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Received for review March 9, 1981. Revised manuscript received May 22, 1981. Accepted May 22, 1981. Published with the approval of the Director of the Arkansas Agricultural Experiment Station. This research was conducted primarily with funds provided to the U.S. Fish and Wildlife Service by the Agency for International Development under PASA's "Control of Vertebrate Pests: Rats and Noxious Birds", ID/TAB-473-1-67.

Dihydropergillin: A Fungal Metabolite with Moderate Plant Growth Inhibiting Properties from *Aspergillus ustus*

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Dihydropergillin, $C_{15}H_{18}O_4$, is a newly discovered metabolite isolated from cultures of *Aspergillus ustus* found growing on seed of *Pisum sativum* var. *macrocarpon*. It is structurally related to pergillin and significantly inhibited wheat coleoptile growth at 10^{-3} and 10^{-4} M. Structural determination was by single-crystal X-ray diffraction analysis.

We recently characterized and described a fungal metabolite, from *Aspergillus ustus*, that possessed moderate plant growth inhibiting properties, to which was assigned the trivial name pergillin (Cutler et al., 1980). Further studies with extracts of the fungus have led to the isolation of a new metabolite, dihydropergillin, that has greater activity than pergillin in our assays.

We now report the isolation and identification of dihydropergillin (I) (Figure 1) and its effects in wheat coleoptile bioassays.

MATERIALS AND METHODS

Production, Purification, and Isolation of Dihydropergillin. *A. ustus* (Bainier) Thom & Church (ATCC accession no. 38849) was isolated from greenhouse-produced pea seeds *Pisum sativum* var. *macrocarpon* (cv. Oregon Sugarpod) grown in Georgia. The fungus was grown on potato dextrose agar slants for 14 days at 26 °C and further maintained at 5 °C until used. Shredded wheat medium, in Fernbach flasks (2.8 L), consisted of 100 g of shredded wheat, 200 mL of Difco mycological broth (pH 4.8), 2% yeast extract, and 20% sucrose per flask (Kirksey and Cole, 1974). The medium was inoculated with the fungus and incubated at ~26 °C for 12 days. After the addition of 300 mL of acetone, the contents of each flask were macerated with a Super Dispax homogenizer, and the resulting pulp was strained through cheesecloth. The liquid phase was filtered through Whatman No. 1 filter paper on a Buchner funnel and then reduced in volume under vacuum at 50 °C to an aqueous phase. The water phase was extracted twice with an equal volume of ethyl acetate, and the ethyl acetate fractions were combined and dried over anhydrous sodium sulfate. This was followed by distillation of the ethyl acetate fraction, under vacuum, to produce a crude extract. The crude extract was added to the top of a silica gel (70–230 mesh) chromatography column (9.0 × 10 cm) that had been slurry packed in benzene and eluted stepwise with 1.0 L each of benzene, ethyl ether, ethyl acetate, acetone, and methanol. Each solvent was allowed to drain to the top of the silica gel before addition of the next sequential solvent. Each bulk fraction was reduced in volume, under vacuum, and aliquots were bioassayed with etiolated wheat coleoptiles. Since the ethyl ether fraction inhibited wheat coleoptile growth, it was reduced in volume and further fractionated with a silica gel (70–230 mesh) chromatography column (4.0 × 50 cm) that had been slurry packed in hexane, and 800 mL of hexane was percolated through the column and discarded. A linear gradient of hexane to ethyl acetate (1.0 L of hexane and 1.0 L of ethyl acetate) was then used, and 20-mL fractions were collected and evaporated to ~2 mL, and 25-μL aliquots were bioassayed. Tubes 31–60 (1.42–2.00 L of total solvent) were active in the assay system. These were combined, reduced in volume, and again placed on a freshly prepared silica gel (70–230 mesh) column (4.0 × 50 cm) that had been slurry packed in benzene. Six-hundred milliliters of benzene was allowed to percolate through the column and discarded, a linear gradient of benzene to ethyl acetate (1.0 L of benzene and 1.0 L of ethyl acetate) was used, and 20-mL fractions were collected. Since bioassays detected plant growth inhibition in tubes 41–65 (1.42–1.90 L of total solvent used), they were combined, reduced to a suitable volume, and loaded onto an RP2 (silica gel 60 silanized, 70–230 mesh, E. Merck) column (3.5 × 45 cm) that had been slurry packed in acetonitrile–water (1:1 v/v). Of the same solvent, 600 mL was allowed to percolate through the column, and 20-mL fractions were collected. Biological activity was associated with tubes 1–22 (0.62–1.04 L of total solvent), and these were combined and placed on a silica gel (70–230 mesh) chromatography column that had been slurry packed in ethyl acetate–benzene (55:45 v/v). Solvent was allowed to percolate through the silica gel, and the various fluorescent bands were noted at 366 nm. As the first fluorescent band started eluting, 5-mL fractions were collected. Biological activity was obtained in fractions 4–10 (20–50 mL of solvent after the first UV–visible band started exiting the column). These fractions were reduced, under vacuum at 50 °C, to the aqueous phase and partitioned against ethyl acetate. The ethyl acetate was dried over anhydrous sodium sulfate and reduced to a small volume. This fraction was added to the top of a C_{18} reverse-phase (obtained by cutting open a Waters Associates Prep PAK-500 C_{18}) chromatography column (2.5 × 11 cm)

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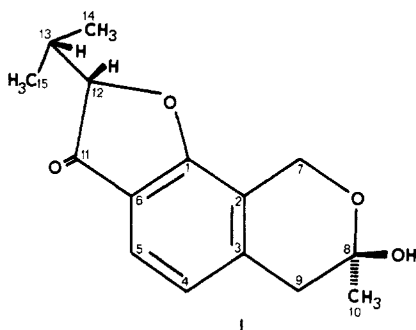

Figure 1. Numbering system and structure for dihydropergillin.

packed in acetonitrile–water (50:50 v/v). The identical solvent was passed through the column, and fluorescent and absorbing bands were observed at 366 nm. Each eluting band was collected in a test tube, but in order to keep the bands eluting sequentially from the column, the polarity of the solvent was changed to acetonitrile–water (75:25 v/v) at tube 21. When no further bands eluted, neat acetonitrile was added to the column (at tube 29), and biological activity was subsequently observed in tube 30.

Tube 30 was stored at 4 °C, and within 1 week, crystals of I formed as large blocks from the hydrated acetonitrile mixture.

Physical and Chemical Analyses. The ultraviolet (UV) spectrum of dihydropergillin was obtained from 10^{-5} M solutions in 95% ethanol with a Beckman Model 35 spectrophotometer. Infrared (IR) spectra were obtained from samples prepared as thin films on KBr windows by using a Beckman IR 4210 equipped with a 4× beam condenser.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a Varian Associates XL-100-12 NMR spectrometer. The samples were run in $CDCl_3$ solution (3 mg/0.4 mL) containing a small amount of tetramethylsilane (Me_4Si) as the internal reference.

Low-resolution (LRP) and high-resolution (HRP) mass spectra were obtained with a Varian MS 902. Samples were introduced into the instrument by the direct-probe method, and ionization was by electron impact at 70 eV. Perfluorokerosene was used as the internal standard.

Melting points for I were determined on a Kofler micro melting point block and were uncorrected. Single-crystal X-ray analyses were made from selected crystals, and data were collected with a Syntex $P2_1$ automatic four-circle diffractometer using Cu Kα radiation. The symmetry of the unit cell was $P2_12_12_1$.

Purification of I was analyzed by thin-layer chromatography on silica gel 60 plates, F-254 (E. M. Laboratories, Inc.) with a developing solvent of toulene–ethyl acetate–formic acid (5:4:1 v/v/v). I was visualized under UV at 254 and 366 nm.

Bioassay. Wheat seeds (*Triticum aestivum* L. cv. Wakeland) were grown on moist sand, in trays, for 4 days in the dark at 22 ± 1 °C (Hancock et al., 1964). Etiolated seedlings were removed from the trays, and the shoots were cut off and saved. The shoot apexes were placed in a Van der Weij guillotine, and the apical 2 mm was extruded, cut off, and discarded. The next 4 mm of each coleoptile was saved for bioassay. Approximately 25 μL of each fraction obtained from column chromatography was added to individual test tubes and reduced to dryness under a stream of nitrogen at 60 °C, and then 2 mL of phosphate–citrate buffer containing 2% sucrose at pH 5.6 (Nitsch and Nitsch, 1956) was added to each test tube. Ten coleoptiles were put into each test tube, and the tubes were placed in a roller–tube apparatus and rotated for 24 h at 0.25 rpm in the dark at 22 °C. All procedures involving test plants were carried out under a green safelight (Nitsch and Nitsch, 1956). After incubation, the coleoptiles were placed on a glass plate, and their images (×3) were projected, measured (Cutler and Vlitos, 1962), and statistically analyzed (Kurtz et al., 1965). Dihydropergillin (I) was assayed at 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M by formulating a stock solution, at 10^{-3} M, in which an appropriate amount of the metabolite was dissolved in 50 μL of acetone and made to 10 mL with buffer (Cutler, 1968).

RESULTS AND DISCUSSION

Physical and Chemical Characteristics. *A. ustus* produced 11.4 mg of dihydropergillin from 72 flasks of shredded wheat medium in 12 days. Those fractions exhibiting biological activity have already been mentioned in the section relating to column chromatography. However, in addition to the manifestation of biological activity, there was the concomitant observation of a fluorescent band on the RP2 chromatography column which aided in the isolation of the metabolite. An identical band was associated with activity on the silica gel column (3.0 × 23 cm) and again with the C_{18} reverse-phase column.

Earlier (Cutler et al., 1980) we reported that tubes 31–60 from the first silica gel linear gradient chromatography column (hexane to ethyl acetate) contained biologically active material and that pergillin precipitated from tubes 52–58 after storage for 1 week at 5 °C. Since the wheat coleoptile assay cannot discriminate between individual inhibitors, on a gross scale, it was impossible to determine whether activity in tubes 31–51 was attributable to dilute amounts of pergillin (specific activity data were unavailable at that time) or another inhibitor. It is now obvious that tubes 31–51 contained dihydropergillin. Furthermore, after the crystallization of pergillin from tubes 52–58, there were recoverable traces of dihydropergillin in the supernatant liquid.

The thin-layer R_f values for I were 0.76–0.82 on silica gel 60 plates developed with toluene–ethyl acetate–formic acid (under 254-nm lamps). The compound appeared to be deep blue absorbing, while at 366 nm it appeared as a bright blue fluorescent spot. The uncorrected melting point for I was 122–126 °C. The ultraviolet spectrum in ethanol solution was λ_{max}^{EtOH} 218 (log ϵ = 4.33) and 264 nm (log ϵ = 4.08). The IR spectrum yielded the following: 3370 (sharp, OH) 2960, 2930, 2850 (all very small, CH_3, CH_2), 1685 (strong, carbonyl), 1598 (phenyl), 1432 (CH_2), 1370 (CH_3), 1328, 1255 (ether), 1210, 1155, 1082 (ring ether), 1035, 968, 948, 872, 805, 760, 702 cm^{-1}. The major difference between dihydropergillin and pergillin is the sharp OH absorption at 3370 as opposed to the broad OH observed with pergillin at 3360 cm^{-1} (Cutler et al., 1980).

The HRP mass spectral analysis of I showed a molecular ion peak (M^+) at m/e 262.1214 when matched with perfluorokerosene ($C_{15}H_{18}O_4$ requires mass 262.1204) with fragment ions at m/e 244 ($M^+ - H_2O$), 220 ($M^+ - C_3H_6$), and 202 ($M^+ - C_2H_4O_2$). As with pergillin, the latter fragment is most probably derived from O7A, C8, the subtended C10 and O8A, and associated protons (Figure 2).

The X-ray diffraction experiments with single crystals of I indicated that the cell constants were a = 7.093 (1), b = 9.300 (1), and c = 21.693 (4) Å for a calculated density of 1.22 g/cm^3 with Z = 4. Of the 1148 unique reflections measured with Cu Kα radiation, 955 (83%) were observed ($I > 3\sigma I$) and corrected for Lorentz and polarization effects. The problem was solved by using a standard multisolution tangent formula approach (Main et al., 1978) and refined by using full matrix least squares by minimizing $\sum[\omega(|F_o|$

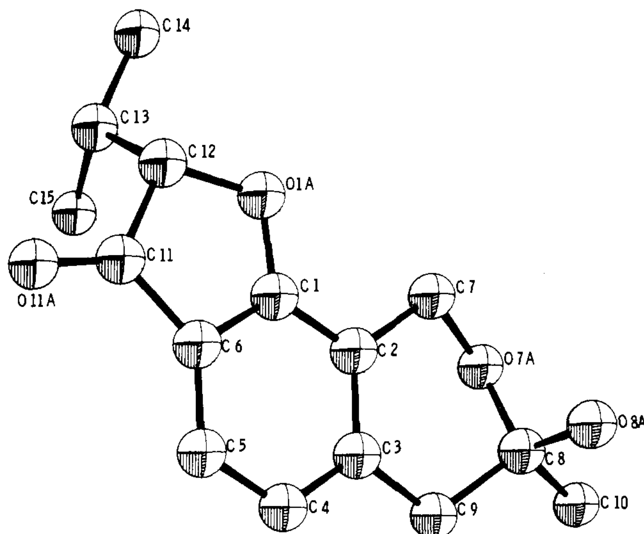

Figure 2. Computer-generated perspective drawing of dihydropergillin with hydrogens omitted for clarity.

Table I. ^{1}H NMR Parameters for Dihydropergillin[a]

proton	δ
4	6.79 (J = 8.0 Hz)
5	7.45 (J = 8.0 Hz)
7	4.94
9	2.97
10	1.62
12	4.41 (J = 4.0 Hz)
13	2.33
14	0.86 (J = 7.0 Hz)
15	1.14 (J = 7.0 Hz)

[a] In $CDCl_3$; in ppm downfield from internal Me_4Si.

$- |F_c|)^2]$ with $\omega = 1/(\sigma F_o)^2$ (Stewart et al, 1972). The final unweighted residual index was 0.045. Tables Is, IIs, and IIIs (see paragraph at end of paper regarding supplementary material) contain the final fractional coordinates and temperature parameters, bond distances, and bond angles respectively; Figure 2 is a perspective drawing of I (Johnson, 1970). Thus the structure for I was unambiguously derived by single-crystal X-ray diffraction analysis.

The only close intermolecular contact is a hydrogen bond of 2.81 Å which includes O8A and O11A. Not surprisingly, conformation of the substituted pyran ring in I is virtually identical with that of pergillin (Cutler et al., 1980).

Data obtained from ^{1}H NMR spectra supported the structure derived by X-ray crystallography and are presented in Table I. Not enough sample remained to obtain ^{13}C spectra.

Bioassay Results. Etiolated wheat coleoptile segments were significantly inhibited ($P < 0.01$) by solutions of I at 10^{-3} and 10^{-4} M (Figure 3). While pergillin inhibited coleoptile growth at 10^{-3} M 50% relative to controls (Cutler et al., 1980), dihydropergillin (I) inhibited coleoptiles 100%. At 10^{-4} M, while the relative inhibitions appear to be close, there is a 7% greater inhibition by dihydropergillin. Thus, the addition of two hydrogen atoms to the C12–C13 double bond of pergillin, to yield diphydropergillin, enhances the inhibition response in coleoptiles.

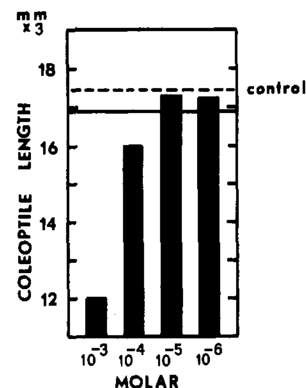

Figure 3. Inhibition of etiolated wheat coleoptiles (*T. aestivum* L. cv. Wakeland) by dihydropergillin. Control: dotted line. Significant inhibition: below solid line ($P < 0.01$).

Structurally, this reduction of the double bond allows the isopropyl group to form a staggered conformation with the substituted furan ring. It is possible that the added flexibility of this end of the molecule accounts for the enhanced biological activity. It is interesting to speculate that other modifications in the molecule could lead to a series of compounds for unique uses in agriculture or medicine.

Supplementary Material Available: Fractional coordinates and temperature factors (Table Is), bond distances (Table IIs), and bond angles (Tables IIIs) (2 pages). Ordering information is given on any current masthead page.

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