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Cytokinins in *Corynebacterium fascians* Cultures

ISOLATION AND IDENTIFICATION OF 6-(4-HYDROXY-3-METHYL-*CIS*-2-BUTENYLAMINO)-2-METHYLTHIOPURINE[1]

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DONALD J. ARMSTRONG,[2] EMANUEL SCARBROUGH, AND FOLKE SKOOG[3]
Institute of Plant Development, Birge Hall, University of Wisconsin, Madison, Wisconsin 53706

DOUGLAS L. COLE[4] AND NELSON J. LEONARD[3]
Roger Adams Laboratory, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801

ABSTRACT

In addition to the four cytokinins, 6-(3-methyl-2-butenylamino)purine, 6-methylaminopurine and the *cis* and *trans* isomers of 6-(4-hydroxy-3-methyl-2-butenylamino)purine, reported earlier from our laboratories, three cytokinin-active fractions have been obtained from the aqueous medium of 6-day-old *Corynebacterium fascians* cultures. One of these has been identified as 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-2-methylthiopurine (2-methylthio-*cis*-zeatin, *c*-ms^{2}io^{6}Ade).

The elution volumes of the other two fractions correspond to those of authentic 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine and 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, indicating the presence of trace amounts of these two ribonucleosides.

Fasciation and modified growth of seedlings closely resembling symptoms of infection with *Corynebacterium fascians* have been induced in *Pisum* by soaking the seeds in high concentrations of kinetin (20). That in fact these symptoms of infection by the bacterium might be due to its production of cytokinin was indicated by the ability of extracts of pure cultures to release lateral buds of *Pisum* from apical dominance in the same manner as kinetin (25). Isolation of cytokinin-active material from aqueous cultures of the same stock[5] was achieved by solvent extraction and chromatography. Three crystalline fractions with cytokinin activity as monitored in the tobacco bioassay were obtained (8, 10). The most active fraction was identified as 6-(3-methyl-2-butenylamino)purine (i^{6}Ade) (6), known at that time only as a synthetic, highly active cytokinin (5, 9). The second fraction contained nicotinamide. Inasmuch as synthetic nicotinamide was inactive in the tobacco bioassay, the activity of the isolated material was ascribed to a contaminant. This cytokinin was recently identified as 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)purine (*cis*-zeatin, *c*-io^{6}Ade) (21). The third active fraction found in 1966 contained N^6-methyladenine, a weakly active cytokinin (12, 23), but in amounts less than required to account for the activity of this fraction in the tobacco bioassay. Hence, another more potent cytokinin was assumed to be present as a contaminant and to be mainly responsible for the cytokinin activity in this fraction. We now report the purification and characterization of this cytokinin from *Corynebacterium fascians* culture medium as 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-2-methylthiopurine (2-methylthio-*cis*-zeatin, *c*-ms^{2}io^{6}Ade).

MATERIALS AND METHODS

Growth of *Corynebacterium fascians* Cultures. The origin of the *C. fascians* strain used in this work and the culture methods employed have been described (21). Cultures were harvested after 6 days growth at about 28 C (about 24 hr after reaching the stationary phase).

Isolation of Cytokinins from the Culture Medium. The preliminary purification and fractionation of the cytokinins present in the medium from *C. fascians* cultures have been described in detail (21). In summary, the following procedures were used: the cells were separated from 100 liters of stationary phase cultures by centrifugation. The supernatant was acidified, filtered, and fractionated by Dowex 50 chromatography. The NH_4OH eluate from the Dowex columns was evaporated to dryness *in vacuo* and the dry solids were extracted with water-saturated ethyl acetate. The extracts were taken to dryness, redissolved in 35% ethanol, and chromatographed on Sephadex LH-20 columns in 35% ethanol. Three peaks of cytokinin activity were detected in the elution profile from the LH-20 columns. In order of elution, these peaks corresponded to *c*-io^{6}Ade (1.4 bed volumes), i^{6}Ade (2.1 bed volumes), and an unidentified cytokinin (2.4 bed volumes). Purification of the latter compound was achieved by a combination of Sephadex LH-20 chromatography and paper chromatography.

Sephadex LH-20 Chromatography. Chromatography on Sephadex LH-20 (Pharmacia) columns was carried out essentially as described by Armstrong *et al.* (1). Details of chromatographic procedures are given in the legends to the figures. Ethanol was redistilled prior to use, and water was double distilled from a Pyrex distillation apparatus.

Paper Chromatography. Whatman No. 1 filter paper, washed as described (21), was used. Cytokinin samples were dissolved in small volumes of 95% ethanol and applied to the paper as 5-cm streaks. The chromatograms were developed by ascending chromatography over 30 cm. UV-absorbing bands were recovered from the chromatograms in small volumes (about 0.5-1 ml) by

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[2] Present address: Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331.

[3] To whom requests for reprints should be sent.

[4] Present address: Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065.

[5] A culture of *Corynebacterium fascians* was kindly given to us in 1964 by Professor K. V. Thimann.

elution with 95% ethanol. Ethanol was redistilled prior to use, and water was triple distilled.

Bioassay of Cytokinin Activity. Cytokinin activity was determined in the tobacco bioassay as described (10, 19) and activity is expressed as kinetin equivalents (KE, the μg of kinetin [6-furfurylaminopurine] required to give the same growth response as the test sample under the specified bioassay conditions).

Procedures for Identification of Cytokinin in Fraction 7 of Figure 2. Ultraviolet absorption spectra in absolute methanol were determined with a Cary model 15 spectrophotometer. Mass spectra were obtained with Varian MAT CH-5 and Varian MAT 731 spectrometers. Side chain geometry was determined by high performance liquid chromatography on a column of Bio-Rad Aminex A-5 cation exchange resin as reported by Cole *et al.* (2).

The syntheses and properties of authentic *c*-io^{6}Ade, *t*-io^{6}Ade, *c*-ms^{2}io^{6}Ade, and *t*-ms^{2}io^{6}Ade used as standards in the identification of cytokinins in this study have been described (17, 27).

RESULTS

Isolation of Cytokinins. A cytokinin-active peak that eluted after i^{6}Ade, when partially purified extracts from 100 liters of *C. fascians* culture medium were fractionated on Sephadex LH-20 columns in 35% ethanol (21), was recovered from the column eluates by evaporation *in vacuo* at 37 C. The solids were rechromatographed on Sephadex LH-20 in 9.5% ethanol. The elution profile for this column is shown in Figure 1. The cytokinin activity eluted as a single peak.

Repeated attempts to purify the cytokinin further by paper chromatography in aqueous ethanol solutions eliminated some colored material but failed to separate the cytokinin activity from contaminating fluorescent material. Therefore, the cytokinin sample was rechromatographed on a Sephadex LH-20 column in distilled H_2O. The elution profile for this column is shown in Figure 2. Two peaks of UV-absorbing compounds were observed, but cytokinin activity was associated with only the second peak. A synthetic sample of the cytokinin 2-methylthiozeatin, applied to the column after the fractionation was completed, eluted at the same position as the cytokinin-active peak.

Further purification of this *C. fascians* cytokinin was achieved by paper chromatography in 19% ethanol. A single UV-absorbing band was observed at about R_F 0.5. To remove traces of fluorescent material, the compound was chromatographed three times under the above conditions. The UV-absorbing band eluted from the final chromatogram was taken to dryness in a stream of N_2. This sample which contained about 1 A_{260} unit was used for structure determination. Using the extinction coefficient for ms^{2}io^{6}Ade (λ_{max}^{EtOH}) 279 nm (ϵ15,800) and correcting for the fraction of the material used in bioassays, the final yield was equivalent to about 0.001 μmol/l of extracted culture medium.

Identification of Isolated Cytokinin. The above dried preparation (about 1 A_{260} unit) dissolved in absolute methanol had an UV-absorption spectrum practically identical with that of authentic *c*-ms^{2}io^{6}Ade. The data in Table I show the congruence of λ_{max} and λ_{min} values of the two compounds and the close agreement in the ratios of their molar absorption values at the absorption maxima. Assuming a molar extinction coefficient of 2500 at 240 nm (28), the UV spectrum indicated that about 10 μg of sample were available for chemical structure determination.

A 20% aliquot was evaporated to dryness in a gold crucible at 50 C and was inserted into the direct inlet of a Varian MAT CH-5 mass spectrometer. The fragmentation patterns derived from the spectra of this sample and from that of authentic *c*-ms^{2}io^{6}Ade are shown in Table II. After subtraction of background, the two spectra are identical, but the peak at *m/e* 265 is very weak in the spectrum of the isolated cytokinin.

The quantity of isolated cytokinin was insufficient for obtaining high resolution mass spectra, but its mol wt was confirmed by its field desorption mass spectrum. The dried sample was taken up in 20 μl of absolute methanol. The field emitter wire of a Varian MAT combination electron impact-field desorption ion source was dipped into the solution and the spectrum was determined on a Varian MAT 731 mass spectrometer. At an emitter current of 23 mamp, the oscillographically recorded spectrum consisted of a single peak at *m/e* 265. This peak was observed at

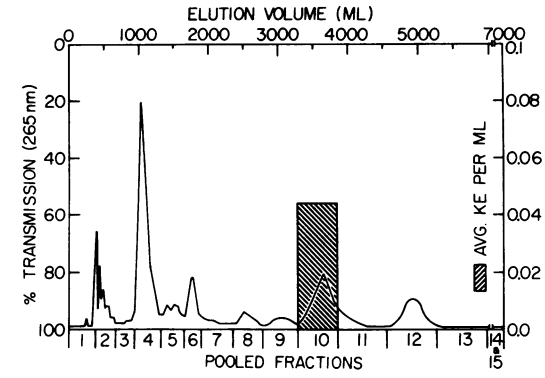

FIG. 1. Chromatography of *C. fascians* cytokinin on Sephadex LH-20 in 9.5% ethanol. The partially purified cytokinin preparation was dissolved in 20 ml of 9.5% ethanol and chromatographed on a Sephadex LH-20 column (125 g, 3.6 × 50 cm, about 530 ml bed volume) in the same solvent. Fractions, each 10 ml, were collected and pooled as indicated. Aliquots (5%) of the pooled fractions were tested for cytokinin activity in the tobacco bioassay.

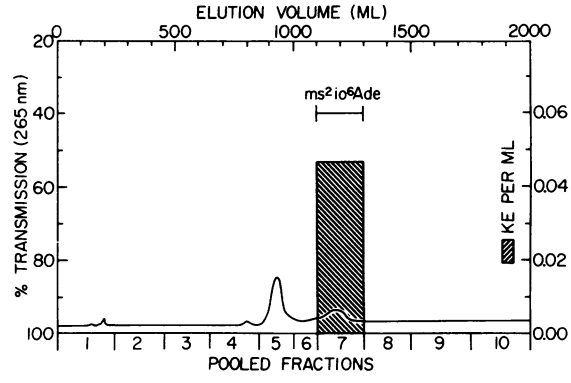

FIG. 2. Chromatography of the *C. fascians* cytokinin on Sephadex LH-20 in distilled H_2O. The partially purified cytokinin preparation from Figure 1 was dissolved in 4 ml of distilled H_2O and chromatographed on a Sephadex LH-20 column (30 g, 1.9 × 43.3 cm, about 123 ml bed volume). Fractions, each 10 ml, were collected and pooled as indicated. Aliquots (7%) of the pooled fractions were tested for cytokinin activity in the tobacco bioassay. The elution position of ms^{2}io^{6}Ade was determined with a synthetic standard subsequently chromatographed on the same column.

Table I. Ultraviolet Absorption Spectra of Isolated Cytokinin and *c*-ms^{2}io^{6}Ade

	Isolated Cytokinin	*c*-ms^{2}io^{6}Ade
	nm	
λ_{max}	278	279
λ_{max}	240	242
λ_{min}	257	257
λ_{min}	221	220
$\frac{\epsilon(\text{long } \lambda)}{\epsilon(\text{short } \lambda)}$	0.61	0.64

all emitter current values. Other masses were seen only intermittently at lower temperatures, indicating that complete purification had not been achieved by Sephadex LH-20 chromatography.

To determine the isomeric configuration of the N^6-side chain, the remaining sample was subjected to high performance liquid chromatography by methods developed by Cole *et al.* (2). The elution volumes of authentic *cis* and *trans* isomers of ms^2io^6Ade and of the isolated cytokinin together with properties of the column and conditions used are given in Table III. The peak of isolated material which eluted from the Bio-Rad Aminex column at 223 ml, as compared with 222 for authentic *c*-ms^2io^6Ade, was concentrated to a minimal volume. An aliquot part was then freeze-loaded onto an emitter wire by the technique of Olson *et al.* (15) for determination of the field desorption mass spectrum. The spectrum at 10-mamp emitter current showed a single peak at *m/e* 266 (protonation due to acidic conditions lead to strong M + H^+ peaks), confirming that the high performance liquid chromatography peak was due to ms^2io^6Ade.

The isolated cytokinin was thus proved to be 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-2-methylthiopurine.

Cytokinin-active Ribonucleosides. In a confirmatory experiment with active material extracted from 200 liters of 6-day-old *C. fascians* culture medium, bioassays revealed two additional peaks of cytokinin activity in the profile from the Sephadex LH-20 column eluted with 35% ethanol. These peaks corresponding to the elution volumes of io^6Ado (111 bed volumes) and i^6Ado (1.7 bed volumes), and distinct from the elution volumes of io^6Ade, i^6Ade, and ms^2io^6Ade (1.4, 2.1, and 2.4 bed volumes, respectively), suggest that trace amounts of these two ribonucleosides are also present in free form in the cultures.

DISCUSSION

This study brings to five the number of cytokinin-active N^6-substituted adenine bases isolated from *C. fascians* cultures, four of which have been chemically characterized: *c*-ms^2io^6Ade in this report, and i^6Ade, *c*-io^6Ade, and the weakly active m^6Ade in earlier reports. The presence of *t*-io^6Ade was indicated by bioassays of chromatographically separated isomers of isolated io^6Ade in our earlier report (21), but the amount of this isomer was not enough for chemical characterization. We now also report indirect evidence for trace amounts of two cytokinin-active ribonucleosides, io^6Ado and i^6Ado, in the culture medium.

Table II. Electron Impact Mass Spectral Fragmentation Patterns of Isolated Cytokinin and c-ms^2io^6Ade

Data for both compounds obtained with a Varian-MAT CH-5 electron impact mass spectrometer with solids probe. Probe temperature was 185 C.

Isolated Cytokinin m/e	c-ms^2io^6Ade m/e
265	265
250	250
248	248
247	247
232	232
206	206
200	200
182	182
181	181
165	165
151	151
135	135
119	119

Table III. High Performance Liquid Chromatography of the Isolated Cytokinin and t - and c-ms^2io^6Ade

Column: Bio-Rad Aminex A-5 cation exchange resin, 13 ± 2 µM diam., 1.3 x 18.5 cm, 70 C, pH 4.1, 0.08 M NH_4HCO_3 buffer, 25% DMF, 0.8 ml/min, 22 atm.

Compound	Elution Volume
	ml
t-ms^2io^6Ade	228
c-ms^2io^6Ade	222
Isolated Cytokinin	223

No meaningful quantitative estimate can be made of total cytokinin activity or amounts of various species in the living cells on the basis of the observed biological activities or amounts isolated from the culture medium. The extent to which the above seven cytokinins may reflect the full spectrum of species in *C. fascians* also remains to be determined. The results suggest that this bacterium produces all species of cytokinin which have been found free or as macromolecular constituents in higher plants. The failure so far to detect ms^2i^6Ade and ms^2i^6Ado may be due merely to the relatively high retention of these methiolated purine derivatives on the Sephadex column and their relatively low activity in bioassays, especially of the *cis* isomers, rather than to their absence or exceptionally low content in the cultures.

As to the origin of free cytokinins in the cultures, they may be nucleic acid degradation products of moribund cells or excreted normal cell metabolites possibly from the same source. Rathbone and Hall (18) have confirmed the presence of i^6Ado in hydrolyzed *C. fascians* tRNA (11) and they found several times more extractable i^6Ade in acidified cultures than the approximate 2 µg/l found in neutral controls. They ascribe the increase in i^6Ade to its being released from tRNA in the extraction process. Even this tentative interpretation seems unwarranted on the basis of the methodology and measurements provided, and except by implication, it leaves open the source of the i^6Ade in the normal cultures. The presence of both io^6Ado and i^6Ado in free form as well as the observed preponderance of the *cis* isomers of both io^6Ade (21) and ms^2io^6Ade would be expected if the free cytokinins derive from tRNA, but alternative sources of these ribonucleosides are not excluded by these findings.

Apparently, the symptoms of *C. fascians* pathogenicity (*e.g.* fasciation) may be brought about by bacteria on the plants' surface, *i.e.* without the need for the microbes to penetrate the epidermis, and can be mimicked by applied cytokinin. The disease, therefore, may be caused by the cytokinins released by the bacteria. The exceptionally high cytokinin activity generated by *C. fascians* is illustrated by the striking growth of cytokinin-dependent tobacco callus or excised pith tissue initiated and sustained by a colony of this bacterium no larger than a pinhead growing on cytokinin-free nutrient agar at distances at least 2 cm away from the plant tissue. Some other plant pathogens (4, 7, 14, 27) and symbionts (3, 13, 16) associated with overgrowth in host tissues similarly have been shown to have relatively high cytokinin contents, although in mycorrhiza at least this relationship may not be obligatory (3). Presumably, other types of microorganisms produce cytokinins of kinds and in quantities ineffective for pathogenicity and not so readily detectable by present assay methods.

Zeatin and its derivatives (cytokinins with one hydroxylated methyl group in the N^6-isopentenyl side chain of adenine) have been considered to occur normally in autotrophic plants (22) including algae (24), but generally not in fungi and bacteria, and not in animals (22). Their presence in saprophytic microorganisms has been restricted to pathogens and symbionts or species otherwise associated with overgrowth in plants. However, one apparent exception to such a functional association, the presence of ms^2io^6Ado in *Pseudomonas aeruginosa* tRNA, has been reported (26).

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