetic 5-[(methylamino)methyl]-2-selenouridine and 10000 cpm of natural ^{75}Se-labeled nucleoside (less than 0.0001 A_{313} unit) were used in each analysis. Panels a–c show the result of 2 M HCl treatment for 0, 30, and 50 min, respectively. Selenonucleosides in 2 M HCl (60-μL volume) were heated anaerobically at 100 °C for the indicated times, neutralized by the addition of 6 M KOH, and subjected to HPLC. Panel d shows the effect of injecting the untreated selenonucleosides (60-μL volume) onto the HPLC column in 1 M Tris-HCl buffer, pH 8.0. In each analysis, HPLC was performed as described under Experimental Procedures. Radioactivity and absorbance at 313 nm were measured in 0.5-mL (0.5-min) fractions.

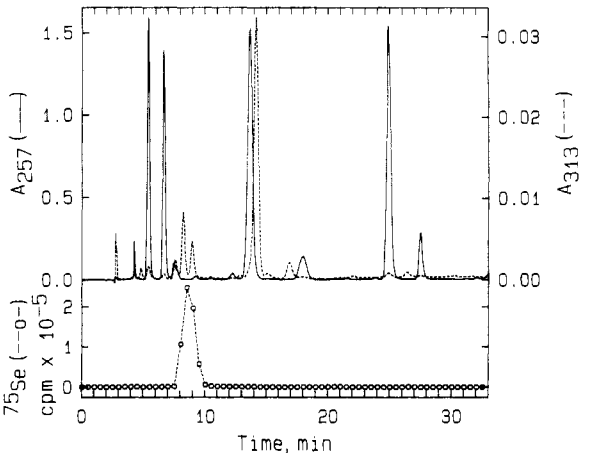


FIGURE 4: Nucleoside analysis of ^{75}Se-labeled tRNA from *C. sticklandii*. Bulk *C. sticklandii* tRNA (3.4 A_{260} units, 60000 cpm) was digested to nucleosides and analyzed by HPLC as described under Experimental Procedures. The four major peaks of 257-nm absorbance are, in order of elution, cytidine, uridine, guanosine, and adenosine. The two peaks of 313-nm absorbance eluting between 8 and 10 min are due to the two selenonucleosides. The later eluting of these selenonucleosides was identified as 5-[(methylamino)methyl]-2-selenouridine by cochromatography with the authentic compound. The prominant peak of 313-nm absorbance at 14.1 min is due to 4-thiouridine.

Comparison of ^{75}Se-Labeled Nucleoside with Authentic 5-[(Methylamino)methyl]-2-selenouridine. The UV spectra of the natural selenonucleoside (Figure 2a) and those of the synthetic compound (Figure 2b) show the same shifts at acidic, neutral, and basic pH values. The two compounds cochromatographed when subjected to HPLC analysis under two different conditions (Figure 3a,d). Furthermore, they showed similar behavior when heated anaerobically in 2 M HCl (Figure 3b,c).[2] These observations strongly support the identification of the natural selenonucleoside as 5-[(methylamino)methyl]-2-selenouridine (**5**).

Identification of 5-[(Methylamino)methyl]-2-selenouridine in the tRNAs from Two Species of Anaerobic Bacteria. In view of the identification of the single selenonucleoside in the tRNAs of *E. coli* as 5-[(methylamino)methyl]-2-selenouridine, its possible presence in the seleno-tRNAs of two strictly anaerobic bacteria, *C. sticklandii* and *M. vannielii*, was examined. Nucleoside analysis of ^{75}Se-labeled bulk tRNA preparations isolated from *C. sticklandii* showed that 40–60% of the selenium was present in a compound that eluted from the reversed-phase HPLC column in the same position as mnm^{5}se^{2}U (Figure 4).[3] The remaining ^{75}Se applied to the column was found in a second selenonucleoside that eluted just ahead of mnm^{5}se^{2}U. Although the UV spectrum of this unidentified selenonucleoside resembles that of mnm^{5}se^{2}U, it is clearly distinguishable from the spectrum of the latter compound (data not shown).

A sample of pure ^{75}Se-labeled tRNA^{Glu} (0.48 A_{257} unit, 10300 cpm of ^{75}Se, 93% pure according to glutamate-accepting activity), the most prominent seleno-tRNA species of *C. sticklandii* (Ching & Stadtman, 1982), was digested to nucleosides as described under Experimental Procedures. Reversed-phase HPLC of the resulting nucleoside mixture (data not shown) indicated a single ^{75}Se-labeled compound. This material cochromatographed with authentic mnm^{5}se^{2}U, and its UV spectrum was identical with that of the reference selenonucleoside (Figure 2b). Thus, the single selenium-containing moiety in this tRNA^{Glu} species is mnm^{5}se^{2}U. One 4-thiouridine residue per seleno-tRNA^{Glu} molecule was also evident as a result of this analysis, but no 2-thio- or 4-selenouridine derivatives were detected. This is a further demonstration of the specificity of selenium modification of tRNAs. In *E. coli*, the previously identified tRNA^{Glu} species lack 4-thiouridine and contain a single thionucleoside, 5-[(methylamino)methyl]-2-thiouridine, which is located in the first position of the anticodon (Ohashi et al., 1970; Uziel & Weinberger, 1975).

The selenonucleoside composition of ^{75}Se-labeled bulk tRNA preparations isolated from *M. vannielii* consists of about 60% mnm^{5}se^{2}U and 40% of another selenonucleoside with a much longer retention time on the reversed-phase HPLC column (Ching et al., 1984). This selenonucleoside that eluted later from the HPLC column is a unique component of the tRNA species that eluted later in the profile from the RPC-5 reversed-phase column whereas the seleno-tRNAs that were

[2] When the synthetic compound (**5**) was heated anaerobically in 2 M HCl and the reaction mixture was examined by HPLC analysis, among the multiple degradation products there was only one peak that had significant 313-nm absorption. This was identified as **2** on the basis of its retention time and UV spectral properties. All the other unidentified peaks absorbed below 300 nm, indicating the loss of Se from the molecule.

[3] Abbreviations: mnm^{5}se^{2}U, 5-[(methylamino)methyl]-2-selenouridine; Tris, tris(hydroxymethyl)aminomethane.

eluted earlier in the profile contain only mnm^5se^2U.

In both *C. sticklandii* and *M. vannielii*, the unidentified selenonucleoside that accounted for the remaining selenium present in each tRNA digest exhibited UV absorbance spectra characteristic of a 2-selenouridine. However, these two unidentified species were readily distinguishable on the basis of their HPLC elution properties. These selenonucleosides might be forms representing intermediate steps in the biosynthesis of the 5-[(methylamino)methyl] moiety or analogues of other known 2-thiouridines.

Analysis of Bacillus subtilis tRNAs. Among the bacterial species we have investigated, the strictly anaerobic methane-producing organism *M. vannielii* contains the highest amount of selenium-modified tRNAs (Ching et al., 1984). Thus, it was of interest to examine a strictly aerobic species, *Bacillus subtilis*, for the presence of seleno-tRNAs. A tryptophan-requiring strain [168 Tryp C 2; originally strain 60001 of Spezizen (1958)] that has been used extensively for tRNA compositional studies (Vold et al., 1982) was selected. Little or no ^{75}Se-labeled tRNA was detected in cells grown in a rich medium (Luria broth) supplemented with $^{75}SeO_4^{2-}$ or a glucose/mineral salts medium (Spezizen, 1958) containing tryptophan, a mixture of amino acids lacking cysteine and methionine, and $^{75}SeO_4^{2-}$. Although suggestive, similar studies with other obligately aerobic microorganisms would be required to evaluate the effects of aerobic conditions per se on biosynthesis of seleno-tRNAs.

Is 4-Selenouridine a tRNA Component? Although earlier reports indicated that 4-selenouridine is a constituent of *E. coli* tRNAs (Hoffman & McConnell, 1974; Prasado Rao & Cherayil, 1974; Young & Kaiser, 1979), the ion-exchange and thin-layer chromatographic procedures available at the time lacked the resolving power necessary to distinguish between selenonucleosides and the numerous degradation products generated by the prolonged alkaline digestion procedures that were employed. In the present studies using rapid enzymic digestion procedures and the much more powerful analytical methods now available, we have not detected 4-selenouridine in the tRNA populations of *E. coli*, *C. sticklandii*, or *M. vannielii*. Since all three organisms contain high and similar amounts of 4-thiouridine in their tRNAs (A_{257}:$A_{333} \approx 50$), it is possible that under certain growth conditions (e.g., when selenium to sulfur ratios are abnormally high) some 4-selenouridine may be formed by nonspecific substitution of selenium for sulfur. From the in vitro studies of Lipsett and collaborators (Abrell et al., 1971; M. N. Lipsett, personal communication), it is known that the *E. coli* sulfur transferase can utilize selenocysteine in place of cysteine as donor with the resultant formation of a 4-selenouridine residue in the tRNA substrate. Thus, in vivo, the relative availability of selenocysteine could be an important determinant in the ability of the organism to form the 4-seleno analogue. Although the immediate selenium donor utilized for synthesis of 2-selenouridine residues in the bacterial tRNAs has not been identified, it is clear that one or more steps in the overall process effectively discriminate between selenium and its far more abundant chemical relative sulfur.

Registry No. **1**, 91425-20-6; **2**, 91425-21-7; **3**, 58381-23-0; **4**, 91443-58-2; **5**, 89314-80-7; Se, 7782-49-2.

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