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Isolation and Identification of Four Cytokinins from Wheat Germ Transfer Ribonucleic Acid*

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ABSTRACT: Four ribonucleosides responsible for cytokinin activity in wheat germ tRNA have been isolated and identified as 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine, and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. tRNA was extracted from defatted wheat germ and was hydrolyzed enzymatically. The ribonucleosides were extracted with ethyl acetate, and fractionated on Sephadex LH-20 columns and on paper. Cytokinin activity in the eluates was followed by the tobacco callus bioassay. The structures of these compounds were assigned on the basis of their chromatographic properties and ultraviolet and mass spectra, which were identical with those of the corresponding synthetic compounds.

Preparations of tRNA from various sources have been shown to possess cytokinin-active ribonucleosides (for references, see Skoog and Leonard, 1968, and Armstrong *et al.*, 1969a,b). In a preliminary report (Hecht *et al.*, 1969b), one of the active cytokinins in wheat germ tRNA was identified as 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. This paper reports fully on the isolation, identification, and biological activity of four cytokinin-active ribonucleosides from wheat germ tRNA.

Experimental Section

Extraction of tRNA. Whole wheat germ (Viobin Corp.) was extracted twice with 5 volumes of ethanol–ether (1:1, v/v), and the solvent was removed by filtration. After drying overnight at room temperature the defatted wheat germ was ground to a powder and extracted with 3 volumes of cold 0.025 M Tris-HCl buffer (pH 7.3) and 2.25 volumes of buffer-saturated phenol. The aqueous phase was reextracted twice with buffer-saturated phenol (0.5 volume/volume of supernatant). To the aqueous phase was added 0.1 volume of 20% potassium acetate and the RNA precipitated with 2 volumes of cold 95% ethanol. The precipitate was resuspended in 0.3 M sodium acetate (0.5 ml/g of defatted wheat germ), and the RNA was precipitated with 2 volumes of cold 95% ethanol. The precipitate was triturated in the cold with 0.5% CTAB[1] in 0.45 M NaCl (Brown *et al.*, 1961) (0.33 ml/g of defatted wheat germ), and the tRNA was precipitated from the supernatant by the addition of 2 volumes of 0.5% CTAB in distilled water at room temperature. The tRNA was converted into its sodium salt by repeated treat-

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[1] Abbreviation used is: CTAB, cetyltrimethylammonium bromide.

ment with 0.4 M sodium acetate (0.33 ml/g of defatted wheat germ) followed by ethanol precipitation until all the precipitate had dissolved in the sodium acetate (Ralph and Bellamy, 1964). The final precipitate was dissolved in 0.1 M Tris-HCl buffer (pH 7.3) (1 ml/3 g of defatted wheat germ) and applied in the cold to a DEAE-cellulose column which had been equilibrated with the same buffer (1-ml column volume/10 g of defatted wheat germ). The column was washed with 30 volumes of 0.1 M Tris-HCl buffer containing 0.2 M NaCl, and the tRNA was eluted with the same buffer containing 1.0 M NaCl (Brunngraber, 1962). The eluate was extracted twice with 0.5 volume of phenol; the aqueous phase was washed twice with 0.5 volume of ether and the tRNA precipitated with 2 volumes of cold 95% ethanol. The precipitate was washed twice with ether and dried *in vacuo* at room temperature.

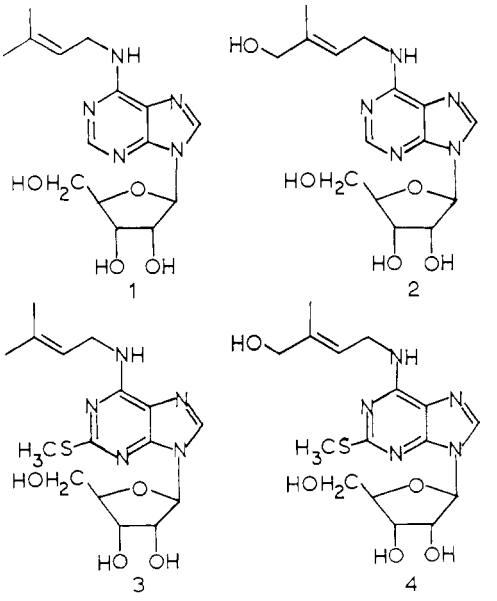
Polyacrylamide gel disc electrophoresis indicated that the preparation referred to as tRNA corresponds mainly to 4S RNA with a small amount of 5S RNA. It is essentially free of high molecular weight RNA and DNA. Amino acid acceptor activity assays for phenylalanine were strongly positive.

The tRNA (2.16 g, 19.7 A_{260} units/mg) was dialyzed against distilled water for 48 hr at 4° and hydrolyzed enzymatically (Burrows *et al.*, 1969). The ribonucleoside mixture was extracted and fractionated by the procedure of Armstrong *et al.* (1969c); *i.e.*, the lyophilized hydrolysate was extracted six times with 25-ml volumes of the upper phase of a mixture of ethyl acetate–water (5:1). The pooled extracts were lyophilized and the solid material was dissolved in 4.0 ml of 35% aqueous ethanol and fractionated on a Sephadex LH-20 column (152 g, 52 × 3.65 cm) which had been equilibrated with the same solvent. The eluates from all columns were monitored at 265 nm. The apparent high background represents a base-line adjustment; the actual absorption was negligible. The fractions were further purified by chromatography on acid-washed Whatman No. 1 paper. For determination of cytokinin activity in fractions from a column, or in the 95% ethanol eluates from the paper chromatograms, samples of the eluates were concentrated, dissolved in 5.0 ml of distilled water, acid hydrolyzed by the standard procedure (Armstrong *et al.*, 1969a), and incorporated into the bioassay medium.

Synthesis of 6-(4-Hydroxy-3-methyl-trans-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**4**).

A. FROM 2,6-BISMETHYLTHIO-9-(2′,3′,5′-TRI-*O*-BENZOYL)-β-D-RIBOFURANOSYLPURINE. To 657 mg (1 mmol) of 2,6-bismethylthio-9-(2′,3′,5′-tri-*O*-benzoyl)-β-D-ribofuranosylpurine (Ikehara *et al.*, 1963) was added 5.0 g (50 mmol) of crude 4-hydroxy-3-methyl-*trans*-2-butenylamine (Shaw *et al.*, 1966). The solution was heated to reflux for 24 hr. The cooled product was debenzoylated in methanolic ammonia and purified by chromatography over cellulose and Sephadex LH-20 to afford a white solid which was recrystallized from absolute ethanol to give white crystals of **4**: yield 22 mg (6%), mp 155–156° (uncor); λ_{max}^{EtOH} (pH 7) 282 nm (ε 17,200) and 243 nm (ε 24,300), λ_{min} 258 nm (ε 9400) and 223 nm (ε 8900); λ_{max}^{EtOH} (pH 1) 285 nm (ε 15,500) and 245 nm (ε 18,500), λ_{min} 263 nm (ε 12,900) and 223 nm (ε 10,300); λ_{max}^{EtOH} (pH 12) 282 nm (ε 17,300) and 243 nm (ε 24,700), λ_{min} 258 nm (ε 9600) and 226 nm (ε 12,600); nuclear magnetic resonance spectrum (δ) from tetramethylsilane (dimethyl sulfoxide-d_6–D_2O): 1.71 (3 H, s, CH_3C), 2.51 (3 H, s, CH_3S), 3.5–3.7 (2 H, m, C-5′ protons), 3.81 (2 H, s, CCH_2O), 4.18 (4 H, m, CCH_2N, C-3′ and C-4′ protons), 4.59 (1 H, m, C-2′ proton), 5.50 (1 H, t, C=CH), 5.85 (1 H, d, C-1′ proton), and 8.21 (1 H, s, Ad-C_8-H). *Anal.* Calcd for $C_{16}H_{23}N_5O_5S$: C, 48.35; H, 5.83. Found: C, 48.27, H, 5.82.

B. FROM 2,6-BISMETHYLTHIO-9-β-D-RIBOFURANOSYLPURINE. To 257 mg (0.75 mmol) of 2,6-bismethylthio-9-β-D-ribofuranosylpurine (Ikehara *et al.*, 1965) was added 5.0 g (50 mmol) of crude 4-hydroxy-3-methyl-*trans*-2-butenylamine. The solution was heated to reflux under a stream of nitrogen for 48 hr. Water was added to the cooled solution and colored material was removed by ether extraction. The aqueous layer containing the product was purified by chromatography over cellulose (eluent 95% EtOH) and Sephadex LH-20 (eluent H_2O) to afford a white solid which was analytically pure, yield 52 mg (18%).



Results

Isolation of Cytokinins. The elution profile of the ribonucleosides (Figure 1) as followed by ultraviolet absorbance was similar to that reported by Burrows *et al.* (1969) for *Escherichia coli* tRNA. Bioassay of 2% of the eluate from each fraction revealed three peaks of cytokinin activity, fractions 4 and 5, 7 and 8, and 12, corresponding to the elution volumes of 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**), 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**1**), and 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**), respectively.

A. FROM FRACTIONS 4 AND 5. The combined solid material from fractions 4 and 5 was dissolved in 2.0 ml of distilled water and fractionated on a Sephadex G-10 column (150 g, 65.5 × 2.4 cm) which had been equilibrated with the same solvent (Figure 2). The eluates in tubes 63–72 were combined, lyophilized, and purified by ascending chromatography on paper in 20% aqueous ethanol. An ultraviolet-absorbing band at R_F 0.48 was eluted. The ultraviolet spectrum of this cytokinin-active material was indicative of an N^6-alkyladenosine. The low-resolution mass spectrum compared

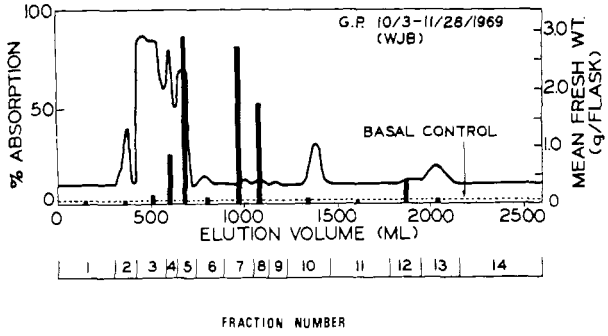


FIGURE 1: Cytokinin activity in the elution profile of the ethyl acetate soluble ribonucleosides from wheat germ tRNA hydrolysate. The ribonucleoside mixture from 2.16 g of tRNA was dissolved in 4.0 ml of 35% aqueous ethanol and applied to a Sephadex LH-20 column (152 g, 52 × 3.65 cm) equilibrated with the same solvent. The column was eluted with 35% ethanol, flow rate 70 ml/hr, and fractions of 10 ml were collected. Cytokinin activity (mean fresh weight of tobacco callus) is represented by vertical bars.

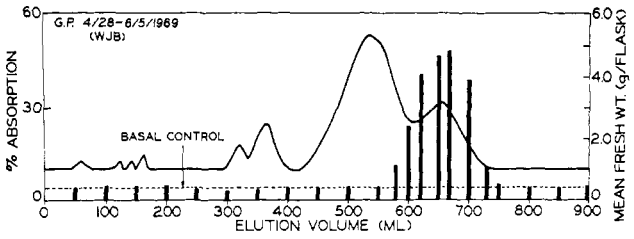


FIGURE 2: Distribution of cytokinin activity in the elution profile of the combined solid material in fractions 4 and 5 (Figure 1). The solid material from these fractions was dissolved in 2.0 ml of distilled water and applied to a Sephadex G-10 column (150 g, 65.6 × 2.4 cm) equilibrated with the same solvent. The column was eluted with distilled water, flow rate 110 ml/hr, and fractions of 10 ml were collected.

favorably with the spectrum of 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (Letham and Miller, 1965; Shannon and Letham, 1966; Shaw *et al.*, 1966; Hall *et al.*, 1967) having abundant ions at *m/e* values of 351 (M^+), 248, 202, 188, 148, and 135. The compositions of the major peaks as determined by high-resolution mass spectrometry are shown in Table I. The accumulated data indicated the structure of this cytokinin to be 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**).

B. FROM FRACTIONS 7 AND 8. The combined eluate was lyophilized and the solid product was dissolved in distilled water and purified by chromatography on a Sephadex LH-20 column (20 g, 15 × 2.4 cm), previously equilibrated with the same solvent (Figure 3). Following ascending chromatography of the solid material in fraction 5 (Figure 3) in 10% aqueous ethanol, the ultraviolet-absorbing band at R_F 0.65 was eluted. The ultraviolet spectrum of the cytokinin suggested it to be an N^6-alkyladenosine. Inspection of the low-resolution mass spectrum revealed substantial peaks at *m/e* values of 335 (M^+), 203, 188, 160, and 135, characteristic of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (Zachau *et al.*, 1966; Biemann *et al.*, 1966; Robins *et al.*, 1967; Madison and Kung, 1967; Madison *et al.*, 1967; Staehelin *et al.*, 1968; Hecht *et al.*, 1969a; Burrows *et al.*, 1969). The compositions of the major peaks identified by high-resolution mass spectrometry are shown in Table I. From these data this cytokinin was assigned the structure 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**1**). The solid from fraction 7 (Figure 3) was purified by ascending paper chromatography in 20% aqueous ethanol, and the ultraviolet-absorbing band at R_F 0.68 was eluted. The ultraviolet spectrum of this material resembled that of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**) (Burrows *et al.*, 1968, 1969) but was chromatographically different on Sephadex LH-20. Since the ultraviolet spectra of ring-substituted N^6-isopentenyladenosines have been shown to be dependent on the nature and position of ring substituents (Robins, 1958; Montgomery *et al.*, 1959; Hecht *et al.*, 1970) but not particularly on the identity of the N^6-alkyl group it seemed reasonable to conclude that this active cytokinin was an N^6-alkyl-2-methylthioadenosine.

The low-resolution mass spectrum of the cytokinin revealed substantial peaks at 397 (M^+), 294, 265, 248, 234, 194, and 181. The series closely paralleled the fragmentation pattern of ribosylzeatin (Letham and Miller, 1965; Shannon and Letham, 1966; Shaw *et al.*, 1966; Hall *et al.*, 1967) but at 46 mass units greater, characteristic of a ring substituent (Burrows *et al.*, 1968, 1969; Hecht *et al.*, 1969b). The high-resolution mass spectrum (Table I) had a molecular ion peak at 397.139 (calcd 397.1420) corresponding to $C_{16}H_{23}N_5O_5S$. The accumulated data strongly suggested the structure of this cytokinin to be 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (ribosyl-2-methylthiozeatin) (**4**).

The synthesis of ribosyl-2-methylthiozeatin was carried out by two methods. The first involved the condensation of 2,6-bismethylthio-9-(2′,3′,5′-tri-*O*-benzoyl)-β-D-ribofuranosylpurine (Ikehara *et al.*, 1963) with 4-hydroxy-3-methyl-*trans*-2-butenylamine (Shaw *et al.*, 1966) 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% aqueous ethanol).

The second method of synthesis was similar to the first, involving the condensation of 2,6-bismethylthio-9-β-D-ribofuranosylpurine (Ikehara *et al.*, 1965) with an excess of 4-hydroxy-3-methyl-*trans*-2-butenylamine at reflux under

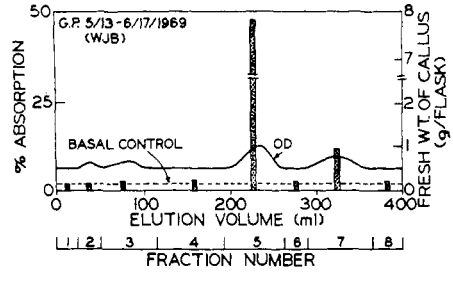


FIGURE 3: Cytokinin distribution in the elution profile of the combined solid material in fractions 7 and 8 (Figure 1). The solid material in these fractions was dissolved in distilled water and chromatographed on a Sephadex LH-20 column (20 g, 15 × 2.4 cm) equilibrated with the same solvent. The column was eluted with distilled water, flow rate 30 ml/hr, and fractions of 10 ml were collected.

TABLE I: Composition of Fragment Ions from the High-Resolution Mass Spectra of 6-(3-Methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**1**), 6-(4-Hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**), 6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**), and 6-(4-Hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**4**).

Fragments	Compounds			
	1	**2**	**3**	**4**
M^+	335.157 (335.1594) $C_{15}H_{21}N_5O_4$	351.153 (351.1543) $C_{15}H_{21}N_5O_5$	381.143 (381.1471) $C_{16}H_{23}N_5O_4S$	397.139 (397.1420) $C_{16}H_{23}N_5O_5S$
$(M - C_3H_5O_3)^+$	246.140 (246.1355) $C_{12}H_{16}N_5O$	262.129 (262.1304) $C_{12}H_{16}N_5O_2$	292.126 (292.1232) $C_{13}H_{18}N_5OS$	308.124 (308.1181) $C_{13}H_{18}N_5O_2S$
$(M - C_4H_7O_3)^+$	232.124 (232.1198) $C_{11}H_{14}N_5O$	248.116 (248.1148) $C_{11}H_{14}N_5O_2$	278.112 (278.1076) $C_{12}H_{16}N_5OS$	294.099 (294.1025) $C_{12}H_{16}N_5O_2S$
$(M - C_5H_8O_4)^+ = (B + 1)^+$	203.119 (203.1171) $C_{10}H_{13}N_5$	219.111 (219.1120) $C_{10}H_{13}N_5O$	249.104 (249.1048) $C_{11}H_{15}N_5S$	265.102 (265.0997) $C_{11}H_{15}N_5OS$
$[(B + 1) - CH_3]^+$[a] or $[(B + 1) - CH_2OH]^+$	188.093 (188.0936) $C_9H_{10}N_5$	204.091 (204.0885) $C_9H_{10}N_5O$ 188.093 (188.0936) $C_9H_{10}N_5$	234.086 (234.0813) $C_{10}H_{12}N_5S$	250.080 (250.0763) $C_{10}H_{12}H_5OS$ 234.082 (234.0813) $C_{10}H_{12}N_5S$
$[(B + 1) - C_3H_7]^+$[a] or $[(B + 1) - C_3H_7O]^+$	160.062 (160.0623) $C_7H_6N_5$	160.063 (160.0623) $C_7H_6H_5$	206.045 (206.0500) $C_8H_8N_5S$	206.048 (206.0500) $C_8H_8N_5S$
$[(B + 1) - C_4H_7]^+$[a] or $[(B + 1) - C_4H_7O]^+$	148.064 (148.0623) $C_6H_6N_5$	148.064 (148.0623) $C_6H_6N_5$	194.046 (194.0500) $C_7H_8N_5S$	194.049 (194.0500) $C_7H_8N_5S$
$[(B + 1) - C_5H_8]^+$[a] or $[(B + 1) - C_5H_8O]^+$	135.053 (135.0545) $C_5H_5N_5$	135.057 (135.0545) $C_5H_5N_5$	181.038 (181.0422) $C_6H_7N_5S$	181.041 (181.0422) $C_6H_7N_5S$

[a] According to the oxygen content of the respective side chains. M^+ and $(B + 1)^+$ refer to the fragment ions of ribonucleosides and bases, respectively, as defined by Biemann and McCloskey (1962).

nitrogen. The product was purified by successive chromatography on cellulose and Sephadex LH-20.

The synthetic and natural products had identical low-resolution mass spectra (Figure 4). The ultraviolet spectra of the natural and synthetic products were the same (absorption maxima (nanometers) in absolute ethanol, 283 and 243; (H^+), 286 and 246; (OH^-), 282 and 242), as were the elution volumes on Sephadex LH-20 in two solvents.

C. FROM FRACTION 12. Ascending paper chromatography of the solid material in fraction 12 in 20% aqueous ethanol gave one ultraviolet-absorbing band at R_F 0.52. The ultraviolet spectrum of the material eluted from this band resembled that of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**) (Burrows *et al.*, 1968, 1969). It seemed reasonable, therefore, to suggest that this cytokinin was an N^6-alkyl-2-methylthioadenosine. The low-resolution mass spectrum had intense peaks at *m/e* values of 381 (M^+), 366, 278, 249, 234, 206, 194, and 181, characteristic of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**) (Burrows *et al.*, 1968, 1969). The compositions of the major peaks identified by high-resolution mass spectrometry are shown in Table I. From these data it was concluded that this cytokinin was 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**).

Discussion

It should be noted that positive identification of the cytokinins reported above rests on mass spectral analysis and total synthesis. Ultraviolet spectra would not suffice; for example, the two 2-methylthio-substituted ribonucleosides (**3** and **4**) are distinguished neither by their ultraviolet spectra nor by uptake of labeled sulfate-^{35}S and methionine-*methyl*-^{14}C. Similarly, the chromatographic procedures are inadequate for positive identification. Examples of this are the following pairs of compounds which have the same R_F values in numerous chromatographic solvents: zeatin and 6-(3-hydroxy-3-methylbutylamino)purine, 6-(3-methyl-2-butenylamino)purine and 6-(3-methyl-3-butenylamino)purine. The same is true of the 9-β-D-ribofuranosides of these compounds.

Specific or "unique" reactions such as the cyclization of **1** or its base (Chen and Hall, 1969) to a 6,6-dimethyl-6,7,8,9-tetrahydro-5a-azonia-1,3,4,9-tetraaza-3*H*-benz[*e*]indene salt or the formal addition of water to the double bond may not provide sufficient identification in light of the existence of other cytokinins. For example, 6-(3-methyl-3-butylamino)-9-β-D-ribofuranosylpurine and its base undergo precisely the same reactions to give the same products (Leonard *et al.*, 1968a,b). All these cited compounds can be identified by mass spectrometry.

Table I illustrates not only that each of the four cytokinins can be characterized by mass spectrometry, but that the fragmentation is systematic and common to all members of the set. Thus the initial event was, in each case, fragmentation of the ribose moiety. Loss of the 2-substituent occurred only at relatively low *m/e* values and the side chains underwent fragmentation to give fragment ions at intervals characteristic of the particular (hydroxy)isopentenyl side chain, but at absolute *m/e* values characteristic of the additional

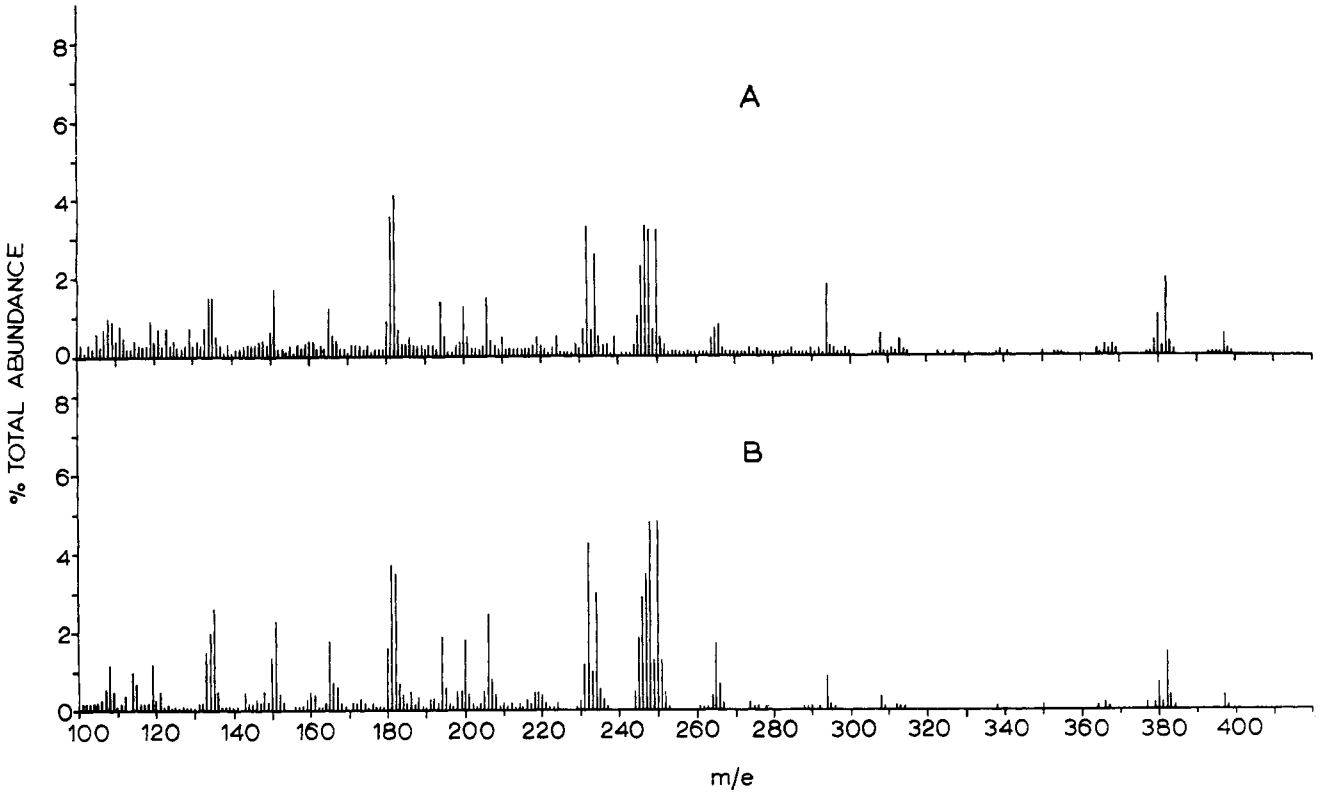


FIGURE 4: Mass spectra at 70 eV of naturally occurring (A) and synthetic (B) 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methythio-9-β-D-ribofuranosylpurine (**4**).

2 substituent. This pattern has been shown to be general for 2-substituted N^6-isopentenyladenines (Hecht *et al.*, 1970) and adenosines and may well prove useful in future structure elucidations.

Only in unusual cases, *e.g.*, differentiation of certain isomeric compounds (S. M. Hecht, in preparation) or geometrical isomers, does mass spectrometry fail. Such a case is the determination of the geometrical configurations of the side chains in 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**) and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**4**), which are unknown in the natural materials reported here. The *trans* isomer of 6-(4-hydroxy-3-methyl-2-butenylamino)-purine is reported to occur free in plant tissues (Letham and Miller, 1965; Shaw *et al.*, 1966; Miller, 1967). Its ribonucleoside, 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**), has since been isolated from the tRNA of sweet corn, spinach, and garden peas and has been assigned *cis* geometry in the side chain (Hall *et al.*, 1967).

It was not possible to estimate the quantities of the four ribonucleosides in the wheat germ preparation. However, it was apparent that 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**) was the most abundant and that 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**) was present in least amount. This report extends the range of known occurrence of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**) to plants. 6-(4-Hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**) has so far been found only in higher plants and fungi and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**4**) has not been reported from any other source.

Hydroxylation of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**1**) to give 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**) has been reported, but at the purine level (Miura and Miller, 1969), and its methylthiation to give 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**3**) has been postulated (Harada *et al.*, 1968). The order of the stepwise processes involved in the presumed biosynthesis of 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**4**) from 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**1**) is not yet known. The discovery of the 2-methylthio derivatives suggests the possible natural occurrence of other 2,6-disubstituted derivatives such as the 2-amino and 2-hydroxy derivatives, the bases of which are both highly active cytokinins (Hecht *et al.*, 1970).

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