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CYTOKININS: SYNTHESIS AND GROWTH-PROMOTING ACTIVITY OF 2-SUBSTITUTED COMPOUNDS IN THE N^6-ISOPENTENYLADENINE AND ZEATIN SERIES*

SIDNEY M. HECHT† and NELSON J. LEONARD

Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois 61801, U.S.A.

and

RUTH Y. SCHMITZ and FOLKE SKOOG

Institute of Plant Development, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

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Abstract—Fourteen compounds were tested for relative promotion of cell division and growth (cytokinin) activity in the tobacco bioassay. The results suggested that 2-substituted-N^6-(hydroxy)isopentenylaminopurines were generally less active than their unsubstituted counterparts. Thus, a 2-OH substituent greatly lowered cytokinin activity in both the isopentenyl and hydroxyisopentenyl (zeatin) series, while 2-NH_2 and 2-SCH_3 groups had a lesser effect on activity and a 2-Cl substituent had a negligible effect. Mass spectra were determined for all of the 9-β-D-ribofuranosides and for a number of the purines as well; the fragmentation patterns were consistent and characteristic, providing a possible systematic approach to the identification of new 2-substituted-N^6-(hydroxy)isopentenyladenines.

INTRODUCTION

TWO NUCLEOSIDES responsible for cytokinin activity in *Escherichia coli* tRNA's have been isolated and identified in these laboratories as 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine [N^6-(Δ^2-isopentenyl)-2-methylthioadenosine] (ms2iPA) (IIc)[1, 2] and 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine[N^6-(Δ^2-isopentenyl)adenosine] (2iPA) (II, R = H).[2] To facilitate the identification of other 2-substituted adenine derivatives possibly occurring in tRNA and to establish relationships between natural occurrence, substitution, and cytokinin activity, we have synthesized and tested a series of N^6-(3-methyl-2-butenyl)adenines (Ia–d) and adenosines (IIa–c). In addition, we have examined a similar series of 2-substituted compounds related to zeatin, 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine (I, R = H, R′ = OH).[3–9] The compounds selected had 2-hydroxy

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† National Institutes of Health Predoctoral Fellow, 1967-70. Present address: Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin, U.S.A.

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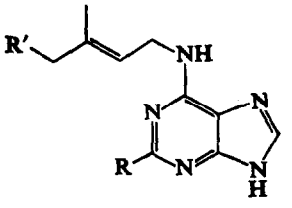
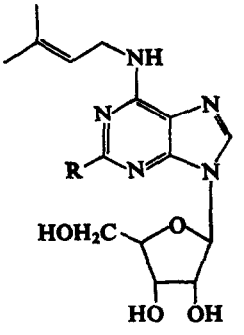
substitution, related to isoguanosine;[10-12] 2-amino substitution, related to 2,6-diaminopurine and its riboside;[12-14] 2-methylthio substitution;[1,2] and 2-chloro substitution, since biological activity had been found in this series.[15] The 2-hydroxy-6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (IIb) is also interesting because of its possible intermediacy in the biochemical conversion of 2iPA (II, R = H) to ms2iPA (IIc).



(I)

(a) R = Cl, R′ = H
(b) R = NH_2, R′ = H
(c) R = OH, R′ = H
(d) R = SCH_3, R′ = H
(e) R = Cl, R′ = OH
(f) R = NH_2, R′ = OH
(g) R = OH, R′ = OH
(h) R = SCH_3, R′ = OH



(II)

(a) R = NH_2
(b) R = OH
(c) R = SCH_3

RESULTS AND DISCUSSION

Chemistry of 2-Substituted Compounds

The syntheses of the test substances, where they were previously unknown, were carried out by unexceptional methods, based upon the preparation of the appropriate 2-substituted 6-chloro-or 6-methylthiopurine and treatment of the intermediate[16] with 3-methyl-2-butenylamine or 4-hydroxy-3-methyl-*trans*-2-butenylamine. The ribosides were obtained via the chloromercuriderivatives of their corresponding purines, which were treated with 1-bromo-2,3,5-tribenzoylribofuranose according to the general method of Davoll and Lowy.[13] Debenzoylation of the protected nucleosides was effected in anhydrous 15 *N* methanolic ammonia to afford the free ribosides.

Mass Spectrometry of 2-Substituted Compounds

The need for an unambiguous and highly sensitive method to facilitate the identification of new 2-substituted adenine derivatives prompted us to determine the mass spectra of several of the 2-substituted adenines synthesized for testing in the tobacco bioassay. The spectrum of 2-amino-6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (IIa) was typical of the set of spectra. The major fragments in this spectrum resulted from stepwise cleavage of the sugar and side-chain moieties. Thus, fragmentation of the sugar moiety led

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to ions of *m/e* 261 $(M-89)^+$, 247 $(B+30)^+$, and 218 $(B+1)^+$,[17] which was followed by fragmentation of the side-chain to give peaks at *m/e* 203, 175, 163, 150, and 134 (Fig. 2).[18] The fragment ions in Fig. 2 still possessed the same 2-substituent as in the molecular ion. This feature was common to the spectra of all compounds studied: loss of the 2-substituent occurred only at relatively low *m/e* values.[1] Moreover, since the spectra of the ribosides at 70 eV were essentially those of the corresponding free bases, a fragmentation pattern common to all these compounds may be represented as in Scheme 1. The side-chain underwent fragmentation in the same fashion as was observed for unsubstituted 6-(hydroxy)-isopentenylaminopurines, resulting in fragment ions at intervals characteristic of the particular side-chain, but at absolute *m/e* values characteristic of the additional 2-substituent (Fig. 3).

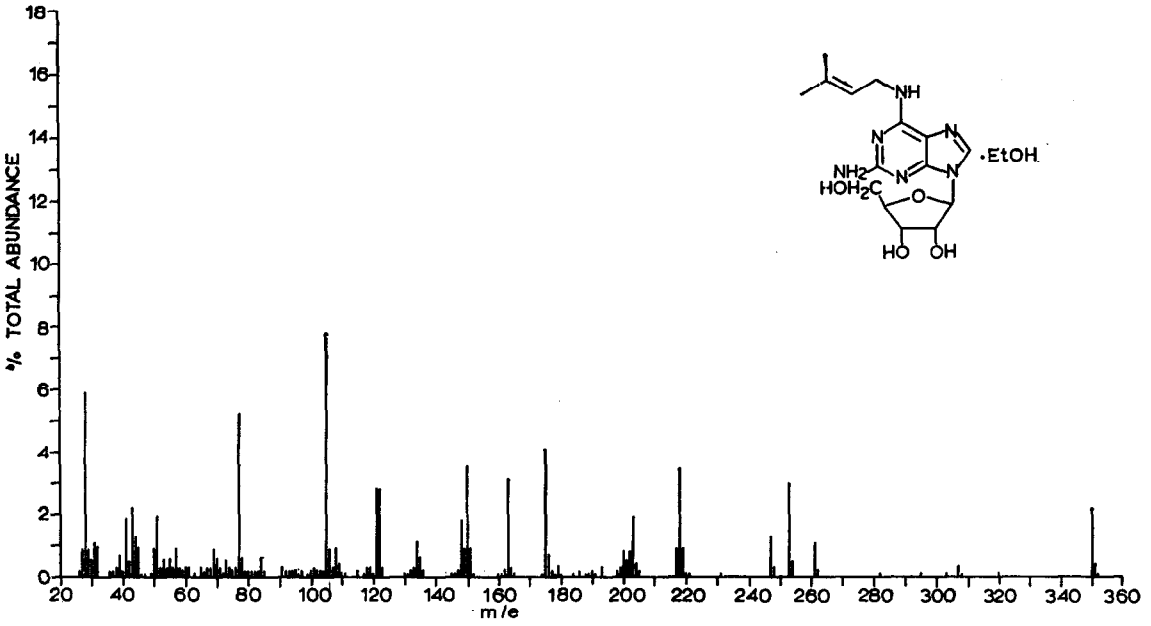

FIG. 1. MASS SPECTRUM AT 70 eV OF 2-AMINO-6-(3-METHYL-2-BUTENYLAMINO)-9-β-D-RIBOFURANOSYL-PURINE (IIa).

Thus, the characteristic mass spectrum associated with the 2-substituted N^6-isopentenyladenines, which provided the first indication of the 2-methylthio substituent in ms2iPA[1,2], appears to be general for all members of the series and may well prove useful in future structure elucidations. In addition, since the characteristic fragments would arise from the purine moiety of a nucleoside, comparison of the mass spectra of synthetic purines with the spectrum of the unknown nucleoside should yield considerable structural information.

Cytokinin Activity of 2-Substituted Compounds

The average relative cytokinin activities of the 2,N^6-disubstituted adenines and adenosines are summarized in Fig. 4. The 2-substituted zeatin derivatives were more active in

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each case than the corresponding derivatives of 2iP. This is in agreement with the slight difference in activity of the unsubstituted compounds themselves. For both the zeatin and the 2iP derivatives the activities were in the order $Cl > NH_2 \geqslant CH_3S$ w OH. The chloro derivatives were consistently the most active; 2-chloro zeatin was no less, and possibly more, active than zeatin itself. The other 2-substituted derivatives were less active than their unsubstituted counterparts. The corresponding 2-amino and 2-methylthio derivatives were about equally active, and the 2-hydroxy derivatives were distinctly less active (cf. Ic, g).

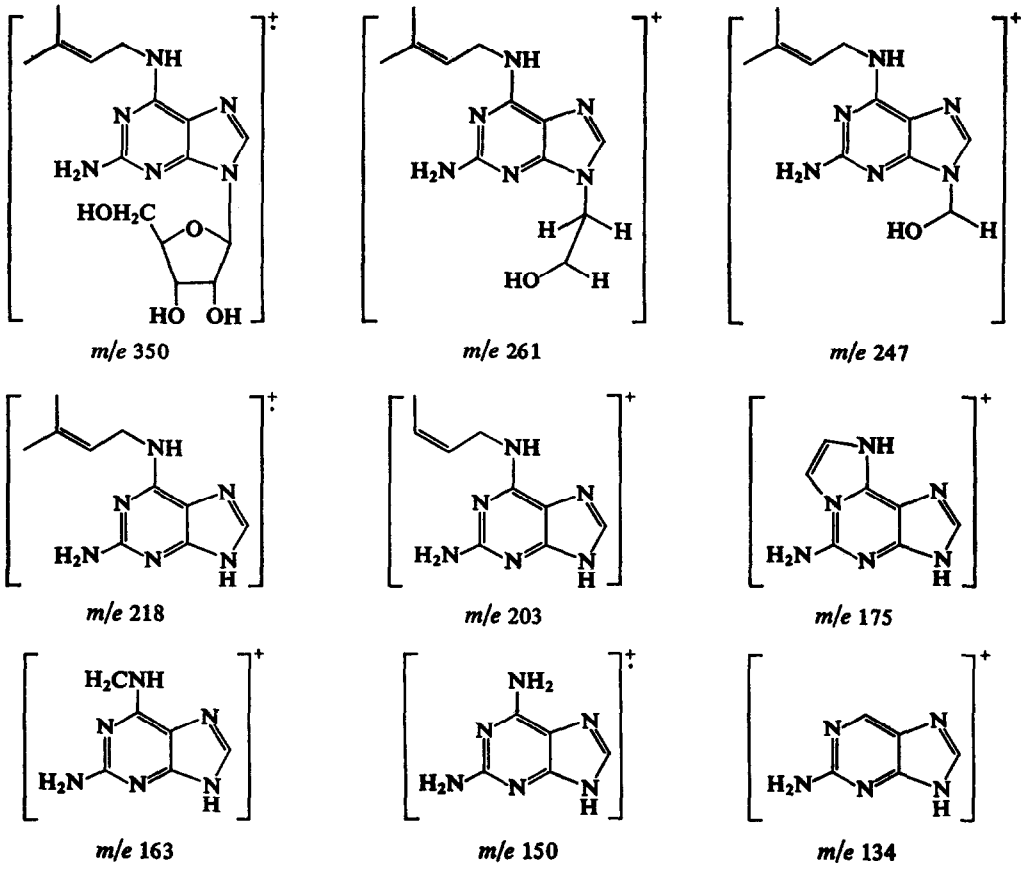


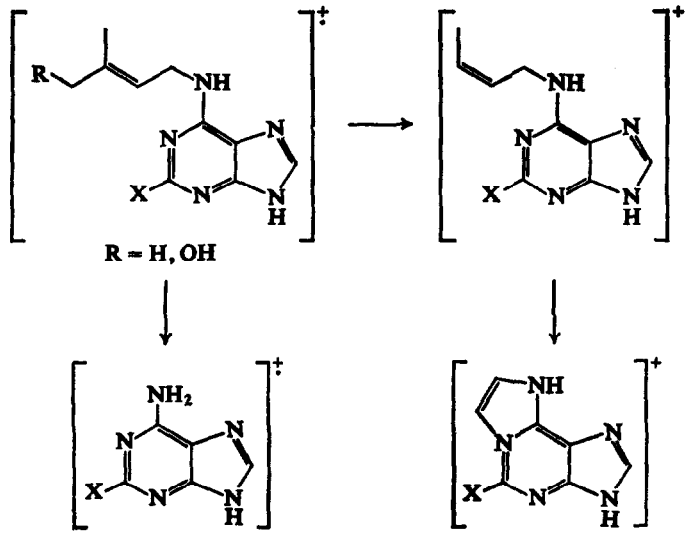
FIG. 2. FRAGMENTS OF 2-AMINO-6-(3-METHYL-2-BUTENYLAMINO)-9-β-D-RIBOFURANOSYLPURINE (IIa) DETERMINED BY MASS SPECTROMETRY AT 70 eV.

Quantitative comparisons of activities of the ribosides and their respective bases are complicated by permeability differences and possible release of free bases from the ribosides during the course of the assay.

Gefter and Russell,[19] comparing the UAG- and UAU-dependent binding to ribosomes of *Escherichia coli* su_{II}^{+} and su_{III}^{+} $tRNA^{Tyr}$ species containing either adenosine, or 2iPA, or (presumably) ms2iPA in the site adjacent to the 3′ end of the anticodon, found that the latter two species were 4 and 7 (with UAG), or 3 and 4 times (with UAU) as efficiently bound to the

[19] M. L. GEFTER and R. L. RUSSELL, *J. Mol. Biol.* 39, 145 (1969).

ribosomes, respectively, as the adenosine-containing species. They concluded that "the modification of the base adjacent to the anticodon in $tRNA^{Tyr}$ is essential for its efficient binding to ribosomes but not for its efficient charging".



SCHEME 1. GENERALIZED FRAGMENTATION PATTERN FOR 2-SUBSTITUTED-6-(HYDROXY) ISOPENTENYLAMINOPURINES AND RIBOSIDES.

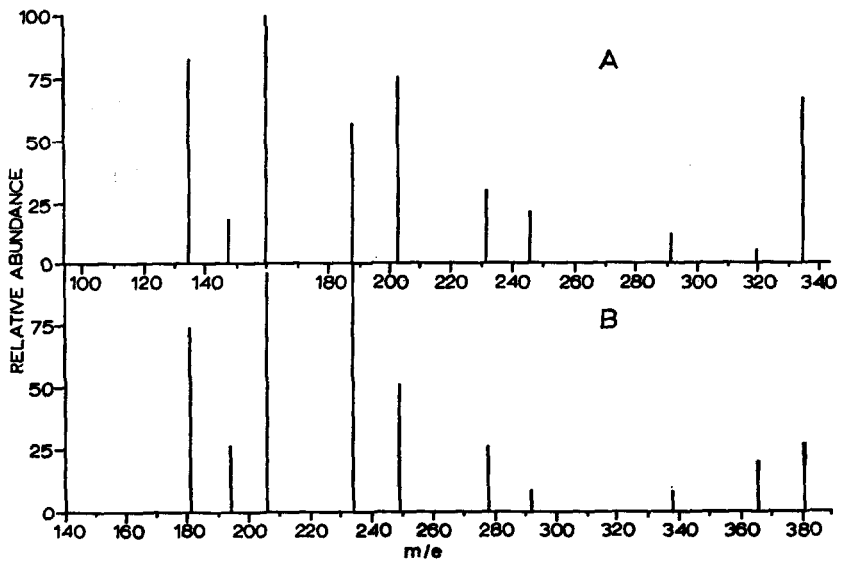


FIG. 3. MASS SPECTRAL COMPARISON AT 70 eV OF (A) 6-(3-METHYL-2-BUTENYLAMINO)-9-β-D-RIBOFURANOSYLPURINE (2iPA) AND (B) 6-(3-METHYL-2-BUTENYLAMINO)-2-METHYLTHIO-9-β-D-RIBOFURANOSYLPURINE (ms2iPA).

If cytokinins have a function as constituents of tRNA in promoting plant growth, the increased binding efficiency associated with presence of ms2iPA and 2iPA rather than

adenosine correlates well with the fact that cytokinin activity is associated with ms2iPA and 2iPA, but not with adenosine. In the tobacco bioassay, however, 2-substitution as in ms2iPA lowers the activity from that observed for 2iPA. Since the test systems are complex and different properties of the molecules may be limiting, the failure to obtain quantitatively parallel effects does not exclude a common function for the cytokinin in the two tests.

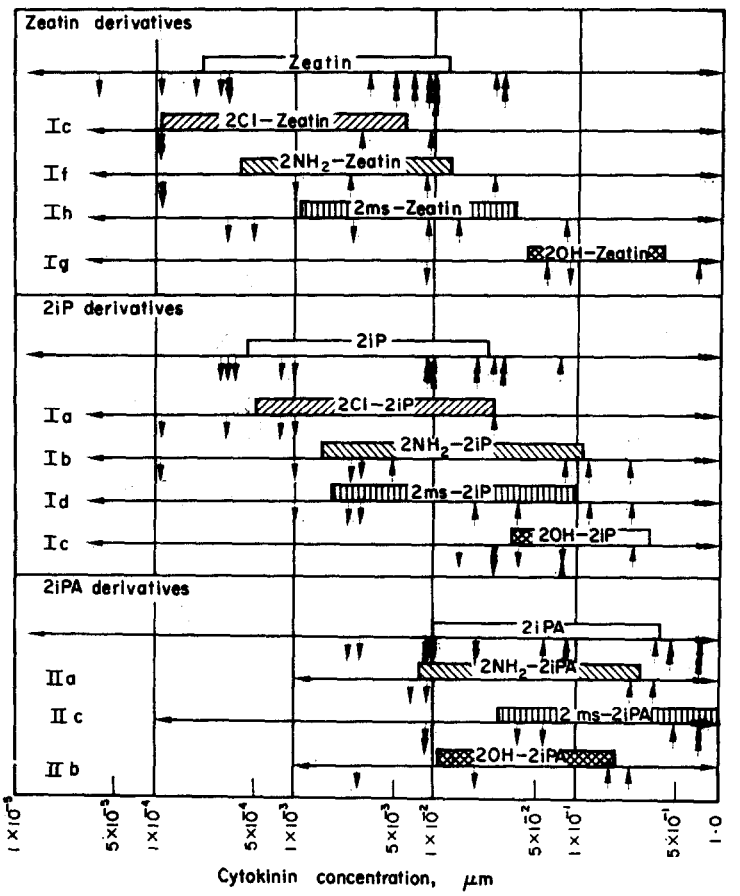


FIG. 4. RELATIVE CYTOKININ ACTIVITIES OF 2,N^6-DISUBSTITUTED ADENINES AND ADENOSINES.

The bars represent average values of the range in which growth increases as a linear function of the log of concentration. The base lines represent tested concentration ranges, and the arrows under the base lines represent the start and end points of the linear growth response in individual experiments. Numbers have been substituted when more than three arrows occur at one point.

EXPERIMENTAL

Synthesis of Test Substances

Syntheses of the following compounds have been reported elsewhere: 2-amino-6-(3-methyl-2-butenylamino)purine (Ib),[12] 2-hydroxy-6-(3-methyl-2-butenylamino)purine (Ic),[12] 6-(3-methyl-2-butenylamino)-2-methylthiopurine (Id),[1,2] 2-amino-6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (IIa),[12] and 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (IIc).[1,2] Compounds Ic, IIa and IIc were characterized by mass spectra in addition to the usual comparison of observed and published physical properties in each case. Identifying u.v. spectra for the new compounds are listed in Table 1.

TABLE 1. ULTRAVIOLET ABSORPTION SPECTRA

	pH 1				pH 7				pH 12			
	λ_{max}^{EtOH}		λ_{min}		λ_{max}^{EtOH}		λ_{min}		λ_{max}^{EtOH}		λ_{min}	
Compound	nm	$\epsilon \times 10^{-3}$	nm	$\epsilon \times 10^{-3}$	nm	$\epsilon \times 10^{-3}$	nm	$\epsilon \times 10^{-3}$	nm	$\epsilon \times 10^{-3}$	nm	$\epsilon \times 10^{-3}$
Ia	273	(15·1)	234	(2·0)	272	(18·6)	231	(2·0)	278	(17·2)	246	(2·8)
Ie	273	(17·1)	232	(2·8)	272	(19·2)	231	(2·7)	278	(17·8)	243	(4·1)
If	282	(12·9)	262	(7·6)	282	(12·3)	262	(6·8)	289	(11·8)	256	(4·1)
	248	(13·0)	236	(11·3)	248	(10·7)	237	(9·9)				
Ig	289	(16·1)	252	(2·9)	284	(12·0)	260	(6·6)	288	(17·5)	256	(4·1)
					243	(11·5)	233	(10·5)				
Ih	292	(17·4)	276	(13·6)	279	(17·5)	257	(11·2)	286	(16·5)	256	(6·7)
	252	(24·2)	216	(9·3)	241	(28·4)	219	(9·3)	231	(34·0)	221	(29·1)
IIc	282	(10·5)	248	(3·3)	272	(13·0)	233	(3·2)	272	(13·5)	237	(4·7)
					279	(sh)			279	(sh)		

2-Chloro-6-(3-methyl-2-butenylamino)purine (*Ia*). To 1·01 g (5·3 mmoles) of 2,6-dichloropurine was added 4·41 g (52 m-moles) of 3-methyl-2-butenylamine, and the solution was heated at reflux for 45 min. The cooled product was treated with H_2O and filtered. Trituration of the solid with portions of hot EtOH afforded white crystals, m.p. 248–249° dec., yield 0·87 g (69%). (Found: C, 50·79; H, 5·33; N, 29·47. Calc. for $C_{10}H_{12}ClN_5$: C, 50·53; H, 5·09; N, 29·47%.)

2-Amino-6-(3-methyl-2-butenylamino)purine (*Ib*) *Hydrochloride*. This compound was made by a different procedure from that employed by Myles and Fox.[12] To 1·70 g (10 mmoles) of 2-amino-6-chloropurine was added 8·00 g (94 mmoles) of 3-methyl-2-butenylamine. The solution was heated at reflux for 12 hr and the cooled product was treated with EtOH. Saturation of the ethanolic solution with Et_2O caused the precipitation of about 10 mmoles of 3-methyl-2-butenylamine hydrochloride, which was removed by filtration. Removal of excess Et_2O under diminished pressure followed by treatment of the ethanolic mother liquors with conc. HCl afforded white crystals, which were isolated by filtration, yield 2·08 g (82%), m.p. 249–250·5° (lit.[12] 250–251°).

2-Chloro-6-(4-hydroxy-3-methyl-trans-2-butenylamino)purine (*Ie*). To 378 mg (2·0 mmoles) of 2,6-dichloropurine was added 2·52 g (25 mmoles) of crude 4-hydroxy-3-methyl-*trans*-2-butenylamine.[20] The solution was heated at reflux for 3 hr and the cooled product was purified by chromatography over 50 g of silica gel, with elution by an ethyl acetate–EtOH gradient. The desired fraction was concentrated and crystallized from absolute EtOH to give white crystals, yield 182 mg (36%), m.p. 226·5–227·5°. (Found: C, 47·22; H, 4·86; N, 27·78. Calc. for $C_{10}H_{12}ClN_5O$: C, 47·35; H, 4·77; N, 27·60%.)

2-Amino-6-(4-hydroxy-3-methyl-trans-2-butenylamino)purine (*If*) *Hydrochloride*. To 340 mg (2·0 mmoles) of 2-amino-6-chloropurine was added 3·0 g (30 mmoles) of crude 4-hydroxy-3-methyl-*trans*-2-butenylamine. The solution was heated at reflux for 24 hr and the cooled product was acidified with conc. HCl, concentrated under diminished pressure, and purified by chromatography over 50 g of silica gel, with elution by an ethyl acetate–EtOH gradient. The fraction containing the desired product was concentrated to a small volume, which yielded crystalline material on standing. Recrystallization from EtOH–Et_2O with decolorization gave white crystals, m.p. 228·5–229·5°; yield 37 mg (7%); $C_{10}H_{15}ClN_6O$: $[(M\text{-}HCl)^+$ calculated 234·1229; found 234·123]; mass spectrum *m/e* 219·097, 202·094, 175·075, 150·067, 135·053, 134·048.

2-Hydroxy-6-(4-hydroxy-3-methyl-trans-2-butenylamino)purine (*Ig*). To 0·51 g (2·8 mmoles) of 2-hydroxy-6-methylthiopurine was added 3·54 g (35 mmoles) of crude 4-hydroxy-3-methyl-*trans*-2-butenylamine and 5 ml EtOH. The mixture was heated at reflux for 3 hr and the white solid that formed on cooling was filtered and triturated with 200 ml of boiling EtOH, m.p. 285-286° dec.; yield 178 mg (27%); $C_{10}H_{13}N_5O_2$ (M^+ calculated 235·1069; found 235·106); mass spectrum: *m/e* 220·086, 204·087, 176·058, 164·058, 151·051, 135·031 $(C_5H_3N_4O)^+$, 119·038.

6-(4-Hydroxy-3-methyl-trans-2-butenylamino)-2-methylthiopurine (*Ih*). To 309 mg (1·5 mmoles) of 2,6-*bis*-(methylthio)purine[21] was added 2·02 g (20 mmoles) of crude 4-hydroxy-3-methyl-*trans*-2-butenylamine.

[20] G. SHAW, B. M. SMALLWOOD and D. V. WILSON, *J. Chem. Soc.* 921 (1966).
[21] J. A. MONTGOMERY, L. B. HOLUM and T. P. JOHNSTON, *J. Am. Chem. Soc.* **81**, 3963 (1959).

The mixture was heated at reflux for 24 hr and the cooled product was purified by chromatography over 250 g of silica gel. Elution with ethyl acetate removed all traces of starting material from the column and was followed by elution with 3:1 ethyl acetate–EtOH, which removed the desired compound. Recrystallization from aqueous EtOH with decolorization afforded a white solid, m.p. 218–221°, yield 34 mg (9%). (Found: C, 49·98; H, 5·62. Calc. for $C_{11}H_{15}N_5OS$: C, 49·79; H, 5·70%.)

2-Hydroxy-6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (IIc). To 30 ml of 50% aqueous EtOH was added 657 mg (3 mmoles) of 2-hydroxy-6-(3-methyl-2-butenylamino)purine and 120 mg (3 m-moles) NaOH. To the hot solution was added a hot solution of 816 mg (3 mmoles) of $HgCl_2$ in 15 ml EtOH.[13] The combined solution was cooled and the precipitate was filtered and dried, yield 1·34 g (98%).

To a solution of 1·65 g of 1-acetyl-2,3,5-tribenzoylribofuranose in 3 ml CH_2Cl_2 was added 9 ml of 30–32% HBr in acetic acid. The solution was allowed to stand for 1·5 hr and was then concentrated under diminished pressure and co-distilled with five portions of dry toluene.

The mercury salt was dispersed in 30 ml of boiling xylene and the sugar, dissolved in 30 ml of xylene, was added. The mixture was heated at reflux for 1 hr and the cooled solution was reduced in volume and saturated with 100 ml of light petroleum. The suspension was refrigerated for several hours and the solid product was filtered and triturated with hot $CHCl_3$. The $CHCl_3$ extract was washed with 30% KI and H_2O and dried (Na_2SO_4). The dried solution was concentrated to a reddish liquid, fractionated over 500 g of silica gel, eluted with ether, and the desired fraction was debenzoylated with 100 ml of anhydrous 15 *N* methanolic NH_4OH in a pressure bottle. Chromatography of the free nucleoside over 750 g of cellulose and elution with EtOH were followed by crystallization from ethanol–ether to give white crystalline material, m.p. 101–104°, in low yield; $C_{15}H_{21}N_5O_5$ (M^+ calculated 351·1542; found 351·158); mass spectrum: *m/e* 219·111, 204·089, 176·055, 164·057, 151·052, 135·029 $(C_5H_3N_4O)^+$, and 135·054 $(C_5H_5N_5)^+$.

Bioassay Procedures

Cytokinin activity was determined on the basis of fresh weight yields in the tobacco callus bioassay.[22] Small pieces of stock tobacco callus were cultured on media containing sufficient nutrients for sustained growth (including 2 mg/l of indole-3-acetic acid), but lacking a cytokinin; to these media were added three-fold serial dilutions of each test substance over a concentration range extending below the lowest biologically-detectable amount and well beyond that resulting in optimal callus growth. Three pieces of callus weighing about 40 mg each were placed on 50 ml of medium in each flask, and four replicate cultures per treatment were used. The measurements of activity, the number of tests, etc. were as indicated in the legend to Fig. 4.

To minimize degradation by heat the chemicals were dissolved in dimethylsulfoxide (DMSO) and added to the autoclaved media after they had cooled nearly to the gelation point. By this procedure filter sterilization could be omitted. The final concentration of DMSO in the media was uniformly 0·5 ml/l; this concentration of DMSO was well within the range which can be used without affecting the yield of tissue.[23]

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[23] R. Y. SCHMITZ, unpublished results.