*CYTOKININS: SYNTHESIS, MASS SPECTRA, AND BIOLOGICAL ACTIVITY OF COMPOUNDS RELATED TO ZEATIN**

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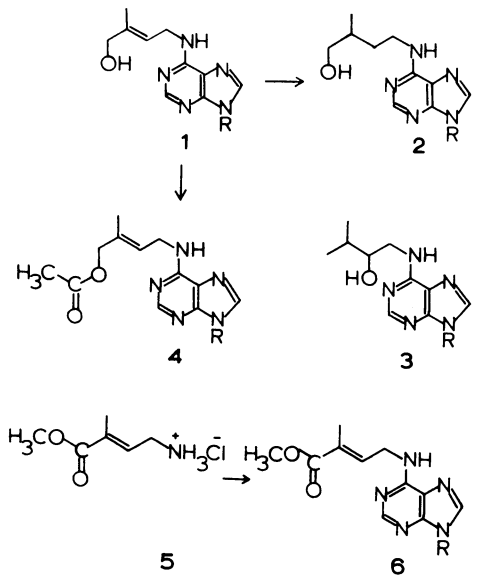
Abstract.—Compounds related to dihydrozeatin that define the influence of the location of the hydroxyl group along the side chain have been synthesized and tested for cytokinin activity. The compounds compared are in the series: 6-(X-hydroxy-3-methylbutylamino)purines and their ribosides, where X = 2, 3, and 4.

Hydroxy substitution on the 4-position of the side chain enhances, but in the 2-, 3-, or 2- and 3- positions, decreases cytokinin activity as compared with the unsubstituted isopentyl (or isopentenyl) chains. This differential influence of the position of the hydroxyl group in the N^6-chain holds also for the similarly related 9-β-D-ribofuranosides. The relatively higher activity of 3,4-dihydroxy as compared with 2,3-dihydroxy derivatives is consistent with this position effect.

Compounds related to zeatin possessing side-chain ester moieties have also been synthesized and tested comparatively. Among these, 6-(4-acetoxy-3-methyl-*trans*-2-butenylamino)purine is at least as active as zeatin, the most active presently known cytokinin in the tobacco bioassay, whereas the analog, methyl 2-methyl-4-(purin-6-ylamino)-*trans*-crotonate, with the ester function effectively reversed, has vastly lower activity, and its riboside is practically inactive.

Zeatin, 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine (**1a**),[1-3] cell-division factor isolated from young sweet corn (*Zea mays*) kernels,[1-9] is the most active cytokinin[10-13] tested to date in the tobacco bioassay.[14, 15] Only slightly lower activity is shown by 6-(3-methyl-2-butenylamino)purine (alternate names: 6-(γ,γ-dimethylallylamino)purine and 6-(Δ^2-isopentenylamino)purine) (2iP),[16, 17] which has been found as the free base in culture filtrates of *Corynebacterium fascians*.[18, 19] The ribosides of both substituted purines have also been found in nature.[6, 20-31] In a continuing investigation of structure and biological activity,[11, 14] we have synthesized a number of substituted 6-isopent(en)ylaminopurines and their ribosides and have studied the effect of systematic variation in the side chain.[15, 32, 33] We have now determined, for a series of compounds related to dihydrozeatin, the influence on cytokinin activity of the location of the hydroxyl group along the side chain, and, for compounds related to zeatin, the effect of the presence of ester groupings in the side chain.

The synthesis and testing of 6-(3-hydroxy-3-methylbutylamino)purine (iP(HOH)) and its riboside, 6-(3-hydroxy-3-methylbutylamino)-9-β-D-ribofuranosylpurine (iP(HOH)A), have been reported previously.[15, 33] The isomeric base and riboside pair, 6-(4-hydroxy-3-methylbutylamino)purine (**2a**)[34, 35] and 6-(4-hy-



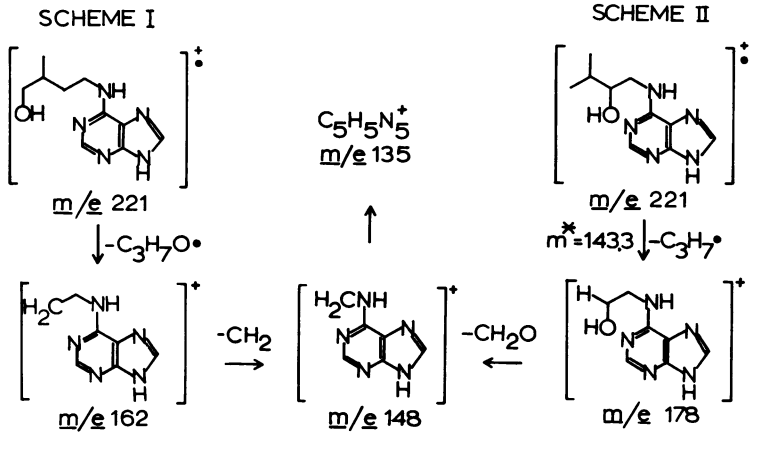
a. R=H; b. R=$C_5H_9O_4$ (β-D-RIBOFURANOSYL)

droxy-3-methylbutylamino)-9-β-D-ribofuranosylpurine (**2b**) were obtained by hydrogenation of zeatin (*trans*) (**1a**) and zeatin riboside (**1b**),[29] respectively, over 5 per cent palladium-on-carbon. (−)-Dihydrozeatin is naturally occurring, having been isolated by extraction of immature lupin seeds (*Lupinus luteus*).[34, 35] Compound **2b** was recrystallized from ethanol as colorless crystals, mp 167–169°, yield 46 per cent; λ_{max}^{EtOH}(pH 1) 265 mμ (ε 16,500), λ_{min}234 (2,400); λ_{max}^{EtOH}(pH 7) 268 (17,000), λ_{min} 229 (200); λ_{max}^{EtOH}(pH 10) 268 (17,200), λ_{min}232 (1,200); $[\alpha]_D^{25}$ −20° (*c* 0.65, EtOH); NMR δ from TMS (DMSO-d_6-D_2O): 0.91 (3H, d, CH_3—C), 1.62 (3H, m, CH_2-CH), 3.28 (2H, m, CH_2—O), 3.6 (4H, m, CH_2—N and C-5′ protons), 4.08 (2H, m, C-3′ and C-4′ protons), 4.56 (1H, m, C-2′ proton), 5.83 (1H, d, C-1′ proton), 8.07, 8.18 (2H, s, Ad-$C_{2,8}$-H's). (*Analysis:* Calculated for $C_{15}H_{23}N_5O_5$: C, 50.98; H, 6.56; N, 19.82. Found: C, 51.29; H, 6.74; N, 19.59.)

6-(2-Hydroxy-3-methylbutylamino)purine (**3a**) and 6-(2-hydroxy-3-methylbutylamino)-9-β-D-ribofuranosylpurine (**3b**) were synthesized according to the following sequence. Isobutyraldehyde cyanohydrin was prepared in theoretical yield according to the method of DeLaet,[36] and the crude product was treated with 2 moles of lithium aluminum hydride in ether solution for one hour. After destruction of the excess reducing agent, the ether solution of the product was dried, filtered, and treated with gaseous hydrogen chloride. The precipitate was filtered, dried, and recrystallized from ethanol-ether as colorless crystalline 2-hydroxy-3-methylbutylamine hydrochloride, mp 109.5–110.5°, yield 61 per cent; NMR δ (DMSO-d_6): 0.91 (6H, d, $(CH_3)_2C$), 1.68 (1H, m, C—CH—C), 2.82 (2H, m, C—CH_2—N), 3.59 (1H, m, C—CH—O). (*Analysis:* Calculated for $C_5H_{14}ClNO$: C, 43.01; H, 10.11; N, 10.03. Found: C, 42.99; H, 10.15; N, 10.23).

Condensation of 2-hydroxy-3-methylbutylamine hydrochloride with 6-chloropurine was effected in *n*-butanol and triethylamine at reflux for one hour. The cooled reaction mixture was concentrated under diminished pressure, and the residue was chromatographed over silica gel. The desired fraction was treated with charcoal and lyophilized successively from water and benzene to afford **3a** as a deliquescent white solid in theoretical yield, mp 140–142° (sealed capillary); $C_{10}H_{15}N_5O$ (M^+ calculated: 221.1277; found: 221.129); λ_{max}^{EtOH} (pH 1) 275 mμ (ε 12,300), λ_{min}235 (2,800); λ_{max}^{EtOH}(pH 7) 268 (13,100), λ_{min}228 (2,000); λ_{max}^{EtOH}(pH 10) 283 (sh), 275 (12,700), λ_{min}241 (2,700); mass spectrum: *m/e* 221.129 (M^+), 178.072, 148.063 (cf. Scheme II); NMR δ (DMSO-d_6-D_2O): 0.95 (6H, m, $(CH_3)_2C$), 1.72 (1H, m, C—CH—C), 3.59 (3H, m, CH_2—CH—O), 8.21, 8.28 (2H, s, Ad-$C_{2,8}$-H's). The riboside **3b** was prepared by condensation of 2-hydroxy-3-methylbutylamine hydrochloride with 6-chloropurine riboside in ethanol and triethylamine at reflux for two hours.[37] The work-up was similar to that employed for the free base **3a**, except that cellulose was employed as the chromatographic support. The product, a white, deliquescent solid, was obtained in 90 per cent yield, mp 74–76° (sealed capillary); $C_{15}H_{23}N_5O_5$ ((M-ribose)$^+$ calculated: 221.1277; found: 221.125); λ_{max}^{EtOH}(pH 1) 266 mμ (ε 12,300), λ_{min}237 (2,900); λ_{max}^{EtOH}(pH 7) 267 (12,300), λ_{min}232 (2,000); λ_{max}^{EtOH}(pH 10) 268 (12,400), λ_{min}235 (2,500); mass spectrum: *m/e* 221.125 (M-ribose)$^+$, 250.130, 178.072, 148.063, 119.035 (cf. Scheme II); NMR δ (DMSO-d_6-D_2O): 0.95 (6H, m, $(CH_3)_2C$), 1.74 (1H, m, C—CH—C), 3.67 (5H, m, CH_2—CH—O and C-9′ protons), 4.1–4.2 (2H, m, C-3′ and C-4′ protons), 4.70 (1H, m, C-2′ proton), 5.99 (1H, d, C-1′ proton), 8.30, 8.43 (2H, s, Ad-$C_{2,8}$-H's).

The mass spectra of **2b** and **3b** have been determined. The fragmentation patterns corresponding to these spectra, which may be represented as shown in Schemes I and II, are particularly interesting since they affirm an earlier con-



SCHEMES I and II.—Partial fragmentation pattern for **2b** (Scheme I) and **3b** (Scheme II) as determined by mass spectrometry at 70eV.

clusion[33] that an intense peak at *m/e* 178 is indicative of a 2-hydroxyl group on the isopentyl side chain, while one at *m/e* 162 reflects the absence of this group.

Moreover, the fragment ion at m/e 190 in the spectrum of **2b** corresponding to the loss of CH_2OH•[1, 3, 38] correlates well with earlier findings for 4-hydroxyl groups on the isopentyl side chain.[33]

The synthesis of 6-(4-acetoxy-3-methyl-*trans*-2-butenylamino)purine (zeatin-*O*-acetate) (**4a**)[29] was effected by direct acetylation of zeatin in an application of the general procedure of Lapidot and Khorana,[39] and recrystallization from ethanol-acetonitrile afforded colorless crystals, mp 166–168° (reported[29] 168–169°); yield 45 per cent; λ_{max}^{EtOH}(pH 1) 276 mμ (ϵ 12,700), λ_{min}234 (1,600); λ_{max}^{EtOH}(pH 7) 268 (13,100), λ_{min}227 (100); λ_{max}^{EtOH} (pH 10) 283 (sh), 275 (13,500), λ_{min}241 (2,400); NMR δ (DMSO-d_6): 1.75 (3H, s, CH_3—C), 2.04 (3H, s, CH_3—C=O), 4.23 (2H, m, C—CH_2—N), 4.41 (2H, s, C—CH_2—O), 5.59 (1H, m, C=CH), 8.07, 8.17 (2H, s, Ad-$C_{2,8}$-H's).

The analog of the acetate with the ester function effectively reversed, namely, methyl 2-methyl-4-(purin-6-ylamino)-*trans*-crotonate (**6a**), was obtained from methyl 4-amino-2-methyl-*trans*-crotonate hydrochloride (**5**), triethylamine, and 6-chloropurine in *n*-butanol[37] and was purified by recrystallization from ethanol, mp 224.5–225.5°; yield 18 per cent; λ_{max}^{EtOH}(pH 1) 278 mμ (ϵ 19,300), λ_{min}238 (3,700); λ_{max}^{EtOH}(pH 7) 269 (19,000), λ_{min}233 (3,800); λ_{max}^{EtOH}(pH 10) 283 (sh), 275 (16,000), λ_{min}242 (4,300); NMR δ (DMSO-d_6): 1.88 (3H's, CH_3—C), 3.60 (3H, s, CH_3—O), 4.26 (2H, m, C—CH_2—N), 6.65 (1H, m, C=CH), 7.98, 8.07 (2H, s, Ad-$C_{2,8}$-H's). (*Analysis:* Calculated for $C_{11}H_{13}N_5O_2$: C, 53.44; H, 5.30; N, 28.32. Found: C, 53.63; H, 5.58; N, 28.45.) The corresponding riboside, methyl 2-methyl-4-(9-β-D-ribofuranosylpurin-6-ylamino)-*trans*-crotonate (**6b**), was prepared similarly from **5** and 6-chloropurine riboside, purified by chromatography on cellulose, and recrystallized from ethanol, mp 102–104°; yield 44 per cent; λ_{max}^{EtOH}(pH 1) 269 mμ (ϵ 17,700), λ_{min}238 (4,500); λ_{max}^{EtOH}(pH 7) 269 (19,100), λ_{min}234 (4,100); λ_{max}^{EtOH} (pH 10) 269 (18,900), λ_{min}235 (4,500); $[\alpha]_D^{23}$ −38° (*c* 0.87, dimethylformamide); NMR δ (DMSO-d_6-D_2O): 1.93 (3H, s, CH_3—C), 3.69 (5H, m, CH_3—O and C—CH_2—N), 4.29 (4H, m, C-3′, C-4′, and C-5′ protons), 4.68 (1H, m, C-2′ proton), 5.96 (1H, d, C-1′ proton), 6.72 (1H, t, C=CH), 8.27, 8.37 (2H, s, Ad-$C_{2,8}$-H's). (*Analysis:* Calculated for $C_{16}H_{21}N_5O_6$: C, 50.66; H, 5.58; N, 18.46. Found: C, 50.59; H, 5.56; N, 18.38.) Compound **5**, $C_6H_{12}ClNO_2$, mp 197–199°, was obtained by hydrazinolysis of methyl 4-(N-phthalimido)-2-methyl-*trans*-crotonate, $C_{14}H_{13}NO_4$, mp 119–121°, followed by treatment with hydrochloric acid, and the earlier precursor was made by condensation of methyl γ-bromotiglate[29] with potassium phthalimide in dimethylformamide. The two *trans*-crotonate derivatives were characterized by microanalyses and NMR spectra.

For comparison with 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (2iPA), with variation of hydroxyl substitution in the sugar moiety, 6-(3-methyl-2-butenylamino-9-(2-deoxy-β-D-ribofuranosyl)purine (2iPdA) was synthesized by 1-alkylation of 2′-deoxyadenosine with γ,γ-dimethylallyl bromide and rearrangement of the 1-substituted deoxyadenosine intermediate to the N^6-substituted product, following the procedure first used[30] for the synthesis of 2iPA. The 2iPdA was obtained as a white hygroscopic solid, mp 48–49° (sealed, evacuated capillary); λ_{max}^{EtOH}(pH 1) 265 mμ (ϵ 16,300), λ_{min}234 (4,100);

λ_{max}^{EtOH}(pH 7) 267 (16,300), λ_{min}229 (2,800); λ_{max}^{EtOH}(pH 10) 267 (16,600), λ_{min}232 (3,100); $[\alpha]_D^{25}$ −9.9° (*c* 0.81, EtOH); NMR δ (DMSO-d_6-D_2O): 1.73 (6H, s, $(CH_3)_2$-C), 3.66 (2H, m, C-5′ protons), 4.08 (4H, m, C—CH_2—N, C-3′ and C-4′ protons), 4.55 (2H, m, C-2′ protons), 5.36 (1H, m, C—CH=C), 6.45, (1H, m, C-1′ proton), 8.28, 8.37 (2H, s, Ad-$C_{2,8}$-H's). (*Analysis:* Calculated for $C_{15}H_{21}N_5O_3 \cdot H_2O$: C, 53.40; H, 6.87; N, 20.76. Found: C, 53.33; H, 6.98; N, 20.90.)

Cytokinin Activity.—Cytokinin activity was determined on the basis of fresh weight yields in the tobacco bioassay.[40] Aqueous solutions of the compounds to be tested were filter-sterilized and added to the autoclaved media, which had been cooled to near the gelation point. The methyl crotonate (**6a**) and the zeatin-*O*-acetate (**4a**), which have low solubility in water, were dissolved in small volumes of DMSO and added to the nutrient medium to give a maximum final concentration of 0.8 ml/liter DMSO at the highest concentration of the former, and 0.04 ml/liter at the highest concentration of the latter compound. All other test substances were dissolved in cold water.

The results of individual experiments have been represented by curves in which the fresh weight yield of tissue is plotted against the logarithm of the cytokinin concentration (Fig. 1). A nearly linear relationship is obtained starting soon after the point at which activity can first be detected and continuing nearly to the point of maximum yield. This linear range has been determined in several experiments and the average value is presented as a bar for convenience in graphing the relative activities (Fig. 2*a* and *b*). The extended base line of the bar represents the tested concentration range. The number of experiments on which each bar is based and the degree of variation may be judged by the arrows inserted below the base line indicating the start and end points of the linear ranges in individual experiments. The amount of tissue produced by each compound at its optimum concentration is generally the same. Exceptions are the weakly active crotonate (**6a**) and its riboside (**6b**). It should be noted that the minimum detectable concentration, used as an index of activity in earlier reports, is lower than the starting point of the bar.

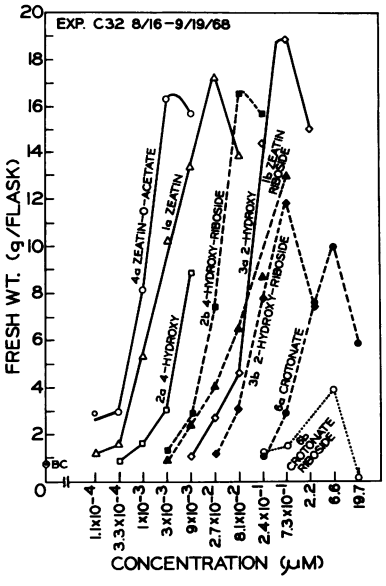


FIG. 1.—Effect of concentration of zeatin-related compounds on growth of tobacco callus.

The most striking feature of hydroxylation in the chain is the increase in activity conferred by a hydroxyl group in the 4-position as against the decrease in activity brought about by the hydroxyl group in the 2- or 3- position, as compared with its absence in corresponding isopentyl or isopentenyl chains. Thus, in Figure 2*a* and *b:*

zeatin (**1a**) > 2iP
4-hydroxyisopentylaminopurine (**2a**) > isopentylaminopurine
zeatin riboside (**1b**) > 2iPA
4-hydroxyisopentylaminopurine riboside (**2b**) > isopentylaminopurine riboside.

The enhancement of activity by the hydroxyl group in the 4-position runs counter to the original finding, on the basis of the compounds then available, that

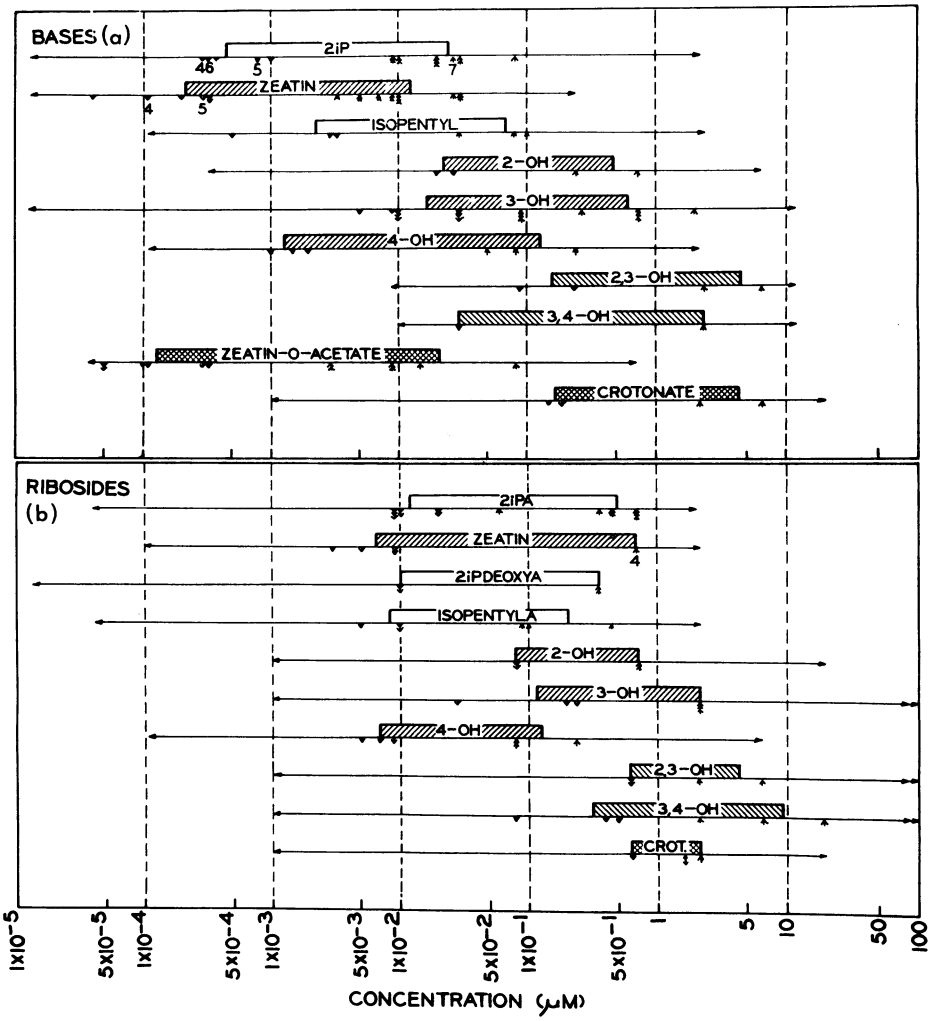


FIG. 2.—Relative cytokinin activities of zeatin-related compounds with different hydroxyl substitution or ester linkages in the side chain.

(*a*) Free bases; (*b*) 9-β-D-ribosides. *Bars* represent average values of the ranges 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.

▨ *Striped bars*, monohydroxy; ▨ *reversed striped bars*, dihydroxy; ⊠ *crossed bars*, ester-containing side chains.

polar groups in the side chain diminish activity.[11] The importance of the position of the hydroxyl group on the side chain is shown impressively when members of the hydroxylated series are compared with each other. In Figure 2*a* and *b:*

Monohydroxy series:	4-hydroxy- $\gg$ 3- and 2-hydroxy-
Dihydroxy series:	3,4-dihydroxy- > 2,3-dihydroxy-
Monohydroxy riboside series:	4-hydroxy- $\gg$ 3- and 2-hydroxy-
Dihydroxy riboside series:	3,4-dihydroxy- > 2,3-dihydroxy-

In each instance the 4-hydroxylated chain is more active than the corresponding unsubstituted isopentyl chain, which in turn is much more active than the 2- or 3-hydroxy-substituted chains.

Activities of two zeatin-related compounds with ester linkages in the side chain (**4a** and **6a**, and the riboside of the latter) are included in Figures 1 and 2*a* (2*b*). That zeatin-*O*-acetate (**4a**) is as active or more so than zeatin itself is of interest in several respects. It might indicate that the hydroxyl group need not be free. On the other hand, the acyl group may serve to "stabilize" the molecule, which then by gradual hydrolysis would provide for a prolonged release of active zeatin in the medium and/or tissue. Nor is the ester function alone the important activity-donating feature. This is shown by the fact that orientation of the ester grouping in the opposite sense at the end of the side chain, as in the crotonate analog (**6a**), whose probable hydrolysis product would be the crotonic acid derivative, drastically cuts down the biological activity.

The effect of the hydroxyl groups in the sugar moiety appears to be less critical, for example, 2iPA and 2iPdA (Fig. 2*b*) have about equal activity.

The results described above, together with earlier evidence,[14] suggest that the physical properties of the side chain rather than a specific chemically reactive group confer high cytokinin activity on N^6-substituted adenine derivatives.

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Note: The desirability of examining a series of branched-chain purinealkanols has been mentioned by Fawcett, C. H., and S. T. C. Wright, *Phytochemistry,* **7,** 1719 (1968).

Abbreviations used are as follows: 2iP, 6-(3-methyl-2-butenylamino)purine; iP(HOH), 6-(3-hydroxy-3-methylbutylamino)purine; iP(HOH)A, 6-(3-hydroxy-3-methylbutylamino)-9-β-D-ribofuranosylpurine; 2iPA, 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine; 2iPdA, 6-(3-methyl-2-butenylamino-9-(2-deoxy-β-D-ribofuranosyl)purine; IAA, indole-3-acetic acid; DMSO, dimethylsulfoxide.

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