Chaetoglobosin K: A New Plant Growth Inhibitor and Toxin from *Diplodia macrospora*

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A new metabolite, identified as chaetoglobosin K, was isolated from cultures of *Diplodia macrospora*. It inhibited the growth of wheat coleoptiles to 10^{-7} M. The metabolite was toxic to day-old chicks with an LD_{50} between 25 and 62.5 mg/kg.

While surveying fungi for the production of plant growth inhibiting substances, we observed that extracts of *Diplodia macrospora*, a pathogen that causes ear rot and stalk rot of corn (*Zea mays* L.), produced a metabolite that was both a potent inhibitor of wheat coleoptile growth and a mycotoxin. Further work indicated that the metabolite was a cytochalasin and the physical and chemical data specifically suggested a chaetoglobosin (that is, a cytochalasin possessing an indol-3-yl attached group).

The cytochalasins appear to induce selective responses in plants. For example, cytochalasin B isolated from *Phoma exigua*, was phytotoxic to chicory, *Cichorium intybus* L., and to periwinkle, *Vinca minor* L. (Bousquet and Barbier, 1972), whereas we have observed only moderate inhibition of wheat coleoptiles. However, cytochalasin H induced a remarkable effect in tobacco (*Nicotiana tabacum* L. cv. Hick's) and acted as an apparent juvenile agent so that flowering was greatly delayed. Corn plants (*Zea mays* L.) were generally unaffected by cytochalasin H, but bean plants (*Phaseolus vulgaris* L.) responded to treatment by exhibiting bending of petioles and longitudinal rolling of the leaves to form cylinders (Wells et al., 1976). Furthermore, it was active at concentrations of 10^{-6} M in wheat coleoptile assays. Other effects of the cytochalasins on plant growth have been reported (Herth et al., 1972).

The effects of cytochalasins on animal cells are diverse and have been adequately documented to show that they induce unique responses (Beno et al., 1977). Cytochalasin H was toxic to chicks and oral administration gave an LD_{50} of 12.5 mg/kg (Wells et al., 1976).

We now wish to report the isolation of a new metabolite, chaetoglobosin K (I) (Figure 1) and the effects that it induces in plants and chicks.

MATERIALS AND METHODS

Production and Purification of Chaetoglobosin K. *Diplodia macrospora* (ATCC accession No. 36896) was isolated from infected corn plants (*Zea mays* L.) in Turrialba, Costa Rica. The fungus was cultured on potato dextrose agar slants at approximately 26 °C for 10 days and was then maintained at 5 °C until transferred to Fernbach flasks (2.8 L), each containing 100 g of shredded wheat, 200 mL of Difco mycological broth (pH 4.8), 2% yeast extract, and 20% sucrose (Kirksey and Cole, 1974) for production of the toxin. Inoculated flasks were incubated in the laboratory at about 24 °C for 19 days, then 300 mL of acetone was added to each flask. The mycelia and substrate were macerated with a Super Dispax homogenizer, and the suspension was strained through cheesecloth to remove the pulp. The liquid was next filtered through Whatman No. 1 filter paper on a Buchner funnel and the clarified filtrate was reduced under vacuum at 50 °C to yield an aqueous phase. This was extracted twice with ethyl acetate; each volume of solvent was equal to twice that of the aqueous portion. Combined ethyl acetate extracts were dried over anhydrous sodium sulfate, reduced to a small volume under vacuum, then placed on a silica gel (70–230 mesh) chromatography column (9.0 × 10 cm) that had been slurry packed in benzene. There followed stepwise elution by 1.0 L each of benzene, ethyl ether, ethyl acetate, acetone, and methanol. Individual solvents drained to the top of the silica gel before addition of the next sequential solvent. The acetone fraction, which exhibited biological activity, was further fractionated by first reducing it to a small volume under vacuum at 50 °C and placing it on silica gel (70–230 mesh) in a column (4.0 × 50 cm) that had been slurry packed in benzene. Then 250 mL of benzene was added to the top of the packing material and immediately a linear gradient of benzene (a further 1.0 L) to acetone (1.0 L) was used for elution. Twenty-milliliter fractions were collected, evaporated to approximately 2 mL and further examined by thin-layer chromatography and bioassay.

Physical and Chemical Analyses. The ultraviolet (UV) spectrum of chaetoglobosin K was determined in 95% ethanol solution with a Beckman Model DK-2 recording spectrophotometer. Infrared (IR) spectra were obtained with a Beckman IR 4210 spectrophotometer using a 4X beam condenser. Samples were prepared as thin films on KBr windows.

Proton and ^{13}C nuclear magnetic resonance spectra were obtained on a Varian Associated XL-100-12 spectrometer equipped with the 620 L disk data system. The sample was prepared in a 5-mm tube as a 40 mg/0.5 mL solution in $CDCl_3$ with tetramethylsilane (Me_4Si) as an internal reference. Proton spectra were recorded in the continuous wave mode while ^{13}C spectra were recorded in the Fourier transform mode with the following parameters: spectral width 5-KHz pulse angle 30°, repetition time between pulses 2 s, and 2-KHz noise modulated proton decoupling. Single-frequency, off-resonance, proton decoupled (sford)

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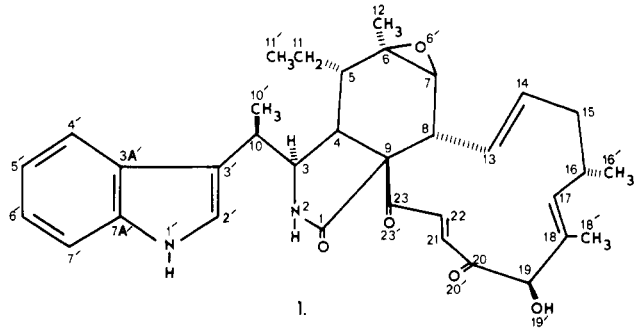

Figure 1. Structure and numbering system of chaetoglobosin K.

^{13}C spectra were obtained by offsetting the decoupler 500 Hz upfield from Me_4Si in order to aid in the assignment of the carbon chemical shifts.

Low-resolution (LRP) mass spectra were obtained on a Hewlett-Packard Model 5985 spectrometer. High-resolution (HRP) data were gathered with an AEI MS-9 spectrometer and perfluorokerosene was used as the internal standard. Samples were introduced into the instrument by direct probe method. Ionization was by electron impact at 70 eV. Melting points were observed with a Hoover capillary melting point apparatus and are uncorrected.

Progress of purification of the metabolite was followed by thin-layer chromatography on silica gel 60, F-254 (E.M. Laboratories, Inc.) with a toluene/ethyl acetate/formic acid (5:4:1, v/v/v) developing solvent. The metabolite was seen as a dark spot under short-wave UV.

Bioassays. Wheat seeds (*Triticum aestivum* L. cv. Wakeland) were sown on moist sand in trays and grown in the dark at 22 ± 1 °C for 4 days (Hancock et al., 1964). The etiolated seedlings were removed from the trays, and the roots and caryopsis were removed from the shoots. The latter were placed in a Van der Weij guillotine and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for bioassay. All manipulations were done under a green safelight (Nitsch and Nitsch, 1956). Fractions to be assayed for biological activity were added to test tubes (approximately 20 μL/tube) and evaporated under nitrogen to dryness. Chaetoglobosin K was assayed at concentrations of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M. Two milliliters of phosphate–citrate buffer containing 2% sucrose at pH 5.6 (Nitsch and Nitsch, 1956) was added to each test tube. Following the placement of ten coleoptiles in each test tube, the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. The coleoptiles were measured by projecting their images (×3) from a photographic enlarger (Cutler and Vlitos, 1962). Data were statistically analyzed (Kurtz et al., 1965).

Greenhouse grown plants were treated with aqueous solutions of chaetoglobosin K formulated as a fine suspension in 10% acetone and 0.1% Tween 20 at concentrations ranging from 10^{-2} to 10^{-4} M. Test solutions (1 mL) containing 5560, 556, and 55.6 μg of the metabolite (10^{-2}, 10^{-3}, and 10^{-4} M, respectively) were sprayed in aerosol, once, onto 6-week-old tobacco seedlings, *Nicotiana tabacum* L. cv. Hick's. Week-old corn seedlings, *Zea mays* L., cv. Norfolk Market White, were treated with 100 μL of each test solution pipetted into each leaf whorl at 10^{-2}, 10^{-3}, and 10^{-4} M (556, 55.6, and 5.56 μg, respectively). Four corn plants were grown in each pot. All experiments were replicated three times.

One-day-old chicks were used for the vertebrate bioassay in testing the toxin. Chicks were dosed via crop intubation with corn oil as the inert carrier (1 mL of corn oil/chick). Samples were prepared for dosing by first dissolving the toxin in acetone. Corn oil was added and the acetone was removed under vacuum on a rotary evaporator at 70 °C. After apparent removal of all the acetone (when no further acetone distilled over) the evaporator reservoir was emptied and dried. Again the sample was placed under vacuum and subjected to further rotary evaporation to ensure complete removal of the acetone (Kirksey and Cole, 1974). Controls were prepared in an identical way.

RESULTS AND DISCUSSION

Physical and Chemical Characteristics. *Diplodia macrospora* produced 87.7 mg of chaetoglobosin K from 50 flasks of shredded wheat media in 19 days. Biological activity was first observed in the acetone fraction obtained from stepwise elution silica gel column chromatography. On further separation, with a linear gradient elution series from benzene to acetone on silica gel, activity was associated with tubes 16–21 (320–420 mL of total solvent). They were combined, reduced in volume under vacuum, and stored in a refrigerator at 5 °C. After several weeks in acetone, yellow crystals were precipitated with great difficulty and were collected on a fine porosity fritted glass funnel under vacuum. The crystals were washed with acetone at −17 °C, then collected, and dried at room temperature.

The R_f value of chaetoglobosin K (I) was 0.53–0.56 on silica gel 60 thin-layer plates developed with toluene/ethyl acetate/formic acid and it was observed as a dark spot under shortwave UV. The uncorrected melting point was 264–266 °C, though the crystals turned white at 60 °C and most probably indicated the loss of acetone of crystallization.

UV analysis of the toxin in ethanol showed λ_{max} (EtOH) 219 nm (log ε 4.65). Strong solutions of the metabolite yielded slight shoulders at λ_{max} (EtOH) 274, 282, and 290 nm and suggested the possibility of an indole ring.

The major IR bands and probable assignments were 3340 (fairly sharp: indole and OH), 2970 (CH_3), 2930 (CH_2), 2880 (CH_2), 1725 (shoulder), 1685 (strong), 1655 (shoulder: carbonyls), 1615 (indole), 1455 (CH_3, indole), 1427, 1375 (CH_3), 1250, 1156, 1105, 1048, 972, 875, and 742 (four adjacent hydrogens on aromatic ring) cm^{-1}.

The LRP mass spectral analysis of I gave a molecular ion peak (M^+) at m/e 556.3, and the HRP analysis using perfluorokerosene as the internal standard indicated that the M^+ was 556.2935 (calculated mass for $C_{34}H_{40}O_5N_2$, 556.2935). Fragment ions were observed at m/e 459 ($M^+ - C_5H_7NO$), 413 ($M^+ - 143$, most probably a loss of 3-ethyleneindole, $C_{10}H_8N$), 412 ($M^+ - 144$, most probably a loss of 3-ethylindole, $C_{10}H_9N$).

The ^1H and ^{13}C NMR spectra of chaetoglobosin K were similar to those obtained for the cytochalasins (Graf et al., 1974; Cox and Ashline, 1977; Ashline, 1977) and the chaetoglobosins examined previously (Cox and Ashline, 1977; Ashline, 1977). Chemical shifts are given in Table I. The ^{13}C assignments were made on the basis of the sford spectrum and comparison with the assignment of the ^{13}C NMR spectrum of chaetoglobosin C (Ashline, 1977). The ^{13}C chemical shifts of chaetoglobosin K were in excellent agreement with those of chaetoglobosin C, taking into account the additional methyl substituents at C(10′) and C(11′) and the double bonds at C(21) and C(23) in chaetoglobosin K. The assignments of the ^1H chemical shifts of chaetoglobosin K (Table I) were based on decoupling experiments and comparison with the assignments given for chaetoglobosin A (Sekita et al., 1973).

Confirmation of the structure by single-crystal X-ray

Table I. Carbon-13 and ^{1}H NMR Parameters for Chaetoglobosin K[a,b]

	carbon/chemical shift			carbon/chemical shift	
1	173.20 s		16'	12.65 q	(1.12, J = 7.1)
2		(6.23)	17	140.11 d	(5.56, J = 8.4)
3	56.69 d	(3.89, J = 4.4)	18	125.87 s	
4	49.23 d	(3.14, m)	18'	20.95 q	(1.29, s)
5	36.29 d	(1.90, m)	19	81.58 d	(4.99, J = 4.5)
6	57.23 s		20	190.04 s	
7	61.58 d	(2.74, J = 5.3)	21	130.99 d	(7.65, J = 16.5)
8	44.03 d	(2.16, m)	22	136.38 d	(6.49, d)
9	63.86 s		23	193.92 s	
10	44.03 d	(3.14, m)	1'		(8.53)
10'	10.57 q	(1.16, J = 7.3)	2'	122.28 d	(6.90)
11	21.73 t	(1.51, m)	3'	116.52 s	
11'	13.77 q	(1.01, J = 7.0)	3A'	132.21 s	
12	19.58 q	(1.29, s)	4'	118.41 d	(7.0-7.5, m)
13	133.19 d	(6.09, J = 10.2, 15.6)	5'	119.72 d	(7.0-7.5, m)
14	128.19 d	(5.01, m)	6'	121.71 d	(7.0-7.5, m)
15	41.90 t	(2.16, m)	7'	111.53 d	(7.0-7.5, m)
16	31.94 d	(2.35, m)	7A'	136.38 s	(7.0-7.5, m)

[a] In ppm downfield from internal tetramethylsilane. [b] Proton chemical shifts and coupling constants given in parentheses.

Table II. Percent Inhibition of Wheat Coleoptiles (*Triticum aestivum* L. cv. Wakeland), Relative to Controls, Obtained with Cytochalasins

compd	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
cytochalasin A	44[a]	15[a]	0	0	
cytochalasin B	65[a]	62[a]	0	0	
cytochalasin E	16[a]	0	0	0	
cytochalasin H	84[a]	80[a]	76[a]	10[a]	0

[a] Significant inhibition: $P < 0.01$.

crystallography will be the subject of another communication and unequivocally establishes the structure to be that of a chaetoglobosin with the unique presence of one methyl group at C(10') and another at C(11') (Figure 1) (Springer et al., unpublished results).

Bioassay Results. Wheat coleoptiles were significantly inhibited ($P < 0.01$) by solutions of chaetoglobosin K at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M (Figure 2). The intensity of the activity is greater than that obtained with the standard plant growth inhibitor (±)-abscisic acid and may represent the most active inhibitor of coleoptile growth yet reported. Chaetoglobosin K is more inhibitory at 10^{-6} and 10^{-7} M than cytochalasin H and is the most active cytochalasin that we have yet assayed (Table II). Of particular interest is the possible role of the indole attachment in the expression of the biological response, especially when the potential for splitting the molecule to yield a simple indole substituted at the C(3') position exists. The general rule indicates that these compounds are promoters of coleoptile growth (Pilet, 1961). In fact, consideration of the entire molecule, from another perspective, leads to the interesting proposition that certain indol-3-yl compounds may be strong inhibitors of coleoptile growth at extremely low concentrations.

Neither tobacco nor corn plants appeared to be affected by direct applications of chaetoglobosin K. Since the metabolite was applied as a very fine suspension it has not been determined whether the inactivity was due to lack of uptake. But certainly further tests, wherein the metabolite is introduced into corn leaf mesophyll, to determine the possible correlation with phytotoxic lesions (Latterell, 1976) will be made.

During the 4-day test period with chicks, the clinical signs observed at all dosage levels except 25 mg/kg were lethargy and anorexia. General deterioration continued until death which occurred from 2 to 4 days after dosing. From these data the LD_{100} was observed at levels as low as 62.5 mg/kg. No mortalities were observed at 25 mg/kg, and therefore the LD_{50} was most probably between these two values. A more accurate LD_{50} could not be determined because of an insufficient quantity of chaetoglobosin K.

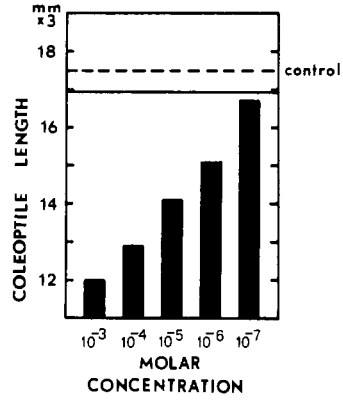

Figure 2. Inhibitory effect on the growth of wheat coleoptiles (*Triticum aestivum* L. cv. Wakeland) by chaetoglobosin K. Control, dotted line. Significant inhibition, below solid line ($P < 0.01$).

Our study indicates that both the plant growth regulating and toxic properties of chaetoglobosin K need expanded investigation and, furthermore, that the presence of the metabolite in corn infected with *D. macrospora* has yet to be established.

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Clearance of the Synthetic Prostaglandin Cloprostenol ("Estrumate") from the Milk of Cows

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The concentration of the synthetic prostaglandin cloprostenol ("Estrumate") has been measured in the milk of three cows following an intramuscular dose of 0.5 mg of [^{14}C]cloprostenol. The maximum observed concentration of cloprostenol in milk was 0.270 ng/mL (2.7×10^{-4} ppm) at 4 h after dosing. At 24 h after dosing, the concentration of cloprostenol in milk had declined to 0.002 ng/mL (2×10^{-6} ppm). The excretion of radioactivity in milk and urine and the proportion of cloprostenol in milk at doses of 0.5 mg and 10 mg of cloprostenol were similar, demonstrating that the drug is cleared by apparent first-order processes over the dose range studied. The similarity in the time course of the clearance from milk of cloprostenol and the endogenous prostaglandin, $PGF_{2\alpha}$, is discussed.

Cloprostenol ("Estrumate", trademark the property of Imperial Chemical Industries Ltd.) (see Figure 1) is a synthetic prostaglandin which is of commercial benefit for the synchronization of estrus and treatment of infertile conditions in cows (Cooper and Furr, 1974; Cooper and Rowson, 1975; Jackson and Cooper, 1976). Studies of the clearance of this potent agent from the milk and edible tissues of the cow have been previously reported (Reeves, 1978). This author reported that the elimination of total radioactivity in milk was a minor route of excretion following intramuscular administration of [^{14}C]cloprostenol. Milk is a tissue of importance with respect to residues for new animal drugs (Perez, 1977), and studies have been undertaken to characterize the components which constitute the radioactivity in milk after dosing [^{14}C]cloprostenol. This paper describes studies undertaken to determine the concentration of the drug in milk using isotope dilution analysis. These studies have been carried out following intramuscular dosing of [^{14}C]cloprostenol at the therapeutic dose (0.5 mg) and an elevated dose (10 mg).

METHODS

Animal Studies. Three Friesian dairy cows (body weight approximately 500 kg) were used. [^{14}C]Cloprostenol prepared as previously described with a radiochemical purity >99% (White, 1977) was administered intramuscularly to the cows at two dose levels. The higher dose was chosen to facilitate the determination of cloprostenol in milk at 24 h after dosing. Each cow received a single injection of 0.5 mg of [^{14}C]cloprostenol (sp act., 91.9 μCi/mg) in 2 mL of citrate buffer. Urine (via a urethral catheter) and milk were collected for 24 h after dosing. Following a 48-h recovery period, the procedures were repeated following administration of 10 mg of [^{12}C/^{14}C]-cloprostenol (ratio ^{12}C/^{14}C forms, 1:2; sp act. of [^{14}C]cloprostenol, 91.9 μCi/mg). Urine and milk were collected for 24 h after dosing.

Determination of the Proportion of [^{14}C]Cloprostenol in Milk. Milk (20 mL) collected from cows up to 8 h after dosing with [^{14}C]cloprostenol was spiked with nonlabeled drug (5 mg) as carrier and [^{3}H]cloprostenol (generally labeled in the phenyl ring; sp act., 33 μCi/mg) such that the ratio of ^{3}H/^{14}C in each sample was known. Standards were prepared in the same manner. The milk was acidified with sulfuric acid (0.5 M, 2 mL), and diethyl ether (25 mL) was added. The sample was mixed and centrifuged and the supernatant removed and concentrated. The extract was redissolved in ether (10 mL) and washed with dipotassium hydrogen phosphate (0.05 M, 10 mL). The aqueous phase was removed and acidified with orthophosphoric acid (1 M, 0.5 mL), and the sample was extracted with ether. After mixing and centrifuging, the organic phase was removed, concentrated, and redissolved in phosphate buffer (pH 6).

For milk samples collected during 8–24 h after dosing, 1 L of milk was taken for analysis. The procedures were modified accordingly.

Aliquots of the buffered solution were assayed for ^{3}H and ^{14}C by liquid scintillation counting. The remainder of the sample was analyzed by high-pressure liquid chromatography using "Spherisorb O.D.S." as column packing (solvent, methanol/water/acetic acid, 60:40:0.5, v/v). The peak eluted with a retention time equivalent to cloprostenol was concentrated and counted for ^{3}H and ^{14}C. The chromatographic procedure was then repeated for each sample using methanol/water/acetic acid, 55:45:0.5 (v/v), then 50:50:0.5 (v/v). The proportion of cloprostenol in each sample was estimated from the changing ^{3}H/^{14}C ratio with successive chromatographic analysis.

Radiochemical Techniques. Urine and milk were assayed for radioactivity as previously described (Reeves, 1978). Samples containing ^{3}H and ^{14}C were assayed using the Triton scintillant described by Reeves and a Packard 3320 or Intertechnique SL40 scintillation counter adjusted for counting dual-labeled samples. All samples were

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