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Cytokinin Activities of N^6-Benzyladenosine Derivatives Hydroxylated on the Side-Chain Phenyl Ring

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Abstract. Cytokinin activities of N^6-benzyladenosine (bzl^{6}Ado) and its derivatives hydroxylated on the side chain phenyl ring in *ortho*, *meta*, and *para* positions were compared in four bioassays based on stimulation of growth of tobacco callus, retention of chlorophyll in excised wheat leaves, dark induction of betacyanin synthesis in *Amaranthus* cotyledons, and release of lateral buds of pea from apical dominance. In all these bioassays hydroxylation of the phenyl ring of bzl^{6}Ado in *ortho* and *para* positions significantly decreased cytokinin activity. Compared with bzl^{6}Ado, the activity was decreased about 10× in the tobacco callus bioassay and wheat leaf chlorophyll retention test, 100× in the *Amaranthus* betacyanin bioassay, and 20× and 200×, respectively, in the pea bud test. Hydroxylation of the phenyl ring in *meta* position increased activity in the tobacco callus, and wheat leaf chlorophyll retention bioassays, 9× and 1.7×, respectively, decreased activity about 2.4× in the pea bud test and was without effect in the *Amaranthus* bioassay. Cytokinin activity of the *meta* hydroxy derivative, N^6-(*m*-hydroxybenzyl) adenosine, was as high as that of *trans*-zeatin in all four bioassays. Possible regulation of biological activities of cytokinins by positionally specific hydroxylation of the side chain phenyl ring is discussed with respect to the reported occurrence of natural purinyl cytokinins with aromatic side chains.

The first naturally occurring purinyl cytokinin having an aromatic side chain was isolated from mature leaves of poplar by Horgan et al. (1973, 1975) and identified as 6-(*o*-hydroxybenzylamino)-9-β-D-ribofuranosylpurine (*o*-OH-bzl^{6}Ado). It was also found in the fruits of *Zantedeschia aethiopica*, together with its glucofuranosyl derivative (Das Neves and Pais 1980a,b). The occurrence and physiological effects of *o*-OH-bzl^{6}Ado indicate that this cytokinin is

involved in the regulation of metabolism and development of mature tissues rather than in the stimulation of cell division of growing plant organs (Horgan et al. 1975, Thompson et al. 1975, Hewett and Wareing 1973, Pais and Das Neves 1982/83). This hypothesis is supported by relatively low cytokinin activity of *o*-OH-bzl^{6}Ado in the soybean callus bioassay compared to zeatin riboside and benzyladenosine, while its activity in the radish leaf senescence test is only slightly lower than that of the two reference cytokinins (Horgan et al. 1975). Position of the substituents on the phenyl ring of both purinyl and urea-type cytokinins has a significant effect on cytokinin activity (Wilcox et al. 1978, Matsubara 1980, Iwamura et al. 1980). With respect to the recently reported occurrence of 6-benzylaminopurine riboside (bzl^{6}Ado) in anise cells cultured *in vitro* (Ernst et al. 1983), the position-specific hydroxylation of this cytokinin on the phenyl ring might represent a mechanism of regulating the activities of natural cytokinins with the aromatic side chain. This paper describes meaningful changes in cytokinin activities in four different bioassays elicited by hydroxylation of the phenyl ring of bzl^{6}Ado in *ortho, meta* (*m*-OH-bzl^{6}Ado), and *para* (*p*-OH-bzl^{6}Ado) positions.

Materials and Methods

Bioassays

For the tobacco callus bioassay, cytokinin-dependent callus tissue derived from *Nicotiana tabacum* L. cv. Wisconsin 38 was used. Tissues were cultivated in 100-ml Erlenmeyer flasks containing 50 ml RM-1965 medium (Murashige and Skoog 1962, Linsmaier and Skoog 1965) supplemented with 2 mg/L IAA. Ten replicates were prepared for each cytokinin concentration. Cytokinin activity is expressed as average fresh weight of tissue per flask after 5 weeks of cultivation in darkness at 26°C.

The chlorophyll retention bioassay was performed using primary leaves of spring wheat (*Triticum aestivum* L. cv. Jara). Seeds were planted into vermiculite saturated with Knop's solution, germinated for 48 h in the dark at 26°C, and then transferred to an illuminated growth chamber (7000 lx, day/night period 18 h/6 h, 20°C). After 10 days of cultivation, when the first leaf was fully developed and the second was partially formed, the leaves were cut off 7 cm from their apical tips. Four leaf pieces were placed with their basal ends down in a test tube (1.5 × 12 cm, flat bottom) containing 0.5 ml test solution. Cytokinins were dissolved in 0.1 *M* HCl; the solution was diluted and neutralized with NaOH (final NaCl concentration 0.01 *M*). Four replicates were prepared from each variant. Test tubes without stoppers were kept in an incubator for 4 days at 26°C in darkness. For chlorophyll estimation, 4 leaf pieces were transferred into calibrated test tubes containing 10 ml 80% EtOH (v/v) and a boiling chip. Chlorophyll was extracted by heating the test tubes in a water bath at 80°C for 8 min. The optical absorbance of the extract was measured at 665 nm.

For the *Amaranthus* bioassay, a modification of the methods of Biddington and Thomas (1973) and Elliot (1979a,b) was used. The seeds of *Amaranthus caudatus* L. var. *atropurpurea* were surface-sterilized in 10% aqueous chlora-

mine B solution (w/v) for 10 min and washed 6 times in distilled water. Seeds were germinated aseptically in 11-cm Petri dishes on 4 layers of Whatman No. 3 filter paper moistened with 20 ml distilled water in the dark at 37°C for 46 h. Explants consisting of the upper portion of the hypocotyl plus cotyledons were allowed to "age" for 3 h in distilled water at 26°C in darkness and were then transferred onto two layers of Whatman No. 1 filter paper in a 5-cm Petri dish containing 1 ml incubation medium. The medium consisted of 10 m*M* Na_2HPO_4–KH_2PO_4 (pH 6.8), 5 m*M* tyrosine, and the cytokinin tested. Each Petri dish contained 25 explants, and 4 replicates were used for each treatment. After incubation in the dark at 24°C for 46 h, betacyanin was extracted with 3.33 m*M* acetic acid (4 ml/20 explants) using the freezing and thawing procedure repeated twice. The concentration of betacyanin was determined from the difference spectra $\Delta A = A_{537\ nm} - A_{620\ nm}$.

The activity of cytokinins in releasing lateral buds of pea from apical dominance was assayed as described by Skoog and Abdul Ghani (1981) with minor modifications. The seeds of *Pisum sativum* L. cv. Bohatýr were surface-sterilized for 3 min in 70% EtOH and washed for 12 h under running tap water. They were then germinated for 48 h on perforated plastic plates covered with distilled water–saturated cellulose wool in a dessicator continuously aerated by a stream of air saturated with water vapor. Selected germinating seeds were planted into vermiculite saturated with Knop's solution and cultivated in an illuminated growth chamber (1600–1800 lx, day/night period 16 h/8 h, 25°C). After 8 days, the seedlings were cut off above the second node, the first developed leaf was removed, and the remaining shoot was placed with its base down in a scintillation vial containing Knop's solution. Vials were covered with aluminum foil with a hole for the pea shoot. Cytokinins were dissolved in 50% EtOH, and 10 μl solution was applied to the fourth lateral bud—i.e., the bud at the axil of the second developed leaf from the stem base. Shoots were incubated for 4 days under the same conditions as the seedlings, and the lengths of the treated lateral buds were then measured under a microscope.

Activities of tested cytokinins were compared on the basis of E_{50} values, defined as the concentration at which 50% of the maximum response was recorded (tobacco callus bioassay), or on the basis of minimum concentration required for positive response (all the other bioassays).

Synthesis of Compounds

Cytokinins *o*-OH-bzl^{6}Ado, *m*-OH-bzl^{6}Ado, and *p*-OH-bzl^{6}Ado were synthesized as described by Kuhnle et al. (1977) by heating of 6-chloro-9-β-D-ribofuranosylpurine with the appropriate primary amine in ethanol containing excessive amount of triethylamine. For preparation of *o*-hydroxy-, *m*-hydroxy-, and *p*-hydroxybenzylamine, the appropriate hydroxybenzaldehyde was heated with hydroxylamine hydrochloride and triethylamine. The resulting oxime of hydroxybenzaldehyde was crystallized and converted to the corresponding primary amine by hydrogenation in EtOH in the presence of palladium catalyst under a pressure of 2 atm. The final products were recrystallized from EtOH to

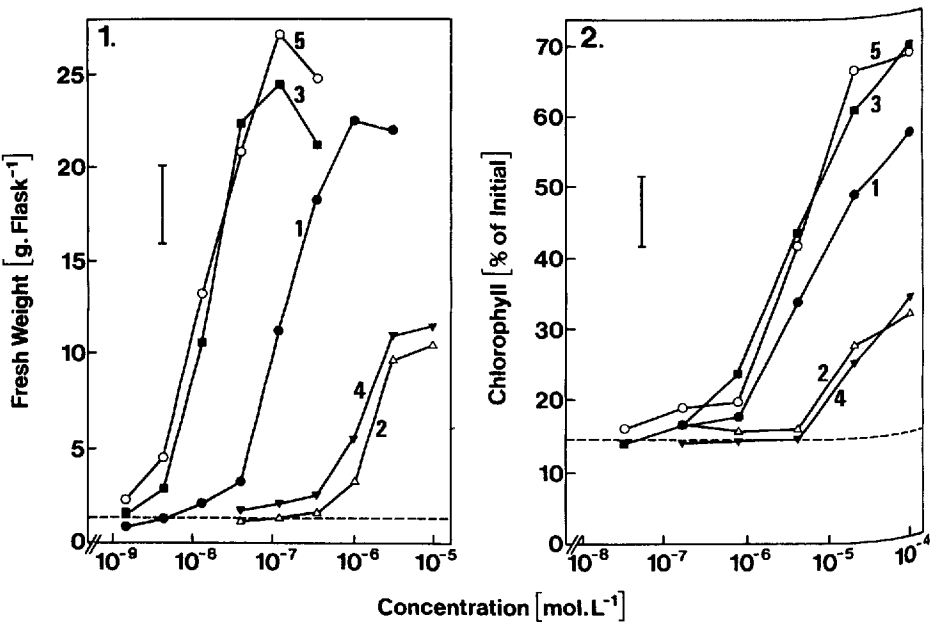

Fig. 1. Effect of cytokinins on fresh weight yield of tobacco callus culture: (1) N^6-benzyladenosine (bzl^{6}Ado); (2) N^6-(*o*-hydroxybenzyl)adenosine (*o*-OH-bzl^{6}Ado); (3) N^6-(*m*-hydroxybenzyl)adenosine (*m*-OH-bzl^{6}Ado); (4) N^6-(*p*-hydroxybenzyl)adenosine (*p*-OH-bzl^{6}Ado); (5) *trans*-zeatin (*t*-io^{6}Ade). Vertical bar indicates LSD.

Fig. 2. Effect of bzl^{6}Ado (1), *o*-OH-bzl^{6}Ado (2), *m*-OH-bzl^{6}Ado (3), *p*-OH-bzl^{6}Ado (4), and *t*-io^{6}Ade (5) on retention of chlorophyll in excised wheat leaves. Other details are in Fig. 1.

constant mp. Their purity and structure were determined by HPLC and ^{1}H NMR spectroscopy, respectively.

Results

Activities of positional isomers of OH-bzl^{6}Ado were compared with those of the parent compound and *trans*-zeatin (*t*-io^{6}Ade). In all bioassays, hydroxylation of the phenyl ring of bzl^{6}Ado in *ortho* and *para* positions decreased cytokinin activity. On the other hand, *m*-OH-bzl^{6}Ado exhibited a very high cytokinin activity being as active as t-io^{6}Ade in all four bioassays (Figs. 1–4).

In the tobacco callus bioassay, the activities of *o*-OH-bzl^{6}Ado and *p*-OH-bzl^{6}Ado were about 10× lower and those of *m*-OH-bzl^6-Ado and *t*-io^{6}Ade about 9× higher than the activity of bzl^{6}Ado when compared on the basis of E_{50} values (Fig. 1). The same order of activities was found in the wheat leaf senescence bioassay, where hydroxylation of the phenyl ring of bzl^{6}Ado in *ortho* and *para* positions decreased and in *meta* position increased the activity by a factor of 10 and 1.7, respectively (Fig. 2). A similar order of activities was

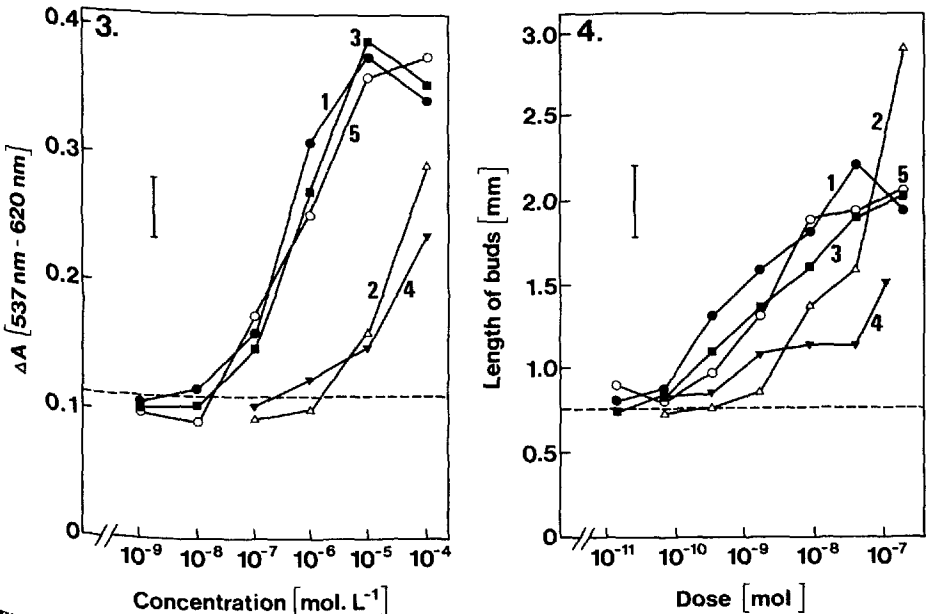

Fig. 3. Effect of bzl^{6}Ado (1), *o*-OH-bzl^{6}Ado (2), *m*-OH-bzl^{6}Ado (3), *p*-OH-bzl^{6}Ado (4), and *t*-io^{6}Ade (5) on dark induction of betacyanin synthesis in *Amaranthus caudatus* cotyledon-hypocotyl explants. Other details are in Fig. 1.

Fig. 4. Effect of bzl^{6}Ado (1), *o*-OH-bzl^{6}Ado (2), *m*-OH-bzl^{6}Ado (3), *p*-OH-bzl^{6}Ado (4), and *t*-io^{6}Ade (5) on growth of lateral pea buds. Other details are in Fig. 1.

recorded in the *Amaranthus* betacyanin bioassay, where *o*-OH-bzl^{6}Ado and *p*-OH-bzl^{6}Ado were about 100× less active than bzl^{6}Ado, whereas *m*-OH-bzl^{6}Ado exhibited the same activity as bzl^{6}Ado and *t*-io^{6}Ade (Fig. 3). In the bioassay based on the release of pea lateral buds from apical dominance, the following order of activities was found: bzl^{6}Ado > *m*-OH-bzl^{6}Ado = *t*-io^{6}Ade > *o*-OH-bzl^{6}Ado > *p*-OH-bzl^{6}Ado (Fig. 4). In agreement with the observation of Kefford et al. (1966), this bioassay yielded more variable results. Effective concentrations of *o*-OH-bzl^{6}Ado induced much faster growth of released buds than all the other tested cytokinins.

Discussion

Position of the hydroxy group in the side chain of cytokinins usually has a significant effect on their biological activity (Kaminek et al. 1979, Iwamura et al. 1980, Matsubara 1980). The structure-activity relationships of cytokinins have been extensively investigated, yet the effects of all monohydroxy substitutions of the phenyl ring of bzl^{6}Ado have not until now been compared in any bioassay for cytokinins. The results obtained with other substituents indicate the presence of a position-specific effect. Corresponding to the results ob-

tained in this study, substitutions on the phenyl ring enhance the activity in the order *meta* > *ortho* ≧ *para* in most bioassays for purinyl and urea-type cytokinins (Iwamura et al. 1980, Matsubara 1980).

Decrease of the activity of bzl^{6}Ado after hydroxylation of the phenyl ring in the *ortho* position seen in the soybean callus bioassay (Horgan et al. 1975) is even more significant in the tobacco callus test. A high increase of the cytokinin activity due to hydroxylation of the phenyl ring of bzl^{6}Ado in the *meta* position as reported here was not found for the corresponding base (Iwamura et al. 1980) or diphenylurea (DPU) in the tobacco callus test. However, hydroxylation of one phenyl ring of DPU in the *ortho* and *para* positions significantly reduced the activity of the parent compound (Bruce and Zwar 1966). The hydroxylated derivatives of *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (Thidiazuron), a compound with high cytokinin activity, showed decreasing activity in order *m* > *o* > *p* in the *Phasoleus lunatus* callus assay (Mok and Mok 1985). Substitution of the phenyl ring in the *meta* position by several other substituents increased the activity of 6-benzyl-oxypurines (Me and OMe substituents) (Wilcox et al. 1978), DPU (Cl, Br, NO_2), and monophenylurea (Cl, Br, P, CF_3, Me, NO_2, OEt, OMe) (Bruce and Zwar 1966). Substitutions in the *ortho* and *para* positions generally decreased their activity.

Most of the cytokinin bioassays based on retention of chlorophyll are more sensitive to bzl^{6}Ade and its riboside than to *t*-io^{6}Ade (Varga and Bruinsma 1973, Kuhnle et al. 1977, Kamínek and Luštinec 1978, Dumbroff and Walker 1979, Tao et al. 1983). The conditions of the test used in our experiments were optimized for *t*-io^{6}Ade. This test yielded the same order of activities of tested cytokinins as the tobacco callus bioassay. The low activity of *o*-OH-bzl^{6}Ado as compared to bzl^{6}Ado corresponded to that reported by Horgan et al. (1975), Biddington and Thomas (1978), and Kuhnle et al. (1977) in senescence bioassays. A different order of activities was obtained by Wilcox et al. (1978) for positional isomers of 6-benzyloxypurines substituted with Me (*o* > *m* > unsubstituted > *p*) and OMe (unsubstituted > *m* > *p* ≧ *o*).

The orders of activities of different cytokinins in the *Amaranthus* betacyanin bioassay often differ from those obtained in some other tests (Gasque 1982, Tao et al. 1983). When compared with the tobacco callus and wheat leaf senescence bioassays, the only difference was the lack of promotion of activity of bzl^{6}Ado after its hydroxylation in the *meta* position. However, in this test, *m*-OH-bzl^{6}Ado showed the same activity as *t*-io^{6}Ade.

Hydroxylation of the phenyl ring of bzl^{6}Ado decreased the activity in the order *meta* > *ortho* > *para* in the pea bud bioassay, where the parent compound was more active than *t*-io^{6}Ade. High activity of bzl^{6}Ado in this test resembled that found by Roussaux et al. (1977) and Skoog and Abdul Ghani (1981) for bzl^{6}Ade. The effect of various cytokinins on the release of buds from apical dominance may be species-specific. Comparing the activities of bzl^{6}Ade and *m*-OH-bzl^{6}Ado in induction of growth of lateral buds in poinsettia and gerbera daisy, we found the latter to be the more active (Kamínek et al. 1987). The lack of correlation in the activity of cytokinins in the initiation of lateral bud growth of pea and stimulation of cell division in tobacco pith (Kefford et al. 1966) is probably related to unequal affinities of different cytokinins for the hypothetical receptor site in the two assay systems (Roussaux et al. 1977).

The present results indicate that the position of the hydroxy substituent on the phenyl ring of bzl^{6}Ado may effectively influence the biological activity of the parent compound. The activity of the corresponding base is decreased in tobacco pith assay when substituted in any position on the phenyl ring with Cl, Me, OMe, or CF_3 (Iwamura et al. 1980). Our preliminary results (not shown) indicate that the *m*-hydroxy derivative of the base is slightly more active than the corresponding riboside in the *Amaranthus* assay, whereas the two compounds exhibit about the same activity in wheat senescence test. The biosynthetic route of *o*-OH-bzl^{6}Ado in plants is not known. However, recent identification of bzl^{6}Ado in anise cell culture (Ernst et al. 1983) supports the speculation that this compound might be a percursor of this cytokinin. If so, *ortho*-hydroxylation may represent a deactivation step in the regulation of cytokinin activity. Hydroxylation of bzl^{6}Ado in *meta* position might have an opposite effect. High activity of *m*-OH-bzl^{6}Ado is of practical importance. It exhibits the same cytokinin activity as the most active natural cytokinin *t*-io^{6}Ade while being much cheaper and probably also stable when applied to plants. It has already been proved of great use in increasing branching and stem cutting production in poinsettia and gerbera daisy stock plants for vegetative propagation (Kamínek et al. 1987).

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