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STRUCTURES OF CYTOKININS INFLUENCE SYNERGISTIC PRODUCTION OF ETHYLENE*

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Key Word Index—Cytokinins; structural variants; synergism; ethylene production; bioassay.

Abstract—Twenty-five naturally occurring cytokinins and structurally related compounds were tested for their ability to promote ethylene production synergistically in the presence of either indole-3-acetic acid (IAA) or Ca^{2+}. The structural modifications tested were, with regard to the side chain: the presence or absence of a double bond in the 2′-position and the presence and configuration of a 4′-hydroxyl group; with regard to the purine nucleus: substitution by 2-methylthiolation, by 2-hydroxylation and by 9-ribosidation. The following activity relationships were observed: (a) hydrogenation or the change from a *trans* to a *cis* configuration of the side chain at the N^6-position had little effect, (b) 9-ribosidation either had no effect or caused a slight decrease in activity, (c) 2-methylthiolation caused a moderate decrease in activity, (d) simultaneous 2-methylthiolation and 9-ribosidation brought about a great decrease in activity, (e) 2-hydroxylation eliminated the activity, (f) purinylcarbamoyl threonine was devoid of activity and (g) the effect of structure upon activity in the cytokinin + IAA system paralleled that in the cytokinin + Ca^{2+} system. Except that 9-ribosidation greatly decreased activity as measured by the growth of callus tissue, the structure–activity relationship for cytokinins was nearly identical for the callus tissue responses reported by others and the stimulation of ethylene production. It is proposed that the synergistic promotion of ethylene production may be advantageously adapted as a primary and supplemental bioassay for cytokinins, using Ca^{2+} as synergist.

INTRODUCTION

Since the isolation of kinetin, several compounds have been identified as free cytokinins or as constituents of *t*RNA species from higher plants, animals, fungi and bacteria [1, 2]. With the exception of certain substituted diphenyl ureas [3], all the naturally occurring cytokinins are adenine derivatives with an isopentenyl or hydroxyisopentenyl side chain at the N^6-position [1, 2, 4]. This side chain can be modified by hydrogenation (saturation), or by change in the configuration of the 4′-hydroxyisopentenyl (*cis* and *trans* forms) group [4]. The purine nucleus also can be modified, by 2-hydroxylation, 2-methylthiolation, 9-ribosidation [4, 5] and by 9-glucosidation [6]. The effect of specific modification of chemical structure upon the biological activity of such compounds has been measured in assays based upon callus growth [5, 7] and upon the retardation of leaf senescence [8, 9]. Lau *et al.* [10] have recently suggested a bioassay based on the ability of cytokinins to stimulate ethylene production synergistically with IAA or with Ca^{2+}. The present study provides a further test of this assay by examining its linearity of response and the influence of structural differences on the activities of 25 natural and synthetic cytokinins. The results are compared to published data from similar studies with previously established cytokinin assays.

RESULTS

The following structural modifications were tested with respect to the side chain: the presence or absence of a double bond in the 2′-position, the presence and the configuration of the 4′-hydroxyl group, and the substitution of carbamoylthreonine (PCLT) for the isopentenyl side chain. Tested with respect to the adenine nucleus were: substitution by 2-methylthiolation, by 2-hydroxylation and by 9-ribosidation. We have utilized 25 compounds representing these modifications (Fig. 1), and have compared their activities in synergism with either IAA or Ca^{2+} upon the stimulation of ethylene production in the mungbean hypocotyl [11, 12]. With very active cytokinins, such as kinetin, it has been determined that over the range of 1–100 μM the stimulation of ethylene production was approximately a linear function of the log of the concentration of the added cytokinin [10, 12]. We have used routinely a concentration of 10 μM for the present study. A time course of ethylene production by the cytokinin + Ca^{2+} system is shown in Fig. 2. The lag period of the cytokinin + IAA system was shorter than that of the cytokinin + Ca^{2+} system, as reported previously [12]. In both systems, the rate of ethylene production declined after 9 hr. The activities of the cytokinins tested with either Ca^{2+} or IAA are summarized in Table 1. It is apparent that the differences in activity were not random, but rather were determined systematically by the nature and combination of the structural modification.

The general result of modification of the free bases, particularly in the Ca^{2+}-mediated system, was a reduction of activity varying from slight to complete. With respect to side chain modifications, 4′-hydroxylation (i^6Ade to *t*-$(ioh^4)^6$Ade) slightly increased activity with IAA, but decreased activity with Ca^{2+}. Saturation of the side chain (i^6Ade to $(ih)^6$Ade) had no effect in the IAA-

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CYTOKININ	STRUCTURE		
			
ABBREVIATION	R_1	R_2	R_3
bzl^{6}Ade	$-CH_2-C_6H_5$	H	H
(bzloh^2)^{6}Ade, (bzloh^2)^{6}A	$-CH_2-C_6H_4-OH$	H	H, $C_5H_9O_4$
i^{6}Ade, i^{6}A	$-CH_2-CH=C(Me)(Me)$	H	H, $C_5H_9O_4$
ms^{2}i^{6}Ade, ms^{2}i^{6}A		Me S	H, $C_5H_9O_4$
oh^{2}i^{6}Ade		HO	H
(ih)^{6}Ade	$-CH_2-CH_2-CH(Me)(Me)$	H	H
t-(ioh^4)^{6}Ade, *t*-(ioh^4)^{6}A	$-CH_2-C(CH_2OH)=CH-Me$	H	H, $C_5H_9O_4$
t-ms^2(ioh^4)^{6}Ade, *t*-ms^2(ioh^4)^{6}A		Me S	H, $C_5H_9O_4$
t-oh^2(ioh^4)^{6}Ade		HO	H
c-(ioh^4)^{6}Ade, *c*-(ioh^4)^{6}A	$-CH_2-CH=C(Me)-CH_2OH$	H	H, $C_5H_9O_4$
c-ms^2(ioh^4)^{6}Ade, *c*-ms^2(ioh^4)^{6}A		Me S	H, $C_5H_9O_4$
c-oh^2(ioh^4)^{6}Ade		HO	H
(ihoh^4)^{6}Ade, (ihoh^4)^{6}A	$-CH_2-CH_2-CH(Me)-CH_2OH$	H	H, $C_5H_9O_4$
ms^2(ihoh^4)^{6}Ade, ms^2(ihoh^4)^{6}A		Me S	H, $C_5H_9O_4$
oh^2(ihoh^4)^{6}Ade		HO	H
PCLT	$-C(=O)-NH-CH(COOH)-CH(OH)-Me$	H	H

Fig. 1. Chemical structure of cytokinins tested. $C_5H_9O_4$, ribofuranosyl group.

mediated system and slightly decreased activity in the Ca^{2+}-mediated system. Where the hydroxyl group was present, double bond saturation (*t*-(ioh^4)^{6}Ade to (ihoh^4)^{6}Ade) also resulted in a slight reduction of activity, as did change of configuration from the *trans* form of the 4'-hydroxyisopentenyl group to the *cis* form (*t*-(ioh^4)^{6}Ade to *c*-(ioh^4)^{6}Ade). Substitution of the carbamoylthreonine group (PCLT) for the isopentenyl side chain, however, resulted in total loss of the activity.

Compared to the unmodified bases, each modification of the purine ring (9-ribosidation, 2-methylthiolation, simultaneous 2-methylthiolation and 9-ribosidation, or 2-hydroxylation) resulted in systematically decreased activity. Although the extent of the depression of activity by these modifications varied slightly among the different bases, the following generalizations are valid: 9-ribosidation of the purine ring only slightly decreased the activity, methylthiolation moderately depressed the activity, simultaneous methylthiolation–ribosidation decreased the activity more extensively, and 2-hydroxylation produced the least active compound in any given series. The suppression of activity by ribosidation and/or methylthiolation of the purine ring was most marked in the (ihoh^4)^{6}Ade series, and least in the i^{6}Ade series.

Linearity of response and the concentration–activity relationship of *trans*-zeatin and its analogs in the presence of Ca^{2+} is illustrated in Fig. 3. With regard to side chain modifications, saturation of the double bond (*t*-(ioh^4)^{6}Ade to (ihoh^4)^{6}Ade) and change of configuration from the *trans* form to the *cis* form (*t*-(ioh^4)^{6}Ade to *c*-(ioh^4)^{6}Ade) resulted in a 10-fold decrease in activity; the linear range of activity for (ihoh^4)^{6}Ade or *c*-(ioh^4)^{6}Ade was from 1 to 100 μM, whereas that for *t*-(ioh^4)^{6}Ade was 0.1–10 μM. With regard to the modifications of the purine ring, 9-ribosidation, 2-methylthiolation, simultaneous ribosidation–methylthiolation and 2-hydroxylation resulted in about 6-, 15-, 100- and 1000-fold reduction of activity, respectively.

Using i^{6}Ade as the cytokinin, the linearity of response over a range of concentrations, with indication of the range of statistical variation, is shown in Fig. 4. The statistical reliability of the method was further tested in experiments using sets of six replicates, with i^{6}Ade as cytokinin and Ca^{2+} as synergist. A single ethylene determination from each sample after a 15 hr incubation period was found to be adequate. Coefficients of variation were within the 10–12% range, which is acceptable for a cytokinin assay.

DISCUSSION

The cytokinins which we have tested can be classified into four groups according to their synergistic activity with Ca^{2+}: (1) the very active cytokinins, including all of the free bases (bzl^{6}Ade, (bzloh^2)^{6}Ade, i^{6}Ade, (ih)^{6}Ade, *t*-(ioh^4)^{6}Ade, *c*-(ioh^4)^{6}Ade, (ihoh^4)^{6}Ade) and i^{6}A, which caused a 7- to 8-fold increase of ethylene production above the sum of the ethylene produced in response to

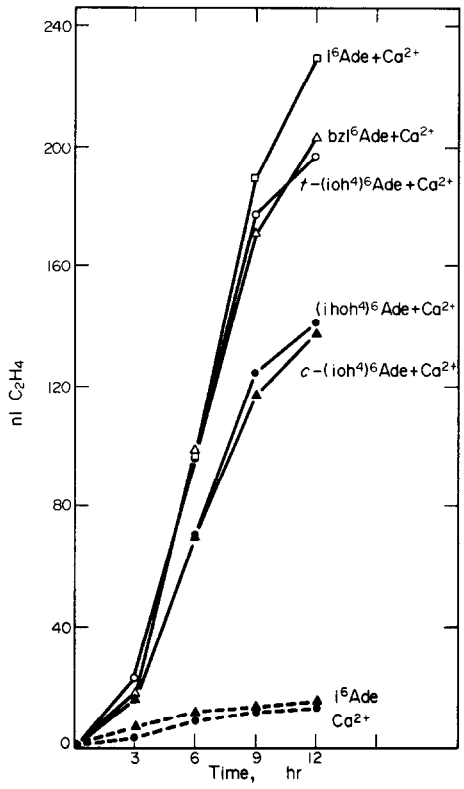

Fig. 2. Time courses of ethylene production from mung bean hypocotyls treated with Ca^{2+} (10 mM) plus a cytokinin base (10 μM).

Ca^{2+} alone plus that produced in response to the cytokinin alone; (2) the moderately active cytokinins, including the methylthiolated or ribosidated cytokinins ((bzloh^2)^{6}A, ms^{2}i^{6}Ade, *t*-(ioh^4)^{6}A, *t*-ms^2(ioh^4)^{6}Ade, *c*-(ioh^4)^{6}A, *c*-ms^2(ioh^4)^{6}Ade, (ihoh^4)^{6}A, ms^2(ihoh^4)^{6}Ade) and ms^{2}i^{6}A; (3) the slightly active cytokinins, including the simultaneously methylthiolated and ribosidated forms (*t*-ms^2(ioh^4)^{6}A, *c*-ms^2(ioh^4)^{6}A) and oh^{2}i^{6}Ade, which caused little ethylene production but still had an obviously synergistic effect; and (4) the inactive cytokinins, including those hydroxylated at the 2-position (*t*-oh^2(ioh^4)^{6}Ade, *c*-oh^2(ioh^4)^{6}Ade, oh^2(ihoh^4)^{6}Ade), ms^2(ihoh^4)^{6}A and PCLT, which had little or no synergistic effect. Although the net increase in ethylene production with the cytokinin + IAA system was greater than that with the cytokinin + Ca^{2+} system, the latter demonstrated a greater synergistic ratio of stimulation (Table 1).

Modifications of the cytokinin side chain, whether by hydroxylation, saturation (hydrogenation) or *cis–trans* isomerization, resulted in relatively little change in ability to stimulate ethylene production synergistically. All modifications of the purine moiety lowered cytokinin activity. Modification by 9-ribosidation, by 2-methylthiolation, by simultaneous ribosidation–methylthiolation or by 2-hydroxylation resulted, respectively, in little to slight, moderate or severe reduction of activity. The kinetin series was not included in this study, but it has been previously demonstrated [7] that kinetin is as active as i^{6}Ade in this system and that kinetin riboside is only slightly less so. This is in agreement with the conclusions drawn in the present study. Our classification of the effects

Table 1. Ethylene production from mungbean hypocotyls in the presence of cytokinin (10 μM) plus IAA (10 μM) or cytokinin (10 μM) plus $CaCl_2$ (10 mM)

Cytokinins	Ethylene production (nl/12 hr)			
	−IAA	+IAA	$-Ca^{2+}$	$+Ca^{2+}$
None	7	99	4	13
bzl^{6}Ade	19	518	15	203
(bzloh^2)^{6}Ade	14	455	14	127
(bzloh^2)^{6}A	12	330	7	13
i^{6}Ade	18	456	15	229
i^{6}A	18	455	19	200
ms^{2}i^{6}Ade	11	361	14	116
ms^{2}i^{6}A	9	308	15	91
oh^{2}i^{6}Ade	9	182	7	44
(ih)^{6}Ade	17	448	15	188
t-(ioh^4)^{6}Ade	17	497	16	197
t-(ioh^4)^{6}A	13	344	10	173
t-ms^2(ioh^4)^{6}Ade	13	342	8	98
t-ms^2(ioh^4)^{6}A	8	210	7	49
t-oh^2(ioh^4)^{6}Ade	9	114	5	18
c-(ioh^4)^{6}Ade	14	420	15	137
c-(ioh^4)^{6}A	8	309	6	106
c-ms^2(ioh^4)^{6}Ade	11	329	8	73
c-ms^2(ioh^4)^{6}A	7	151	6	27
c-oh^2(ioh^4)^{6}Ade	8	94	3	14
(ihoh^4)^{6}Ade	14	437	14	141
(ihoh^4)^{6}A	11	255	7	101
ms^2(ihoh^4)^{6}Ade	8	229	5	56
ms^2(ihoh^4)^{6}A	5	109	5	13
oh^2(ihoh^4)^{6}Ade	9	102	4	13
PCLT	10	99	4	10

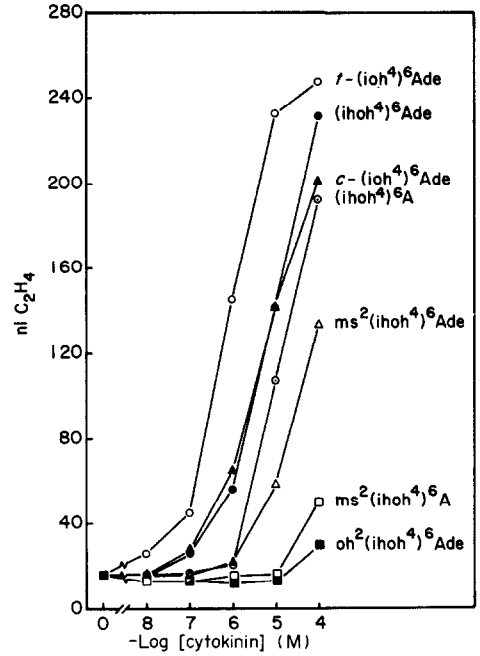

Fig. 3. Effect of the concentration of zeatin and its analogs in the presence of 10 mM Ca^{2+} on the production of ethylene in 15 hr.

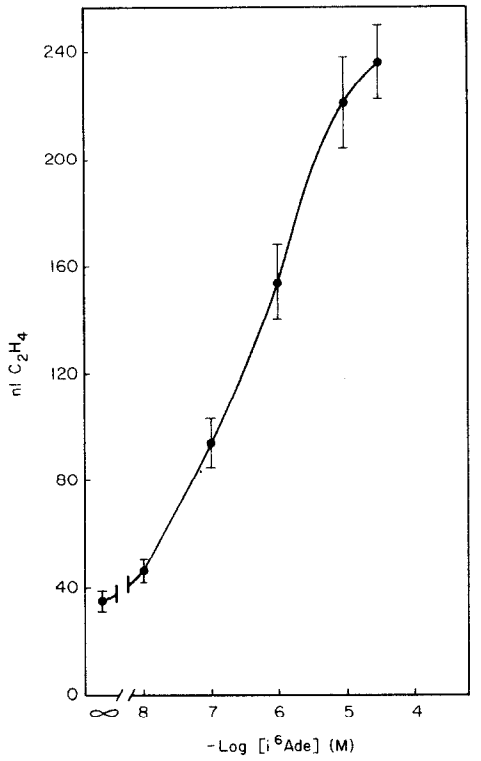

Fig. 4. Dependence of ethylene production on the concentration of i^{6}Ade in the presence of 10 mM Ca^{2+} after 15 hr incubation at 27°. Bars represent standard deviation.

of specific structural modification provides a basis for predicting the activity of previously untested cytokinins of known structure upon ethylene production.

The relationship between structure and activity of cytokinins in the ethylene system shows excellent selectivity, but is not identical to that of either the tobacco callus assay [5, 7] or the chlorophyll retention assay [8, 9]. The effect of saturation (hydrogenation) of the side chain in callus assays varied with the test tissue used. In tobacco callus, *t*-(ioh^4)^{6}Ade was more active than (ihoh^4)^{6}Ade, and i^{6}Ade was more active than (ih)^{6}Ade [4, 5], but in *Phaseolus vulgaris* callus [13] the saturated cytokinins were more active than the corresponding unsaturated form. Slight enhancement of activity by the addition of a hydroxyl group to the 4′-position of the isopentenyl side chain was noted in tobacco callus, while in the ethylene system neither saturation nor hydroxylation of the isopentenyl side chain exerted a significant effect. Considering the purine ring, 2-methylthiolation decreased activity in both the callus assay and in the ethylene production system, but the decrease was of greater magnitude in the callus assay. In callus, it was noted that 2-methylthiolation of the purine ring resulted in slight (3- to 5-fold) loss of activity in i^{6}Ade and *t*-(ioh^4)^{6}Ade, but a greater loss in activity in (ihoh^4)^{6}Ade, in which the side chain is saturated. The response of the ethylene system was similar in direction, but smaller. The major deviation between the two systems is that the ribosidation of free bases resulted in at least an 8-fold loss of activity in the callus assay, while it resulted in slight or no decrease in activity as measured by ethylene production. The ethylene production response differed from the chlorophyll retention assay in that methylthiolation reduced i^{6}Ade and (ihoh^4)^{6}Ade activity (*c*-(ioh^4)^{6}Ade remained the same) in the present ethylene production assay but enhanced it in the chlorophyll retention assay [8].

The biological response to an exogenous cytokinin depends on its uptake, translocation and metabolism in the tissue, as well as upon its function at action site(s). It is not clear whether differences in the effect of structure upon activity in these several assays are due to differences in uptake and/or metabolism or are caused by differences in structural requirements for cytokinin activity in different plant tissues or for different biological processes. In these experiments, we have included 2-hydroxylated cytokinins, which were reported to enhance *t*RNA ribosomal binding efficiency [14, 15], and PCLT, which occurs in *t*RNA adjacent to the 3-anticodon [1, 16], but which has shown no cytokinin activity in several bioassays. Each of these compounds was inactive in the ethylene production system, and it appears very unlikely that the ability of cytokinins to stimulate ethylene production synergistically is related to their presence in *t*RNA.

The standard bioassay for cytokinin activity is based on the influence upon the induction of cell division and the promotion of growth in tissue cultures. Because tissue culture bioassays are laborious and time consuming, other more rapid cytokinin responses have been used. These assays, however, suffer from limited specificity [2, 9]. The technique described here is much simpler and more rapid than the callus assay, and yet it is capable of detecting a wider range of cytokinins, including ribosides which are known to have very low activity in the tobacco callus assay. Sensitivity, linearity and reliability of the present method have been shown to be adequate. Although the net increase in ethylene production with cytokinin + IAA was greater than with the cytokinin + Ca^{2+} system, the latter provided a greater stimulation when expressed in terms of the synergistic ratio. The coefficient of variation calculated from a series of six replicates using i^{6}Ade with Ca^{2+} is supportive of our conclusion that the synergistic increase in ethylene production in response to a cytokinin plus Ca^{2+} provides a convenient bioassay for the cytokinins.

EXPERIMENTAL

Mung bean seeds (*Vigna radiata* L.) were purchased from a local market. For indicated experiments, seeds of the variety, Berken, were obtained from Ekroat Seed Co., Oklahoma City. The seeds were thoroughly washed, imbibed in aerated H_2O for 12 hr, and then grown in vermiculite for 3 days in darkness at 25°. Twenty 2 cm-long hypocotyl segments, cut at a point 1 cm below the hook, were incubated in a 50 ml Erlenmeyer flask in 5 ml of medium containing (for the cytokinin + IAA system) 2% sucrose, 50 μg/ml chloramphenicol, 10 μM cytokinin where specified, and 10 μM IAA, all in 50 mM MES buffer (pH 6.1), or (for the cytokinin + Ca^{2+} system) 2% sucrose, 50 μg/ml chloramphenicol, 10 mM cytokinin where specified, and 10 mM $CaCl_2$, all in 50 mM K-Pi buffer (pH 5.4). The flasks were flushed with air, sealed with serum caps and incubated at 27°. Every 3 hr the C_2H_4 content of the gas phase was determined by GC [11]. The flasks were then flushed with air and resealed until the next C_2H_4 determination. Experiments comparing different cytokinins were replicated, and the data reported here represent a typical experiment. Although there were quantitative variations in C_2H_4 production from one experiment to another, the relative

efficacy of different cytokinins was constant. One set of 6 replicates was used to test statistical variation of the method. 2-Hydroxy-*cis*-zeatin (*c*-oh^2(ioh^4)^{6}Ade) was synthesized as follows: a mixture of 0.03 mol of 4-hydroxy-3-methyl-*cis*-buten-2-ylamine, 0.01 mol of 2-hydroxy-6-methylthiopurine and 15 ml of 2-ethoxyethanol was refluxed for 4 hr, when analysis by HPLC showed the reaction to be 86% completed. After cooling, the white crystals (1.9 g) were collected, triturated with 250 ml of boiling EtOH and recrystallized from H_2O–EtOH. The mp (Kofler hot stage) was 257–260° (dec.). (Found: C, 50.3; H, 5.64; N, 28.9. $C_{10}H_{13}N_5O_2$ requires: C, 51.05; H, 5.57; N, 29.77%). Syntheses of all other cytokinins used in this study have been described elsewhere [8] or in refs cited therein. The identity of each compound was confirmed by NMR spectra (100 MHz) and the purity was determined by HPLC.

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