Cytokinins: Synthesis and Biological Activity of Geometric and Position Isomers of Zeatin

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ABSTRACT

Geometric and position isomers of zeatin and of ribosyl-zeatin and other compounds closely related to zeatin have been tested in the tobacco (Nicotiana tabacum var. Wisconsin No. 38) bioassay. None was more active than zeatin itself. There was a much greater difference in activity (> 50-fold) between trans- and cis-zeatin than between trans-isozeatin [6-(4-hydroxy-2-methyl-2-butenylamino)purine] and cis-isozeatin [6-(4-hydroxy-2-methyl-cis-2-butenylamino)purine], the latter being less active than cis-zeatin and trans-isozeatin. Higher concentrations were required for equivalent callus growth stimulated by the 9-ribosyl derivatives, which followed an order of decreasing activity: ribosyl-trans-zeatin > ribosyl-cis-zeatin > ribosyl-trans-isozeatin > ribosyl-cis-isozeatin, corresponding roughly to that of the bases. The effect of side chain, double bond saturation was to diminish the activity, and in the dihydro series the shift of the methyl group from the 3- to the 2-position in going from dihydrozeatin to dihydroisozeatin [6-(4-hydroxy-2-methylbutylamino)purine] resulted in a 70-fold decrease in activity. cis-Norzeatin [6-(4-hydroxy-cis-2-butenylamino)purine], which was less than one-fifth as active as cis-zeatin, showed the effect of complete removal of the side chain methyl group, and cyclic-norzeatin [6-(3,6-dihydro-1,2-oxazin-2-yl)purine] was about 1/500 as active as cis-norzeatin. These findings delineate completely the effect on the cytokinin activity of zeatin of variation in side chain geometry, presence and position of the methyl substituent, presence and geometry of hydroxyl substitution, presence of the double bond, and of side chain cyclization.

There is a marked influence of side chain substitution and configuration on the cytokinin activity of compounds related to 6-(3-methyl-2-butenylamino)purine (I), 2IP, (1, 2, 5, 7, 16), which is especially evident in the difference in activity between trans- and cis-zeatin (II, III) (9) both of which have been found as their 9-ribosyl derivatives in tRNA (13, 18). Because of the variety of modification of terpenoid structures in nature and in order to extend our knowledge of the influence of side chain geometry on biological activity, we have determined the structure-activity relationship of trans- and cis-isozeatin (V, VI) and their 9-ribosyl derivatives (XIV, XV), together with dihydrozeatin (IV) and its 9-ribonucleoside (XIII), dihydroisozeatin (VII), cis-norzeatin (VIII), and cyclic-norzeatin (IX), all compared in the tobacco callus bioassay with the naturally occurring 6-(3-methyl-2-butenylamino)purine, 2IP (I), and its 9-ribonucleoside, 2IPA (X).

MATERIALS AND METHODS

Bioassay Procedure. The tobacco bioassay (12) was used to determine the cytokinin activity. The medium contained the specified mineral salts (Table 6, part A, of ref. 12) and the following organic constituents: 30 g/liter of sucrose, 10 g/liter of Difco agar, 100 mg/liter of myoinositol, 2 mg/liter of indole-3-acetic acid, and 0.4 mg/liter of thiamine hydrochloride. The chemicals to be tested were dissolved in dimethylsulfoxide and added to the cooling autoclaved media at the uniform rate of 0.05% (v/v), 0.025 ml of (CH₃)₂SO solution to each flask with 50 ml of medium). The use of (CH₃)₂SO as specified does not affect growth of the tobacco tissue, and permits the addition of the test substances in sterile solutions after autoclaving, thus protecting the compounds from possible degradation by heat (14).

Stock callus tissue (Nicotiana tabacum var. Wisconsin No. 38), was maintained on the above medium supplemented with 300 μg/liter of kinetin, and put through two 3-week passages on medium supplemented with 30 μg/liter of kinetin before use for bioassays. For each treatment 12 pieces of callus, about 40 mg each, were planted three apiece in 125-ml Erlenmeyer flasks containing 50 ml of medium. The cultures were kept at 28 C and were oc

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2 Abbreviations: Zeatin or trans-zeatin: 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine; ribosyl-cis-zeatin: 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine; ribosyl-trans-zeatin: 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine; cis-isozeatin: 6-(4-hydroxy-cis-2-butenylamino)-9-β-D-ribofuranosylpurine; trans-isozeatin: 6-(4-hydroxy-2-methyl-2-butenylamino)-purine; cis-norzeatin: 6-(4-hydroxy-cis-2-butenylamino)purine; cyclic-norzeatin: 6-(3,6-dihydro-1,2-oxazin-2-yl)purine; 2IP: 6-(3-methyl-2-butenylamino)purine or N⁴-(3-isopentenyl)adenine; 2IPA or, in shortened form, 2IPA: 6-(3-methyl-2-butenylamino)9-β-D-ribofuranosylpurine or N⁴-(3-isopentenyl)adenosine.
casionally exposed to light of low intensity to permit observation during the 5-week growth period.

Compounds. Recently described are the preparations of ribosyl-
cis-zeatin (XII) (13), trans-isozeatin (V) (11), cis-isozeatin (VI), and dihydroisozeatin (VII) (8). Dihydroisozeatin (IV), which is naturally occurring, has been synthesized (3, 4), along with its 9-
ribonucleoside (7). The syntheses of several new compounds in the series are described below.

cis-Norzeatin, 6-(4-hydroxy-2-methyl-trans-2-
butenylamino)-9-β-D-rubofuranosylpurine (VIII)
c and cyclic-norzeatin, 6-(3,6-dihydro-1,2-oxazin-2-yl)purine (IX).
The procedure of Wichterle and Novak (19) was used to prepare 4-hydroxy-cis-2-butenylamine and 3,6-dihydro-1,2-oxazine. A mixture of 1.5 g of the two resulting from an incomplete zinc-
acetic acid reduction of the oxazine, 650 mg of 6-chloropurine, and 125 ml of 1-butanol was heated under nitrogen at reflux for 4 hr. Silica (10 g) was added, and the butanol was removed in vacuo.

The resulting powder was applied to a 300-g silica column and eluted with chloroform-methanol, 9:1 (v/v). Complete separation of VIII and IX was achieved, the appropriate fractions were pooled, the solvent was removed, and the residues were recrystallized from ethanol. For analytically pure VIII, the yield was 160 mg (18%), m.p. 207 to 208 C; ultraviolet λmax 268 nm (ε 18,600), λmin 229; λ1% N HCl 273 (17,510), λmin 233; λ1% NaOH 274 (18,010), 283(sh), λmin 240; nuclear magnetic resonance (CD3)2SO δ 4.22 (m, 4H), 4.82 (m, 1H), 5.65 (m, 2H), 7.72 (m, 1H), 8.14 (s, 1H), 8.24 (s, 1H); mass spectrum m/e = 205, 188, 174, 160, 136, 119, 108, 93, 66.

C9H12N2O
Calculated: C 52.67, H 5.40, N 34.13
Found: C 52.38, H 5.47, N 33.97

For IX, yield 110 mg (13%), m.p. 267 to 268 C (dec.); ultraviolet λmax 281 nm (ε 16,890), λmin 235; λ1% N HCl 286 (19,110),

\[ \lambda_{\text{min}} \text{241; } \lambda_{\text{1% N NaOH}} \text{283 (14,760), } \lambda_{\text{min}} \text{240: nuclear magnetic resonance ((CD3)2SO)} \delta 4.52 (m, 2H), 4.70 (m, 2H), 6.10 (m, 2H), 8.42 (s, 1H), 8.47, (s, 1H); mass spectrum m/e = 203, 186, 174, 135, 119, 92, 65.

**C12H14N2O7**
Calculated: C 53.19, H 4.46, N 34.47
Found: C 53.38, H 4.37, N 34.27

Ribosyl-trans-isozeatin, 6-(4-hydroxy-2-methyl-trans-2-
butenylamino)-9-β-D-rubofuranosylpurine (XIV). A mixture of 1.0 g of 6-chloro-9-β-D-rubofuranosylpurine, 2.0 g of 4-hydroxy-2-
methyl-trans-2-butenylamine (11), and 10 ml of triethylamine with 20 ml of ethanol was stirred at reflux under nitrogen for 2 hr. Silica (3 g) was added, and the mixture was evaporated to a dry paste which was applied to a 100-g silica column and eluted with chloroform-methanol, 9:1 (v/v). The last component to come off the column was the desired product, and the appropriate fractions were pooled and reduced in vacuo to a semisolid. This was dissolved in methanol, decolorized with charcoal, recrystallized, filtered, and the solid was washed successively with methanol, ethyl acetate, and ether, and then vacuum dried at room temperature. Yield 110 mg, m.p. 193 to 195 C; ultraviolet λmax 267 nm (ε 19,200), λmin 231; λ1% N HCl 264 (19,300), λmin 234; λ1% N NaOH 268 (19,350), λmin 233.

**C12H14N2O7**
Calculated: C 51.27, H 6.02, N 19.93
Found: C 51.09, H 6.07, N 20.14

Ribosyl-cis-isozeatin, 6-(4-Hydroxy-2-methyl-cis-2-buteryl-
amino)-9-β-D-rubofuranosylpurine (XV). A similar preparation was carried out using a crude mixture of cis- and trans-aminokol,
4-hydroxy-2-methyl-2-butenylamine (8), followed by adsorption chromatography on silica. The three compounds isolated were, in order of elution, 6-chloro-9-β-D-rubofuranosylpurine, ribosyl-cis-isozeatin (XV), and ribosyl-trans-isozeatin (XIV). Purification and recrystallization yielded pure XV, m.p. 178 to 183 C (dec.); ultraviolet λmax 268 nm (ε 18,990), λmin 231; λ1% N HCl 264 (19,130), λmin 234; λ1% N NaOH 267 (19,850), λmin 233.

**C12H14N2O7**
Calculated: C 51.27, H 6.02, N 19.93
Found: C 51.09, H 6.07, N 20.01

The relationship between the ribosyl-cis- and trans-isozeatins XV and XIV as geometrical isomers is reflected in their nuclear magnetic resonance spectra [(CD3)2SO], as shown in Table I.

| Table I. Comparative Nuclear Magnetic Resonance Spectra (8) of Ribosyl-cis- and -trans-Isocetains |
|---|---|---|
| Protons | cis (XV) | trans (XIV) |
| CH3 | 1.73(s) | 1.64(s) |
| CH2O | 3.9-4.5(m) | 3.85-4.3(m) |
| CHN | 5.35-5.6(m) | 5.3-5.6(m) |
| NH | 7.94(t) | 8.00(t) |
| 2,8-H's | 8.25(s) | 8.24(s) |
| 2,8-H's | 8.42(s) | 8.40(s) |

The ribosyl protons are omitted.
RESULTS AND DISCUSSION

Representative examples of relationships between concentration and growth of tobacco callus tissue for the substances in individual experiments are shown in Figures 1 and 2, and a graph indicating their relative activities as based on average values of the "linear growth/log concentration ranges" for all experiments is presented in Figure 3.

Fig. 1. Effect of serial concentrations of trans-, cis-, and iso-"zeatins" in the tobacco bioassay. The number designations of the compounds are as given in the text, and their configurations can be compared by the partial structures shown in Figure 3. Each curve in Figure 1 is based on a single assay; all except curves I, V, and VI were from experiment C 148 (7-8-71 to 8-11-71).

Fig. 2. Effect of serial concentrations of trans-, cis-, and iso-"ribosylzeatins" in the tobacco bioassay. The numbers of the compounds are given in the text, and the partial structures in Figure 3. The curves for all compounds except XIII are from one assay (C 145, 5-27-71 to 6-3-71).

Fig. 3. Summary of cytokinin activities of trans-, cis-, and iso-"zeatins" and "ribosylzeatins". The compounds are numbered as in the text. For easy reference the configuration of the substituent in the 6-position has also been indicated in the margin. The base lines represent the tested concentration ranges, the bars represent mean values of the concentration range over which growth increased as a nearly linear function of the log of concentration of added cytokinin, and the arrows under the base lines represent the start and end points of this range in individual experiments. An incomplete bar indicates that the compound was not tested at a high enough concentration to yield maximum growth.

All the compounds represented in Figures 1 and 2 except cyclic-norzeatin (IX) were capable of giving a full growth response when supplied in concentrations up to 20 μM, and they gave roughly parallel curves when yields were plotted against the logarithm of the concentration. Cyclic-norzeatin (IX) was still in the exponential phase at 20 μM, the highest concentration tested. For comparison of activities based on all the available data the ranges in which growth was a nearly linear function of the log of concentration were plotted as described in the legend to Figure 3. It may be seen that no derivative was more active than zeatin (II) itself. As reported earlier (9) zeatin (II) was at least 50 times more active than its cis isomer (III). There was much greater difference in activity between the trans- and cis- zeatins than between the trans- and cis-isozeatins V and VI, in which the methyl group is in the 2- instead of the 3-position of the side chain, but the relative configurations have been preserved. The activities of cis-zeatin (III) and of trans-isozeatin (V) are actually in the same range, and that of cis-isozeatin (VI) is measurably lower than both. Letham (10) has recently reported, on the basis of a different bioassay involving the promotion of expansion of excised radish cotyledons, that 6-(4-hydroxy-2-methyl-trans-2-butenylamino)purine, trans-isozeatin (V), has about half the activity of zeatin (II) at 10 μM.

The 9-ribosyl derivatives of the trans-zeatin, cis-zeatin, transisozeatin, and cis-isozeatin series (XI, XII, XIV, and XV, respectively) followed an order of decreasing activity in the tobacco bioassay corresponding roughly to that of the bases. However, higher concentrations were required for activity and they are
closely grouped on the logarithmic scale on which they are pre-
present. The decrease in activity was least marked in the case of
the isomers which already exhibited low activity. It should be
noted that there is no means of distinguishing between the bio-
logical activity which may derive from the intact ribonucleosides
and from bases set free by hydrolysis in the course of the bioas-
say. Possible differences in rates of hydrolysis are, inter alia, an
additional complicating factor in attempts to compare biological
activities of the ribonucleosides.

The effect of saturation of the side chain double bond of zeatin
has been considered before (7, 10, 15) and may be observed in the
comparison of activities of II and IV in Figure 3. Comparison of
dihydrozeatin (IV) with dihydrodioszatin (VII) showed that a
shift of the methyl group from the 3- to the 2-position resulted in
a 70-fold decrease in activity. This decrease compares closely
with that resulting from shifting the terminal methyl group in
zeatin (II) to give trans-isozeatin (V).

The effect of removing the methyl group completely was ob-
served in the case of cis-norzatin (VIII), which was less than a
fifth as active as the related cis-zeatin (III). The cyclic form of this
compound, 6-[(3, 6-dihydro-1, 2-oxazin-2-yl)purine, cyclic-nor-
zeatin (IX) had $\frac{1}{2}$ of the activity of norzatin and, while still a
cytokin, had only $\frac{1}{2}$ of the activity of zeatin. The effect may
also be related to the isomerization of N6 in IX since double
substitution is known to lower cytokinin activity (16, 17).

These results confirm and amplify previous findings on the in-
fluence of size, configuration, degree of saturation, etc., of the side
chain on the cytokinin potency of N6-substituted adenine deriva-
tives, and they permit a more detailed examination of their rela-
tive importance in compounds closely related to zeatin. As ex-
pected, reduction in chain-branching, as in cis-norzeatin (VIII),
or rearrangement of the carbon skeleton, as in trans- and cis-
isozeatin (V and VI) and dihydrodioszatin (VII), markedly low-
ered activity. The loss of activity associated with saturation, ear-
lier noted by the differences between the isopentyl and iso-
portenyl adenosines (16) or between zeatin and dihydrozeatin (7)
was valid also for trans- or cis-isozeatin and dihydrodioszatin.

The importance of the relative position of the hydroxyl group in
the side chain, i.e., its enhancing effect on biological activity
when in the 4-position and its depressing effect when in the 2- or 3-
position or both of the isopentyl chain (7) was further demon-
strated here by the 50-fold difference in activity between zeatin
(II) and cis-zeatin (III), and by the relatively low activity of ribo-
syl-cis-zeatin (XII) compared with ribosyl-trans-zeatin (XI). In
fact, the present studies show that the 50-fold decrease in activity
which resulted from removal of the side chain hydroxyl group, as
in going from zeatin to 2iP (I), is a relatively minor loss as
compared with the 50-fold decrease brought about by shifting the
hydroxyl group in zeatin (II) from the trans- to the cis-position
(III).

The magnitude of any one structural modification on the bio-
logical activity of a cytokinin molecule may be strongly in-
fluenced by a second concomitant modification. One instance of
this is the difference in effect of saturating the side chain in 2iP
(I) and zeatin (II); that is, in the absence and presence of the
trans-4-hydroxyl group. Saturation of the former resulted in a 10-
fold loss in activity (16) while saturation of the latter resulted only
in a 4-fold loss (7, 15), in the tobacco bioassay. The activity of
6-(4-hydroxy-3-methylbutylamino)purine (IV) actually falls be-
 tween the activities of trans- and cis-zeatin (Figure 3). The 10-
fold increase in activity in relating cis-zeatin to the dihydro deriva-
tive common to both geometric isomers was not, therefore, the
result of saturation but of relieving the restriction of the 4-hydroxyl
den orientation with respect to the N6-CH3. The effect is not
as dramatic in the isozeatin series.

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