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Biological Activity of Several 9-Nonglycosidic-Substituted Natural Cytokinins

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Abstract. A series of 9-substituted derivatives of four common naturally occurring cytokinins was tested for growth response in the soybean callus assay. The 9-substituents, 2-carboxyethyl ($-C_2CO_2H$), 2-carbo-*t*-butoxyethyl ($-C_2CO_2t$Bu), and 2-nitriloethyl ($-C_2CN$), all reduced the biological activity of the parent compound. The order of activities was, in general, $-C_2CO_2H > -C_2CO_2t$Bu $> -C_2CN$ and followed the unsubstituted cytokinin response pattern of E-zeatin (E-Z) > Z-zeatin (Z-Z) > ≅ (R,S)-dihydrozeatin [(diH)Z] > *N*-(3-methyl-2-butenyl)-1H-purin-6-amine (iP). The nature of the dose–response curves indicate that the activities of the derivatives are a function of their affinities to a common receptor site and that their steric and polar properties determine that affinity.

The 9-(2-carboxyethyl) cytokinins provide a practical alternate source of haptens for the raising of cytokinin antibodies, since sugar-cleavage oxidations are avoided in the preparation of the antigens. E-9-(2-carboxyethyl)-O-β-D-glucopyranosylzeatin (E-ZOG9C_2CO_2H) was synthesized for the purpose of making antibodies specific for E-O-β-D-glucopyranosylzeatin (E-ZOG) and was also assayed for its growth response.

The results obtained with stable 9-substituted cytokinins are useful in defining guidelines for the design of ligands available to explore receptor-site topology.

Structure–activity studies for small organic molecules have been very rewarding in a number of fields—medicine, pesticide research, and the study of

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a wide range of plant growth regulators. Similarly, much work has been done with the cytokinins. After the discovery of kinetin (Miller et al. 1956), there was a flurry of syntheses that led to the finding of N^6-benzyladenine (BA), an active, easily obtainable cytokinin. A tremendous amount of research in plant physiology was done with BA, much of which may not be closely related to actual plant biochemistry. The isolation and identification of the natural cytokinin E-Z (Letham et al. 1964) led to another surge of syntheses as well as the search for other natural cytokinins. The search for high-activity synthetic cytokinins with potential use in agriculture was dampened by the reports that BA was found in the tRNA of callus treated exogenously with BA (Armstrong et al. 1976). However, the usefulness of structure–activity relationships still remains, both to elucidate the mechanism of cytokinin action and to provide information about the receptor sites involved in the diverse physiological responses.

Curiously, very little research has been done on nonglycosidic substitution at the 9-position in natural cytokinins (Matsubara 1980), although these derivatives are of both theoretical and methodological interest. Two members of this class do occur naturally, lupinic acid (LA) (the 9-alanyl conjugate of E-Z) and dihydrolupinic acid (Duke et al. 1978).

The two goals of this research were as follows. The first goal sought to explore a neglected region of the natural cytokinins—the vicinity of the 9-position—to determine what influence substituents might have on dose–activity relationships. A short series of related substituents was selected for observation. The 9-(2-carboxyethyl)-, 9-(2-carbo-*t*-butoxyethyl)- and 9-(2-nitriloethyl)-derivatives of E-Z, Z-Z, (diH)Z, and iP were synthesized and assayed (soybean callus) for cytokinin activities.

The second goal, closely related to the first from a chemical standpoint, was to develop a general method of synthesizing haptens useful in preparing specific immunochemical reagents for the analysis and localization of cytokinins, particularly the glycosides. The established method of synthesizing cytokinin haptens is to oxidize the ribose ring of the corresponding nucleoside to a dialdehyde with periodate. The dialdehyde is then coupled to a carrier protein, and the resulting aldimine is reduced with borohydride to complete the antigen (Erlanger and Beiser 1964, Hacker et al. 1972). It is obvious that other sugar moieties would not survive the oxidation steps.

Cytokinin analysis is especially complicated since there are many diverse forms that must be estimated individually (Letham et al. 1983), and the immunoreagent specificities, or lack of them, must be taken into account. Of necessity, a number of reagents may be required for analysis, even if some determinations can be made by difference (e.g., analyses done before and after treatment with a specific enzyme) (Badenoch-Jones et al. 1984). Alternatively, a preliminary separation of cytokinins may be done (HPLC), and then the assay performed using appropriate conversion factors (Badenoch-Jones et al. 1987, Sayavedra-Soto et al. 1988). A direct and specific analysis of individual components is preferred, and precise structuring of haptens can lead to monoclonal immunoreagents that will make this possible.

Table 1.[a] Chemical characteristics of cytokinins, derivatives and precursors.

Base	Functional group	Melting points (°C)[b]	Empirical formula	Calculated %			Found %		
				C	H	N	C	H	N
(diH)Z	$9C_2$ CN	113–114	$C_{13}H_{18}N_6O$	56.92	6.61	30.64	57.0	6.60	30.7
	$9C_2CO_2tBu$	80–81	$C_{17}H_{27}N_5O_3$	58.43	7.79	20.04	58.4	7.80	20.0
	$9C_2CO_2H$	172–173	$C_{13}H_{19}N_5O_3$	53.23	6.53	23.88	53.4	6.58	23.8
iP	$9C_2CN$	148–150	$C_{13}H_{16}N_6$	60.92	6.29	32.79	60.8	6.23	32.7
	$9C_2CO_2tBu$	90–91	$C_{17}H_{25}N_5O_2$	61.61	7.60	21.13	61.6	7.61	21.2
	$9C_2CO_2H$	193–194	$C_{13}H_{17}N_5O_2$	56.71	6.23	25.44	56.8	6.26	25.6
Z-Z	$9C_2CN$	170–172	$C_{13}H_{16}N_6O$	57.34	5.92	30.86	57.6	5.95	30.7
	$9C_2CO_2tBu$	111–112	$C_{17}H_{25}N_5O_3$	58.77	7.25	20.16	58.5	7.08	20.1
	$9C_2CO_2H$	204–206	$C_{13}H_{17}N_5O_3$	53.60	5.88	24.04	53.6	5.99	23.8
E-Z	$9C_2CN$	123	$C_{13}H_{16}N_6O$	57.34	5.92	30.86	57.3	5.85	30.5
	$9C_2CO_2tBu$	95–97	$C_{17}H_{25}N_5O_3$	58.77	7.25	20.16	58.9	7.23	19.9
	$9C_2CO_2H$	182	$C_{13}H_{17}N_5O_3$	53.60	5.88	24.04	53.6	5.86	23.7
6-ClP	$9C_2CO_2CH_3$	93.5	$C_9H_9ClN_4O_2$	44.92	3.77	23.28	45.1	3.93	22.9
	$9C_2CO_2tBu$	92	$C_{12}H_{15}ClN_4O_2$	50.97	5.35	19.82	51.1	5.54	19.8
	$9C_2CO_2H$	163–165	$C_8H_7ClN_4O_2$	42.40	3.11	24.72	42.7	3.40	24.7
6-MeSP	$9C_2CO_2CH_3$	123–125	$C_{10}H_{12}N_4O_2S$	47.60	4.80	22.21	48.0	4.91	21.8

(diH)Z, (R, S)-dihydrozeatin; iP, *N*-(3-methyl-2-butenyl)-1H-purin-6-amine; Z-Z, Z-zeatin; E-Z, E-zeatin; 6-ClP, 6-chloropurine; 6-MeSP, 6-methylthiopurine.

[a] The NMR spectra were all consistent with the proposed structural assignments.

[b] Melting points were taken on a Kofler hot stage and are uncorrected.

Materials and Methods

Chemicals

Cytokinin standards were synthesized in this laboratory by methods described previously in the literature. Commercial chemicals were obtained as indicated. Analytical and melting-point data are given in Table 1. Proton NMR spectra were measured at room temperature on Varian EM 390 (90 MHz) and Nicolet NMC-200 (200 MHz) spectrometers. Since the modifications of the O-glucoside syntheses are of sufficient importance, these syntheses are described in detail.

The syntheses of 9-substituted cytokinins were carried out in the usual manner by reaction of one of the substituted 6-chloropurines with the amine or amino alcohol of choice, to give the desired product, using 1-BuOH as solvent and *N,N*-diisopropylethylamine as a proton sink. The 9-(2-carboxyethyl) cytokinins were isolated by adjusting the cold, concentrated aqueous solution of the reaction mixture (after removal of the 1-BuOH and amine in vacuo) to a pH of 4.0–4.2. All of the cytokinin derivatives were purified by chromatography on silica using appropriate EtOAc–MeOH gradients.

6-Chloro-9-(2-carbomethoxyethyl)purine, 6-chloro-9-(2-carbo-*t*-butoxyethyl)purine, and 6-methylthio-9-(2-carbomethoxyethyl)purine were synthesized by the Michael addition of methyl acrylate and *t*-butyl acrylate to the 6-substituted purine using conditions reported by Baker and Tanna (1965) for the addition of acrylonitrile to 6-chloropurine.

6-Chloro-9-(2-carboxyethyl)purine

A solution of 10.4 g (0.0432 mol) 6-chloro-9-(2-carbomethoxyethyl)purine in 400 ml dioxane, 20 ml H_2O, and 50 ml N NaOH was stirred 2 h at room temperature. Nearly all the solvent was removed by roto-evaporation and 50 ml N HCl was added. The mixture was chilled and the precipitate collected; 9.0 g (92%); recrystalized MeOH-pet ether; melting point (mp) 163–165°C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 8.80 (1H, s, 7 or 8-H), 4.52 (2H, t), 2.97 (2H, t, CH_2CO).

Attempts to hydrolyze the *t*-butyl group from 6-chloro-9-(2-carbo-*t*-butoxyethyl)purine by aqueous acid catalysis resulted in the simultaneous displacement of the 6-chloro group to yield 9H-hypoxanthin-9-ylpropionic acid (Baker and Tanna 1965). Treatment of an anhydrous ethyl ether solution of the *t*-butyl ester with anhydrous HCl in ether gave a precipitate that, when isolated, also gave 9-H-hypoxanthin-9-ylpropionic acid upon treatment with ice-H_2O.

E-2-Methyl-4-phthalimidobut-2-enyl 2,3,4,6-tetra O-acetyl-β-D-glucopyranoside

The general procedure of glucuronide formation of Conrow and Bernstein (1971) was followed. A mixture of 11 g (0.048 mol) E-2-methyl-4-phthalimi-

dobut-2-enol (mp 104–105°C; Corse and Kuhnle 1972) and 17.2 g $CdCO_3$ (Johnson and Matthey, Seabrook, NH, USA) in 1200 ml toluene was dried by distilling about 200 ml toluene from the flask (stirring). A solution of 39.7 g (0.1 mol) of 2,3,4,6-tetra-O-acetyl-α-glucopyranosyl bromide (acetobromoglucose) in 200 ml dry toluene was added to the stirred, refluxing mixture over 1 h. The hot mixture was filtered through a Celite pad, and the toluene was removed in vacuo from the filtrate. The resulting tan gum (46 g) contained the desired product, starting alcohol, and its acetate. Purification was simplified by acetylating the entire mixture with 50 ml acetic anhydride, 135 ml pyridine, and 1 g 4-dimethylaminopyridine (steam bath, 30 min). The volatiles were removed in vacuo, the residue was dissolved in EtOAc, and the solution washed with H_2O, diluted HCl, $KHCO_3$ solution, and H_2O. After drying and removal of the solvent, 29.8 g remained, which was divided into four portions and chromatographed on silica [J. T. Baker, flash chromatography grade, 40 μm, 50 cm × 4 cm column, hexane-EtOAc (65-35)]; yield 14.36 g (53.3%); mp 118–120°C; ^1H NMR identical to that reported by Duke et al. (1978).

E-2-Methyl-4-phthalimidobut-2-enyl-β-D-glucopyranoside

A solution of 4 g E-2-methyl-4-phthalimidobut-2-enyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside in 500 ml MeOH was stirred 16 h with 5 g Amberlyst A-26 (OH) prepared in MeOH (Reed et al. 1981). The catalyst was removed by filtration, the volatiles removed in vacuo, and the residue recrystalized from MeOH-Et_2O; yield 2.27 g (74%); mp 143–145°C; m/z (chemical ionization using NH_3) 411.1745 (calculated for $C_{19}H_{27}N_2O_8$: 411.1767); ^1H NMR (200 MHz, DMSO-d_6) δ 7.86 (4 H, m, Ar), 5.50 (1H, t, C=CH), 5.03 (1 H, d, glucose OH), 4.96 (2 H, m, glucose OH), 4.46 (2 H, t, CH_2N), 4.22 (2 H, dd, CH_2OH, J = 28), 4.12 (1 H, anomeric H, J = 8), 3.63 (1H, q, glucose OH), 2.8–3.4 (4 H, m, glucose H), 1.78 (3H, s, CH_3).

E-9-(2-Carboxyethyl)-O-β-D-glucopyranosylzeatin

E-2-Methyl-4-phthalimidobut-2-enyl-β-D-glucopyranoside [1.17 g; (3 mmol)] dissolved in a mixture of 26.9 ml 2-PrOH and 4.54 ml H_2O was stirred overnight with 0.57 g sodium borohydride (Osby et al. 1984). Glacial AcOH (3.14 ml) was added and the solution heated 2 h at 80°C and then evaporated to dryness in vacuo. The dry residue was triturated several times with 5 ml portions of ether to remove phthalide. The remainder was heated 10 h at 100–105°C with 0.6 g 6-chloro-9-(2-carboxyethyl)purine, 20 ml 1-BuOH, and 2 ml *N,N*-diisopropylethylamine. The volatiles were removed in vacuo, the residue dissolved in 4.5 ml DMSO, and the filtered solution applied to a 21.4 mm × 50 cm HPLC column (C-18, 8 μm). Elution was carried out with CH_3CN-H_2O (6–94%); yield 0.199 g (14.6%); mp 195–198°C (dec) with sintering at 165°C; m/e 453.1839 (calculated for $C_{19}H_{27}N_5O_8$: 453.1758); ^1H NMR (200 MHz, DMSO-d_6 + D_2O) δ 8.20 (1H, s, 7 or 8-H), 8.03 (1H, s, 7 or 8-H), 7.38 (1H t, NH), 5.62 (1H, t, =CH), 4.36 (2H, t, NCH_2C), 4.21 (2H, d, CH_2 O),

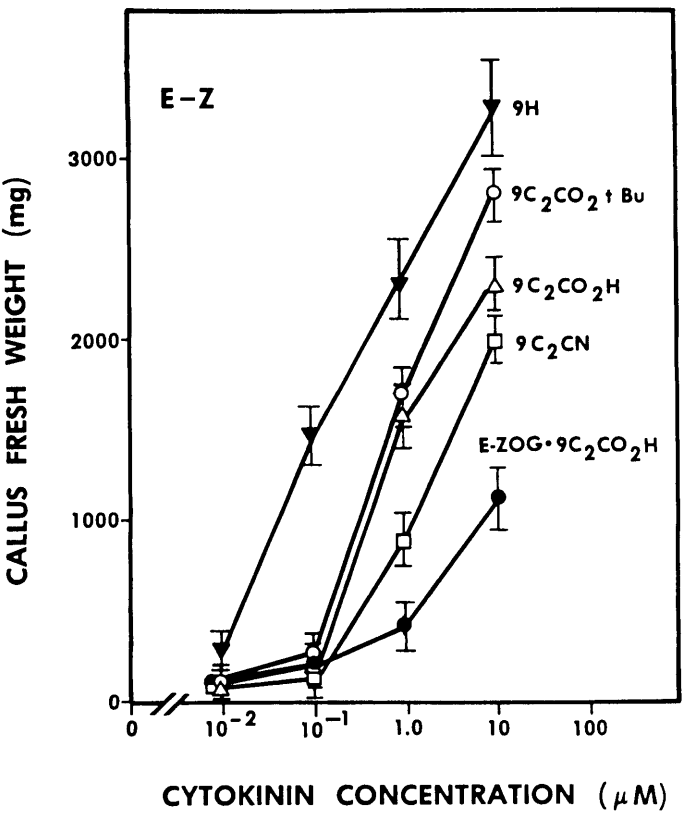

Fig. 1. Mean fresh weight of *Glycine max* (L.) Merr (cv Acme) callus (8 replicates/treatment) 28 days after subculture in the presence of E-Z and 9-substituted E-Z derivatives. Bars indicate confidence intervals ($p < 0.05$).

4.14 (H, d, anomeric H), 4.02 (2H, dd, CH_2O, J = 25), 3.54 (2H, q, glucose H), 2.9–3.3 (3 H, complx, glucose H), 2.85 (2H, t, CH_2CO_2), 1.73 (3H, s, CH_3).

Bioassays

The cytokinins were dissolved in DMSO (Aldrich Chem. Co., Milwaukee, WI, USA), and these solutions were incorporated into the basal medium (Miller 1963) to achieve final concentrations of 10, 1.0, 0.1, and 0.01 μM. The concentrations of all test solutions were checked by UV spectrophotometry using published or calculated ∈ values. A basal medium control, in triplicate, was included with each assay replication.

The soybean (*Glycine max* (L.) Merr cv. Acme) callus, derived from the cotyledons of germinating seedlings was a gift from Dr. S. S. Hua (WRRC, Albany, CA, USA) and has been maintained for over eight years in our labora-

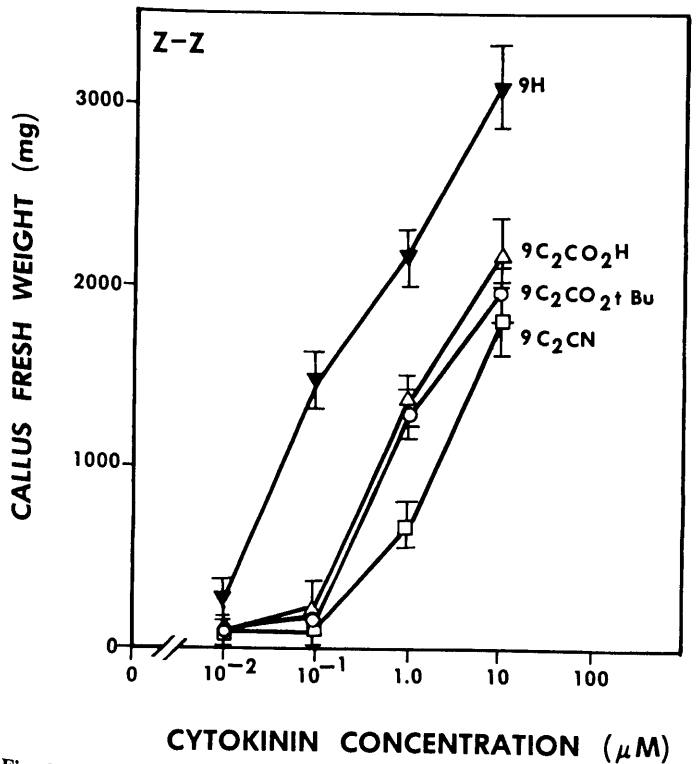

Fig. 2. Mean fresh weight of *Glycine max* (L.) Merr (cv Acme) callus (8 replicates/treatment) 28 days after subculture in the presence of Z-Z and 9-substituted Z-Z derivatives. Bars indicate confidence intervals ($p < 0.05$).

tory. This callus was cytokinin-dependent and has been maintained on basal medium solidified with agar (1.0%, w/v) and supplemented with α-naphthaleneacetic acid (2 mg L^{-1}) and kinetin (0.6 mg L^{-1}). All cultures were grown at 28°C in the dark. Callus tissue for the bioassay was used 28 days after subculture.

Cytokinin bioassays were performed according to Miller (1963). Three callus explants, ~30 ± 4 mg each, were placed in each 125 ml Erlenmeyer flask containing 30 ml medium. Test solutions of cytokinins were added to autoclaved (120°C for 20 min) medium in individual flasks just prior to gelation (~45°C). The concentration of DMSO in the final medium was kept below 0.35% (v/v), and DMSO was also added to the control flasks. Callus fresh weights were determined after growth for 28 days at 28°C in the dark, and the three callus weights from each flask were averaged. Each week, for eight weeks, a total of 83 flasks were set up in a complete replication of the experimental design (4 compounds × 4 derivatives × 5 concentrations + 3 control flasks).

Means and variances were determined for each compound × derivative × concentration combination (80 total treatments with 8 replicates per treat-

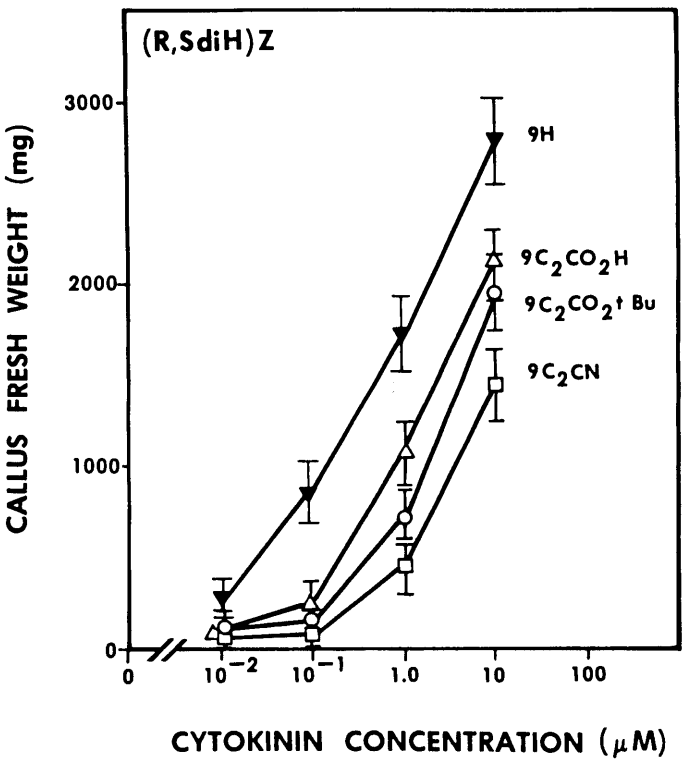

Fig. 3. Mean fresh weight of *Glycine max* (L.) Merr (cv Acme) callus (8 replicates/treatment) 28 days after subculture in the presence of (diH)Z and 9-substituted (diH)Z derivatives. Bars indicate confidence intervals ($p < 0.05$).

ment). Transformation of the means and variances for each treatment to $\log_{10}$ values was used in an analysis of variance (ANOVA) to determine main effects, independent of concentrations, using the procedures of the Statistical Analysis Systems (Freund and Littell 1981). Means and confidence intervals ($p < 0.05$) were computed and back-transformed for data presentation. To measure these interactions, ANOVA was performed separately on untransformed data at 1.0 and 10 μM incorporated cytokinin, and to verify statistical differences, comparisons between treatment means were performed using Student's *t* test ($p < 0.05$).

Results and Discussion

All of the 9-β-substituted ethyl derivatives of the four natural cytokinins tested showed considerable activity (Figs. 1–4). The derivatives were all less active than the parent cytokinins, but the relative activities differed depending upon the structure of the isoprenoid side-chain. The pattern of activities was fairly uniform: H > $-C_2CO_2H$ > $-C_2CO_2tBu$ > $-C_2CN$, except for E-Z, wherein the *t*-butyl ester was more active than the free acid. The underlying general ac-

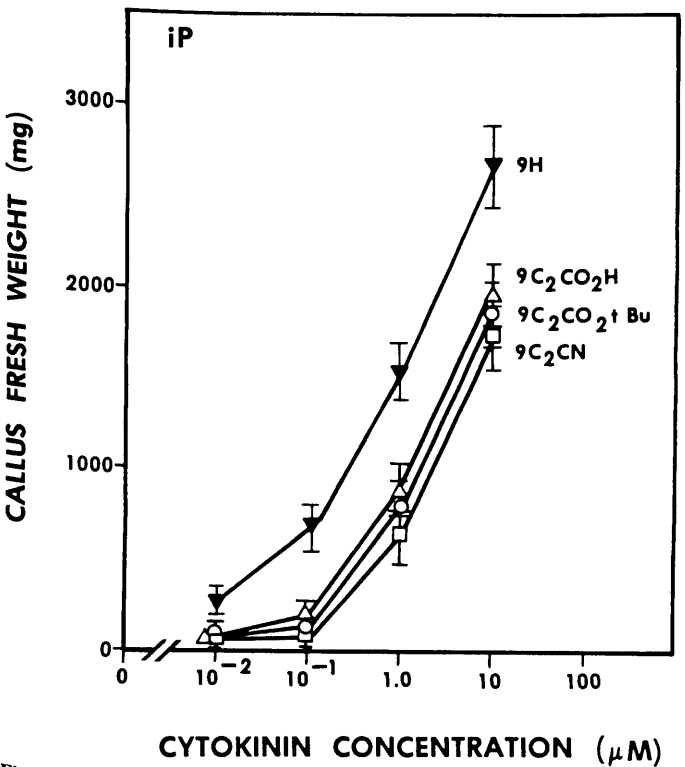

Fig. 4. Mean fresh weight of *Glycine max* (L.) Merr (cv Acme) callus (8 replicates/treatment) 28 days after subculture in the presence of iP and 9-substituted iP derivatives. Bars indicate confidence intervals ($p < 0.05$).

tivity series of E-Z > Z-Z ≅ (diH)Z > iP for the parent cytokinins was followed in the derivatives. Thus, the activities of the 9-(2-carboxyethyl) cytokinins were E-Z9C_2CO_2H > Z-Z9C_2CO_2H ≅ (diH)Z9C_2CO_2H > iP9C_2CO_2H.

The very low activity (Fig. 1) of E-9-(2-carboxyethyl)-O-β-D-glucosylzeatin is surprising in view of the reported response in assays of the parent O-glucoside (Palni et al. 1984, Van Staden and Papaphilippou 1977). A difference in the soybean callus we used toward glycosidic hydrolysis could account for the results. Mok et al. (1978, 1982) and Palni et al. (1984) have shown quite divergent chemical reactivities in strains of related calluses. For this reason we have used a callus line sensitive to 9-substitution and kept variations in the 6-position limited to those occurring naturally. Further, the 9-substituent used here cannot be hydrolyzed by glucosidases that might unduly complicate the results.

The results clearly show the importance of 9-position steric and polarity effects in implementing cytokinin response. It appears that steric influence (size), [-C_2CO_2tBu > -C_2CO_2H > -C_2CN (Fig. 5A and B)] was of less importance than polarity, (-C_2CO_2H > -C_2CO_2tBu > -C_2CN). The 9-alanyl conjugates of E-Z (LA) and (diH)Z occur naturally as metabolic products of exogenously supplied E-Z (MacLeod et al. 1975). The cytokinin activity of LA is

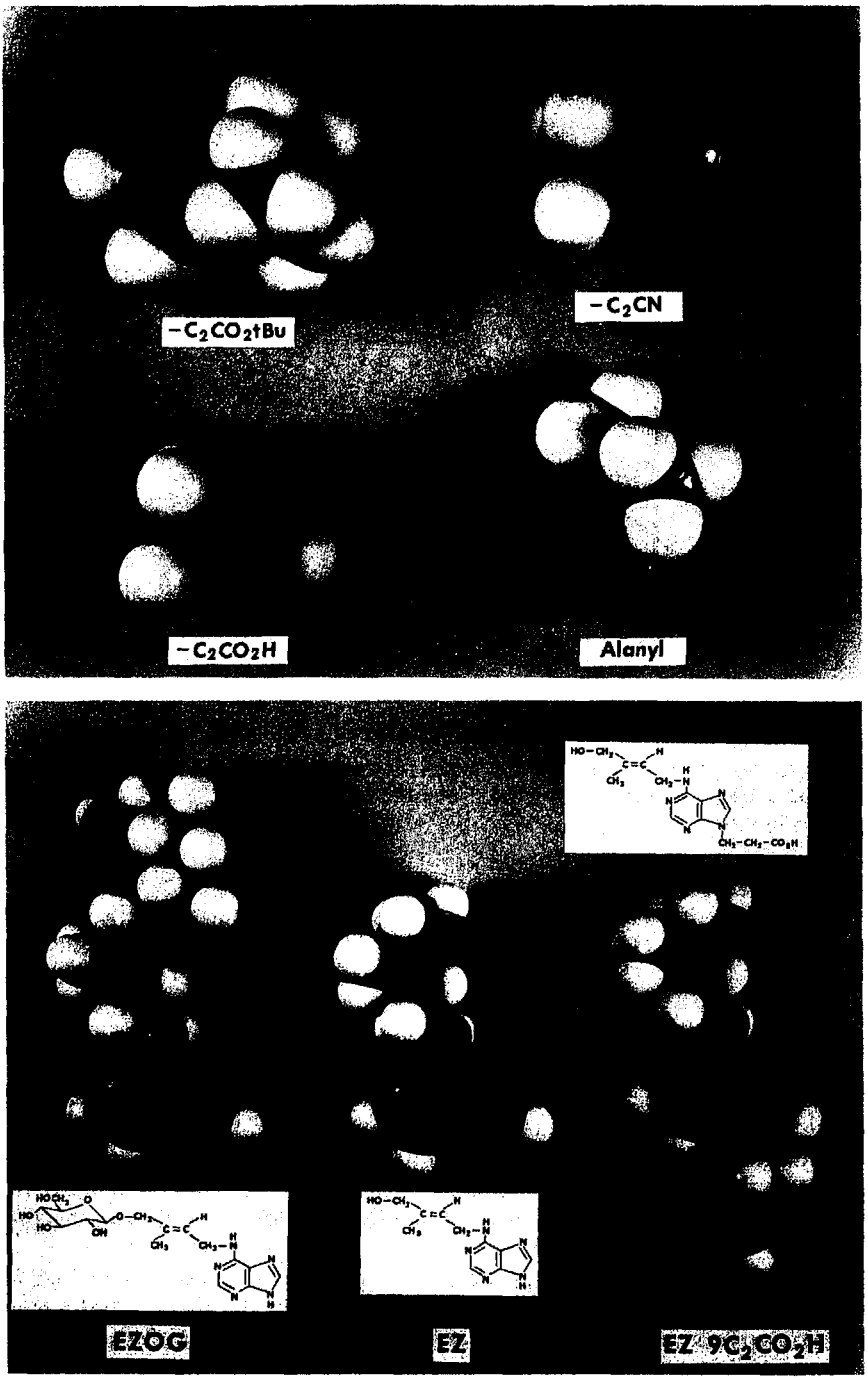

Fig. 5. **(A)** Corey-Pauling-Koltun molecular models of the 9-substituents $-C_2CO_2H$, $-C_2CO_2tBu$, $-C_2CN$, and alanyl. **(B)** CPK molecular models of E-Z, E-ZOG, and E-Z9C_2CO_2H.

dependent on the type of bioassay, being inactive in the tobacco-callus and radish-cotyledon assays but having appreciable activity in the soybean-callus (Palni et al. 1984) and *Amaranthus* assays (Letham et al. 1983). The alanyl group is very slightly larger than the $-C_2CO_2H$ group (Fig. 5A), but much more polar because of the zwitterion contribution to structure. A direct correlation of the activity of LA with our results is hardly possible because of differences in the soybean callus line.

A number of 9-substituted BA derivatives have been reported but no correlations with the present work can be drawn about structure–activity relationship due to the diversity of bioassay methods used in testing. For example, 9-(2-carboxyethyl)BA and its ethyl ester were found to be inactive in the radish-cotyledon assay; the ethyl ester showed slight activity in the oat-leaf senescence assay (Letham et al. 1983). Neither compound was tested on soybean callus.

In the wide variety of cytokinin assays commonly used (callus growth, betacyanidin formation in *Amaranthus,* cotyledon expansion, seed germination, chlorophyll retention, or ethylene production), the plant response plotted against log [cytokinin] gives similarly shaped curves. For a given assay the slopes of the curves in the maximum response sections are parallel, as in this research. These results may be interpreted as being due to structure-dependent affinities for receptor sites in the test tissues (Corse et al. 1983, see Romanov et al. 1988 for discussion and references). Firn (1986) described the changes in dose–response curves obtained using the same growth substance and measuring the same response, in which there are changes in the numbers or affinities of hormone receptors. The curve in Fig. 1B (Firn 1986) illustrates a situation wherein the receptor has 10 times less affinity for the growth substance; the dose–response curve is thus displaced along the x axis but the shape remains the same. This is formally analogous to decreasing the binding between growth substance and receptor by 10-fold due to changes in the structures of growth substances. The use of the word "affinity" in this paper refers to Firn's "growth substance–receptor interaction" and not to the changes of that interaction due to modifications of the receptors themselves.

If it is necessary for biochemical reactions to occur (oxidation, hydrolysis, etc.) in order to convert a derivative or precursor to a form of the hormone with high affinity to the receptor site, the slopes of the activity curves would vary if those reactions were slow in comparison with the biological response of the test tissue (see Fig. 7, Letham et al. 1983). If those reactions were very fast, as with the oxidation of the 9-methyl group to carbon dioxide in the 9-methyl cytokinins, the derivatives would exhibit the properties of the parent (Fox et al. 1971, Pietraface and Blaydes 1981, Shaw et al. 1968). It follows then, if one cytokinin is not as active as another and they have the same activity–concentration slopes, they are probably binding to the same receptor and the differences in activity reflect the differences in affinity. With these guidelines we can extend probe design to explore receptor-site topology and estimate parameters for molecular modeling. The intensive research on adenylate cyclase receptors (summarized by Ukena et al. 1987) provides an excellent example of the information about receptors that can be gained from structure–activity studies. A sound basis for cytokinin binding (Matsubara 1980)

has already been laid by the wide research led by Letham since his first characterization of E-Z (Letham et al. 1964), by the productive cooperation between the groups of Leonard at the University of Illinois and Skoog at the University of Wisconsin, and by similar cooperation among the groups of Matsubara, Fujita and Koshimizu at the Kyoto Universities.

The localization of cytokinins in maize root cells (Zavala and Brandon 1983) with labeled antibodies is puzzling. How can a cytokinin bound to a receptor site still be recognized by an antibody? The answer may well be indicated by the involvement of different regions of even small molecules like cytokinins in antibody and receptor-site specificities. The literature is replete with dose–response data showing the effect of structure on activity (and hence on receptor-site recognition) relevant to 6-substitution in cytokinins. The present work shows that 9-position receptor-site binding is similarly sensitive to structural variations. A polyclonal antibody (as used by Zavala and Brandon 1983) would probably contain antibodies responsive to regions near either the 6- or 9-positions. Thus, a cytokinin bound to a receptor site near one region would still have the other region open for antibody binding. Having monoclonal antibodies of known specificities (Brandon et al. 1987, Trione et al. 1985, 1987) should permit regional specifications of the receptors to be determined.

The 9-(2-carboxyethyl) cytokinins, whose syntheses are described in this report, have proven useful in the preparation of a new generation of haptens with nonhydrolyzable chemical linkages to the immunogen through the 9-position. The successful use of these haptens to prepare polyclonal and specific monoclonal antibodies will be reported elsewhere.

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