the TMC experiment. (1) The mass of material (95%) remains at the site of application, with a sharp gradient existing in the soil for the chemical leaving the posts. (2) Steady, but small amounts of material are contributed to the ecosystem for distribution in the air and through the biota living in close proximity to the treated wood. (3) Of the chemicals tested, creosote components were dispersed to a greater extent than others. (4) The rate of release was too low to evidence acute effects on the vole, but insecticidal activity was pronounced and continuous for HEOD, and both creosote and HEOD had a depressing effect on vole predation of crickets. (5) Because of the high degree of mass accountability and because of adaptability of the test system to temporal and media-specific studies, the TMC-II system appears to be a useful means of studying toxic chemical fate and effects.

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Isolation and Identification of Two New [11]Cytochalasins from *Phomopsis sojae*

Richard J. Cole,* David M. Wilson, James L. Harper, Richard H. Cox, Teena W. Cochran,[1] Horace G. Cutler, and Durham K. Bell

Selected isolates of the soybean pathogen *Phomopsis sojae* produced two new [11]cytochalasin mycotoxins related to cytochalasin H and deacetylcytochalasin H. The mycotoxins were identified spectroscopically as 21-acetoxy-6,7-epoxy-18-hydroxy-10-phenyl-5,6,16,18-tetramethyl[11]cytochalasa-13,19-dien-1-one (epoxycytochalasin H) and 18,21-dihydroxy-6,7-epoxy-10-phenyl-5,6,16,18-tetramethyl[11]cytochalasa-13,19-dien-1-one (epoxydeacetylcytochalasin H). Both metabolites were toxic to day-old chickens and both showed plant growth inhibition in wheat coleoptile bioassay.

The soybean (*Glycine max* L. Merrill) is an important crop used worldwide for oil and protein for human consumption and feedstock for domestic animals. Few toxigenic fungi are known to invade soybeans, and little is known of potential hazards from consumption of molded soybeans. *Diaporthe phaseolorum* (CKe. Å ELL.) var. *sojae* Wehm. (imperfect state *Phomopsis sojae* Leh.) produces a pod and stem blight of soybean that is recognized worldwide. Infection occurs after pod formation and may be severe when harvest is delayed, especially when environmental conditions are warm and moist. Fungal invasion is often severe in fields with high plant populations and extensive lodging (Sinclair and Shurtleff, 1975). Environmental conditions in south Georgia during harvest are frequently favorable for colonization by *P. sojae*.

We recently reported the isolation, chemical characterization, and biological properties of two [11]cytochalasin metabolites, cytochalasin H (I) and deacetylcytochalasin H (II), from culture extracts of *Phomopsis* sp. (Wells et al., 1976; Beno et al., 1977; Cole et al., 1981). Biological assay of culture extracts of eleven selected isolates of *P. sojae* parasitic on soybean seeds produced two isolates that were toxigenic. We now report the chemical structures of

National Peanut Research Laboratory, U.S. Department of Agriculture, Science and Education Administration, Agricultural Research, Dawson, Georgia 31742 (R.J.C.), University of Georgia, Plant Pathology Department, Georgia Coastal Plain Experiment Station, Tifton, Georgia 31793 (D.M.W., J.L.H., and D.K.B.), National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 (R.H.C. and T.W.C.), and Richard B. Russell Agricultural Research Center, Plant Physiology Unit, U.S. Department of Agriculture, Science and Education Administration, Agricultural Research, Athens, Georgia 30613 (H.G.C.).

[1]Present address: Environmental Monitoring Systems Laboratory, Environmental Protection Agency, Research Triangle Park, NC 27711.

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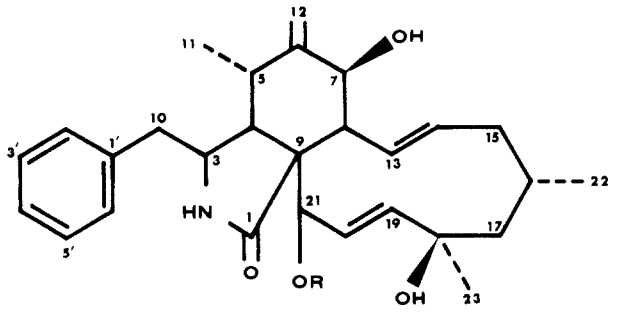
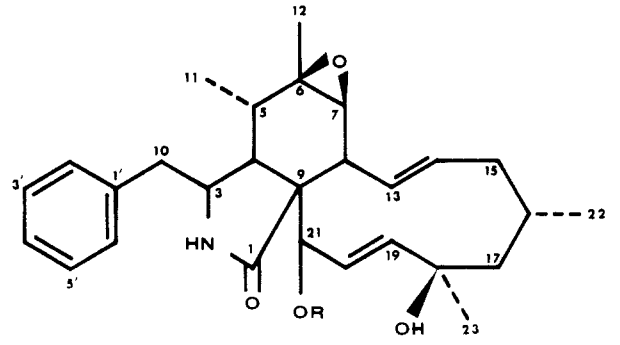


I, R = C(=O)CH_3
II, R = H

two new [11]cytochalasins (III and IV) derived via spec-



III, R = C(=O)CH_3
IV, R = H

troscopic methods. Also presented are preliminary biological properties of the new cytochalasins in plant and animal systems.

EXPERIMENTAL SECTION

Culture and Extraction. The isolates of *P. sojae* were cultured on autoclaved soybean seeds. Seven isolates were grown in Fernbach flasks containing 200 g of soybean seed rehydrated with 80 mL of water. The culture flasks were autoclaved twice for 15 min each time and inoculated with mycelial plugs of *P. sojae* from potato dextrose agar cultures. The cultures were incubated for 21 days at 26 °C, and then they were extracted with an equal volume of hot chloroform on a steam bath. The entire contents of the flasks were removed and homogenized to a fine suspension by using a Super-Dispax homogenizer (Tekmar, Inc.). The suspension was filtered through anhydrous sodium sulfate, and the resulting filtrate was reduced to dryness under vacuum at 60 °C by using a Roto-Vap (Buchi).

Two flasks each of the seven isolates were used for screening for toxigenicity (Kirksey and Cole, 1974). One of the two toxigenic isolates was used for mass production of the toxins in 25 flasks. Five flasks of the other toxigenic isolate were used to compare metabolites from the other toxigenic isolate.

Purification. The crude chloroform extract was fractionated on a silica gel 60 column (4 × 15 cm) packed as a slurry in benzene and eluted with 3 column volumes each of benzene, ethyl ether, ethyl acetate, acetone, and methanol. The ethyl ether and ethyl acetate fractions were highly toxic, and thin-layer chromatography (TLC) analyses suggested the same toxins were involved. These two fractions were combined, concentrated under vacuum at 60 °C, and added to a second silica gel 60 column (4 × 15 cm) packed in ethyl ether. The column was eluted with a linear gradient from ethyl ether to ethyl acetate (240 20-mL fractions were collected). Fractions 4–11 were associated with acute toxicity to day-old chickens. These fractions were combined and reduced to dryness under vacuum at 60 °C. The combined fractions were applied to a C_{18} reverse-phase column (4 × 15 cm) packed in acetonitrile and reequilibrated with 10% acetonitrile–water. The column was eluted with a linear gradient from 10% acetonitrile–water to 100% acetonitrile (150 20-mL fractions were collected). Toxicity was associated with fractions 75–95. On the basis of TLC analyses, the fractions were combined as follows: fractions 75–82 yielded III; fractions 83–86 yielded III and IV; fractions 87–95 yielded IV.

Physical and Chemical Analyses. Purification of III and IV was monitored by TLC by using 5 × 10 cm glass plates precoated with silica gel 60 F-254 (EM Laboratories, Inc.). The TLC developing solvent was toluene–ethyl acetate–formic acid, 5:4:1 (v/v/v). The cytochalasins were visualized on TLC plates by spraying with 50% ethanolic H_2SO_4 and heating at 100 °C for 3 min.

Melting points (mp) were determined on a Kofler micro melting point apparatus and were uncorrected. Ultraviolet spectra (UV) of III and IV were recorded with a Beckman Model DB-G recording spectrophotometer in methanol solution at a concentration of 1.0×10^{-5} M.

Infrared spectra (IR) were recorded with a Perkin-Elmer Model 257 spectrophotometer equipped with a 4X beam condenser. Samples for analyses were coated onto KBr windows.

Proton decoupled ^{13}C NMR (25.1-MHz) spectra were obtained on a Varian Associates XL-100 NMR spectrometer equipped with the 620-L disk data system. Samples were prepared in 5-mm sample tubes to a concentration of 50 mg/0.5 mL in $CDCl_3$ containing 1% Me_4Si as an internal reference. Parameters used to acquire the FT spectra were as follows: spectral width, 5000 Hz; data points, 8K; pulse width, 30°; recycle time, 1 s; line broadening, –0.5; square wave proton decoupling; room temperature. Single-frequency, off-resonance, proton decoupled (sford) ^{13}C NMR spectra were obtained to aid in chemical shift assignments by offsetting the decoupling frequency 500 Hz upfield from Me_4Si. Proton NMR spectra were obtained on a Bruker WM-250 spectrometer. Chemical shift assignments given in Table I are based on comparisons with data for other cytochalasins and the sford spectra.

Mass spectra were obtained on a VG Micromass 70/70 mass spectrometer with a VG 2250 data system. Samples were introduced via direct probe. The mass spectrometer was operated at 70 eV, with a 200-μA emission current, resolution at 3000 (5% peak valley definition), and a scan speed 3s/decade with a scan range from 600 to 20. Data were acquired by using the VG accurate mass measurement (MM2) software with tetraiodoethylene as the secondary reference compound.

Animal and Plant Bioassay. Purification of metabolites III and IV was monitored by using 1-day-old chickens dosed orally via crop intubation according to the method of Kirksey and Cole (1974). Insufficient material was present to obtain an accurate LD_{50} determination on either metabolite.

A wheat coleoptile bioassay was used to detect plant growth inhibition (Hancock et al., 1964). Etiolated wheat seedlings (*Triticum aestivum* L. cv Wakeland) were grown in the dark at 22 ± 1 °C on moist sand for 4 days. Seedlings were removed from the sand, and roots and caryopses were discarded. The seedlings were next placed tip first into a Van der Weij guillotine, and the apical 2 mm was extruded, cut, and discarded. The next 4 mm was cut and retained for bioassay. Toxin III was prepared for bioassay by dissolving 4.93 mg in 50 μL of acetone (Cutler, 1968)

Table I. Carbon-13 NMR Chemical Shifts for III and IV[a-c]

carbon/ compd	III[d]	IV[e]
1	175.3 s	176.4
2	(6.14)	(5.88)
3	54.2 d	54.3 d
4	50.7 d	51.1 d
5	36.7 d	36.6 d
6	57.1 s	57.2 s
7	62.9 d	63.2 d
8	45.2 d	43.9 d
9	53.6 s	54.7 s
10	42.9 t	42.8 t
11	12.6 q (0.83, J = 7.0)	12.8 q (1.05, J = 7.0)
12	19.7 q (1.33)	19.9 q (1.35)
13	127.1 d (5.86, J = 9.6, 16.2)	128.1 d (5.85, J = 10.1, 16.2)
14	138.2 d (5.28, J = 5.5, 16.2)	136.8 d (5.22, J = 6.1, 16.2)
15	45.9 t	46.2 t
16	30.7 d	30.7 d
17	53.7 t	53.7 t
18	74.2 s	74.5 s
19	127.7 d (5.48, J = 2.2, 16.6)	134.8 d (5.72, J = 2.2, 16.9)
20	135.2 d (5.77, J = 2.2, 16.6)	130.4 d (5.95, J = 2.2, 16.9)
21	76.1 d (5.67, J = 2.2)	75.3 d (4.23, J = 2.2)
22	26.4 q (1.04)	26.4 q (1.03, J = 5.4)
23	28.2 q (1.19)	28.2 q (1.22)
1′	136.9 s	137.1 s
2′,6′	129.2 d (7.17, m)	129.0 d (7.17, m)
3′,5′	128.9 d (7.26, m)	128.7 d (7.25, m)
4′	125.4 d (7.26, m)	126.9 d (7.25, m)
CH_3CO	170.2 s	
CH_3CO	20.8 q (2.20)	

[a] In ppm downfield from Me_4Si. [b] Single-frequency, off-resonance proton decoupling results given as s = singlet, d = doublet, etc. [c] Proton chemical shifts given in parentheses. [d] The remainder of the protons appear in multiplets centered at 1.7, 2.1, and 2.7 ppm. [e] The remainder of the protons appear in multiplets centered at 1.9 and 2.7 ppm.

and bringing the volume to 10 mL with phosphate–citrate buffer at pH 5.6 plus 2% sucrose (Nitsch and Nitsch, 1956) to give a 10^{-3} M solution. Similarly, toxin IV was prepared by using 4.51 mg to give a 10^{-3} M solution. From these, serial dilutions were made at 10^{-4}, 10^{-5}, and 10^{-6} M.

Two milliliters of each concentration to be assayed was put into test tubes, and 10 4-mm coleoptile sections were added to each test tube. Tubes were rotated at 0.25 rpm in a roller tube apparatus at 22 °C for 24 h in the dark. All prior manipulations had been carried out under a green safelight (Nitsch and Nitsch, 1956). Following incubation, coleoptiles were measured by projecting their images (×3) from a photographic enlarger (Cutler and Vlitos, 1962), and data were statistically analyzed (Kurtz et al., 1965).

RESULTS AND DISCUSSION

Purified III formed crystals from ethyl ether, mp 128–130 °C, $[\alpha]^{30}_D$ = −84.68 (c 0.33 in chloroform). It migrated on TLC to R_f 0.43 compared to 0.53 for cytochalasin H (I). Crystals of toxin IV melted at 192–194 °C, $[\alpha]^{30}_D$ −49.1 (c 0.19 in chloroform) and migrated on TLC to R_f 0.48. The UV spectra of III and IV were similar to that of cytochalasin H which showed strong end absorption and a series of weak bands in the region of 240–280 nm. The distinguishing feature in the IR spectrum of IV was the absence of carbonyl ester absorption (1735 cm^{-1}) which was present in the IR spectrum of III. This was the same relationship observed for cytochalasin H and deacetylcytochalasin H (Cole et al., 1981).

The LRP electron-impact mass spectra of III gave ions at nominal mass m/e 493 (M^+), 475 ($M^+ - H_2O$), 433 ($M^+ - H_2O - C_2H_2O$), 415 ($M^+ - 2H_2O - C_2H_2O$), 402, 343, 324, 270, 240, 120, and 91 (base). The HRP electron-impact mass spectrum gave a molecular ion peak at 493.2748 (calcd for $C_{30}H_{39}O_5N$ = 493.2828). The LRP spectrum of IV gave a molecular ion at 451 with additional peaks at 433 ($M^+ - H_2O$), 415 ($M^+ - 2H_2O$), 342, 270, 240, 120, and 91 (base). The HRP spectrum gave a molecular ion at 451.2682 (calcd for $C_{28}H_{37}O_4N$ = 451.2722). The mass spectral data for III and IV show that they have the same composition as two previously isolated cytochalasins from *Phomopsis* sp., cytochalasin H (I) and deacetylchtochalasin H (II). However, it is clear from their NMR data that III and IV differ.

The ^{13}C NMR spectrum of III (Table I) shows 28 peaks. Intensity considerations suggest that the peaks at 129.2 and 128.9 ppm are due to two carbons each and suggest a monosubstituted phenyl ring. The 250-MHz ^1H NMR spectrum confirms the presence of a monosubstituted phenyl ring. Comparison of the ^{13}C NMR spectrum of III with that for cytochalasin H indicates that III does not contain an exocyclic double bond at C_6–C_{12} that is present in cytochalasin H. Other differences are an additional methyl group in III and a singlet and doublet (sford) at 57.1 and 62.9 ppm in the spectrum of III. Additional displacements in the chemical shifts of some of the aliphatic carbons are noted for III as compared with cytochalasin H. Comparison of the ^{13}C spectrum of III with those cytochalasins containing an epoxide at C_6–C_7 (cytochalasins A, C, E, and F) suggests that the singlet and doublet at 57.1 and 62.9 ppm in the spectrum of III are due to an epoxide at C_6–C_7. The shifts of C_3–C_8 of III in comparison with their shifts in cytochalasin H are consistent with an epoxide at C_6–C_7 in III. The chemical shifts for the exocyclic double bond in the ^1H spectrum of cytochalasin H are absent in the ^1H spectrum of III plus there is an additional methyl singlet in the ^1H spectrum of III. Thus, the NMR data for III are consistent with the structure 21-acetoxy-6,7-epoxy-18-hydroxy-10-phenyl-5,6,16,18-tetramethyl[11]cytochalasa-13,19-dien-1-one (trivial name epoxycytochalasin H).

The ^{13}C NMR data for IV (Table I) are similar to those of III with the exception that the peaks for the acetate group are absent in IV. In the ^1H spectrum the acetate methyl is absent and the chemical shift for H_{21} is shifted in IV compared to III as expected for the conversion of an acetate into a secondary hydroxyl group. The data for IV are therefore consistent with the structure of 18,21-dihydroxy-6,7-epoxy-10-phenyl-5,6,16,18-tetramethyl[11]-cytochalasa-13,19-dien-1-one (trivial name deacetylepoxycytochalasin H).

Solutions of III significantly inhibited ($P < 0.01$) wheat coleoptiles at 10^{-3}, 10^{-4}, and 10^{-5} M; they were inhibited 81, 63, and 46%, respectively, relative to controls. In addition, coleoptiles were slightly curved at 10^{-3}M and were greatly curved at 10^{-4} and 10^{-5} M but were straight at 10^{-6} M. Metabolite IV inhibited coleoptiles at 10^{-3}, 10^{-4}, and 10^{-5} M, 100, 81, and 33% relative to controls. The 10^{-3} M treated coleoptile segments were straight, but those at 10^{-4} and 10^{-5} M were curved, and the 10^{-6} M treatments, while exhibiting no inhibition, were slightly curved.

Apparently, both III and IV affect coleoptile growth in two ways. First, there is straight growth inhibition, and second, there may be specific inhibition of certain cells in the coleoptile segments so that curvature is induced. The latter phenomenon is puzzling because the coleoptile

segments are constantly rotated during the course of the experiment and they are, theoretically, bathed equally with the solution. Identical curvature was observed with deacetylcytochalasin H (Cole et al., 1981). However, in that case, the 10^{-3} M treated coleoptiles, which were inhibited 100%, were also markedly curved.

When the relative inhibitory activities of cytochalasins previously studied are compared, metabolite III was unlike any already tested (Cutler et al., 1980). Metabolite IV resembled the activity of deacetylcytochalasin H at all concentrations except 10^{-6} M (Cole et al., 1981).

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Spectral Identification, X-ray Structure Determination, and Iron-Chelating Capability of Erythroglaucin, a Red Pigment from *Aspergillus ruber*

George W. Engstrom,* Ronald E. Stenkamp, Donald J. McDorman, and Lyle H. Jensen

A red pigment from *Aspergillus ruber* was purified by thin-layer and column chromatography with three different solvent systems and crystallized from acetone. The pigment had a molecular weight of 300 and an empirical formula of $C_{16}H_{12}O_6$. X-ray crystallographic and spectral analysis showed it to be 1,3,5-trihydroxy-3-methoxy-6-methylanthraquinone, identical with the compound previously named erythroglaucin. The molecule crystallizes in space group *Pnma*, a = 23.862 (5) Å, b = 6.547 (1) Å, c = 8.149 (2) Å, and Z = 4. X-ray data include final atomic parameters, thermal ellipsoids, bond lengths, bond angles, and molecular numbering scheme. With ferric iron, the pigment formed a dark blue complex that was insoluble in chloroform, methanol, ether, water, and dimethyl sulfoxide. The IR spectrum of the complex was different in several areas from that of erythroglaucin, and elemental analysis showed it contained 15.70% iron.

A large number of bacterial and fungal pigments including ferrichrome from *Ustilago sphaerogena* (Neilands, 1952), skizokinen from *Bacillus megaterium* (Byers et al., 1967), and ferrineoaspergillin from *Aspergillus ochraceus* Wilh (Maebayashi et al., 1978) are known to form complexes with iron atoms. Our interest in microbial mineral metabolism led us to investigate the structure and iron-chelating capability of pigments from other sources. We selected the fungus *Aspergillus ruber* (König, Spieckerman, and Bremer) Thom et Church because it was one of the *Aspergillus glaucus* group especially rich in pigments (Raper and Fennell, 1965). Gould and Raistrick (1934) reported that various species of the *A. glaucus* group produced yellow, orange, and red pigments called flavoglaucin, auroglaucin, and rubroglaucin, respectively. Later, Ashley et al. (1939) demonstrated that rubroglaucin was a mixture of two pigments: physcion ($C_{16}H_{12}O_5$) and erythroglaucin ($C_{16}H_{12}O_6$).

Recently, we reported on the iron-chelating ability of physcion, a yellow anthraquinone derivative from ether extracts of *A. ruber* (Engstrom et al., 1980). This was the first report of complexes formed between iron and 1,8-dihydroxyanthraquinones. We have now isolated a dark red crystalline pigment from an ether extract of *A. ruber* grown in a liquid medium that contained adequate iron. This report presents data from the identification and X-ray structure determination of the red pigment which demonstrated that it was identical with erythroglaucin (Suemitsu et al., 1977; Bachmann et al., 1979; Podojil et al., 1979). The report also includes data that show the ability of erythroglaucin to form a complex with ferric iron.

The National Animal Disease Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Ames, Iowa 50010 (G.W.E. and D.J.M), and the Department of Biological Structure, School of Medicine, University of Washington, Seattle, Washington 98195 (R.E.S. and L.H.J.).

EXPERIMENTAL SECTION

Materials. Sources of chemicals and supplies were as follows: potassium bromide from Harshaw Chemical Co. (Cleveland, OH); malt extract and Noble agar from Difco

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