Proc. Nat. Acad. Sci. USA
Vol. 70, No. 12, Part II, pp. 3825–3829, December 1973

Isolation of *cis*-Zeatin from *Corynebacterium fascians* Cultures

(cytokinins/6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)purine/ 6-(3-methyl-2-butenylamino)purine/fasciation)

EMANUEL SCARBROUGH*, DONALD J. ARMSTRONG*, FOLKE SKOOG*, CHARLES R. FRIHART†, AND NELSON J. LEONARD†

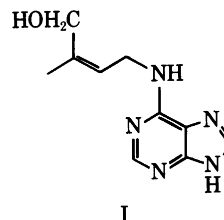
*Institute of Plant Development, Birge Hall, University of Wisconsin, Madison Wisc. 53706; and †Roger Adams Laboratory, School of Chemical Sciences, University of Illinois, Urbana, Ill. 61801

Contributed by Nelson J. Leonard and Folke Skoog, August 28, 1973

ABSTRACT Three cytokinin-active constituents have been isolated from the culture medium of *Corynebacterium fascians*, a plant pathogen causing fasciation. One constituent is now identified as *cis*-zeatin [6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)purine]. It represents the first unequivocal demonstration of the production of a zeatin isomer by a bacterial species. Another constituent was identified earlier as 6-(3-methyl-2-butenylamino)purine. The third constituent, distinct, on Sephadex LH-20 column chromatography, from the above two and their ribosides, has not yet been identified.

The symptoms of the fasciation disease in plants caused by *Corynebacterium fascians* can be imitated by treatment with kinetin (6-furfurylaminopurine) (1, 2), and the presence of a chloroform-soluble, silver-precipitable, cytokinin-active constituent in cultures of this plant pathogen has been demonstrated (2). From large-scale cultures derived from the same stock and following an isolation procedure that involved heat, an acidification step, and chromatographic separation, Klämbt, Thies, and Skoog (3) isolated three cytokinin-active fractions. The main component of each, as a picrate derivative, was identified by Helgeson and Leonard (4). One fraction contained 6-(3-methyl-2-butenylamino)purine, or N^6-(Δ^2-isopentenyl)adenine (2iP or i^6Ade), previously synthesized and recognized as a highly active cytokinin (5). The isolation and identification of i^6Ade from *C. fascians* has recently been repeated, presumably from a stock culture of different origin (6). The second fraction that we isolated contained nicotinamide, which in pure form was devoid of cytokinin activity. Accordingly, the activity in the fraction containing nicotinamide was ascribed to contamination with a highly active cytokinin, the identification of which has remained a challenge. The third fraction contained 6-methylaminopurine (3, 4), but the amount of this low-potency cytokinin was less than could account for the observed biological activity, and it was concluded that this fraction also contained trace amounts of some more active cytokinin(s).

Tests on tRNA hydrolysates of *C. fascians*, based on chromatography and biological activity, provided evidence for the presence of i^6Ade and indication of another cytokinin-active constituent with R_F corresponding to zeatin (7), and it was recognized that the heat and acid treatments in the original extraction procedure could generate the active bases from precursors (3), notwithstanding the fact that the original bacterial cultures did possess high cytokinin activity when not subjected to heat or acid treatment. In a reexamination of the cytokinins present in the medium of *C. fascians* cultures using a milder isolation procedure, we have found and identified *cis*-zeatin [6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)purine] (I) in the chromatographic fraction corresponding to that which yielded nicotinamide (3, 4). Evidence is presented also for the presence of another cytokinin that is distinct from i^6Ade and *cis*-zeatin and their corresponding ribonucleosides (6).



MATERIALS AND METHODS

Growth and Harvest of Cultures. The culture of *Corynebacterium fascians* Cf-1 originated from the Burkholder Collection at Cornell University. It was obtained from Dr. R. S. Dickey by Dr. J. P. Helgeson, University of Wisconsin, who in turn kindly supplied a transfer to us. It came from the same source as the Cf-1 strain used in refs. 2–4 and 7. The cells were cultured as described by Klämbt *et al.* (3) except that distilled water was used and adenine was omitted from the culture medium. Liquid cultures of *C. fascians* were grown in several batches to yield a total of 100 liters of cell suspension. Cultures were harvested after 6 days growth at about 28° (about 24 hr after the stationary phase was reached).

Isolation of Cytokinins from Culture Medium. The cells were separated from the culture medium, without heating, by continuous-flow centrifugation at 35000 × *g* (Sorvall RC2-B centrifuge, rotor SS-34) and a flow rate of 150–200 ml/min. Two centrifugations yielded a clear supernatant which was used exclusively in this study. The supernatant was adjusted to pH 2 with 1 N HCl, allowed to stand overnight at 4°, and then filtered through a porous polyethylene filter candle to remove a light, flocculant precipitate.

The filtrate from the acidified culture medium was frac-

Abbreviations: i^6Ade, N^6-(Δ^2-isopentenyl)adenine or 6-(3-methyl-2-butenylamino)purine, also called 2iP; i^6Ado, N^6-(Δ^2-isopentenyl)adenosine, also called 2iPA; KE, kinetin equivalents; TLC, thin-layer chromatography.

tionated by Dowex 50 column chromatography (see below). The NH_4OH eluates from the columns were combined and evaporated to dryness under reduced pressure at 40°.

The dry solids from the Dowex column eluates were extracted with water-saturated ethyl acetate. Twelve extractions (15 min each) were performed with 5 ml of solvent per liter of original culture medium per extraction. The extracts were combined, divided into four aliquots (each equivalent to 25 liters of original culture medium), and evaporated to dryness under reduced pressure at 37°. The ethyl acetate extracts were chromatographed on Sephadex LH-20 columns in 35% ethanol (see below). The cytokinin-active fractions were rechromatographed on a Sephadex LH-20 column in water and the active material from this column was purified by paper chromatography (see below).

Dowex 50 Chromatography. Dowex 50W-X4, H^+ (50–100 mesh) was prepared for use by extensive washing with 6 *N* NH_4OH followed by doubly distilled water until the column effluent was near neutrality. The columns were then converted to the H^+-form by washing with 6 N HCl followed by 0.01 N HCl until the effluent was at pH 2. Brief washes with intermediate concentrations of acid or base prevented excessive heating when the solvents were changed. After use, the columns were recycled through the above wash procedure. Each column was used twice before the ion-exchange resin was discarded.

For cytokinin purification, aliquots (10 liters each) of the filtrate from the acidified culture medium were applied to 1-liter Dowex 50 columns (6.4 × 31.0 cm) at a flow rate of about 1 liter/hour. Each column was washed with 2 liters of 0.01 N HCl followed by 2 liters of doubly distilled water. The washings were discarded and each column was eluted with 2.5 liters of 2 N NH_4OH followed by 2 liters of 5 N NH_4OH.

Sephadex LH-20 Chromatography was carried out essentially as described by Armstrong *et al.* (8). Details of chromatographic procedures are given in the legends to the figures. Ethanol was redistilled before use, and water was doubly distilled from glass.

The synthetic cytokinin standards were applied to the LH-20 columns after fractionations of isolated materials were complete. The columns were discarded after the elution positions of the standards were determined.

Paper Chromatography. Whatman no. 1 filter paper was prepared for use by successive washes (descending chromatography) with 0.02 N HCl, water, 95% ethanol, and water. The washed paper was dried at room temperature. Redistilled ethanol and triply distilled water were used in the wash procedure and in the preparation of chromatographic solvents used in cytokinin purification.

Cytokinin samples were dissolved in a small volume of 95% ethanol and applied as a 5-cm streak to a sheet of washed paper. The paper chromatograms were developed by ascending chromatography over 30 cm. The UV-absorbing bands were recovered from the chromatograms in small volumes (about 0.5–1.0 ml) by elution with 95% ethanol.

Bioassay of Cytokinin Activity. Cytokinin activity was determined by the tobacco bioassay (9, 10) as described (11). For bioassay purposes, aliquots of column fractions were evaporated to dryness, redissolved in distilled water in a steamer for 30 min, and incorporated into 100 ml of RM-1965 medium (9). The concentrations at which the bioassay samples were tested are expressed as liter equivalents of original *Corynebacterium* medium per liter of tissue culture medium. Cytokinin activity is expressed as kinetin equivalents (KE), defined as the micrograms of kinetin (6-furfurylaminopurine) required to give the same growth response as the test sample under the specified bioassay conditions.

Cytokinin Identification. The cytokinin-active preparation was dissolved in 300 μl of absolute methanol. Of this solution, 100 μl was transferred to a 0.1-cm UV cell and diluted with 200 μl of methanol. The UV spectrum was determined on a Cary model 15 spectrophotometer; the sample was transferred back to the original solution and it was taken to dryness under reduced pressure. The quantity of cytokinin was calculated from the observed absorbance at λ_{max} and the known extinction coefficient of the pure free base. An aliquot of the sample was dissolved in absolute methanol and spotted on silica-gel plates with fluorescent indicator. The spots were eluted with either a mixture of chloroform and methanol (9:1, v/v) (solvent A) (12) or a mixture of chloroform and acetic acid (8:2, v/v) (solvent B) (13). Approximately one-fourth of the sample was placed in a gold crucible for direct inlet into a Varian-MAT CH-5 mass spectrometer equipped with a computer printout. The low-resolution spectrum was compared with that of a synthetic sample of *cis*-zeatin (12). The high-resolution mass spectrum was obtained with the rest of the sample in a similar manner on a Varian-MAT 731.

Controls for Isolation Procedures. The fate of several cytokinin bases and ribonucleosides under the conditions of the isolation procedure described above was determined. The stabilities of *cis*-zeatin (I), *trans*-zeatin, ribosyl-*cis*-zeatin, ribosyl-*trans*-zeatin, and i^6Ade were tested under the following conditions: (*a*) 0.1 N HCl (pH 2) at 4° (also 25°) for 24 hr; (*b*) 5 N NH_4OH at 25° for 24 hr; (*c*) heating an aqueous solution of the compound to 95°, acidification to pH 2, and cooling (the last not used in present isolation). After the test period, aliquots from the reaction mixtures were spotted on silica-gel thin-layer chromatography (TLC) plates which were then eluted with a mixture either of chloroform–methanol (9:1, v/v) or chloroform–acetic acid (8:2, v/v). By use of *cis*-zeatin, *trans*-zeatin, ribosyl-*cis*-zeatin, ribosyl-*trans*-zeatin, i^6Ade, cyclized i^6Ade (14), 6-(3-hydroxy-3-methylbutylamino)purine, and 6-(3,4-dihydroxy-3-methylbutylamino)purine (14) as TLC standards, it was determined that all five test compounds were stable under conditions *a* and *b*. Under conditions *c*, i^6Ade was about 10% converted to 6-(3-hydroxy-3-methylbutylamino)purine; *cis*- and *trans*-zeatin were about 5% converted to 6-(3,4-dihydroxy-3-methylbutylamino)purine; and ribosyl-*cis*- and *trans*-zeatins lost their ribosyl groups almost completely.

RESULTS

The partially purified, cytokinin-active preparation obtained from *C. fascians* culture medium by Dowex 50 chromatography and ethyl acetate extraction (see above) was chromatographed on Sephadex LH-20 columns in 35% ethanol. The elution profile of one of the four replicate columns is shown in Fig. 1. Three peaks of cytokinin activity were detected. Peak I corresponds to the elution position of zeatin. The cytokinin activity present in this peak was equal to about

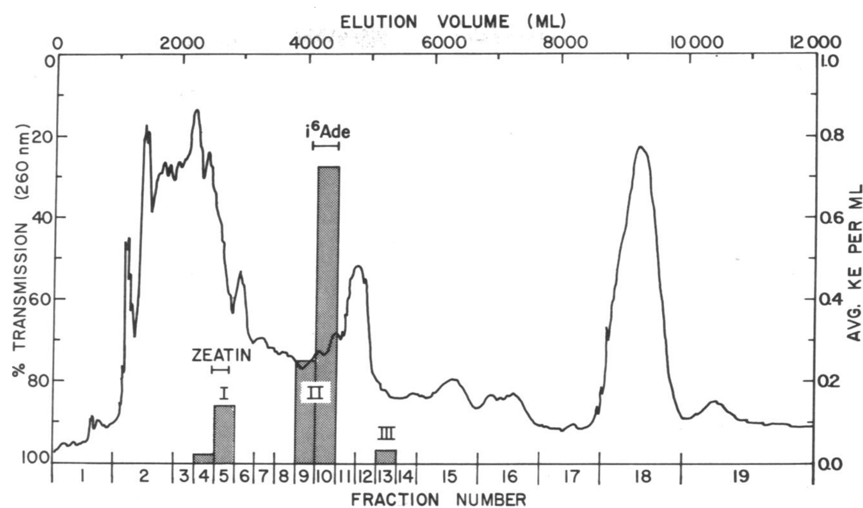


FIG. 1. Chromatography of cytokinins from *C. fascians* medium on Sephadex LH-20 in 35% ethanol. The solids recovered from one-fourth of the total ethyl acetate extract were dissolved in 40 ml of 35% ethanol and applied to a Sephadex LH-20 column (500 g, 4.8 × 91.3 cm, 1.93-liter bed volume) equilibrated with the same solvent. The column was eluted with 35% ethanol, and 20-ml fractions were collected. These fractions were pooled as indicated (1 through 19), and aliquots from the pooled fractions were tested for cytokinin activity in the tobacco bioassay at a maximum concentration of 5 liter equivalents per liter (see *Methods*). Cytokinin activity is indicated by *vertical bars*. The elution positions of cytokinin standards are labeled.

2 KE per liter of harvested *Corynebacterium* culture medium. The isolation and identification of the cytokinin-active component of this peak is described below. Peak II corresponded to the elution position of i^6Ade. The cytokinin activity present in this peak was equal to about 12 KE per liter of culture medium. Rechromatography of this peak on Sephadex LH-20 in 9.5% ethanol (not shown) gave a single peak of cytokinin activity which again corresponded to the elution position of i^6Ade. Peak III contained cytokinin activity of about 0.4 KE per liter of culture medium.

Fractions corresponding to cytokinin peak I from the four replicate Sephadex LH-20, 35% ethanol columns described above (Fig. 1, fraction 5) were combined and evaporated to dryness under reduced pressure at 37°. The solids were chromatographed on a Sephadex LH-20 column in distilled water. The elution profile for this column is shown in Fig. 2. Bioassay revealed cytokinin activity in a single peak (fraction 13′) at the approximate elution position of zeatin. It should be mentioned that neither ethanol nor water as eluant will permit chromatographic differentiation between the *cis* and *trans* isomers of zeatin on a Sephadex LH-20 column.

Fraction 13′ was evaporated to dryness under reduced pressure at 37°, and the solids were chromatographed on Whatman no. 1 paper in 9.5% ethanol as described above. In addition to diffuse fluorescent material, a single UV-absorbing band was observed at R_F 0.57. This R_F value corresponded to that of zeatin chromatographed under the same conditions in a separate experiment. The UV-absorbing band was eluted and rechromatographed twice on Whatman no. 1 paper, first in 9.5% ethanol and then in 12.4% ethanol. The UV-absorbing band from the final chromatogram was eluted, an aliquot was removed for bioassay, and the remainder of the sample was taken to dryness in a stream of nitrogen. This sample was used for the structural determination described below. The final yield of material, based on UV-absorption measurements and assuming an extinction coefficient similar to that of zeatin, was equivalent to about 1.4 μg/liter of original *Corynebacterium* culture medium. The isolated cytokinin was less than one-tenth as active as *trans*-zeatin.

The cytokinin sample (equivalent to 84 liters of culture medium), when dissolved in methanol, exhibited a UV maxi-

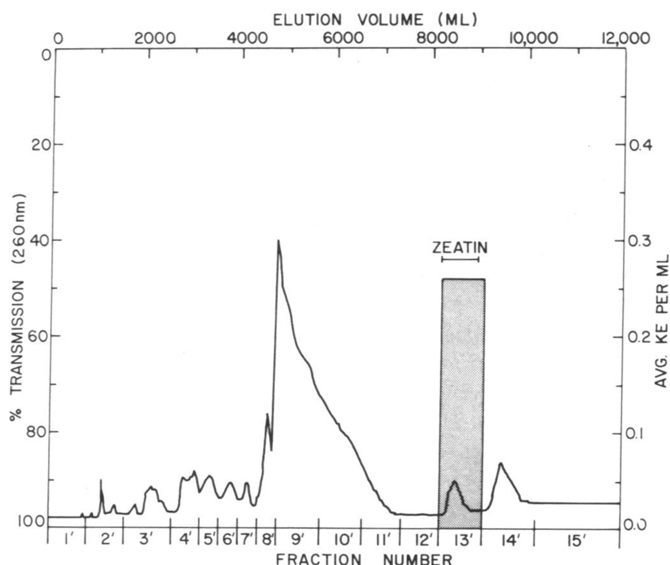


FIG. 2. Chromatography of cytokinin peak I on Sephadex LH-20 in distilled water. The solids recovered after combining and evaporating fraction 5 (Fig. 1) from four replicate columns were dissolved in 20 ml of distilled water and applied to a Sephadex LH-20 column (500 g, 4.8 × 94.8 cm, 2.00-liter bed volume) equilibrated with the same solvent. The column was eluted with distilled water, and 20-ml fractions were collected. These fractions were pooled as indicated (1′ through 15′) and aliquots from the pooled fractions were tested for cytokinin activity in the tobacco bioassay at a maximum concentration of 50 liter equivalents per liter (see *Methods*). Cytokinin activity is indicated by *vertical bars*. The elution position of the zeatin standard is labeled.

mum at 269 nm, typical of an N^6-substituted adenine, and the absorbance corresponded to 115 μg (0.53 μmol). The mass spectrum at 70 eV, probe temperature 160°, gave prominent peaks at *m/e* (%Σ) 219(1.61), 202(6.88), 188(7.87, base peak), 186(2.68), 185(2.42), 174(0.86), 160(4.04), 148(1.58), 136 (5.33), and 135(3.59). The high-resolution spectrum was determined under similar conditions and the peaks listed above had the expected composition (12). The sample had an R_F on silica gel of 0.34 in solvent A and 0.59 in solvent B compared with R_Fs of 0.33 and 0.59 for *cis*-zeatin and 0.24 and 0.50 for *trans*-zeatin in solvents A and B, respectively. The mass spectral and tlc data prove that the isolated compound is 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)purine (12, 16). Although no UV-absorbing spot for the *trans* isomer could be seen (therefore, <5%), a small amount of *trans*-zeatin (about 2% of the *cis*-zeatin content) was indicated in bioassays of the appropriate sections of the TLC plate.

Under the isolation conditions used in this investigation of the cytokinin components from the cultures of *C. fascians*, the bases i^6Ade and *cis*- and *trans*-zeatin and the ribonucleosides i^6Ado and ribosyl-*cis*- and *trans*-zeatin were shown to remain unchanged. The latter two ribosides were also shown to be virtually unaffected during the chromatographic sequences on Dowex as given under *Methods*. Thus, any cytokinin bases isolated from the culture medium in the manner described were actually present as such and were not produced by laboratory manipulation procedures, e.g., possible hydrolysis.

DISCUSSION

The isolation and identification of *cis*-zeatin from *C. fascians* cultures is the *first unequivocal demonstration of the production of a zeatin isomer by bacteria*. The human pathogen of the same genus, *C. diphtheriae*, according to Thimann and Sachs (2), did not give evidence of cytokinin activity in the oat-leaf test that was positive for *C. fascians*. Phillips and Torrey (17, 18) recently reported the presence of a cytokinin with chromatographic properties similar to those of zeatin in extracts of *Rhizobium* cultures. Cultures of other microorganisms with pathogenic or symbiotic functions related to growth in plants also have been shown to contain considerable cytokinin activity. Examples of this are the crown gall organism, *Agrobacterium tumefasciens* (19, 20), in which i^6Ade was indicated by gas chromatography (20), and two strains of bacteria, apparently required symbionts present in the seeds of *Ardisia* species, in which cytokinins were found but not rigorously identified (21). Cultures of fungi associated with mycorrhiza in plants also are rich in cytokinins (22, 23) and from one of these, *Rhizopogon roseolus*, Miller (23) has isolated zeatin as well as its ribonucleoside and ribonucleotide.

The concentration of *cis*-zeatin present in the medium from *C. fascians* cultures was estimated to be about 1.4 μg/liter, as compared with about 1.2 μg/liter of i^6Ade. However, in the tobacco bioassay *cis*-zeatin is a weaker cytokinin (12, 16), so that about 83% of the cytokinin activity recovered from the medium after partial purification and Sephadex LH-20 chromatography may be attributed to i^6Ade. The data for i^6Ade are based on bioassays and the previous finding that it is roughly 10 times as active as kinetin (16, 24). It should be noted, however, that estimates of quantities of individual free bases, ribonucleosides, or more complex forms of cytokinins in the culture medium, with or without the cells, give no accurate indication of the amounts or form of cytokinins in the living cells. To a considerable extent this holds also for estimates based on extracts from cells, especially when old (stationary phase) cultures are used.

The isolation of mainly the *cis* rather than the *trans* isomer of zeatin raises obvious questions concerning the origin of this compound. Zeatin isolated from higher plant sources (25–27) and from cultures of the fungus *Rhizopogon roseolus* (23) has been identified as the *trans* isomer. The *cis* isomer has been identified as a constituent of the tRNA of a number of plant species (28, 29), but it has not previously been isolated in the free form. The possibility that this compound is released from the tRNA of *C. fascians* during the isolation procedure or by autolysis during the growth of the cultures cannot be excluded. The problems associated with determining whether cytokinins present in microbial culture media arise by breakdown of tRNA have been discussed by Rathbone and Hall (6) and by Phillips and Torrey (18). We have run product stability controls of our present isolation procedure, from which we can conclude that *cis*-zeatin (I) would remain unchanged and that no isomerization from *trans*- to *cis*-zeatin would take place. Moreover, ribosyl-*cis*-zeatin would not have been converted to *cis*-zeatin appreciably under the conditions presently used. Although it is not yet certain whether zeatin actually occurs in the tRNA of *C. fascians* (6, 7), it appears likely that this will prove to be the case since the zeatin-like compound found in *Rhizobium* cultures was present in the tRNA as well as in the free form (18). In any event, *cis*-zeatin has now been fully identified as a second cytokinin-active component, along with N^6-(Δ^2-isopentenyl)adenine (3, 4, 6), isolable from the liquid cultures of *C. fascians*, a pathogen known to cause a syndrome of abnormal development in plants resembling that brought about by excessive cytokinin treatments.

We thank Mrs. Anna Hilden at the University of Wisconsin for technical assistance with the bioassays. At the University of Wisconsin this work was supported in part by Grant GB-35260X from the National Science Foundation. At the University of Illinois, the high-resolution mass spectrometer and data processing equipment employed in the present study were provided by NIH Grants CA 11388 and GM 16864 from the National Cancer Institute and the National Institute of General Medical Sciences, respectively.

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