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Cytokinin of Wheat Germ Transfer RNA: 6-(4-Hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine

Abstract. *A new modified nucleoside is responsible, in part, for the cytokinin activity of transfer RNA from wheat germ. The structure as judged by mass spectrometry is 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. Unequivocal synthesis afforded material having ultraviolet, mass spectral, and chromatographic properties identical with those of the natural product.*

Hydrolyzates of wheat germ tRNA (*1*) exhibit cytokinin activity in the tobacco callus bioassay. The activity was due, in part, to a compound which was different from the cytokinins known to be naturally occurring, and the identification of this compound is of considerable interest. The new component was isolated by following the fractionation of wheat germ tRNA by means of the tobacco bioassay.

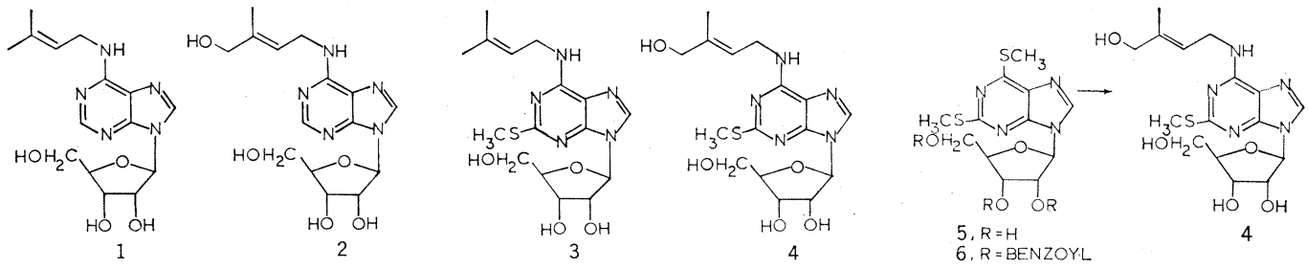
Whole wheat germ (Viobin Corp.) was extracted twice with a mixture of ethanol and ether (1:1, by volume), and the solvent was removed by filtration. The defatted wheat germ was dried overnight at room temperature, ground to a powder, and extracted three times with cold phenol-tris buffer (0.025*M*, *p*H 7.3). Precipitation of the RNA from the aqueous phase was effected with cold 95 percent ethanol. The precipitate was triturated with 0.5 percent CTAB in 0.45*M* sodium chloride (*2*), and the tRNA was precipitated from the supernatant by the addition of two volumes of 0.5 percent CTAB in distilled water at room temperature. The tRNA was converted to its sodium form by repeated treatment with 0.4*M* sodium acetate followed by reprecipitation with three volumes of cold 95 percent ethanol (*3*). The final precipitate was dissolved in cold 0.1*M* tris-HCl buffer (*p*H 7.3) and applied in the cold to a DEAE-cellulose column that had been equilibrated with the same buffer. The column was washed with 0.1*M* tris buffer containing 0.2*M* sodium chloride, and the tRNA was eluted with the same buffer containing 1.0*M* sodium chloride (*4*). The tRNA was treated twice with phenol-tris buffer; the supernatant was washed with ether and precipitated with cold 95 percent ethanol. The precipitate was washed with ether and dried in a vacuum at room temperature.

The tRNA (2.16 g, 19.7 $O.D._{260}$ unit/mg) was dialyzed for 48 hours at 4°C before enzymatic hydrolysis. The hydrolysis procedure (*5*) was similar to that of Hall (*6*). The lyophilized riboside mixture was extracted with water-saturated ethyl acetate, and the soluble material was dissolved in 35 percent ethanol and purified by chromatography on Sephadex LH-20 (152 g; 52 by 3.65 cm) which had been equilibrated with the same solvent (*7*); the elution profile was similar to that observed for the fractionation of ribosides resulting from the hydrolysis of *E. coli* tRNA (*8*). A portion of the cytokinin activity was associated with a region near that in which 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**1**) was known to be eluted. Rechromatography on Sephadex LH-20 (20 g, 15 by 2.4 cm)—elution with water—afforded a symmetrical, ultraviolet-absorbing peak. The material in this fraction was dissolved in 95 percent ethanol and further purified by ascending chromatography on Whatman No. 1 paper in 20 percent ethanol. The ultraviolet-absorbing band (R_F 0.60 to 0.75) was eluted with 95 percent ethanol.

Although the chromatographic values of the compound on Sephadex LH-20 were similar to those of **1**, the ultraviolet spectrum bore greatest resemblance to that of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**) (*5*, *8*). Since it has been shown (*5*, *9*) that the ultraviolet spectra of ring-substituted N^6-isopentenyladenosines were dependent on the nature and position of the ring substituent, but not particularly on the nature of the N^6-alkyl group, it seemed reasonable to conclude that this modified nucleoside might well be an N^6-alkyl-2-methylthioadenosine.

The natural occurrence of **1** (*10*), ribosyl zeatin [6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine] (**2**) (*11*), and **3** (*5*, *8*) emphasized the possibility of the natural occurrence of a ribosyl derivative of 2-methylthiozeatin (*12*), [6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine] (**4**). If the postulated biochemical conversion of **1** to **3** is correct (*9*, *13*), as shown for the conversion of **1** to **2** (*14*), then enzymes necessary to produce **4** from **1** are probably available for its biochemical synthesis. In support of the possibility that structure **4** may be the new cytokinin, the chromatographic behavior of this compound indicated that it was indeed more polar than **3**. Also, it was anticipated that the ultraviolet spectra of **3** and **4** would be the same, on the basis of the spectra of the respective purines (*9*).

The high-resolution mass spectrum of the new cytokinin was indeed con-



sistent with the hypothesis that the cytokinin was ribosyl 2-methylthiozeatin; peaks were observed at intervals characteristic of the fragmentation of zeatin (*11, 15*), but consistently at 46 *m/e* units greater, characteristic of a ring substituent (*5, 8, 9*). The mass spectrum confirmed the presence of sulfur; peaks attributable to a compound with the structural characteristics of **4** included those at *m/e* 308.115 ($C_{13}H_{18}N_5O_2S$), 294.106 ($C_{12}H_{16}N_5O_2S$), 250.079 ($C_{10}H_{12}N_5OS$), 249.102 ($C_{11}H_{15}N_5S$), 248.096 ($C_{11}H_{14}N_5S$), 234.081 ($C_{10}H_{12}N_5S$), 233.074 ($C_{10}H_{11}N_5S$), 224.064 ($C_8H_{10}N_5OS$), 211.057 ($C_7H_9N_5OS$), 210.047 ($C_7H_8N_5OS$), 207.057 ($C_8H_9N_5S$), 206.046 ($C_8H_8N_5S$), 195.054 ($C_7H_9N_5S$), 194.052 ($C_7H_8N_5S$), 182.049 ($C_6H_8N_5S$), 181.042 ($C_6H_7N_5S$), 165.023 ($C_6H_5N_4S$), 136.061 ($C_6H_6N_5$), 135.053 ($C_5H_5N_5$) and 133.048 ($C_5H_9O_4$) (Fig. 1).

Although the molecular ion was not observed initially, the peaks at *m/e* 308 and 294, which reflect the presence of the intact purine nucleus and side chain as well as fragments characteristic of the electron bombardment of a ribose group (*16*), provide sufficient structural information in light of the confirmed presence of an intact ribose group by the peak at *m/e* 133.

The peaks at *m/e* 234, 206, 194, and 181 were thought to correspond to the peaks at *m/e* 188, 160, 148, and 135 in the spectrum of **1** and to indicate a substituted Δ^2- rather than a Δ^3-isopentenyl side chain (*17*).

The synthesis of 2-methylthiozeatin riboside was carried out by two methods. The first involved the condensation of 2,6-bis-methylthio-9-(2′,3′,5′-tri-*O*-benzoyl)-β-D-ribofuranosylpurine (**6**) (*18*) with 4-hydroxy-3-methyl-*trans*-2-butenylamine (*19*) at reflux. The cooled product was debenzoylated in methanolic ammonia, and the free ribonucleoside was purified by chromatography on cellulose (elution with ethanol), and rechromatography on Sephadex LH-20 (elution with 35 percent ethanol).

The second method of synthesis was similar to the first, involving the condensation of 2,6-bis-methylthio-9-β-D-ribofuranosylpurine (**5**) (*20*) with an excess of 4-hydroxy-3-methyl-*trans*-2-butenylamine at reflux under nitrogen. The product was purified by successive chromatography on cellulose and Sephadex LH-20. Both synthetic products had the same chromatographic values as the naturally occurring cytokinin on Sephadex LH-20, in both ethanol and water, and on paper, in a number of solvent systems. The high- and low-resolution mass spectra of the synthetic material were consistent with the data obtained for the natural material. The molecular formula for the synthetic **4** was confirmed as $C_{16}H_{23}N_5O_5S$ by high-resolution mass spectrometry (*21*): found for M^+, 397.143; calculated, 397.1420; found for $(M-89)^+$ (Fig. 1), 308.117; calculated, 308.1181; found for $(B+30)^+$, 294.107; calculated, 294.1025. The qualitative ultraviolet spectra for both natural and synthetic samples were the same [absorption maximums (nm) in absolute ethanol: 283 and 243; (H^+), 286 and 246; (OH^-), 282 and 242]; both were identical with the ultraviolet spectrum exhibited by **3**.

The configuration of the side chain of 2-methylthiozeatin riboside is not yet known. Zeatin riboside was isolated

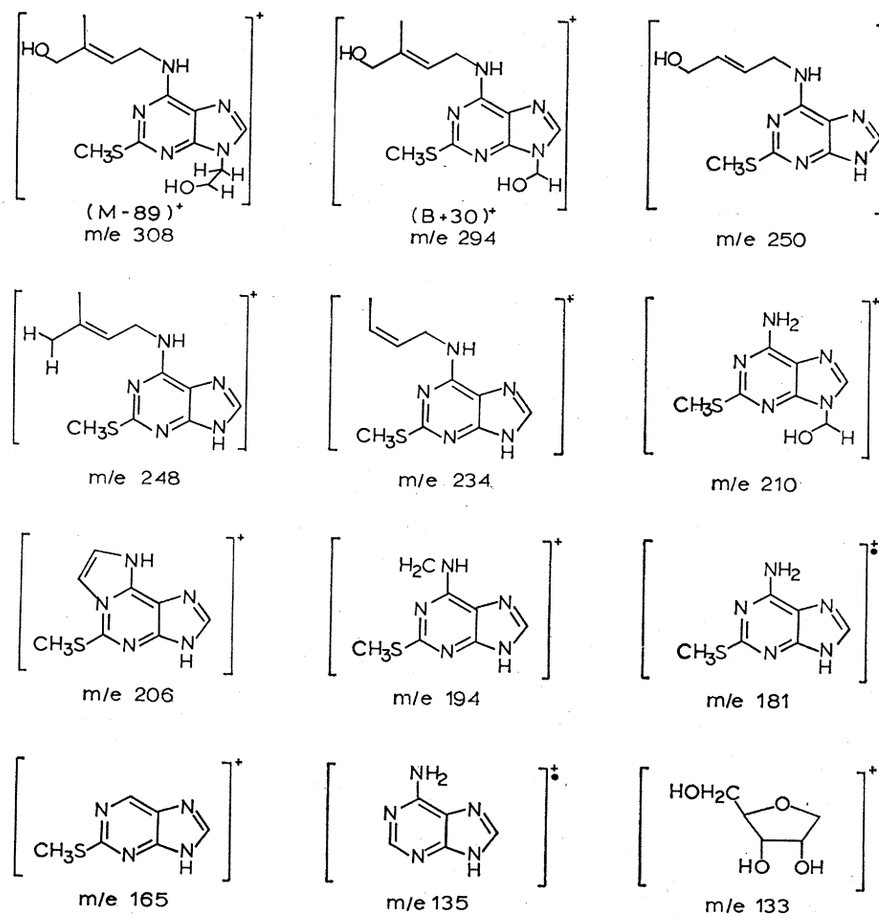


Fig. 1. Mass spectral fragments at 70 ev of a new cytokinin from wheat germ tRNA. $(M-89)^+$ indicates the loss of 89 *m/e* units, which includes three carbons of the ribose unit; $(B+30)^+$ represents the base moiety plus 30 *m/e* units attributable to only the 1′-carbon of the ribose (*16*).

from the tRNA of sweet corn, garden peas, and spinach as the *cis* isomer (*11*), the assignment being based primarily on the melting point of the natural material, which was different from that reported (*19*) for the synthetic product. The isolation of zeatin and zeatin riboside from plant tissue, however, has been reported to afford the *trans* isomer (*19, 22*).

The presence of a second N^6-isopentenyl-2-methylthioadenosine in tRNA may obscure the identity of naturally occurring materials thought to be identical to **3** on the basis of uptake of labeled sulfate-^{35}S and [*methyl*-^{14}C] methionine or on the basis of ultraviolet spectra. In view of the fact that natural systems may not be limited to two N^6-alkyl-2-methylthioadenosines, it would seem judicious to base structural assignments on relatively unambiguous data, for example, mass spectral determination or comparison of physical properties with those of a well-defined synthetic sample.

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References and Notes

1. Abbreviations used in this paper include: tRNA, transfer ribonucleic acid; *m/e*, mass-to-charge ratio; O.D.$_{260}$, optical density at 260 nm; su^+_{III}, mutant strain of *E. coli*; CTAB, cetyltrimethylammonium bromide; tris, tris (hydroxymethyl) aminomethane; DEAE, diethylaminoethyl.
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21. The CEC 21-110 double-focusing mass spectrometer was used at both low and high resolution, and all samples were introduced directly into the ion source. A complete high-resolution mass spectrum, including M^+, has been obtained on a second sample of the natural material, and it is identical with that of synthetic **4** (note added in proof).
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Corticosterone Regulation of Tryptophan Hydroxylase in Midbrain of the Rat

Abstract. *The tryptophan hydroxylase activity in the rat midbrain decreases after adrenalectomy and is restored by treatment with corticosterone. Cycloheximide, administered intracisternally, prevents the restoration of the enzyme activity by corticosterone. Cycloheximide administration to adrenalectomized rats results in a further decrease in the enzyme activity, an indication that the enzyme has a rapid turnover even in the absence of corticosterone.*

Tryptophan hydroxylase is the first of two enzymes responsible for the biosynthesis of serotonin from tryptophan. It is found in the regions of the brain where, as judged by specific histofluorescence, the cell bodies and endings of neurons containing this amine are located (*1*). Serotonin may function as a chemical synaptic transmitter (*2*), and regulatory mechanisms for serotonin formation and utilization are being investigated. Possible effects of adrenal steroids on serotonin concentration in the brain have been studied (*3*), but the results are inconclusive. Therefore, we investigated the role of corticosterone, principal adrenal glucocorticosteroid in the rat (*4*), in controlling the activity of tryptophan hydroxylase in rat brain. We report the effects of adrenalectomy and replacement by corticosterone on the production of this enzyme in the midbrain of the rat, where the cell bodies and therefore the cell nuclei of neurons containing serotonin are located.

Albino male rats (Sprague-Dawley strain, Charles River) (300 to 400 g) were subjected to bilateral adrenalectomies. The animals were maintained in group cages for 1 to 2 weeks on 0.8 percent NaCl and were given free access to standard laboratory chow before use. Corticosterone was administered intraperitoneally in 50 to 200 μl of ethanol; control animals received the same amount of ethanol alone. The rats were decapitated in the early afternoon, and the brains were rapidly removed and chilled. The midbrain was dissected according to the following anatomical landmarks: the anterior boundary was the anterior end of the superior colliculus and the posterior end of the mammillary body; the posterior boundary was the posterior end of the inferior colliculus and the anterior ridge of the pons (isthmus) (*5*).

Tryptophan hydroxylase was assayed by the method of Ichiyama *et al.* (*6*). L-Tryptophan-1-C^{14} (New England Nuclear; specific activity 9 c/mole) was used as substrate, and ^{14}C-CO_2 liberated by the action of L-amino acid decarboxylase on 5-hydroxytryptophan, was measured as the product. Initial experiments established that the amount of labeled CO_2 produced is linearly related to the amount of midbrain homogenate added to the reaction vessel. We also conducted experiments which ruled out that direct inhibition or activation of the enzyme could explain the differences in enzyme activi-