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Received for review August 4, 1980. Accepted December 4, 1980. These studies were supported by Grant No. B-122-NC from the North Carolina Water Resources Research Institute. Paper No. 6612 of the North Carolina Agricultural Research Service Journal Series. Presented at the Second Chemical Congress of the North American Continent, Las Vegas, NV, Aug 24-29, 1980, Division of Environmental Chemistry. This paper partially fulfills the requirements for the MSc. degree for H.S.

Indole Metabolites from a Strain of *Aspergillus flavus*

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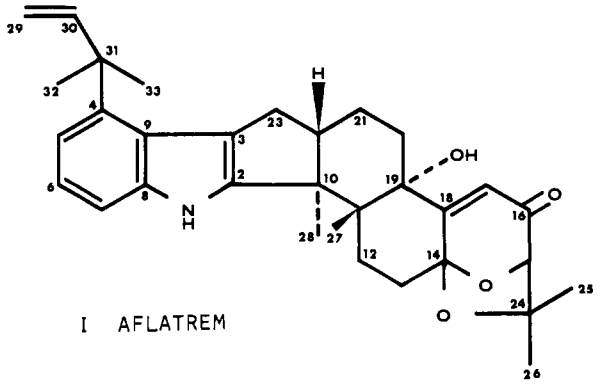
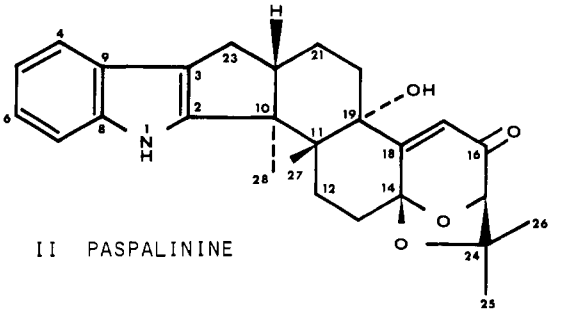
The X-ray structure of dihydroxyaflavinine, a new diterpene indole metabolite from *Aspergillus flavus*, is reported. The fungal tremorgens aflatrem and paspalinine were also isolated from the *A. flavus* isolate. Previously, paspalinine has only been reported from sclerotia of *Claviceps paspali*.

The tremorgenic indole metabolites of *Claviceps paspali* are closely related chemically to aflatrem (I), which is



I AFLATREM

produced by some strains of *Aspergillus flavus* (Cole et al., 1977; Gallagher et al., 1980a). In addition, aflatrem represents the most logical distal product in the biosynthesis of this group of fungal metabolites (Cole, 1980). Because some *A. flavus* isolates can produce this distal product, some of the *C. paspali* type indoles may be present in *A. flavus* cultures. These relationships prompted us to critically examine the indole metabolites of an aflatrem-producing strain of *A. flavus* to determine if the *C. paspali* type metabolites are also metabolites of *A. flavus*. We now report the isolation and identification of the *C. paspali* type tremorgen paspalinine (II) and a new



II PASPALININE

unrelated nontremorgenic indole diterpene metabolite, 20,26-dihydroxyaflavinine (III), from *A. flavus*.

MATERIALS AND METHODS

The *A. flavus* (NRRL 3251) isolate was obtained from the Northern Regional Research Center Culture Collection. The fungus was mass cultured in 2.8-L Fernback flasks,

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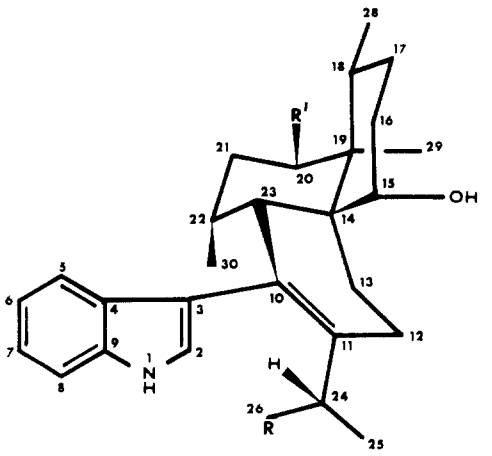

	R	R^1
III DIHYDROXYAFLAVININE	OH	OH
IV AFLAVININE	H	H

each containing 100 g of shredded wheat supplemented with 200 mL of Difco mycological broth (pH 4.8) plus YES medium (Davis et al., 1966). The cultures were extracted with hot chloroform after being grown at 26–29 °C for 2 weeks. The crude chloroform extract was chromatographed on a silica gel column (9.5 × 17 cm) eluted with 2 L each of benzene, ethyl ether, ethyl acetate, and acetone. The ethyl ether and ethyl acetate eluants were combined, evaporated to dryness, and chromatographed on a second silica gel column (3.5 × 40 cm) packed in benzene and eluted with 150 mL of benzene, followed by a linear gradient elution from benzene to ethyl ether (156 17-mL fractions collected). This elution was followed by another linear gradient elution from ethyl ether to ethyl acetate (152 17-mL fractions collected). Aflatrem (I) and another biologically active indole-type metabolite (II) appeared in fractions 47–58. A third indole metabolite (III) eluted in fractions 151–156 of the first gradient and continued in fractions 1–45 of the second gradient. After these fractions were combined and concentrated under vacuum, III crystallized at 5 °C. Fractions 47–58 were combined and chromatographed on a C_{18} reverse-phase column (3.5 × 15 cm) by using a linear elution gradient from 20:80 (v/v) acetonitrile–water to 80:20 (v/v) acetonitrile–water (240 17-mL fractions were collected). Analyses by thin-layer chromatography (TLC) showed that fractions 105–107 contained II, fractions 108–109 contained I and II, and fractions 110–111 contained only I.

The indole metabolites were analyzed by TLC with 5 × 10 cm glass plates coated with silica gel 60 F-254 (EM Laboratories, Inc.). The developing solvents were chloroform–acetone (93:7 v/v) and toluene–ethyl acetate–acetic acid (5:4:1 v/v/v). The indole metabolites were visualized on TLC plates by spraying the plates with 1% ethanolic (dimethylamino)benzaldehyde, followed by 50% ethanolic sulfuric acid, and then heating them at 125 °C for 1.0 min. The color intensity of spots continued to develop after the plates were removed from the oven.

Melting points were determined on a Kofler micro-melting-point apparatus and were uncorrected. Ultraviolet spectra (UV) of the metabolites were taken with a Beckman Model DB-G recording spectrophotometer in methanol solution (1.0×10^{-5} M). Infrared spectra (IR) were taken with a Perkin-Elmer Model 257 IR spectrophotometer equipped with a 4X beam condenser. Samples were analyzed as a thin film coated onto KBr windows.

Proton and ^{13}C NMR spectra were obtained on a Varian Associates XL-100-12 NMR spectrometer equipped with the 620-L disk data system. Single-frequency, off-resonance proton decoupled (sford) ^{13}C NMR spectra were run to aid in the identification of the different types of carbons. Spectra were run in 5-mm sample tubes with a $CDCl_3$–Me_2SO (1:1) solution containing tetramethylsilane (Me_4Si) as an internal reference. Chemical shifts are reported downfield from Me_4SI.

Low-resolution mass spectra were obtained on a Finnigan 3200 spectrometer, and high-resolution spectra were obtained on a VG-Micromass ZAB-2F spectrometer. Samples were introduced into the ion source by direct probe, and ionization was effected by electron impact at 70 eV.

Crystals of III suitable for X-ray diffraction experiments formed from methanol with symmetry $P2_1$ with $a = 9.448$ (2) Å, $b = 10.446$ (2) Å, $c = 12.655$ (2) Å, and $\beta = 96.68$ (1)°. With a formula of $C_{28}H_{39}NO_3$ and $Z = 2$, the calculated density was 1.17 g/cm^3. An automatic four-circle diffractometer with Cu Kα radiation was used to collect 1783 unique pieces of data with $2\theta \leq 114°$. Of these, 1730 (97%) were observed ($I \geq 3\sigma I$) and corrected for polarization and Lorentz effects. A standard multisolution tangent formula approach was used to find initial positions for 30 of the 32 nonhydrogen atoms (Main et al., 1978). Least-squares refinements and difference Fourier calculations revealed the remainder of the atoms. The final crystallographic R factor of 0.039 was calculated after full-matrix, least-squares refinements minimized the function $\sum[\omega(|Fo| - |Fc|)^2]$, with $\omega = 1/(\sigma Fo)^2$ (Stewart et al., 1972).

Samples were tested for tremorgenic activity in 1-day-old chickens dosed orally via crop intubation. Samples were prepared as previously described (Kirksey and Cole, 1974). Purified samples of metabolites were dosed at levels up to 250 mg/kg.

RESULTS AND DISCUSSION

Metabolite I was conclusively identified as aflatrem by direct comparison of TLC, UV, IR, ^{13}C NMR, ^1H NMR, and mass spectra with spectra of standard aflatrem. Metabolite II was likewise identified as paspalinine (Gallagher et al., 1980b). Paspalinine, a tremorgenic metabolite previously isolated only from *C. paspali*, has been implicated as one of three tremogenic metabolites responsible for the clinical signs of "paspalum staggers," the neurological disease of cattle ingesting *Paspalum* spp. grasses infected with *C. paspali* (Cole et al., 1977). Although staggers syndromes are primarily associated with forages (e.g., Bermuda grass tremors, ryegrass staggers, and paspalum staggers), a clinically indistinguishable neurological condition was observed on several different occasions in cattle that had fed on lodged corn (Cole, 1977). No particular substrate other than corn was consistently associated with the disease. Because *A. flavus* can be a significant contaminant of corn, especially in the southeastern United States, the roles of aflatrem, paspalinine, and related tremorgens as possible etiological agents of neurological diseases associated with the ingestion of corn need critical evaluation.

Metabolite III was obtained as colorless platelets, mp 254–256 °C, from ethyl acetate. Purified III was a single reddish purple spot on TLC at R_f 0.44 (toluene–ethyl acetate–formic acid, 5:4:1 v/v/v). The UV spectrum of III, λ_{max} (MeOH) 225 nm (31 200), 282–284 (7400), and 290 (6000), was typical of a 3-substituted indole chromophore. The IR spectrum showed absorptions indicating the presence of OH and indole groups (3250–3450 and 1035 cm^{-1}), *gem*-dimethyl groups (doublet 1370–1380 cm^{-1}), and four adjacent aromatic hydrogens (745 cm^{-1}).

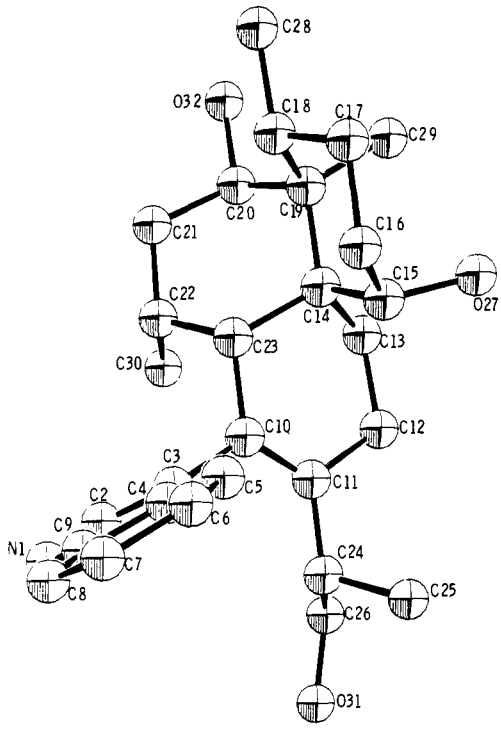

Figure 1. A computer-generated perspective drawing of dihydroxyaflavinine.

The high-resolution mass spectrum of III gave a molecular ion peak at m/e 437.29096 amu corresponding to a molecular formula of $C_{28}H_{39}NO_3$ (calcd 437.29297 amu). In addition to the molecular ion peak in the low-resolution mass spectrum of III, peaks were observed at 419 (M - H_2O), 401 (M - $2H_2O$), 338, and 130. The 100-MHz ^1H NMR spectrum of III showed three doublets (J = 7 Hz) at 0.83, 0.97, and 1.15 ppm and a singlet at 1.22 ppm for the four methyl groups. In addition to multiplets at 1.2–2.7 and 3.2–4.0 ppm, other peaks were observed at 4.3 ppm (broad singlet), 6.7–7.2 ppm (indole ring protons) and 8.9 ppm (N–H).

The ^{13}C NMR spectrum of III showed 10 peaks for the sp^2 carbons at 137.0 (s), 135.8 (s), 127.8 (s), 126.9 (s), 122.1 (d), 120.6 (d), 118.8 (d), 118.3 (d), 116.5 (s), and 111.1 (d) ppm. The hydroxy-bearing carbons appear at 70.5 (d), 68.9 (d), and 65.7 (t) ppm. The remainder of the aliphatic carbons have absorptions at 44.2 (s), 43.1 (s), 38.9, 35.1 (t), 31.1 (d), 30.3 (t), 29.6 (d), 27.3 (t), 21.8 (t), 21.5 (t), 19.4 (q), 19.2 (q), 15.4 (q), and 13.1 (q) ppm.

Single crystal X-ray diffraction experiments determined that III was closely related to aflavinine (IV), a recently reported diterpene indole from *A. flavus* (Gallagher et al., 1980c). Metabolite II is aflavinine with two additional OH groups located on carbons 20 and 26 (III). Therefore, we propose the trivial name dihydroxyaflavinine for III.

The stereochemistry of III is identical in all corresponding carbon atoms with that of IV (Gallagher et al., 1980c). However, the presence of two more hydroxyl groups generates two additional asymmetric centers: 20 (R^*) and 24 (S^*). The conformation of III is virtually identical with that of IV with the two cyclohexane rings in chair conformations, whereas the cyclohexene ring has a distorted half-chair conformation with C13 and C14, 0.17 and −0.58 Å from the least-squares plane formed from the remaining four atoms. The π orbitals of the indole ring are virtually uncoupled to the double bond since the torsional angle C2–C3–C10–C11 is −88°. Three intermolecular hydrogen bonds exist in the crystal lattice: N1–H1···O27, 2.81 Å; O27–H27···O31, 2.71 Å; O31–H31···O32, 2.76 Å. All other close intermolecular distances are van der Waals contacts. Tables I–III (see paragraph at end of paper regarding supplementary material) contain the final fractional coordinates, temperature parameters, bond distances, and bond angles for III, whereas Figure 1 is a perspective drawing (Johnson, 1970) in which the hydrogens have been omitted and no absolute stereochemistry is implied.

Aflatrem (I) and paspalinine (II) were tremorgenic to 1-day-old chickens at levels down to 25 mg/kg (ED_{50} < 25 mg/kg), while dihydroxyaflavinine (III) showed no tremorgenic activity at levels up to 300 mg/kg.

Supplementary Material Available: Tables I, II, and III containing fractional coordinates, temperatures parameters, bond distances, and bond angles for III (4 pages). Ordering information is given on any current masthead page.

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