Phytochemistry, 1972, Vol. 11, pp. 1603 to 1610. Pergamon Press. Printed in England.

# COMPARISON OF CYTOKININ ACTIVITIES OF NATURALLY OCCURRING RIBONUCLEOSIDES AND CORRESPONDING BASES*

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(*Received* 9 *October* 1971)

**Abstract**—Cytokinin activities in the tobacco bioassay have been determined for four adenosine derivatives known to be components of wheat germ *t*RNA: 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine, and 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine. Also determined and compared with the four natural components of *t*RNA were the activities of the four 3-methylbutylamino analogs of the naturally occurring species and the eight substituted purines corresponding to both sets of ribonucleosides. The systematic structural modifications within this group of sixteen compounds were reflected in the variations in cytokinin activity with the level of modification.

## INTRODUCTION

CYTOKININ activity has been found in ribonucleoside components of the *t*RNA of organisms ranging from bacteria and yeasts to higher plants and animals.[1] This activity, in cases in which the structure of specific *t*RNA's has been determined, has been found to correspond to modified adenosines located adjacent to the 3'-end of the anticodon. The presence of these modified ribonucleosides has been limited to species of *t*RNA responding to codons beginning with uridine.

6-(3-Methyl-2-butenylamino)-9-β-D-ribofuranosylpurine [$N^6$-($\Delta^2$-isopentenyl)adenosine (VI)] was the first cytokinin[2,3] to be identified as a component of *t*RNA[4,5] when it was shown to be located adjacent to the 3'-end of the anticodons in two species of *t*RNA$^{Ser}$ in yeast.

* Supported at the University of Wisconsin by NSF Research Grant GB 25812 and by the Research Committee of the Graduate School with funds from the Wisconsin Alumni Research Foundation. Supported at the University of Illinois by NIH Research Grant GM-05829 and GM-12395.

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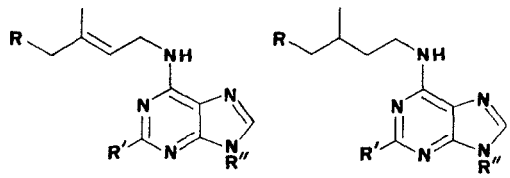
It has also been found in *t*RNA's from many other sources including bacteria, plant tissue, and animal tissue.[6–19]

6-(3-Methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (VIII) has been isolated and identified in *t*RNA from *E. coli*.[12,14] By chromatographic separation and bioassays of cytokinin-active ribonucleosides it has been shown to be present in tRNA species corresponding to each of six amino acids whose codons start with uridine, i.e., $tRNA^{Phe}$, $tRNA^{Leu}$, $tRNA^{Ser}$, $tRNA^{Tyr}$, $tRNA^{Cys}$, and $tRNA^{Trp}$, but is not present in all sub-species which respond to these codons.[20] Its presence in $tRNA^{Phe}$ and $tRNA^{Ser}$ was shown independently by Nishimura *et al.*[21] Its position next to the 3'-end of the anticodon in *E. coli* $tRNA^{Tyr}$ was determined by Harada *et al.*,[22] and confirmed by Gefter and Russell.[23] Compound VIII has also been found in the *t*RNA from wheat germ[15,18] and from *Staphylococcus epidermidis*.[13]



| | R | R′ | R″ | |
|---|---|---|---|---|
| I | OH | H | H | IX |
| II | H | H | H | X |
| III | OH | $SCH_3$ | H | XI |
| IV | H | $SCH_3$ | H | XII |
| V | OH | H | $C_5H_9O_4$ | XIII |
| VI | H | H | $C_5H_9O_4$ | XIV |
| VII | OH | $SCH_3$ | $C_5H_9O_4$ | XV |
| VIII | H | $SCH_3$ | $C_5H_9O_4$ | XVI |

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6-(4-Hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine (V, modified to the *cis* isomer) is the structure assigned to the cytokinin isolated from the tRNA of peas, spinach, and corn,[9,24,25] and 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-9-β-D-ribofuranosylpurine (ribosylzeatin) (V) has been isolated from immature corn kernels[26,27] and from chicory root[28] where it occurs free according to the evidence available. Both 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (V, configuration not assigned) and 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (VII, configuration not assigned) have been found in wheat germ *t*RNA in addition to VI and VIII, as noted above,[15,18] and in tobacco callus *t*RNA.[19] Further studies in these laboratories[29] have shown that the ribosylzeatin in the wheat germ *t*RNA and the tobacco callus *t*RNA has the *cis* configuration, while in the pea epicotyl *t*RNA (Vreman *et al.*,[30] unpublished) it exists as a mixture of both the *cis* and *trans* isomers.

Among the natural cytokinins lacking a ribose group, 6-(3-methyl-2-butenylamino)-purine [$N^6$-($\Delta^2$-isopentenyl)adenine (II)] has been identified as the growth-promoting substance in cultures of the plant pathogen *Corynebacterium fascians*.[31,32] 6-(4-Hydroxy-3-methyl-*trans*-2-butenylamino)purine (I) is zeatin, the highly active stimulant of cell division in plant tissue cultures, which was first isolated from milky stage endosperm of sweetcorn (*Zea mays*).[33–38] The saturated side chain derivative, (—)-6-(4-hydroxy-3-methylbutylamino)purine (IX), has been found in immature seeds of *Lupinus luteus*.[39–41]

Thus all naturally occurring cytokinins which are purine derivatives have been found to contain an isopent(en)yl side chain, which may be further substituted with a 4-hydroxyl group, and to sustain possible modification on the ring with 2-methylthio and 9-β-D-ribofuranosyl groups. Since the compounds which are presently known to occur naturally do not include all possible combinations of the four modifications (4-OH, side-chain saturation, 2-$SCH_3$, 9-$C_5H_9O_4$) and since it seem reasonable to expect that some of these 'unknown' types may yet be found in nature, we have accumulated the set of 16 compounds which encompasses all of the possible modifications. We report here on the syntheses and physical properties of the new compounds and the relative activities of all 16 compounds in the tobacco bioassay. In those cases in which there may be more than one configurational isomer, we have arbitrarily tested the *trans* isomer and the racemic mixture

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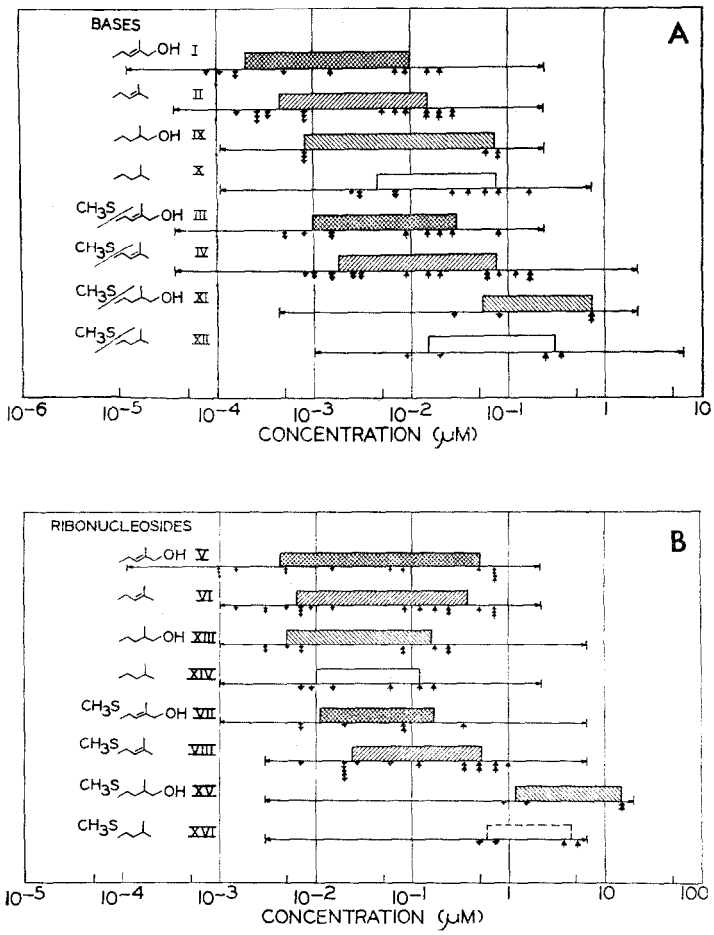


FIG. 1. RELATIVE CYTOKININ ACTIVITIES OF $N^6$-ISOPENT-(EN)YL ADENINE (A) AND ADENOSINE (B) DERIVATIVES.

The compounds are numbered as in the text. For easy reference the substituents on the $N^6$-position, and the methylthio group on the 2-position, are indicated in the margin.

The base lines represent the tested concentration ranges, and the arrows under the base lines represent the start and end points, in individual experiments, of the concentration range over which growth increases as a nearly linear function of the log of concentration of added cytokinin. Bars represent the average range of the linear growth response. A bar outlined by a dashed line indicates that the compound became toxic at a concentration which was too low to support maximal callus growth.

Side chain structure is indicated by shading of the bars as follows: isopentyl group; 4-OH-substituted isopentyl group; Isopentyl group; 4-OH-substituted isopentyl group.

wherever applicable, although we are aware that differences in configuration[42–44] may substantially affect cytokinin activity.

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## RESULTS AND DISCUSSION

The structural features affecting biological activity were, with regard to the side chain, the presence or absence of a double bond in the 2-position and of a hydroxyl group on C-4; with regard to the purine nucleus, the substitution of a methylthio group on the 2-position and a β-D-ribofuranosyl group on the 9-position. The activities of these bases and ribonucleosides are shown in Figs. 1A and B. Growth curves for the eight purine bases as determined in a single experiment are shown in Fig. 2. To simplify the discussion of comparative activities, zeatin (I), the most active of the naturally occurring cytokinins, has been used as a model. The extent to which the above modifications, alone or in combination, depress cytokinin activity can be estimated by noting the concentration range over which the linear relationship between the logarithm of cytokinin concentration and growth response is valid. It can be seen that the relative activities follow certain patterns according to the nature and combination of the modifications.

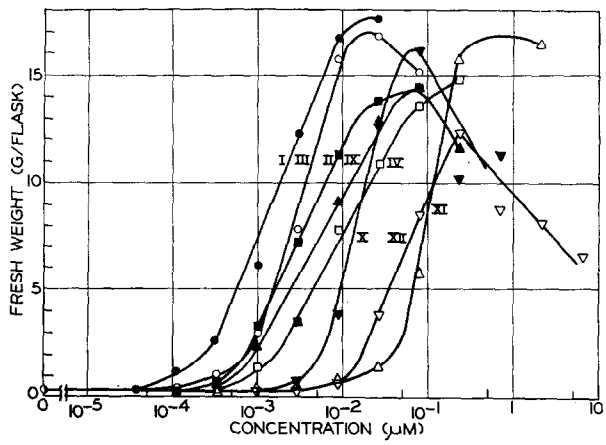


FIG. 2. FRESH WEIGHT YIELD OF TOBACCO CALLUS TISSUE CULTURED ON EIGHT ISOPENT(EN)YL ADENINE DERIVATIVES AS DETERMINED IN A SINGLE EXPERIMENT (C 85, 2–5 to 3–18–70). THE COMPOUNDS ARE NUMBERED AS IN THE TEXT.

### *Double Bond Saturation*

The degree to which saturation lowered activity varied. This effect was marked for all free bases, causing a 4–50-fold reduction in activity. It was even more pronounced for the methylthiolated ribonucleosides, where a 100-fold reduction in activity occurred. In contrast, the non-methylthiolated ribonucleosides showed less than a 2-fold reduction in activity upon saturation of the double bond.

### *Removal of the 4-Hydroxyl Group*

In most cases loss of the hydroxyl group on C-4 resulted in lower activity. In the absence of the methylthio group, the effect was more pronounced for the purine bases, causing about a 3–4-fold decrease, than for the ribonucleosides, where the decrease in activity was 2-fold or less. In the case of compounds combining side chain unsaturation with methylthiolation, the loss was also about 2-fold. In the case of methylthiolated compounds with saturated side chains the removal of the hydroxyl group appears to increase activity as indicated in Fig. 1, but examination of curves XI and XII in Fig. 2 shows that this apparent increase is valid only at the lower end of the activity range. It should be noted that the

results are less systematic for compounds which are only active at high concentrations and which give less than optimal growth at any concentration.

*Addition of the 2-Methylthio Group*

The extent to which methylthiolation of the 2-position of the purine nucleus lowered activity was dependent on other modifications. It was most markedly affected by saturation of the side chain. In the presence of the isopentenyl side chain, loss of activity was only 3–5-fold; but in the absence of the double bond, except in the case of compound XII, the loss in biological activity was about 100-fold.

*Effect of Ribosidation*

In comparing results of bioassays of ribonucleosides it should be noted that the measured activities may in fact derive in part or wholly from the dissociated active bases. The ribonucleosides were all less active than their corresponding purine bases and, with one exception, at least by a factor of eight. The mere 2-fold loss in activity by the ribonucleoside (XIV) of unsubstituted 6-isopentylaminopurine (X) appeared to be due to the relatively low activity of this purine base, as compared with the others, rather than to a relatively high activity of the ribonucleoside. On the logarithmic scale as presented, the ribonucleosides generally differed less in activity among themselves than did the corresponding purines.

On the whole, the relative activities of these sixteen compounds agree with expectations based on previous reports. The enhancement of activity by the formal addition of a hydroxyl group in the 4-position of the isopent(en)yl side chain, the markedly lower activity of compounds with a saturated side chain, the consistently lower activity of the ribonucleosides as compared with the corresponding purine bases[45,46] and the reduction in activity accompanying methylthiolation of the 2-position of the purine nucleus[47] were all consistent with earlier findings.

The effects which are of particular interest are those which deviate from the expected pattern. It appears that any single modification of I, except ribosidation, caused less than a 10-fold reduction in activity. The markedly larger size of the ribonucleosides, as compared with the purines, may produce secondary effects, such as decrease in permeability, which in themselves could obscure their effectiveness as cytokinins.

When two or more modifications occurred simultaneously, the decrease in activity was cumulative. The combination of two factors which caused by far the greatest loss in activity was double bond saturation and methylthiolation. When combined with a third (loss of the 4-hydroxyl group), however, activity appeared to be partially restored in terms of minimum concentration of cytokinin required for a detectable growth response (compounds XII and XVI), but neither of these two compounds was capable of eliciting maximal callus growth over the range of active concentrations. Negative factors may cease to have a cumulative effect when the molecule has been altered so drastically that it is unable to function with full effectiveness as a cytokinin at any concentration.

Present evidence indicates that the modified adenosines are located in a specific site in a particular class of *t*RNA species. It has been shown that while modification of the adenosine located adjacent to the 3′-end of the anticodon does not alter the ability of the *t*RNA molecule to recognize the proper amino acid, it is reported to have an effect on the

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ability of the *t*RNA-amino acid complex to bind to the ribosomal ribonucleic acid.[23,48] Whether the effectiveness of these compounds as cytokinins is directly related to their presence and role in the *t*RNA molecule has not been demonstrated. In contrast to its reported effect on ribosomal binding,[23] the methylthio groups tended to decrease cytokinin activity in the tobacco bioassay (see especially VIII vs. VI). The magnitude of this effect was small, however, and this comparison alone does not provide sufficient evidence to rule out the possibility that the site of action of the cytokinins is in fact at the *t*RNA level.

It is clear from the present findings that formal modifications of zeatin (I) by deoxidation, hydrogenation, and methylthiolation (II–IV, IX–XII), and especially by ribosidation (V–VIII, XIII–XVI), lead to rather systematic decrements in its biological activity. The set of sixteen compounds here examined provides an additional basis for predicting activity as a function of structural modification. Finally, characterization of the previously 'unknown' members by physical means and by relative activities should be useful for identification of any further cytokinins which may be isolated from natural sources.

## EXPERIMENTAL

### *Bioassay Procedures*

The tobacco bioassay, which is based on the increase in fresh weight yield of cytokinin-dependent tobacco callus, was used as the measure of biological activity.[45] In order to avoid possible degradation of the compounds by heat and to improve solubility, each of the compounds was dissolved in dimethyl sulfoxide in a series of three-fold dilutions covering the range of cytokinin activity, and small aliquots were then added to the cooling agar media. The final concentration of dimethyl sulfoxide did not exceed 0·02% (v/v), which does not affect biological activity in this assay system.[49] The range of concentrations over which there was a nearly linear relationship between increase in the fresh weight of the tissue and logarithmic increase in cytokinin concentration was determined. Each compound was tested at least twice. The average values have been represented as bars for ease of comparison of the sixteen compounds (see Fig. 1).

### *Synthesis of Test Substances*

6-(4-*Hydroxy*-3-*methylbutylamino*)-2-*methylthiopurine* (XI). To 212 mg (1·0 m-mol) of 2,6-bis (methylthio)purine[50] was added 825 mg (8·0 m-mol) of 4-hydroxy-3-methylbutylamine.[51] The solution was heated at reflux for 1 hr under $N_2$. The residue was purified by chromatography over 100 g of silica gel, elution with EtOAc–EtOH, followed by chromatography of the appropriate fraction on neutral alumina, elution with EtOAc–EtOH. The appropriate fraction was concentrated and the solid product was recrystallized from aqueous EtOH to afford white crystals of XI, yield 115 mg (43%), m.p. 212·5–214·0°; $C_{11}H_{17}N_5SO$ ($M^+$ calculated 267·1153; found: 267·115); $\lambda_{max}^{EtOH}$ (pH 1) 291 ($\epsilon$ 15600) and 252 nm (21100), $\lambda_{min}$ 275 nm (12100); $\lambda_{max}^{EtOH}$ (pH 7) 279 (15900) and 240 nm (24600), $\lambda_{min}$ 256 nm (9900); $\lambda_{max}^{EtOH}$ (pH 12) 285 nm (14600), $\lambda_{min}$ 256 nm (5600).

6-(3-*Methylbutylamino*)-2-*methylthiopurine* (XII). To 212 mg (1·0 m-mol) of 2,6-bis (methylthio)purine was added 4 ml of isopentylamine. The solution was heated at reflux for 24 hr and evaporated to dryness. The residue was crystallized from EtOH to afford white crystals of XII, yield 101 mg (40%), m.p. 261–263°; $\lambda_{max}^{EtOH}$ (pH 1) 292 ($\epsilon$ 15500) and 251 nm (21700), $\lambda_{min}$ 275 nm (11600); $\lambda_{max}^{EtOH}$ (pH 7) 279 (15800) and 241 nm (24600), $\lambda_{min}$ 256 nm (9400); $\lambda_{max}^{EtOH}$ (pH 12) 286 nm (15200), $\lambda_{min}$ 256 nm (6300). Anal. (Calc. for $C_{11}H_{17}N_5S$: C, 52·56; H, 6·82. Found: C, 52·50; H, 6·78%).

6-(4-*Hydroxy*-3-*methylbutylamino*)-2-*methylthio*-9-*β*-D-*ribofuranosylpurine* (XV). To 344 mg (1·0 m-mol) of 2,6-bis (methylthio)-9-*β*-D-ribofuranosylpurine[52] was added 950 mg (9·2 m-mol) of 4-hydroxy-3-methylbutylamine. The solution was heated at reflux under $N_2$ for 1·5 hr. The cooled product was purified by chromatography over 600 g of cellulose, elution with EtOH, followed by chromatography of the appropriate fraction over 30 g of Sephadex LH-20, elution with 35% EtOH. The product was recrystallized from EtOH

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to afford white crystals of XV in low yield, m.p. 156–158°; $C_{16}H_{25}N_5O_5S$ ($M^+$calculated: 399·1577; found: 399·158); $\lambda_{max}^{EtOH}$ (pH 1) 285 (ε16800) and 245 nm (18500), $\lambda_{min}$ 262 nm (13900); $\lambda_{max}^{EtOH}$ (pH 7) 283 (18200) and 243 nm (25100), $\lambda_{min}$ 257 nm (9500); $\lambda_{max}^{EtOH}$ (pH 12) 283 (18200) and 243 nm (25100); $\lambda_{min}$ 257 nm (9500); MS: *m/e* 399·158, 310·134, 296·120, 267·118, 249·103, 236·095, 208·066, 181·040.[15,46,47,53] Compound XI was also isolated in low yield as a side product.

6-(3-*Methylbutylamino*)-2-*methylthio*-9-β-D-*ribofuranosylpurine* (XVI). To 482 mg (1·4 m-mol) of 2,6-bis-(methylthio)-9-β-D-ribofuranosylpurine was added 5 ml of isopentylamine. The solution was heated at reflux under $N_2$ for 5 hr. The cooled product was evaporated to dryness and purified by chromatography over 600 g of cellulose, elution with EtOH, followed by chromatography of the appropriate fraction over 30 g of Sephadex LH-20, elution with 35% EtOH. The product was recrystallized from EtOH–$H_2O$ to afford white crystals of XVI, yield 152 mg (28%), m.p. 134–135°; $C_{16}H_{25}N_5O_4S$ ($M^+$ calculated: 383·1626; found: 383·163); $\lambda_{max}^{EtOH}$ (pH 1) 285 (ε17100) and 245 nm (19800), $\lambda_{min}$ 263 nm (14100), $\lambda_{max}^{EtOH}$ (pH 7) 283 (18800) and 243 nm (25800), $\lambda_{min}$ 257 nm (10000); $\lambda_{max}^{EtOH}$ (pH 12) 283 (19100) and 242 nm (26000), $\lambda_{min}$ 257 (9700); MS: *m/e* 383·163, 294·139, 280·123, 251·120, 194·050, 181·041.[15,46,47,53]

The syntheses of the following compounds have been reported previously: I,[38] II,[54,55] III,[47] IV,[12,14] V,[58] VI,[3] VII,[15,18] VIII,[12,14] IX,[39,40] X,[53,56] XIII,[46] and XIV.[57]

*Acknowledgement*—We wish to thank Mr. James Chickering for technical assistance with the bioassays.

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*Key Word Index*—Cytokinins; plant hormones, activity comparisons; natural ribonucleosides.