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CYTOKININS: SYNTHESIS AND BIOLOGICAL ACTIVITY OF ZEATIN ESTERS AND RELATED COMPOUNDS*

RUTH Y. SCHMITZ and FOLKE SKOOG

Institute of Plant Development, Birge Hall, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

and

SIDNEY M. HECHT† and NELSON J. LEONARD

Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois 61801, U.S.A.

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Abstract—The esters of the highly active cytokinin zeatin [6-(4-hydroxy-3-methyl-*trans*-2-butenylamino) purine] with formic, propionic, and indole-3-acetic acids and of its 2-chloro-substituted derivative [2-chloro-6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine] with acetic and propionic acids have been synthesized, and their growth-promoting activities in the tobacco bioassay have been determined and compared with those of four known, related compounds: zeatin, 6-(4-acetoxy-3-methyl-*trans*-2-butenylamino)purine, methyl 2-methyl-4-(purin-6-ylamino)-*trans*-crotonate, and 2-chlorozeatin. The five new esters were fully as active as any cytokinins we have tested in the tobacco bioaassy. The three zeatin esters were consistently, although only slightly, more active than zeatin, and each of the 2-chlorozeatin esters showed activity about twice that of zeatin on a molar basis.

INTRODUCTION

EXCEPTIONALLY high biological activity has been found earlier in bioassays of two compounds[1,2] closely related to zeatin (Ia).[3–6] The first of these was an ester of zeatin, 6-(4-acetoxy-3-methyl-*trans*-2-butenylamino)purine (zeatin-*O*-acetate) (IIc), which proved fully as active as zeatin itself in the tobacco bioassay. To determine whether the high activity of the acetate ester was unique to the acetoxy group, the formate (IIb), propionate (IId), and indole-3-acetate (IIa) esters have now been synthesized and tested. The second compound, 2-chloro-6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine (2-chloro zeatin) (Ib), was the most active among a number of 2-substituted cytokinins which we synthesized following the identification of 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine as a

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† National Institutes of Health Predoctoral Fellow, 1967–70. Present address: Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin, U.S.A.

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naturally occuring cytokinin.[7,8] 2-Chlorozeatin was recognized as somewhat more active than zeatin, the most active known cytokinin, in these tests.

In view of the favorable effect of the 2-chloro substitution and esterification on biological activity, two compounds have now been synthesized which combine both modifications in the same molecule, namely 2-chloro-6-(4-acetoxy-3-methyl-*trans*-2-butenylamino)-purine (2-chlorozeatin-*O*-acetate) (IIe) and 2-chloro-6-(4-propionoxy-3-methyl-*trans*-2-butenylamino)purine (2-chlorozeatin-*O*-propionate) (IIf).

RESULTS AND DISCUSSION

Syntheses and Mass Spectra

Syntheses have been reported previously for methyl 2-methyl-4-purin-6-ylamino)-*trans*-crotonate,[1] 2-chloro-6-(4-hydroxy-3-methyl-2-butenylamino)purine (Ib), and 6-(4-acetoxy-3-methyl-*trans*-2-butenylamino)purine (IIc). The *O*-propionyl derivatives (IId) and (IIf) were prepared by treatment of zeatin and 2-chlorozeatin with propionic anhydride in pyridine. The *O*-acetyl derivative (IIe) of 2-chlorozeatin was prepared similarly. The *O*-formyl derivative of zeatin (IIb) was obtained by treatment of zeatin with dicyclohexylcarbodiimide in 10-fold excess and formic acid in 100-fold excess and was purified by chromatography on cellulose. These products were characterized by u.v. spectra and, in representative cases, by NMR spectra.

6-[4-(Indole-3-acetoxy)-3-methyl-*trans*-2-butenylamino]purine (IIa) was prepared by condensation of zeatin with indole-3-acetic acid in pyridine, using dicyclohexylcarbodiimide

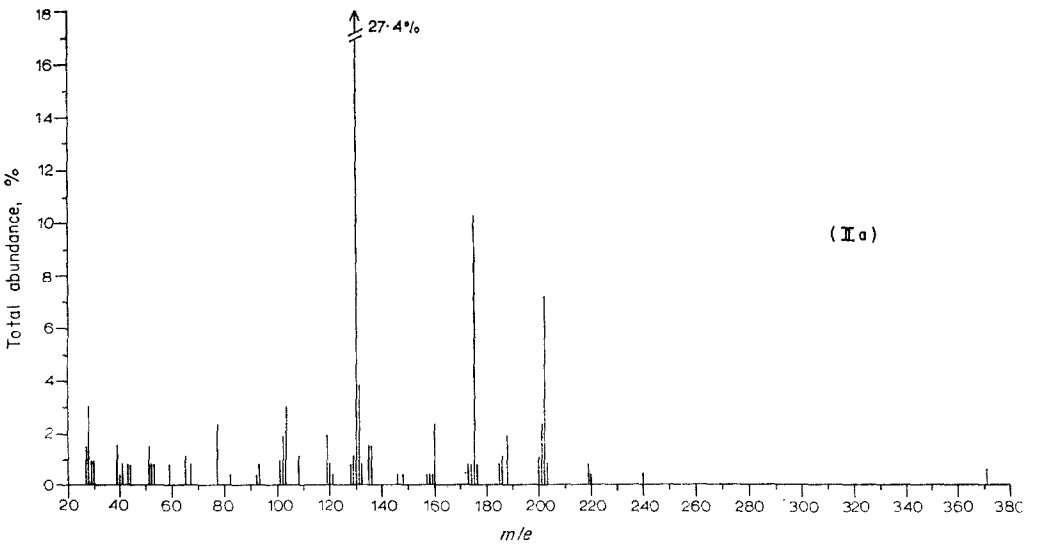

FIG. 1. MASS SPECTRUM AT 70 eV OF 6-[4-INDOLE-3-ACETOXY)-3-METHYL-*trans*-2-BUTENYLAMINO]-PURINE (IIa).

[7] W. J. BURROWS, D. J. ARMSTRONG, F. SKOOG, S. M. HECHT, J. T. A. BOYLE, N. J. LEONARD and J. OCCOLOWITZ, *Science* **161**, 691 (1968).

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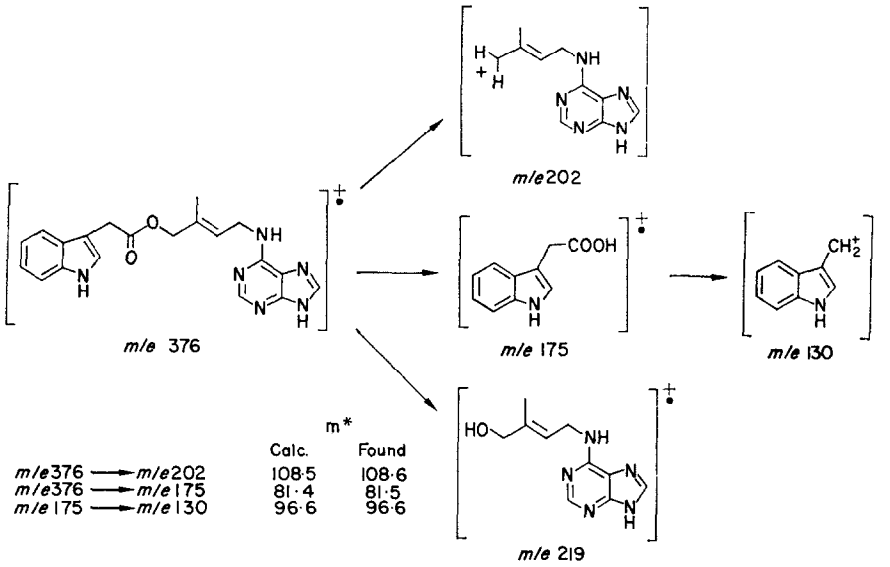


FIG. 2. FRAGMENTS OF 6-[4-INDOLE-3-ACETOXY)-3-METHYL-*trans*-2-BUTENYLAMINO]PURINE (IIa) DETERMINED BY MASS SPECTROMETRY AT 70 eV.

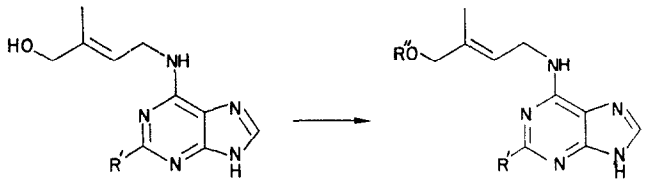
in large excess. This compound was characterized by its u.v. spectrum, which was qualitatively equivalent to the spectrum of an equimolar mixture of zeatin and indole-3-acetic acid, and by its high and low resolution mass spectra (Fig. 1). The fragmentation pattern (Fig. 2) is definitive for the assigned structure. This compound is of special interest because it represents a possible reversible and unique position for auxin attachment in the nucleic acid of plant systems. Details of the syntheses for the new compounds are provided in Experimental.

Cytokinin Activity of Zeatin and 2-Chlorozeatin Esters

The average relative cytokinin activities are summarized in Fig. 3. The esters of zeatin are slightly but uniformly more active than zeatin itself as indicated by the start of the linear portions of the growth curves. From a comparison of the esters it appears that the size of the acid portion has no effect on activity. The greater activity of 2-chlorozeatin (Ib) compared with zeatin (Ia), reported earlier,[2] is confirmed by the present tests, which show the 2-chloro derivative to be about twice as active. The acetate and propionate esters of 2-chlorozeatin (IIe, f) are equally as active as the 2-chlorozeatin itself, so that in this series esterification does not appreciably increase the biological activity.

Although the size of the ester moiety, within the range tested, does not affect activity, the orientation of the ester group has a drastic effect on cytokinin activity. When the orientation is effectively reversed, as in methyl 2-methyl-4-(purin-6-ylamino)-*trans*-crotonate,[1] the cytokinin activity drops to 0·1 per cent that of the true zeatin esters.

The slight but consistent increase in activity conferred on the zeatin molecule by esterification, coupled with the nearly equal activity of all the tested esters, suggests that esterification serves to stabilize the molecule rather than to confer biological activity *per se*. Gradual hydrolysis of the esters probably provides a continuous supply of active zeatin, whereas an equivalent initial supply of unmodified zeatin might be subject to somewhat more rapid loss



(I) (II)

(a) R′=H
(b) R′=Cl

(a) R′=H, R″=indole-3-acetyl
(b) R′=H, R″=formyl
(c) R′=H, R″=acetyl
(d) R′=H, R″=propionyl
(e) R′=Cl, R″=acetyl
(f) R′=Cl, R″=propionyl

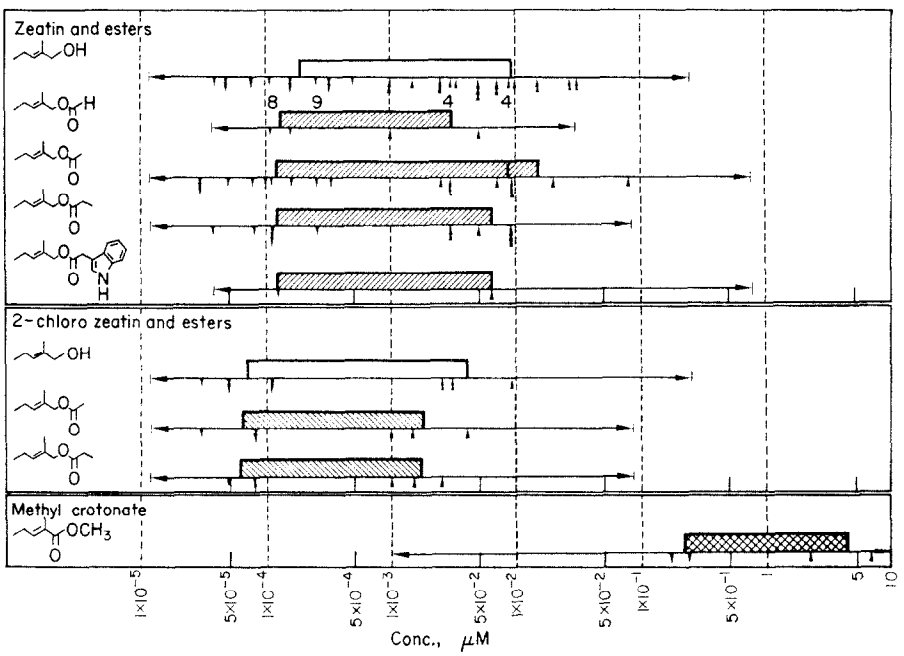


FIG. 3. RELATIVE CYTOKININ ACTIVITIES OF ZEATIN AND 2-CHLOROZEATIN ESTERS.

The first column shows the group attached to N^6 of adenine, representing, in order, the compounds: zeatin (Ia), 6-(4-formyloxy-3-methyl-*trans*-2-butenylamino)purine (zeatin-*O*-formate) (IIb), 6-(4-acetoxy-3-methyl-*trans*-2-butenylamino)purine (zeatin-*O*-acetate) (IIc), 6-(4-propionoxy-3-methyl-*trans*-2-butenylamino)purine (zeatin-*O*-propionate) (IId), 6-[4-(indole-3-acetoxy)-3-methyl-*trans*-2-butenylamino]purine (IIa), 2-chlorozeatin (Ib), 6-(4-acetoxy-3-methyl-*trans*-2-butenylamino)-2-chloropurine (IIe), 2-chloro-6-(4-propionoxy-3-methyl-*trans*-2-butenylamino)purine (IIf), and methyl 2-methyl-4-(purin-6-ylamino)-*trans*-crotonate. The bar represents average values for the range in which growth increases as a linear function of the log of concentration. Note that the starting points of the bars are somewhat higher than the limit of biologically detectable concentrations. 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.

during the long culture period. In the case of the methyl crotonate derivative, hydrolysis would free an acid, which would account for the considerably lower cytokinin activity. Alternatively, or in addition, esterification may affect the entry of the substrate into the tissue. In the case of the 2-chloro derivatives an enhancement by esterification may be too slight for detection in this assay, or the chloro group itself may confer sufficient protection on the zeatin molecule. On the other hand, the enhancement by the 2-chloro group may be related to the actual functioning of the zeatin molecule. Evidence has been presented by Gefter and Russell[9] that the presence of a methylthio group in the 2-position of the purine nucleus in 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine, as found in an *Escherichia coli* tRNA species, increases the binding of that species,[7,8] to ribosomes. If cytokinin activity is in fact related to such a function by the modified adenosines located next to the anticodons in certain tRNA species, then the increased biological activity from 2-chloro substitution of zeatin may be related to ribosomal binding. However, neither 2-methylthiozeatin (II, R′ = CH_3S, R″ = H) nor 6-(3-methyl-2-butenylamino)-2-methylthiopurine, both of which occur as ribonucleosides in certain tRNA's,[7–13] is quite as active as the related zeatin or 6-(3-methyl-2-butenylamino)purine in the tobacco bioassay.[2]

EXPERIMENTAL

Bioaasay Procedures

Cytokinin activity was determined by the tobacco callus bioaassy.[14] This activity has been expressed by bars (Fig. 3) representing that part of the growth curve over which fresh weight increases nearly linearly with the log of cytokinin concentration.[1]

For bioassays the compounds were dissolved in dimethyl sulfoxide (DMSO) in a series of three-fold dilutions and small aliquot portions were added directly to the cooling agar media.[2] This method avoided possible degradation of the synthetic compounds by heat. The final concentration of DMSO in the media was 0·05%, well below concentrations which produce toxic effects.[15]

Synthesis of Test Substances

6-[4-(*Indole-3-acetoxy*)-3-*methyl*-trans-2-*butenylamino*]*purine* (IIa). To 150 mg (0·69 mmole) of 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine (zeatin) (Ia) was added 1·21 g (6·9 mmoles) of indole-3-acetic acid, 1·42 g (6·9 mmoles) of dicyclohexylcarbodiimide and 10 ml of dry pyridine. The solution was stirred at room temp. for 2 days. The suspension was filtered and 10 ml of water was added to the filtrate, which was extracted with pentane. The aqueous layer was evaporated to leave a residue which was purified by chromatography over 750 g of cellulose, elution with ethanol. The desired fraction was then further purified by chromatography over 500 g of silica gel, elution with EtOAc–EtOH (3:1), and by recrystallization from EtOAc–Et_2O to give (IIa) as a white solid, yield 84 mg (33%), m.p. 134–135·5°: $C_{20}H_{20}N_6O_2$ (M^+ calculated 376·1646; found 376·165); λ_{max}^{EtOH} (pH 7) 290 (sh), 269 nm (ε 23,100), λ_{min} 236 (6800); λ_{max}^{EtOH} (pH 1) 289 (sh), 277 (21,900), λ_{min} 237 (6700); λ_{max}^{EtOH} (pH 10) 284 (sh), 275 (23,300), λ_{min} 242 (7400); mass spectrum: *m/e* 376·165 (M^+), 219·112, 202·107, 130·067.

6-(4-*Formyloxy*-3-*methyl*-trans-2-*butenylamino*)*purine* (IIb). To a stirred mixture of 150 mg (0·69 mmole) of 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine, 1·42 g (6·9 mmoles) of dicyclohexylcarbodiimide and 10 ml of dry pyridine was added dropwise 3·20 g (69 mmoles) of 98–100% formic acid. Stirring was continued at room temp. for 2 days. The suspension was filtered and water was added to the filtrate, which was

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extracted with pentane. The residue from the aqueous layer was purified by chromatography over 750 g of cellulose, elution with ethanol, and by crystallization of (IIb) as a white solid from aqueous EtOH, yield 75 mg (44%), m.p. 200–201·5°; λ_{max}^{EtOH} (pH 7) 267 nm (ϵ 18,200), λ_{min} 228 (2600); λ_{max}^{EtOH} (pH 1) 276 (17,200), λ_{min} 235 (3600); λ_{max}^{EtOH} (pH 10) 284 (sh), 276 (17,800), λ_{min} 241 (4100). (Found: C, 53·15; H, 5·25. Calc. for $C_{11}H_{13}N_5O_2$: C, 53·44; H, 5·30%.)

6-(4-*Acetoxy*-3-*methyl*-trans-2-*butenylamino*)*purine* (IIc). Has been described previously.[1,16]

6-(4-*Propionoxy*-3-*methyl*-trans-2-*butenylamino*)*purine* (IId). To 150 mg (0·69 mmole) of 6-(4-hydroxy-3-methyl-2-butenylamino)purine was added 390 mg (3·0 mmoles) of propionic anhydride and 8 ml of pyridine. The resulting solution was allowed to stand overnight at room temp. and was then evaporated several times with EtOH, leaving a pink solid. The solid was purified by chromatography over 750 g of cellulose, elution with EtOH, and by recrystallization from aq. EtOH to afford (IId) as a white solid, yield 64 mg (35%), m.p. 141–142·5°; $C_{13}H_{17}N_5O_2$ (M^+ calculated 275·1382; found 275·137); λ_{max}^{EtOH} (pH 7) 268 nm (ϵ 17,400), λ_{min} 229 (2800); λ_{max}^{EtOH} (pH 1) 276 (16,400), λ_{min} 236 (3400); λ_{max}^{EtOH} (pH 10) 283 (sh), 275 (16,700), λ_{min} 240 (3700); mass spectrum: *m/e* 275·137 (M^+), 218·103, 188·093, 160·065, 135·056; NMR δ (DMSO-d_6-D_2O): 1·06 (3H, t, CH_3-C-C), 1·76 (3H, s, CH_3-C=C), 2·34 (2H, q, C-CH_2-C), 4·24 (2H, d, C-CH_2-N), 4·47 (2H, s, C-CH_2-O), 5·64 (1H, t, C=CH), 8·13, 8·25 (2H, s, Ad-$C_{2,8}$-H's).

6-(4-*Acetoxy*-3-*methyl*-trans-2-*butenylamino*)-2-*chloropurine* (IIe). To 127 mg (0·5 mmole) of 2-chloro-6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine was added 255 mg (2·5 mmoles) of acetic anhydride and 6 ml of pyridine. The resulting solution was allowed to stand overnight at room temp. and was then evaporated several times with EtOH to leave a solid product. This solid was purified by chromatography over 750 g of cellulose, elution with EtOH to afford (IIe) as a white solid, yield 60 mg (41%), m.p. 217–219° λ_{max}^{EtOH} (pH 7) 271 nm (ϵ 18,900), λ_{min} 232 (2700); λ_{max}^{EtOH} (pH 1) 272 (16,800), λ_{min} 233 (2500); λ_{max}^{EtOH} (pH 10) 278 (17,700), λ_{min} 244 (4400). (Found: C, 48·58; H, 4·86; N, 23·32. Calc. for $C_{12}H_{14}ClN_5O_2$: C, 48·74; H, 4·77; N, 23·68%.)

2-*Chloro*-6-(4-*propionoxy*-3-*methyl*-trans-2-*butenylamino*)*purine* (IIf). To 127 mg (0·50 mmole) of 2-chloro-6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine was added 325 mg (2·5 mmoles) of propionic anhydride and 6 ml of pyridine. The resulting solution was allowed to stand overnight at room temp. and was then evaporated several times with EtOH to leave a solid product. Chromatography over 750 g of cellulose, elution with EtOH, afforded a white solid which was recrystallized from EtOH to give colorless crystals of (IIf), yield 44 mg (28%), m.p. 208–209·5°; λ_{max}^{EtOH} (pH 7) 272 nm (ϵ 19,200), λ_{min} 231 (2600); λ_{max}^{EtOH} (pH 1) 272 (17,200), λ_{min} 233 (2500); λ_{max}^{EtOH} (pH 10) 278 (17,800), λ_{min} 244 (4500). (Found: C, 50·43; H, 5·10; N, 22·67. Calc. for $C_{13}H_{16}ClN_5O_2$; C, 50·41; H, 5·21; N, 22·61%.)

Acknowledgement—We wish to thank Mr. James Chickering for technical assistance with the bioassays.

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