4-Hydroxymellein: A New Metabolite of *Aspergillus ochraceus*

Richard J. Cole,* Jack H. Moore,[1] Norman D. Davis,[1] James W. Kirksey, and Urban L. Diener[1]

A new metabolite from an ochratoxin-producing isolate of *Aspergillus ochraceus* has been isolated and identified as 4-hydroxymellein. The chemical similarity of this compound to the dihydroisocoumarin moiety of the ochratoxins implicates it as a possible biosynthetic precursor of the ochratoxins.

Strains of *Aspergillus ochraceus* have been isolated from soils, decaying vegetation, and moldy grains throughout the world (Raper and Fennell, 1965); their metabolites include the mycotoxin, ochratoxin A (van der Merwe *et al.*, 1965), produced by *A. ochraceus*, *A. sulphureus*, *A. melleus*, and also by *Penicillium viridicatum* (Lai *et al.*, 1970; van Walbeek *et al.*, 1969). Mellein, a metabolite of *A. melleus* Yukawa, was isolated by Nishikawa (1933) in 1933. The same year, Yabuta and Sumiki (1933) isolated ochracin from cultures of *A. ochraceus* Wilh. and found it to be identical with mellein.

This paper reports the identification of two metabolites, AO-1 and AO-2, isolated by one of the authors (JHM) during research on the production of ochratoxin A (Davis *et al.*, 1969) by *A. ochraceus* Wilh.

MATERIALS AND METHODS

A. ochraceus Wilh., NRRL 3174, was maintained at 28° C on Czapek's agar slants with 20% sucrose and 0.7% Difco yeast extract (Davis *et al.*, 1969). Nutrient medium was that of Ferreira (1967) modified to the following composition (g/l.): sucrose, 30; L-glutamic acid, 3; KH_2PO_4, 1; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.024; $ZnSO_4 \cdot 7H_2O$, 0.001; $MnSO_4 \cdot H_2O$, 0.011; $CuSO_4$, 0.004; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0025. Medium (pH 5.2) was inoculated with spores of *A. ochraceus* and incubated 7 days at 25° C as stationary cultures. Cultures were filtered and the medium extracted twice with equal volumes of chloroform. The chloroform was evaporated and the crude extract chromatographed on a silica gel column (0.05–0.2 mm mesh) (70 × 2 cm o.d.) with chloroform–*n*-hexane (90:10, v/v) as the eluting solvent. Fractions were monitored by thin-layer chromatography (tlc) with chloroform–acetone (93:7, v/v). Separate column fractions were combined, evaporated to dryness under vacuum, and residues crystallized from chloroform–*n*-hexane to yield colorless crystals (AO-1, mp 126–130° C; AO-2, mp 37–42° C). Tlc revealed a yellow fluorescent contaminant in AO-1. AO-1 was separated from this contaminant by preparative tlc (chloroform–acetone, 93:7, v/v). AO-1 recovered following tlc was chromatographed on a liquid–liquid column with 20% MEOH in benzene as the stationary phase coating 0.8 mm or less silica gel. Elution was with 2% MEOH in benzene. AO-1 fractions were combined and recrystallized from chloroform–*n*-hexane to yield colorless crystals (m.p. 131–132° C). Tlc of AO-2 showed a nonpolar impurity was present. Sublimation of AO-2 yielded colorless crystals (mp 54–55° C).

Infrared spectra (ir) were taken with a Perkin-Elmer 257 ir spectrometer equipped with a 6× beam condenser. Samples were coated onto KBr blocks as a thin flim. Ultraviolet spectra (uv) of AO-1 and AO-2 were taken with a Beckman Model DB-G recording spectrometer in methanol solution. High-resolution and low-resolution mass spectral analyses were made with an A.E.I. MS-9 spectrometer by the Department of Chemistry, Florida State University, Tallahassee, Fla. Samples were introduced by the direct probe method and ionization was effected by electron impact at 70 eV. Nuclear magnetic resonance (nmr) spectra of samples dissolved in $CDCl_3$ were obtained with a Jeolco PS-100 nmr spectrometer. Melting points were taken with a Kofler micro-melting point apparatus and were corrected. Tlc was on glass plates (20 × 20 cm or 20 × 10 cm) coated with 0.25-mm (analytical) or 0.5–1.0-mm (preparative) layers of silica gel GH-R; developing solvents were chloroform–acetone (93:7, v/v) and toluene–ethyl acetate–formic acid (5:4:1, v/v). Detection of AO-1 and AO-2 on tlc plates was by uv fluorescence. The diacetate derivative of AO-1 was prepared by treatment with acetic anhydride and pyridine at 70° C for 1.5 hr under nitrogen.

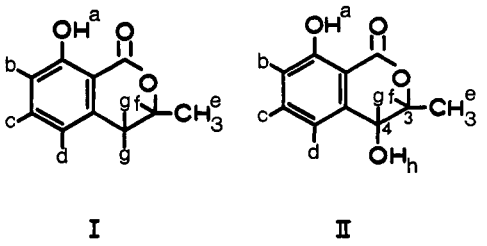
RESULTS AND DISCUSSION

On the basis of uv, ir, mp, tlc, nmr, and mass spectroscopy, AO-2 was identified as mellein (I) (Figure 1). Mellein has been reported to be produced by *A. ochraceus* (Nishikawa, 1933; Yabuta and Sumiki, 1933), so specific reference to data on mellein will be made only as it relates to the subsequent identification of AO-1. AO-1 had an R_f on tlc of 0.38 (chloroform–acetone) and 0.74 (toluene–ethyl acetate–formic acid). Uv analysis of AO-1 showed λ_{max}^{MEOH} 247 and 315 nm (ϵ_{max} 5300 and 4200). This uv spectrum is similar to the spectra of compounds with a dihydroisocoumarin-type structure such as dihydro-oospolactone, λ_{max} 245 and 310 (Yamamoto *et al.*, 1962); and 8-hydroxy-3-methyl-3,4-dihydroisocoumarin (mellein), λ_{max} 246 and 314 nm (Blair and Newbold, 1955).

The ir spectrum of AO-1 showed carbonyl absorption at

National Peanut Research Laboratory, United States Department of Agriculture, Dawson, Georgia 31742

[1] Department of Botany and Microbiology, Auburn University Agricultural Experiment Station, Auburn, Alabama 36830



I II

Figure 1. Structural formulas of mellein (I) and 4-hydroxymellein (II)

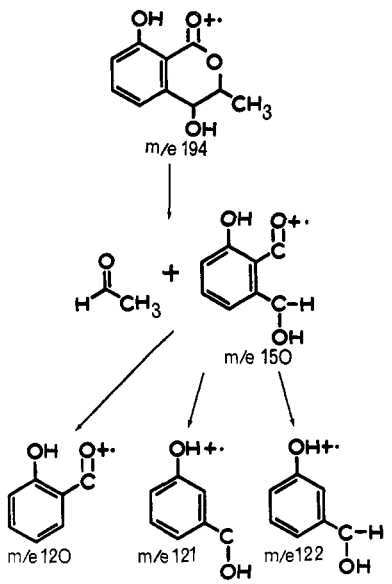


Figure 2. Mass spectrometric fragmentation of 4-hydroxymellein

1670 cm^{-1} similar to that of mellein (1675 cm^{-1}). The carbonyl at 1670 cm^{-1} and OH absorption at 3200 cm^{-1} indicated hydrogen bonding between the 8-hydroxyl group and the carbonyl in the ortho position. OH absorption at 3415 cm^{-1} indicated two hydroxyl groups (chelated and nonchelated). Ir analysis of the diacetate derivative demonstrated that the 1670 cm^{-1} had shifted to a higher wave number and was associated with 1735 cm^{-1} ester absorption. This phenomenon occurred also with mellein, oospolactone (Yamamoto *et al.*, 1961), and oosponol (Yamamoto *et al.*, 1962). Further evidence for a hydroxyl group chelated with an ortho carbonyl was provided by nmr spectra of mellein and AO-1. Both spectra contained a singlet at δ 11.03 due to a D_2O exchangeable proton Ha (I and II) (Figure 1). This extreme paramagnetic shift is characteristic of H-bonded hydroxyl protons. These data suggest that AO-1 has the 8-hydroxy,dihydroisocoumarin structure.

Low-resolution (LRP) and high-resolution (HRP) mass spectral analyses demonstrated a molecular ion peak at *m/e* 194 and an empirical formula of $C_{10}H_{10}O_4$. The basic 8-hydroxy,dihydroisocoumarin structure accounted for nine carbon and three oxygen atoms and data indicated the remaining oxygen was present as an OH and the remaining carbon was probably present as CH_3. Positions of CH_3, OH, and hydrogen atoms were located by comparing the nmr spectra of AO-1 and mellein in conjunction with D_2O exchange and spin–spin decoupling and HRP mass spectral peak matching. Nmr spectrum of mellein showed that two methylene protons (Hg) were coupled with a methine proton (Hf) and appeared as a doublet, δ 2.83, J_{gf} = 3 Hz (I) (Figure 1). The methine proton (Hf) showed a complex signal (δ 4.5, J = 3 Hz) that looked like a sextet and was coupled with Hg and methyl group (He). The hydroxyl proton (Ha) (D_2O exchangeable) on mellein appeared as a singlet at δ 11.03 (shifted downfield due to H-bonding). The three aromatic protons (Hb, Hc, and Hd) appeared as two single proton doublets at δ 6.46, J_{cb} = 4 Hz and δ 6.60, J_{cd} = 4 Hz, coupled with a 1-proton triplet (δ 7.22, J = 4 Hz) (I).

The nmr spectrum of AO-1 showed two single aromatic proton doublets Hb and Hd (II) (Figure 1) (δ 6.82, J = 4 Hz and δ 6.85, J = 4 Hz) coupled with a single proton triplet Hc (II) (δ 7.42, J = 4 Hz). This was similar to the chemical shifts of the aromatic protons of mellein and demonstrated that the CH_3 group and the OH group were not substituted on the aromatic ring. Thus, they could be attached to either carbon-3 or carbon-4 (II). The nmr spectrum contained a D_2O exchangeable proton Ha (II) at δ 11.03 (same as 8-hydroxyl group of mellein) and an additional hydroxyl proton Hh (II) positioned at δ 2.61 initially as a doublet (J = 3 Hz), but shifted to δ 2.45 as a broad singlet upon standing in deuterated chloroform. The coupling between the secondary OH (Hh) was apparently observable initially and subsequently disappeared as a result of complete exchange.

The 2-proton doublet Hg (δ 2.83) (I) (Figure 1) in the nmr spectrum of mellein was not present in the spectrum of AO-1. A 3-proton doublet similar to that of mellein (He) (I) was present at δ 1.22, J = 3 Hz. A complex signal observed at approximately δ 4.4 integrated at two protons and was interpreted from spin–spin decoupling to result from two single proton signals superimposed (Hf and Hg) (II). This signal appeared to be a 1-proton doublet (Hg) (δ 4.45, J = 2 Hz) coupled with a single proton (Hf) (δ 4.40). The signal of the proton (Hf) appeared as a quintet with J = 2.5 Hz. This was supported by decoupling experiments.

Nmr data demonstrated that the CH_3 group and OH group were on different carbon atoms, since the methyl splittings were unaffected by D_2O exchange. Therefore, the CH_3 group could be located on either carbon-3 (same as mellein) or carbon-4 and the OH group must be adjacent to the CH_3. LRP mass spectrum showed a fragment ion at nominal mass *m/e* 150 (base peak) resulting from a loss of *m/e* 44 (supported by requisite metastable ion at *m/e* 116). A fragment ion at nominal mass *m/e* 150 could result from loss of CO_2 or CH_3CHO from the molecular ion. Calculated mass of *m/e* 150 from loss of CO_2 is 150.068075, while a loss of CH_3CHO would produce a fragment ion at calculated mass 150.031690. HRP mass spectral peak matching of the fragment ion at nominal mass *m/e* 150 using the reference peak *m/e* 149.9904 of perfluorokerosene resulted in a value of 150.031497. This demonstrated that the *m/e* 150 fragment ion resulted from loss of CH_3CHO and not from loss of CO_2 (Figure 2).

This pattern is consistent with that expected of a compound containing a CH_3 group attached to a carbon atom bonded directly to an oxygen atom. Rix and Webster (1968) reported electron impact elimination of acetaldehyde from a group of 3-methyl-3,4-dihydroisocoumarin compounds including 6,8 - dihydroxy - 3 - methyl - 3,4 - dihydroisocoumarin. Origin of the acetaldehyde was from elimination of carbon-3 with attached oxygen and CH_3 group as a result of a retro-Diels–Alder reaction. These compounds also lost CO from the molecular ion minus *m/e* 44 fragment ion. AO-1 lost CO (*m/e* 122), COH (*m/e* 121), and COH_2 (*m/e* 120) from the molecular ion minus *m/e* 44 fragment ion (*m/e* 150), which is consistent with the expected fragmentation of carbon-1 (CO) and carbon-4 (COH and COH_2) (Figure 2). Mellein

exhibited a molecular ion minus *m/e* 44 fragment ion and subsequent loss of CO from this ion.

We propose that AO-1 is 3-methyl-4,8-dihydroxy-3,4-dihydroisocoumarin (II) (Figure 1). The chemical similarity of this compound and mellein to the dihydroisocoumarin moiety of the ochratoxins (van der Merwe *et al.*, 1965) implicates them as possible biosynthetic precursors of the ochratoxins. NOTE: During the preparation of this manuscript, a recent publication (Masaoki *et al.*, 1970) reported the isolation of 3-methyl-4,8-dihydroxy-3,4-dihydroisocoumarin from *Aspergillus oniki*.

LITERATURE CITED

Blair, J., Newbold, G. T., *J. Chem. Soc.* 2871 (1955).
Davis, N. D., Searcy, J. W., Diener, U. L., *Appl. Microbiol.* **17,** 742 (1969).
Ferreira, N. P., "Biochemistry of Some Foodborne Microbiol Toxins," R. I. Mateles and G. N. Wogan, Eds., M.I.T. Press, Cambridge, 1967, p 157.
Lai, M., Semeniuk, G., Hesseltine, C. W., *Appl. Microbiol.* **19, 542** (1970).
Masaoki, S., Kaneko, Y., Oshita, K., Takamatsu, H., Asao, Y., Yokatsuka, T., *Agr. Biol. Chem.* **34,** 1296 (1970).
Nishikawa, E., *J. Agr. Chem. Soc. Japan* **9,** 772 (1933).
Raper, K. B., Fennell, D. I., "The Genus Aspergillus," Williams & Wilkins, Baltimore, 1965, p 288.
Rix, M. J., Webster, B. R., *J. Chem. Soc.* **B(3),** 254 (1968).
van der Merwe, K. J., Steyn, P. S., Fourie, L., Scott, de B., Theron, J. J., *Nature (London)* **208,** 1112 (1965).
van Walbeek, M., Scott, P. M., Harwig, J., Lawrence, J. W., *Can. J. Microbiol.* **19,** 542 (1969).
Yabuta, T., Sumiki, Y., *J. Agr. Chem. Soc. Japan* **9,** 1264 (1933).
Yamamoto, I., Nitta, K., Yamamoto, Y., *Agr. Biol. Chem.* **25,** 405 (1961).
Yamamoto, I., Nitta, K., Yamamoto, Y., *Agr. Biol. Chem.* **26,** 486 (1962).

Received for review February 10, 1971. Accepted March 31, 1971. Supported in part by PHS Research Grant FD-00081 from the Food and Drug Administration.