Supracide is, however, one-third that of ethion and carbophenothion.

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Orlandin: A Nontoxic Fungal Metabolite with Plant Growth Inhibiting Properties

Horace G. Cutler,* Farrist G. Crumley, Richard H. Cox, Oscar Hernandez, Richard J. Cole, and Joe W. Dorner

A new metabolite, bis[8,8′-(7-hydroxy-4-methoxy-5-methylcoumarin)], trivial name orlandin, was isolated from *Aspergillus niger* found growing on orange leaves. It was nontoxic to day-old cockerels but significantly inhibited wheat coleoptile growth at 10^{-3}, 10^{-4}, and 10^{-5} M. Orlandin may be a precursor of kotanin, bis[8,8′-(4,7-dimethoxy-5-methylcoumarin)], isolated from *Aspergillus clavatus*. The latter was toxic to day-old cockerels, but did not inhibit the growth of wheat coleoptiles.

In March 1978, while visiting a citrus orchard in Orlando, Florida, we collected leaves from orange trees on which colonies of *Aspergillus niger* were visible. While many trees were diseased, no gross anatomical or physiological differences were observed on leaves infected with *A. niger*. Nevertheless, the organism was examined for plant growth regulator metabolites. Subsequent isolation and extraction of the fungus, from shredded wheat cultures, yielded a crystalline metabolite that inhibited the growth of etiolated wheat coleoptile sections. The metabolite, when compared with the plant growth inhibitor (±)-abscisic acid was as active at 10^{-3} and 10^{-4} M in the coleoptile bioassay, but was less active at 10^{-5} M. (+)-Abscisic acid has been found in many higher plants (Addicott and Lyon, 1969; Milborrow, 1974) and was initially isolated from immature cotton fruits, *Gossypium hirsutum* L. (Ohkuma et al., 1963) and sycamore leaves, *Acer pseudoplatanus* L. (Cornforth et al., 1965). Chemical and physical analyses showed that the new metabolite was bis[8,8′-(7-hydroxy-4-methoxy-5-methylcoumarin)] which we have given the trivial name orlandin (I) (Figure 1).

The simple coumarins have shown a wide range of growth regulating responses in plants from growth promotion to growth inhibition depending on the plant species or plant part treated (Mayer and Poljakoff-Mayber, 1961). In animals, the simple coumarins induce a hypnotic and narcotic response (Dean, 1952; Wawzonek, 1951) and the furanocoumarins are highly toxic to fish (Späth, 1936).

Orlandin is closely related to kotanin (III), bis[8,8′-(4,7-dimethoxy-5-methylcoumarin)], a fungal metabolite isolated from *Aspergillus glaucus* (Büchi et al., 1971) which was later identified as *A. clavatus* (Büchi et al., 1977) found on mold-damaged rice collected from a village in Baan Kota, Thailand. The rice, which was also contaminated with *A. flavus*, *A. niger*, and unidentified *Penicillium*, was implicated in the death of a young boy. However, neither kotanin, nor desmethylkotanin was toxic in rat bioassays. Kotanin did not inhibit the growth of wheat coleoptiles but it was toxic to day-old cockerels in our assay.

MATERIALS AND METHODS

Production and Isolation of Orlandin. *Aspergillus niger* (ATCC accession no. 36626) was isolated from the surface of orange leaves and cultured on potato dextrose agar slants at 26 °C for 7 days. Cultures were 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 metabolite. After 19 days growth at 27 °C, 300 mL of acetone was added to each flask (ethyl acetate in later extractions). Mycelia and substrate were macerated with a Super Dispax Homogenizer. The suspension was strained through cheesecloth to remove the pulp. The filtrate was filtered through Whatman No. 1 filter paper on a Buchner funnel to yield a crude liquid extract. Solvent was removed from the crude extract under vacuum at 50 °C and the resulting aqueous phase was extracted twice with two volumes of ethyl acetate, each equal to the volume of the aqueous phase. Combined ethyl acetate extracts were dried over anhydrous sodium sulfate, reduced

U.S. Department of Agriculture, Science and Education Administration, Agricultural Research, Georgia Coastal Plain Experiment Station, Tifton, Georgia 31794 (H.G.C., F.G.C.), The National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 (R.H.C., O.H.), and U.S. Department of Agriculture, Science and Education Administration, Agricultural Research, National Peanut Research Laboratory, Dawson, Georgia 31742 (R.J.C., J.W.D.).

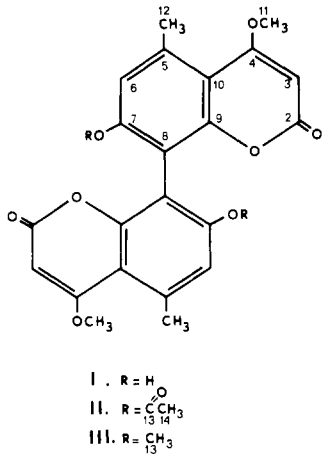

Figure 1. Structure of orlandin.

in volume under vacuum and then placed on a Florisil (100–200 mesh) chromatography column (9.0 × 10 cm) that had been slurry packed in benzene. The ethyl acetate fraction was eluted stepwise by 1.0 L each of benzene, ethyl ether, ethyl acetate, acetone, and methanol. Each solvent drained to the top of the Florisil before the next solvent was added. Each fraction was reduced under vacuum and aliquots were bioassayed with wheat coleoptiles to test for the presence of plant growth inhibitors. The ethyl acetate fraction inhibited coleoptile growth and additional separation of that fraction was effected by further Florisil column chromatography (4.0 × 50 cm). The Florisil was packed as a slurry in benzene, the ethyl acetate fraction was added to the top of the packing material, and 1.5 L of benzene was allowed to percolate through the column. Then a linear gradient of benzene to ethyl acetate (1.0 L of benzene and 1.0 L of ethyl acetate) was used for elution. Twenty-milliliter fractions were collected, evaporated to a small volume under vacuum, and bioassayed.

Synthetic Preparations. Synthesis of the diacetate (II) of orlandin was accomplished by stirring 25 mg of I in a solution of 1 mL of acetic anhydride and 1 mL of pyridine overnight under nitrogen. Evaporation, under vacuum, of the reaction mixture gave an oily residue that was characterized without further purification.

Kotanin (III) was synthesized from orlandin in the following way. Oil-free sodium hydride (8 mg, 0.08 mmol) was suspended in dry dimethylformamide (DMF, 2 mL) under argon atmosphere. Iodomethane (0.5 mL, 0.023 mmol) was added and the suspension was cooled to −15 °C. A solution of orlandin (9.8 mg, 0.023 mmol) in DMF (10 mL) was added dropwise while maintaining a temperature of ca. −15 °C. After stirring for 1 h, the reaction mixture was allowed to warm to room temperature and stirring continued overnight. The mixture was partitioned between chloroform and water and the resulting organic solution was washed with water, 5% sodium bisulfite, water, brine, and then dried over sodium sulfate. Evaporation of the solvent yielded a pale yellow solid with mp >300 °C, that was compared with authentic kotanin.

Physical and Chemical Analyses. Ultraviolet (UV) spectra of orlandin were taken in 95% ethanol solution with a Beckman Model DB-G recording spectrophotometer. Infrared (IR) spectra were made from samples prepared as thin films on KBr windows with a Beckman IR 4210 spectrophotometer equipped with a 4X beam condenser. Proton and natural abundance carbon-13 nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL-100-12 spectrometer equipped with the 620-L data system. Typical operating conditions for obtaining the ^{13}C spectra in the Fourier transform (FT) mode were: pulse angle 15°; pulse delay 3 s; spectral width 5 KHz; data points 8K; exponential broadening 1.0; and broadband proton decoupling. Single frequency, off-resonance proton decoupled (sford) spectra were obtained to aid in the carbon resonance assignment. Samples were prepared in 5-mm tubes with tetramethylsilane as the internal standard and were used for both the ^{1}H and ^{13}C spectra. The sample of I was prepared in dimethyl-d_6 sulfoxide; samples of II and III were prepared in $CDCl_3$. Low-resolution (LRP) mass spectral analyses were gathered with a Finnigan 3300 mass spectrometer, high-resolution (HRP) analyses were gathered with an AEI MS-9 mass spectrometer. Samples were introduced into the instrument by the direct probe method, and ionization was by electron impact at 70 eV. Melting points were determined with a Hoover capillary melting point apparatus.

Compounds I and III were chromatographed on silica gel 60, F-254, (E.M. Laboratories, Inc.) thin-layer plates with a toluene/ethyl acetate/formic acid (5:4:1, v/v/v) solvent system. Synthetic kotanin was compared with an authentic (from *A. clavatus*) sample using Merck silica gel 60 plates and diethyl ether/dichloromethane (1:4, v/v) as the developing solvent. Compounds were visualized under short-wave UV.

Bioassays. Wheat seedlings (*Triticum aestivum* L. cv. Wakeland) were grown in the dark on moist sand for 4 days at 22 ± 1 °C (Hancock et al., 1964). The apical 2 mm of the coleoptiles were removed in a Van der Weij guillotine and discarded. The next 4 mm of the coleoptiles were 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 to dryness under nitrogen. Two milliliters of phosphate-citrate buffer containing 2% sucrose at pH 5.6 (Nitsch and Nitsch, 1956) was added to each test tube. Ten coleoptiles were added to each test tube and the tubes were then placed in a roller-tube apparatus, rotated at 0.25 rpm for 24 h at 22 °C in the dark. Coleoptile lengths were measured by projecting their images (X3) from a photographic enlarger (Cutler and Vlitos, 1962). Orlandin and kotanin (obtained from *A. clavatus*) were assayed at 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M. A 10^{-3} M stock solution of each metabolite was made by dissolving the appropriate weight of 50 μL of acetone (Cutler, 1968) and then adding phosphate-citrate buffer to a 10-mL volume. All data were statistically analyzed (Kurtz et al., 1965).

Samples of orlandin and authentic kotanin were prepared for dosing to 1-day-old DeKalb cockerels by dissolving the samples in methanol, adding the inert carrier (corn oil) and removing the methanol under vacuum at 70 °C. Adequate controls were similarly prepared to insure that all the methanol was removed by this procedure. Cockerels were dosed orally by crop intubation of 62.5 mg/kg body weight of kotanin and 62.5 and 125 mg/kg for orlandin. Chicks were observed daily for 5 days after dosing.

RESULTS AND DISCUSSION

Physical and Chemical Characteristics. In mass culture, *Aspergillus niger* produced 84.8 mg of orlandin from 32 flasks of media in 19 days. During the purification procedure, biological activity was first observed in the ethyl acetate fraction obtained from liquid–liquid partition and again in the ethyl acetate fraction obtained from sequential elution column chromatography on Florisil. Upon further purification by Florisil column chromatography, with linear

Table I. ^1H NMR Spectra of Orlandin and Some Derivatives

proton compd	I	II	III
3	5.53[a]	5.57	5.51
6	6.68	6.90	6.72
11	3.93	3.90	3.93
12	2.62	2.65	2.70
13		1.95	3.80

[a] Chemical shifts in parts per million downfield from internal tetramethylsilane.

gradient elution of benzene to ethyl acetate, activity was associated with tubes 110–160 (2.2–3.2 L, of total solvent). Tubes 110–160 were combined, reduced in volume under vacuum, and then stored in a refrigerator. Crystals, which formed overnight, were filtered and washed with ice-cold acetone and dried. It was noted that crystals left standing at room temperature in acetone deteriorated in 30–60 min.

R_f values for I and III on silica gel 60 thin-layer plates developed with toluene/ethyl acetate/formic acid were 0.37–0.42 and 0.52–0.56, respectively. Both authentic and synthetic kotanin had R_f values of 0.25–0.29 in ether–dichloromethane solvent systems. Spots were visualized as dark spots under short-wave UV. Thermal decomposition started at 285 °C for I and the melting point for III has been reported as >315 °C (Büchi et al., 1971). UV analysis for I were λ_{max}^{EtOH} 311 (log ϵ 4.47) and λ_{max}^{EtOH} 321 (shoulder; log ϵ 4.40). The IR spectra for I gave the following values: 3200–3300 (broad, weak) 1675, 1605, 1590, 1560, 1450, 1360, 1325, 1262, 1205, 1132, 1105, 1087, 1035, 1015, 1002, 970, 795, 785 cm^{-1}.

High-resolution mass spectral analyses for I gave a molecular ion peak (M^+) at m/e 410.1002 (calculated mass 410.1002) and molecular formula $C_{22}H_{18}O_8$. For the orlandin diacetate derivative (II), bis[8,8′-(7-acetoxy-4-methoxy-5-methylcoumarin)], the low-resolution mass spectral analyses showed a molecular ion peak (M^+) at m/e 494 with prominent ion fragment peaks at m/e 452 ($M^+ - CH_2CO$) and 410 ($M^+ - 2CH_2CO$), indicating the loss of acetate groups.

The ^1H NMR spectrum of I (Table I) shows four single peaks. Integration of the peaks and chemical shift considerations suggest the presence of a methyl group, a methoxy group, and two olefinic and/or aromatic hydrogens. The ^{13}C NMR spectrum of I exhibits only 11 carbon resonances (Table II) which were shown by the sford spectrum to be seven quaternary carbons, two methine carbons, and two methyl carbons. Chemical shift considerations confirm the presence of the methyl and methoxy groups suggested by the ^1H spectrum and further establish that the remaining carbons are sp^2 type carbons. The relative simplicity of the ^1H and ^{13}C spectra of I along with the mass spectral data suggest that I is a symmetrical dimer with molecular formula $C_{22}H_{18}O_8$.

The low-field ^{13}C resonances along with the IR spectrum further suggest that I contains a hydroxyl group (phenolic) in each monomeric unit. Accordingly, the acetate derivative (II) of I was prepared. Mass spectral data and the ^1H (Table I) and ^{13}C (Table II) NMR spectra establish the presence of two symmetrical acetate groups in II, thus confirming the presence of a phenolic group in each monomeric unit of I.

At this point, the NMR shift data and other data suggested that orlandin (I) is a highly substituted dicoumaryl or dichromonyl. A literature search yielded two bicoumarins (kotanin and desmethylkotanin) isolated previously from *Aspergillus clavatus* (Büchi et al., 1971, 1977). The similarity between the ^1H spectral data for I and kotanin and desmethylkotanin suggested that I and kotanin might have similar structures, with I having two phenolic hydroxyl groups in place of two of the methoxy groups on kotanin. Thus, I was methylated (III) for comparison with kotanin. The ^1H (Table I) and ^{13}C (Table II) spectra of III, as well as IR spectra and R_f values of III were identical in all respects with those of an authentic sample of kotanin. Therefore, the 8,8′-bicoumaryl structure for I is established. The only remaining question concerning the structure of I is whether the hydroxyl groups are located at the 4,4′ or 7,7′ positions. Comparison of the ^1H and ^{13}C NMR spectral differences among I, II, and III rules out the possibility of the hydroxyl groups being located at the 4,4′ positions and are only consistent with their being located at the 7,7′ (that is, formation of II from I results in significant changes in the chemical shifts of C-5–10 and only minor changes in C-2–4 positions). Therefore, the structure of orlandin (I) is established as bis[8,8′-(7-hydroxy-4-methoxy-5-methylcoumarin)].

Table II. ^{13}C NMR Chemical Shifts of Orlandin and Some Derivatives[a]

carbon compd	I	II	III
2	169.38	168.95	169.64
3	86.28	89.56	87.67
4	153.68	153.01	153.32
5	136.82	138.43	138.34
6	115.26	121.77	111.30
7	158.26	150.45	159.37
8	105.91[b]	112.32[c]	108.36[d]
9	161.43	161.89	162.82
10	105.52[b]	111.16[c]	107.35[d]
11	56.36	56.09	56.05
12	23.11	23.73	24.10
13		168.14	55.84
14		20.71	

[a] In parts per million downfield from internal tetramethylsilane. [b-d] Assignments may be reversed.

The assignment of the ^1H spectra (Table I) of I, II, and III is straightforward based on known substituent effects (Jackmon and Sternhell, 1969). Assignment of the ^{13}C spectra (Table II) is based upon the sford results, known substituent effects (Stothers, 1972) and comparison with model compounds (Giannini et al., 1974; Wenkert et al., 1976). Carbons 2, 3, and 4 were assigned on the basis of comparison with model compounds and their consistency among the compounds. Carbons 11, 12, 13, and 14 were assigned from sford results and from expected chemical shift values (Stothers, 1972). The remaining carbons were assigned from that of coumarin (Wenkert et al., 1976) and from shift differences expected for a hydroxy, methoxy, and acetate substituent on an aromatic ring (Stothers, 1972).

Effects on Plant Growth. Orlandin (I) significantly ($P < 0.01$) inhibited the growth of etiolated wheat coleoptiles 100, 100, and 35% at 10^{-3}, 10^{-4}, and 10^{-5} M, respectively, relative to controls. Kotanin (III) did not inhibit coleoptile elongation at any concentration tested (Figure 2). In comparison it has been shown earlier (Cutler et al., 1978) that (±)-abscisic acid routinely inhibited coleoptile growth 100, 90, and 69% at 10^{-3}, 10^{-4}, and 10^{-5} M.

Effects on Day-Old Chicks. No toxic effects were observed for chicks dosed with orlandin at 62.5 mg/kg and 125 mg/kg body weight. However, kotanin had an LD_{100} of 62.5 mg/kg. Immediately after dosing with kotanin, chicks became lethargic and failed to feed. All chicks dosed with kotanin died between the third and the fourth day

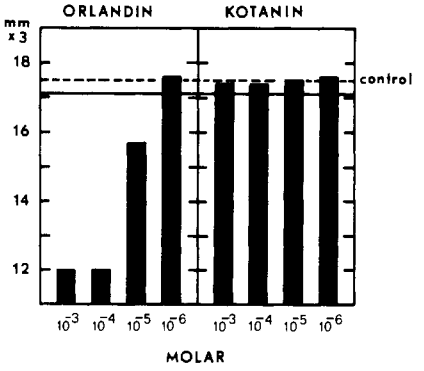

Figure 2. Growth regulating activity of orlandin (I) and kotanin (II) in wheat coleoptile bioassays (*Triticium aestivum* L. cv. Wakeland). Control: dotted line. Significant inhibition: below solid line ($P < 0.01$).

after dosing. Insufficient material was available to obtain an LD_{50}.

Wheat coleoptile and chick bioassays indicate that the biological activity of orlandin in plants, and of kotanin in chicks, is intimately associated with the 7,7′ hydroxyl groups. Methylation of the 7,7′ hydroxyl groups caused the molecule to be inactive in the coleoptile assay but increased toxicity to chicks.

Two points remain to be elucidated. First, does kotanin occur in *A. niger* as does orlandin, and second, is orlandin a biosynthetic precursor to desmethylkotanin and kotanin? Neither of these points has been resolved here. But more importantly the bicoumarin structure offers an interesting model to determine functional group activity in plant and animal bioassays.

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Ergosine, Ergosinine, and Chanoclavine I from *Epichloë typhina*

James K. Porter,* Charles W. Bacon, and Joe D. Robbins

Epichloë typhina, isolated from toxic K-31 tall fescue grass, was shown to produce ergot alkaloids in vitro. Ergosine, ergosinine, and chanoclavine I were isolated and identified by comparison with authentic standards with regard to thin-layer chromatography, ultraviolet absorption, and low-resolution mass spectra analyses. Total alkaloid production, colorimetrically determined as ergonovine maleate, was 5.5 mg/L for 28-day-old cultures. This is the first report of a fungus outside the genera of *Claviceps* and *Balansia* capable of producing alkaloids that are N-peptide-substituted amides of lysergic acid.

The signs of the fescue toxicity syndrome have been described in cattle by Yates et al. (1971), in sheep by Simpson (1975), and associated with the ingestion of a mycotoxin produced on tall fescue, *Festuca arundinacea* Schreb. (Yates et al., 1971). Recently, *Epichloë typhina* (Fries) Tulasne, a clavicipitaceous endophyte of the cultivars and hybrids of tall fescue, has been implicated in the fescue toxicity syndrome in cattle (Bacon et al., 1977). Proof that the isolates of this fungus are toxic depends on the production of toxic compounds in vitro and subsequent isolation of these compounds or their modifications from

Biological Constituents Unit, Field Crops Utilization and Marketing Research Laboratory, Richard B. Russell Agricultural Research Center, United States Department of Agriculture, Science and Education Administration, Athens, Georgia 30604.

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