Proceedings of the National Academy of Sciences
Vol. 68, No. 1, pp. 48–51, January 1971

Chemical Modifications of Transfer RNA Species. Desulfurization with Raney Nickel

SIDNEY M. HECHT*, LESLIE H. KIRKEGAARD†, AND ROBERT M. BOCK

Laboratory of Molecular Biology and Department of Biochemistry, University of Wisconsin, Madison, Wis. 53706

Communicated by N. J. Leonard, October 15, 1970

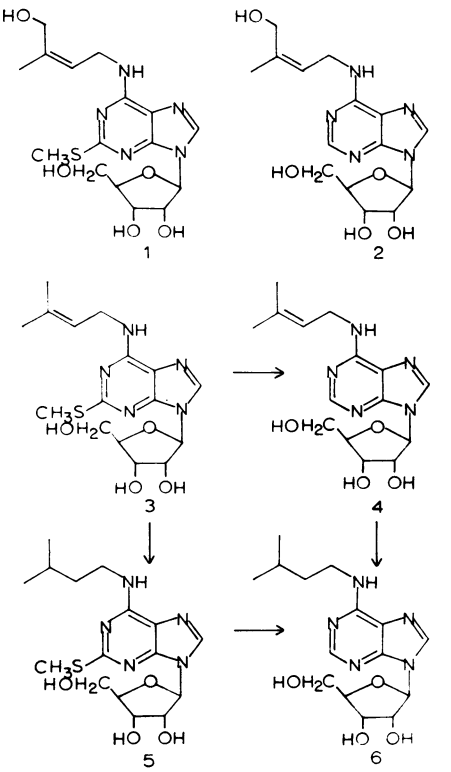
ABSTRACT **A single, modified nucleoside in *Escherichia coli* tRNA, 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine, has been desulfurized with Raney nickel to afford its probable biosynthetic precursor. The limitations of the reaction at the nucleoside and tRNA levels and its lack of inhibition of the amino acid acceptor activity of tRNA are described.**

Certain modified nucleosides found in tRNA are also potent plant growth hormones (1). Where these hormones, or cytokinins, occur in tRNA, they occupy only that position adjacent to the 3′-end of the anticodon triplet, and only in tRNA species that respond to codons beginning with uridine (1, 2). Four cytokinins (**1**–**4**) have been isolated as components of tRNA (1–3).

To facilitate the study of the metabolic function and biosynthesis of one of these modified nucleosides, 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**), a procedure has been developed for hydrogenolysis of the methylthio groups of **3**, where it occurs in *Escherichia coli* tRNA.

The identification of cytokinins as components of tRNA has prompted considerable research into the metabolic importance of these compounds. Studies on *E. coli* tRNA, for example, indicated that cytokinins were located adjacent to the 3′-end of the anticodons in tyrosine (4) and phenylalanine (5) tRNAs, and were also present, presumably adjacent to the anticodon, in cysteine, tryptophan, serine, and leucine tRNAs (6, 7). Gefter and Russell (8) also presented evidence that the cytokinins were important in the specific binding of tRNA to the appropriate ribosome-bound codon triplets, and Fuller and Hodgson (9) hypothesized the importance of this group to proper codon-anticodon interaction in protein synthesis.

Peterkovsky (10) and Hall (11) showed that the mevalonate-requiring bacteria *Lactobacillus acidophilus* and *Lactobacillus plantarum* rapidly incorporated [^{14}C]mevalonate into the side chain of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**4**) in tRNA. Hall (12–14) later showed that tRNA, which had been partially modified by treatment with permanganate, incorporated [^{14}C]Δ^2-isopentyl pyrophosphate to a greater extent than unmodified tRNA. In addition, Miura and Miller (15) demonstrated that the fungus *Rhizopogon roseolus* contained the enzymes necessary to convert exogenous 6-(3-methyl-2-butenylamino)purine (**4**, purine portion) to the hydroxylated derivative **2**.



Prior to the identification of **3** as a component of *E. coli* tRNA, Goodman *et al.* (4) showed that the nucleoside adjacent to the anticodon of *E. coli* tRNA^{Tyr}, now assumed to be **3**, could be labeled with both [^{35}S]sulfate and [methyl-^{14}C]-methionine. Rosenberg and Gefter (16) presented evidence consistent with an iron dependence for the *in vivo* formation of **3** from **4** in tRNA. After the identification of **3** as a tRNA component (17–19), Harada *et al.* (19) suggested that this nucleoside must be formed by the modification of **4** in one or two steps.

To test for the presence of an enzyme(s) that would modify **4** to **3** at the tRNA level, and to investigate the effect of the methylthio group (in **3**) on tRNA function, hydrogenolysis of tRNA that contained **3** was effected with Raney nickel.

MATERIALS AND METHODS

The *E. coli* tRNA used for modification was isolated from *E. coli* K_{12}, from a tryptophan revertant of strain M 72 that possessed the following genotype: $F^- Su^- lac^- try^+ Sm^r T_1^r$.

The Raney nickel catalyst was obtained from the Raney Catalyst Division of W. R. Grace and Co. and was prepared

* National Institutes of Health Predoctoral Fellow, 1967–1970.
† National Institutes of Health Postdoctoral Fellow, 1969–1970.

for use by extensive washing (20). The initial modification of the nucleoside, which produced N^6-isopentyladenosine, was performed on 26 mg (0.07 mmol) of **3**, which was treated with 3.0 ml of absolute ethanol containing 0.3 g of Raney nickel, according to the general method of Ikehara *et al.* (21). The suspension was heated at reflux for 5 hr, the catalyst was filtered through a celite pad, and the solution was evaporated to give 15 mg (66%) of white, crystalline N^6-isopentyladenosine, identical with an authentic sample (22).

Modification of the nucleoside, **3**, with Raney nickel at a concentration comparable to that which would be used for tRNA modification, was performed as follows: to a solution of 1.5 mg (4 μmol) of **3** in 30 ml of water was added 3 g of Raney nickel and 435 mg (3.2 mmol) of KH_2PO_4. The reaction mixture was heated to 60°C and stirred vigorously with a mechanical stirrer. Aliquots were removed after 1, 5, 10, and 15 min. The reaction had gone to completion in no more than 1 min, producing desulfurized product in 80% yield. Longer reaction times did not adversely affect yield.

For the reduction of tRNA, a solution of potassium phosphate (pH 7) was combined with the minimum quantity of catalyst that would desulfurize 4 μmol of **3** in 1 min. The tRNA (0.2–0.6 g) was dissolved in 30 ml of 0.03 M potassium phosphate (pH 7), and the proper amount of Raney nickel was added. The solution was heated to 60°C and stirred vigorously for 10–45 min. The catalyst was filtered through a coarse scintered glass filter and residual catalyst was removed by centrifugation. A portion of the tRNA was then hydrolyzed enzymatically with snake venom phosphodiesterase and alkaline phosphatase, according to the method of Hall (23). The resulting nucleoside solution was extracted with five equal portions of water-saturated ethyl acetate. The ethyl acetate extract was concentrated to give the cytokinin mixture. The mixture was then assayed by paper chromatography. The R_f values for the eight possible products arising from treatment of the known cytokinins with Raney nickel are given in Table 1.

The products were eluted from the paper chromatograms with water and the structural assignments were verified by ultraviolet and mass spectral data. Compounds **4** and **6** were not conveniently separated on paper, but were instead determined by high- and low-resolution mass spectrometry as a mixture; the ratio of the two was deduced from a comparison of the low-resolution mass spectrum with the spectra of defined mixtures of the two pure synthetic materials, determined on the same mass spectrometer under the same operating conditions.

Amino acid acceptor assays on the tRNAs used the assay procedure described previously (24). The acylation reagent had the following composition: 0.1 M NH_4OH–piperazine-*N*,*N*′-bis-(2-ethane sulfonic acid) monosodium monohydrate buffer (pH 7.0), 1.0 mM ATP, 0.4 mM CTP, 0.01 M magnesium chloride, 0.1 M potassium chloride, and 0.5 mM EDTA. [^{14}C]Amino acids (50 Ci/mol) were added to a concentration of 40 μM. The 19 amino acids not tested were present at a concentration of 33 μM. To each 0.6-ml fraction was added 0.1 ml of crude *E. coli* enzyme. The reaction was initiated by the addition of 0.1 ml of tRNA solution containing 60 A_{260} units/ml of modified *E. coli* tRNA. The progress of the reaction was followed by the removal of 0.1-ml aliquots from the reaction mixture at six specified time intervals. The reaction was quenched by adding the aliquots to a paper disc previously treated with 0.1 ml of 0.05 M cetyltrimethylammonium bromide in 1% aqueous acetic acid solution. The discs were washed and counted as described (24).

Crude *E. coli* aminoacyl-tRNA synthetases were prepared from the same strain of *E. coli* from which the tRNA was isolated. 50 g of frozen cells was lysed under toluene. The toluene was removed and the cell paste was treated with 50 ml of a solution that contained 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, and 5 μM β-mercaptoethanol. The cell suspension was dialyzed for 24 hr against the same buffer and centrifuged at 50,000 rpm in a Beckman, titanium, type 50 rotor for 90 min. The supernatant was used as the aminoacyl-tRNA synthetase.

RESULTS AND DISCUSSION

It was initially observed that compound **3** could be converted to N^6-isopentyladenosine (**6**) by treatment with Raney nickel in ethanol at reflux, according to the general method of Ikehara and co-workers (21). It was hoped that under milder conditions, desulfurization of **3** might proceed faster than hydrogenation of the double bond, so that **4**, rather than **6**, would result. Indeed, when **3** was modified at a concentration comparable to that which would be encountered in tRNA modification, it was noted that the relative proportions of compounds **4** and **6** could be controlled by variation in the length of the reaction and the activity of the catalyst. It was thus possible, starting from **3**, to obtain **4** with no significant amounts of **6** present. More vigorous conditions, of course, gave **6** as the sole product. The amount of compound **5** isolated under any set of reaction conditions was very small, reflecting the faster rate of hydrogenolysis (desulfurization) as compared with hydrogenation.

The identification of the nucleoside products arising from treatment with Raney nickel was conveniently monitored by paper chromatography of the product mixture, followed by elution of the appropriate ultraviolet-absorbing bands (see Table 1) and identification of the products by ultraviolet and mass spectral data. The amounts of **4** and **6** were estimated

TABLE 1. *R_f values of the cytokinin ribonucleosides known to occur in tRNA and their possible hydrogenation products*

Compound	R_f values*
6-(4-Hydroxy-3-methyl-*trans*-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**1**)	0.57
6-(4-Hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**)	0.75
6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**)	0.00
6-(3-Methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**4**)	0.63
6-(4-Hydroxy-3-methylbutylamino)-2-methylthio-9-β-D-ribofuranosylpurine†	0.56
6-(4-Hydroxy-3-methylbutylamino)-9-β-D-ribofuranosylpurine	0.75
6-(3-Methylbutylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**5**)†	0.32
6-(3-Methylbutylamino)-9-β-D-ribofuranosylpurine (**6**)	0.66

* Determined on Whatman no. 1 paper in water.

† Synthetic compounds not previously reported (manuscript in preparation).

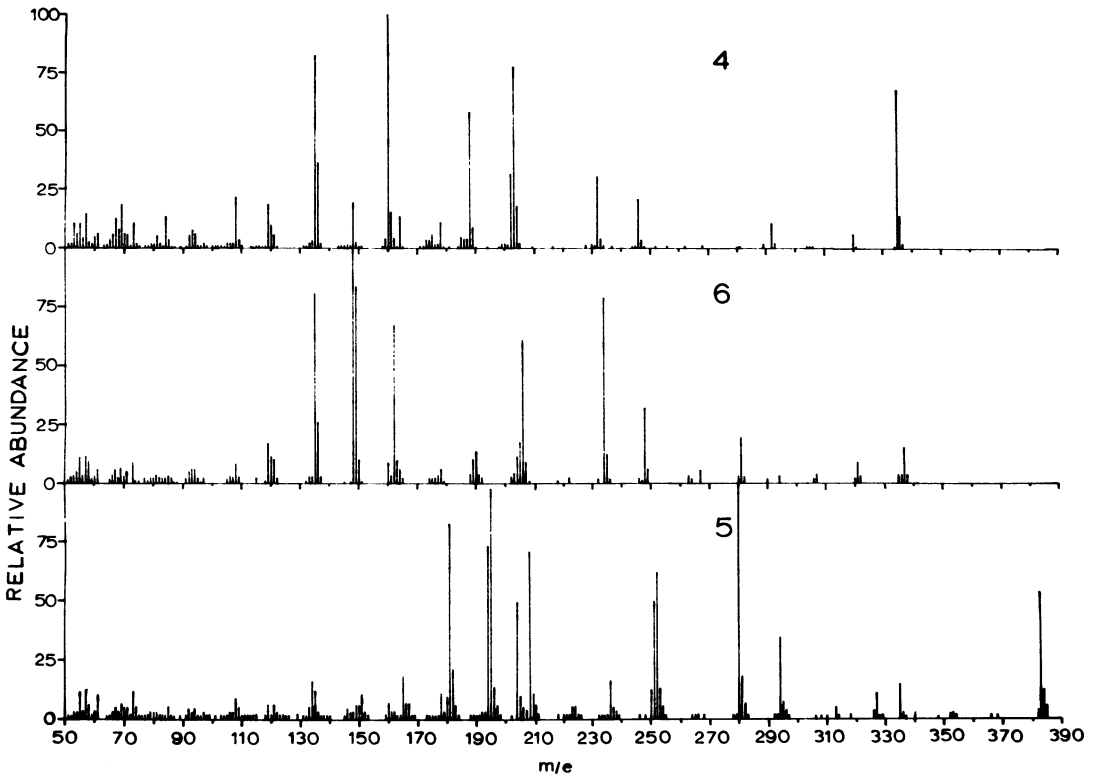


FIG. 1. Mass spectra at 70 eV of synthetic 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**4**), 6-(3-methylbutylamino)-9-β-D-ribofuranosylpurine (**6**), and 6-(3-methylbutylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**5**).

as the mixture by comparison of the low-resolution mass spectrum with the spectra of a series of defined mixtures of the two pure synthetic compounds. The ratios of characteristic peaks due to **4** and **6** were reproducible for each of the product mixtures (see *Methods*), and gave a reliable indication of the proportion of the products, especially where a number of related peaks due to the two compounds were considered. Fig. 1 shows the mass spectra of pure synthetic samples of each of the possible products. Fragmentation patterns corresponding to the major peaks arising from compounds of these types have been reported (18, 25).

Unfractionated *E. coli* tRNA has been shown to contain both **3** and **4**, with **3** being present as the major species (18). Investigation of the specific tRNA sample used for this study confirmed this report. Of the four cytokinins known to occur naturally in tRNA, **3** constituted almost 90% of the total in the sample studied. Most of the rest was compound **4**, with compounds **1** and **2** constituting less than 0.1% of the total, if they occurred at all (manuscript in preparation).

We initially attempted to convert 0.6 g of unfractionated *E. coli* tRNA to tRNA species containing exclusively **4** with a concentration of catalyst sufficient to desulfurize an equi-

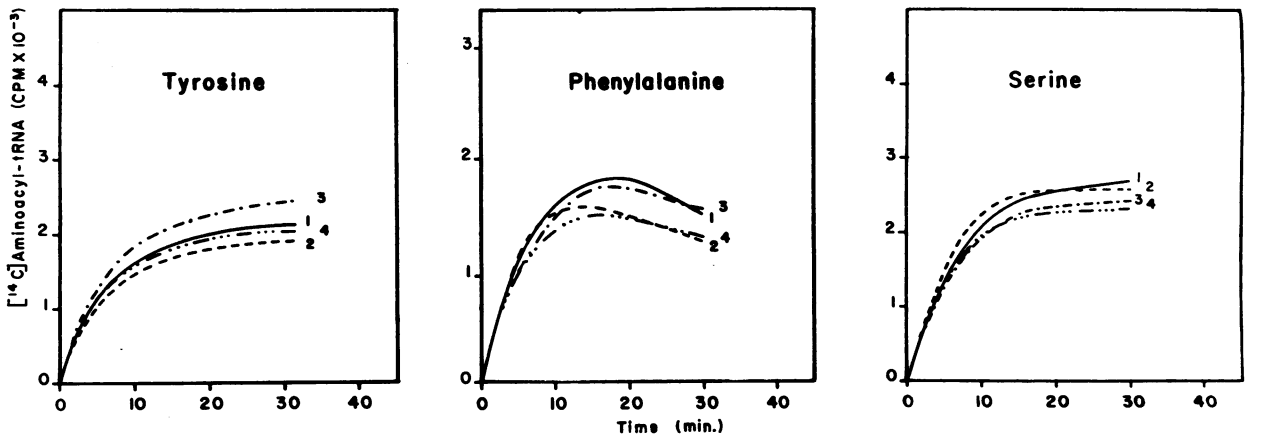


FIG. 2. Amino acid acceptor activity of modified tyrosine, phenylalanine, and serine tRNAs. The solid line, 1, depicts the rate of acylation of unmodified tRNA. The broken lines, 2, 3, and 4, show the acylation rates corresponding to the desulfurized tRNAs. Line 2 corresponds to the product arising from the mildest reaction conditions. Approximately 40–50% desulfurization of **3** was effected with essentially no hydrogenation of the resulting moieties of **4**. Line 3 corresponds to a product of intermediate reaction time, containing **4** as well as smaller amounts of **6**, while line 4 corresponds to species containing only **6**.

TABLE 2. *Composition of the fragment ions from the high-resolution mass spectrum of a mixture of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine* (**4**) *and 6-(3-methylbutylamino)-9-β-D-ribofuranosylpurine* (**6**) *from tRNA*

Fragments*	Assignment of peaks to compounds 4	or 6
M^+	335.158(335.1593) $C_{15}H_{21}N_5O_4$	337.173(337.1750) $C_{15}H_{23}N_5O_4$
$(M-CH_3)^+$	320.139(320.1359) $C_{14}H_{18}N_5O_4$	322.154(322.1515) $C_{14}H_{20}N_5O_4$
$(M-C_3H_7)^+$	292.106(292.1046) $C_{12}H_{14}N_5O_4$	294.122(294.1202) $C_{12}H_{16}N_5O_4$
$(M-C_3H_5O_3)^+$	246.133(246.1355) $C_{12}H_{16}N_5O$	248.152(248.1511) $C_{12}H_{18}N_5O_4$
$(M-C_4H_7O_3)^+$	232.120(232.1198) $C_{11}H_{14}N_5O$	234.136(234.1355) $C_{11}H_{16}N_5O$
$(B+1)^+$	203.120(203.1171) $C_{10}H_{13}N_5$	205.134(205.1327) $C_{10}H_{15}N_5$
$[(B+1)-C_3H_7]^+$	160.062(160.0623) $C_7H_6N_5$	162.078(162.0780) $C_7H_8N_5$

* M^+ and $(B+1)^+$ refer to fragment ions from the ribonucleoside and base, respectively (26).

molar amount of **3**, as the nucleoside, in 1 min. Under these conditions, 40–50% of the tRNA species containing **3** were converted to species containing **4** after 10 min. Only small amounts of **6** were observed. More vigorous conditions (30 min reaction time) resulted in complete desulfurization; less than $^1/_2$ of the resulting cytokinins were present as **6** rather than **4**. Intermediate reaction times gave primarily tRNA species containing compound **4**. Smaller amounts of **3** or **6** were present, depending on the exact reaction conditions.

Essentially all of the tRNA modified by Raney nickel treatment, for any reaction time up to 60 min, was recovered. Longer runs were not attempted. Loss of 4-thiouridine moieties, as judged by changes in A_{328}, ranged from 10–20% depending on reaction conditions. In no case was a significant amount of **5** observed.

The amino acid acceptor activity of the modified tRNA species was assayed with respect to three amino acids: tyrosine, phenylalanine, and serine. These amino acids were chosen because they corresponded to species of tRNA that contained cytokinins in the position adjacent to the anticodon (4–7). The results of these assays, shown in Fig. 2, indicated that no significant change in amino acid acceptor activity accompanied the change in the form of cytokinin in the tested species, and that the tRNA integrity was not affected by the reaction conditions. This result agreed with earlier observations that amino acid acceptor activity was unaffected by modification of the cytokinins with respect to the methylthio group or isopentenyl side chain (8, 13).

The identity of the three products (**3**, **4**, and **6**) isolated from the tRNA at various stages of modification has been verified by their R_f values and by ultraviolet and low- and high-resolution mass spectroscopy. Thus, the compound thought to be identical with **3** in the partially desulfurized tRNA sample had peaks at m/e 381.145 (M^+, $C_{16}H_{23}N_5O_4S$, calculated: 381.1471), 313.087 ($C_{11}H_{15}N_5O_4S$, 313.0845), 278.108 ($C_{12}H_{16}N_5OS$, 278.1076), 249.105 ($C_{11}H_{15}N_5S$, 249.1048), 234.081 ($C_{10}H_{12}N_5S$, 234.0813), 206.051 ($C_8H_8N_5S$, 206.0500), 194.049 ($C_7H_8N_5S$, 194.0500), 181.041 ($C_6H_7N_5S$, 181.0422).

The compounds thought to correspond to **4** and **6** were determined by high-resolution mass spectrometry of an approximately 1:1 mixture isolated from tRNA after one of the longer modification attempts. The determination (summarized in Table 2) was made on the mixture primarily to demonstrate that the pairs of peaks at m/e 335 and 337, 246 and 248, 232 and 234, 160 and 162, etc., which had been used in the determination of the ratio of the two compounds, were actually attributable to fragments arising uniquely from **4** and **6**, respectively.

Studies intended to characterize the enzymic reactions involved in the biosynthesis of **3** are in progress, utilizing both the modified *E. coli* tRNA described here and unmodified yeast tRNA, which contains **4** but not **3** (manuscript in preparation).

We thank Dr. Dieter Söll, Yale University, New Haven, Conn., for providing the strain of *E. coli* used in these experiments, and Dr. Rodger Foltz, Battelle Memorial Institute, Columbus, Ohio, for the high-resolution mass spectral determinations.

This work was supported by grant GM-12395 from the U.S. Public Health Service.

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