expensive identifications be used to screen resistance factors in native regions and gene banks. Associational resistance, i.e., a benefit from the chemical defense in other plants, is another part of the natural chemical defense of plants (Atsatt and O'Dowd, 1976). These methods could also be used to mix crops in the most favorable defense combinations.

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

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A new metabolite, $C_{15}H_{16}O_4$, was isolated from cultures of *Aspergillus ustus* found growing on seeds of *Pisum sativum* var. macrocarpon. It was nontoxic to day-old chicks but significantly inhibited wheat coleoptile growth at 10^{-3} and 10^{-4} M. The trivial name pergillin was given to the metabolite.

A further survey of fungi for the production of plant growth inhibiting substances revealed that cultures of *Aspergillus ustus*, found growing on the testa of pea seeds, *Pisum sativum* var. macrocarpon (Oregon Sugarpod), produced a metabolite that significantly inhibited the growth of wheat coleoptiles. The metabolite was nontoxic to day-old chicks.

Certain strains of *A. ustus* have yielded several metabolites toxic to vertebrates. Among those reported are austamide (Steyn, 1971), austidiol (Vleggaar et al., 1974), and the highly complex polyisoprenoid austin (Chexal et al., 1976).

We now report the isolation of a new metabolite, to which we have assigned the trivial name pergillin (I) (Figure 1). The effects induced by pergillin in plants are also described.

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MATERIALS AND METHODS

Production, Isolation, and Purification of Pergillin. *A. ustus* (Bainier) Thom & Church (ATCC accession no. 38849) was isolated from greenhouse-produced pea seeds *P. sativum* var. macrocarpon (cv. Oregon Sugarpod) grown in Georgia. The fungus was cultured on potato-dextrose-agar slants at 26 °C for 14 days and then maintained at 5 °C. Cultures were then 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. Inoculated flasks were incubated in the laboratory for 12 days at about 26 °C. Then 300 mL of acetone was added to each flask. The substrate and mycelia were macerated with a Super Dispax homogenizer, and the resulting suspension was strained through cheesecloth to remove the pulp. The filtrate was filtered through Whatman No. 1 filter paper on a Buchner funnel, and the clarified filtrate was reduced under vacuum at 50 °C to an aqueous phase. The aqueous phase was extracted twice with 2 volumes of ethyl acetate, and each volume of solvent used was equal to the volume of the aqueous phase. Ethyl acetate extracts were combined, dried over anhydrous sodium sulfate, and reduced in volume under vacuum at 50 °C. This crude extract was placed on a silica gel (70-230 mesh) chromatography column (9.0 × 10 cm) that had been slurry packed in benzene. The extract 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 silica gel before addition of the next sequential

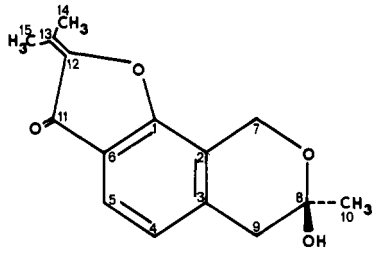

Figure 1. Structure and numbering system of pergillin.

solvent. The individual solvents containing the eluted compounds were each reduced under vacuum, and aliquots were bioassayed for the presence of plant growth inhibitors with wheat coleoptiles. The ethyl ether fraction inhibited coleoptile growth and was further fractionated by reducing it to a small volume and placing it on silica gel (70–230 mesh) in a column (4.0 × 50 cm) that had been slurry packed in hexane. Then, 800 mL of hexane was allowed to percolate through the column and discarded, after which a linear gradient of hexane–ethyl acetate (1.0 L of hexane and 1.0 L of ethyl acetate) was used for elution. Twenty-milliliter fractions were collected and evaporated to about 2 mL, and 20-μL aliquots were bioassayed.

Physical and Chemical Analyses. The ultraviolet (UV) spectrum of pergillin was determined in 95% ethanol solution with a Beckman Model 35 spectrophotometer. Infrared (IR) spectra were obtained with a Beckman IR 4210 spectrophotometer with a 4× beam condenser and samples were prepared as thin films on KBr windows.

Proton and ^{13}C nuclear magnetic resonance spectra were obtained on a Varian Associates XL-100-12 spectrometer equipped with the 620 L disk data system. The sample was prepared in a 5-mm tube with tetramethylsilane as the internal standard and was used for both ^{1}H and ^{13}C spectra; a mixture of $CDCl_3$–dimethyl-d_6 sulfoxide (1:1) was used to dissolve the sample. Proton spectra were recorded in the continuous wave mode, and ^{13}C spectra were recorded in the Fourier transform mode. Operating parameters were pulse angle 30°, pulse delay 2 s, spectral width 5 kHz, data points 8K, exponential broadening –0.5, and broadband proton decoupling.

Low-resolution (LRP) mass spectra were obtained with a Hewlett-Packard 5985 spectrometer, and high-resolution (HRP) spectra were obtained with a Varian MAT-731 spectrometer using perfluorokerosene as the internal standard. Introduction of the samples into the instrument was by the direct probe method, and ionization was by electron impact at 70 eV.

Pergillin crystals were recrystallized from hot acetone, and uncorrected melting points were taken on a Kofler micro melting point block. Suitable crystals for single-crystal X-ray analyses were formed from ethyl acetate with symmetry $P2_1/c$. Data were collected with a Syntex $P2_1$ automatic four-circle diffractometer using Cu Kα radiation.

Steps in the purification of the metabolite were monitored 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. Plates were observed in daylight, under shortwave and longwave UV, and after being sprayed with anisaldehyde (Stahl, 1965).

Bioassays. Wheat seeds (*Triticum aestivum* L., cv. Wakeland) were cultured on moist sand for 4 days in the dark at 22 ± 1 °C (Hancock et al., 1964). The apical 2 mm of the etiolated coleoptiles was removed in a Van der Weij guillotine and discarded. The next 4 mm of the coleoptiles was used for the bioassay. Fractions assayed for biological activity were added to test tubes (approximately 20 μL/tube) and evaporated to dryness under nitrogen at 60 °C.

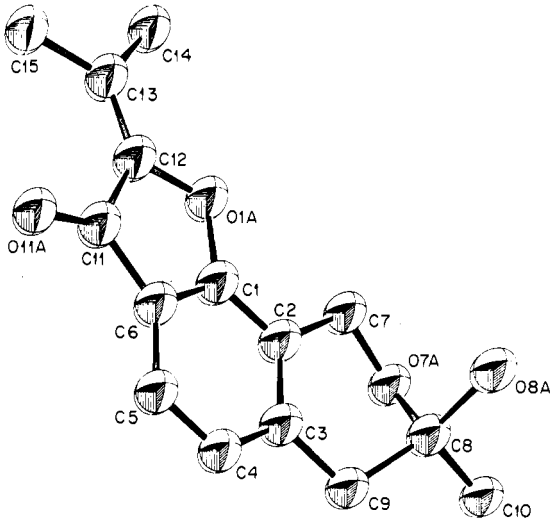

Figure 2. Computer-generated perspective drawing of pergillin. Hydrogens are omitted for clarity.

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 placed in each test tube, and the tubes were placed in a roller-tube apparatus and rotated at 0.25 rpm for 24 h at 22 °C in the dark. All manipulations were 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). Pergillin was assayed at 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M. The original 10^{-3} M stock solution was formulated by dissolving the appropriate weight in 50 μL of acetone (Cutler, 1968) and adding phosphate–citrate buffer to a volume of 10 mL. Data were analyzed statistically (Kurtz et al., 1965).

For the vertebrate bioassay, 1-day-old chicks were used. They were dosed by crop intubation using corn oil as the carrier. Samples were prepared by dissolving pergillin in acetone and then adding corn oil. The acetone was removed under vacuum at 70 °C, and, after apparent removal of all acetone, the evaporator reservoir was emptied and dried. The sample was subjected to vacuum and heat again to ensure complete removal of the solvents (Kirksey and Cole, 1974), and controls were identically prepared. Chicks were dosed with 1 mL of corn oil/chick up to a maximum concentration of 250 mg/kg of body weight.

RESULTS AND DISCUSSION

Physical and Chemical Characteristics. *A. ustus* produced 151.9 mg of pergillin from 72 flasks of shredded wheat medium in 12 days. Plant growth inhibitory properties were first noted in the ethyl acetate fraction obtained from liquid–liquid partition and again in the ethyl ether phase obtained from open column chromatography (9.0 × 10 cm column). Assay results gathered from fractions obtained from the linear gradient of hexane–ethyl acetate indicated biological activity in tubes 31–60 (1.42–2.00 L of total solvent). Test tubes containing the active fractions were stored at 5 °C for 1 week and crystals precipitated from tubes 52–58. They were collected on a fine porosity fritted glass funnel under vacuum and yielded pale yellow crystals after recrystallization.

The R_f values for I were 0.65–0.69 on silica gel 60 thin-layer plates developed with toluene–ethyl acetate–formic acid, and in daylight it was observed as a yellow spot. Under UV at 254 nm, spots were deep bronze, and at 366 nm they were bright fluorescent yellow and tailed upward. When sprayed with anisaldehyde and heated to 100 °C, transient deep orange spots appeared which shortly turned

Table I. Proton and Carbon-13 Parameters for Pergillin[a,b]

carbon	^{1}H	^{13}C
1		159.9
2		118.6
3		131.0
4	6.83 (J = 8.0 Hz)	120.9
5	7.60 (J = 8.0 Hz)	123.1
6		120.5
7	4.90	56.8
8		93.8
9	2.92, 3.0	39.2
10	1.55	28.6
11		182.7
12		141.7
13		144.7
14	2.10	19.9
15	2.33	17.1
OH	5.72	

[a] In $CDCl_3$-Me_2SO-d_6 (1:1) solution. [b] In ppm downfield from internal Me_4Si.

to purple. The uncorrected melting point was 171–172 °C. UV spectra obtained from ethanol solutions showed λ_{max}^{EtOH} 225 (log ϵ = 3.98) and 287 nm (log ϵ = 4.03). The IR spectra gave the following values: 3360 (broad, OH), 2980, 2920, 2850 (all weak, CH_3, CH_2), 1670, 1645 (carbonyl), 1595 (phenyl), 1430 (CH_2), 1365 (CH_3), 1327, 1287, 1255 (ether), 1228, 1165, 1110, 1072 (ring ether), 1038, 938, 868, 830–820 (doublet, two adjacent hydrogens on aromatic ring), 780 cm^{-1}.

The LRP mass spectral analysis of I showed a molecular ion peak (M^+) at m/e 260.105, and HRP analysis with perfluorokerosene as the internal standard indicated that the M^+ was at m/e 260.1047 (calculated mass for $C_{15}H_{16}O_4$, 260.1048). Fragment ions were observed at m/e 242 ($M^+ - H_2O$) and 200 ($M^+ - C_2H_4O_2$). The latter fragment is derived from O7A, C8, the subtended C10, and O8A, plus associated protons (Figure 2).

Initial X-ray diffraction experiments with single crystals of I indicated that the cell constants were a = 12.856 (3) Å, b = 14.142 (2) Å, c = 14.190 (2) Å, and β = 93.70 (1)° with Z = 8 for a calculated density of 1.34 g/cm^3. Of the 3470 unique reflections measured with $2\theta \leq 114°$, 2634 (76%) were observed ($I \geq 3\sigma I$). The structure was solved with routine application of direct methods (Main et al., 1978) and Fourier techniques (Stewart et al., 1972). Isotropic temperature parameters for the hydrogens were assigned from the temperature factors of the atoms to which they were bonded. The function $\sum w(|F_o| - |F_c|)^2$ with $w = 1/(\sigma F_o)^2$ was minimized to give a final residual index of 0.050. Figure 2 is a computer drawing of one of the unique molecules of I in the unit cell (Johnson, 1970). Details for obtaining supplemental crystallographic data properties are given in the final paragraph. Two intermolecular hydrogen bonds were found in the crystal structure: one between O8A and O11A of 2.84 Å and the other between O8A′ and O11A′ of 2.91 Å.

The ^{1}H and ^{13}C NMR spectra obtained were consonant with the structure obtained by X-ray crystallography. Values for both sets of data are shown in Table I.

Bioassay Results. Wheat coleoptiles were significantly inhibited ($P < 0.01$) by solutions of pergillin at 10^{-3} and 10^{-4} M (Figure 3). While the expression of activity is not equal to that of the standard plant growth inhibitor (±)-abscisic acid, which inhibits coleoptile growth at concentrations as low as 10^{-6} M (Cutler et al., 1978), it is nevertheless activity that cannot be ignored and the metabolite should be assayed in other plant systems, including fungi, when enough pergillin can be isolated.

Pergillin was nontoxic to chicks at rates up to 250 mg/kg. Observations were made on treated chicks up to

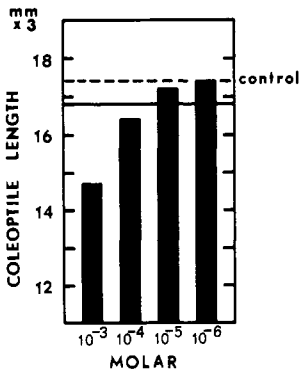

Figure 3. Inhibitory effect of pergillin on the growth of wheat coleoptiles (*T. aestivum* L., cv. Wakeland). Control: dotted line. Significant inhibition: below solid line ($P < 0.01$).

1 week after dosing the metabolite. Because of the nontoxic properties of pergillin, both pergillin and synthetic derivatives should be further evaluated as plant growth regulators.

Pergillin could be reasonably formed from the condensation of a pentaketide and one molecule of mevalonic acid. One observation of interest is that I has no optical activity even though it possesses one asymmetric carbon, C8. This hemiketal results from the union of the two ends of the pentaketide chain.

Supplementary Material Available: Fractional coordinates and temperature factors (Table Is), bond distances (Table IIs), and bond angles (Table IIIs) (5 pages). Ordering information is given on any current masthead page.

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