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3′-Hydroxyzearalenones, Two New Metabolites Produced by *Fusarium roseum*

Sadanand V. Pathre, Stuart W. Fenton, and Chester J. Mirocha*

Two new metabolites related to zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone] were isolated from the rice cultures of *Fusarium roseum* and were shown to be diastereomeric 3′-hydroxyzearalenones [6-(3,10-dihydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone]. The accumulation of 3′-hydroxyzearalenones as the culture ages suggests that these hydroxy derivatives are the product of zearalenone metabolism by *F. roseum*.

Fusarium roseum under certain conditions of moisture and temperature infects maize and produces a mycotoxin called zearalenone (Urry et al., 1966; Stob et al., 1962) [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone]. When such corn is fed to swine, it causes the estrogenic syndrome which involves primarily the genital system (Christensen et al., 1965; Mirocha et al., 1971; Mirocha and Christensen, 1974).

During the search on new metabolites related to zearalenone, Mirocha et al. (1974) reported the isolation of two derivatives, namely, F-5-3 and F-5-4, which were thought to be 3′-hydroxyzearalenones. However, Jackson et al. (1974) revised the structures of F-5-3 and F-5-4 as diastereomeric 8′-hydroxyzearalenones (Bolliger and Tamm, 1972). Further, Mirocha et al. (1971) reported that a significant estrogenic activity was found in a number of components of *Fusarium* culture extracts. Some of those components were more polar than 8′-hydroxyzearalenones. Steele et al. (1976) reported the isolation of 6′, 8′-dihydroxyzearalene, presumably produced through the metabolism of zearalenone by *Fusarium*. We isolated two minor metabolites; their isolation and characterization are the subject of this paper.

RESULTS AND DISCUSSION

The extensive chromatographic separation of extracts of 2.5 kg of rice cultures of *Fusarium roseum* yielded 30 mg of crystalline F-5-3, (8′*R*,10′*S*)-8′-hydroxyzearalenone (II) and 40 mg of F-5-4, (8′*S*,10′*S*)-8′-hydroxyzearalenone (III). The UV, IR, and mass spectra of these compounds were identical with those of the authentic samples isolated by Jackson et al. (1974).

Chromatographic fractions containing a fluorescent component more polar than F-5-3 were pooled and were purified by preparative thin-layer chromatography on silica gel repeatedly developed in chloroform-absolute ethanol (97:3, v/v). The successive chromatographic separation by preparative thin-layer chromatography yielded 6 mg of a pure compound. The ^1H and ^{13}C NMR spectra in acetone-d_6 indicated that the unknown fluorescent compound was related to zearalenone. The compound appeared to be homogeneous (single component) on TLC when developed in several different solvent systems. However, it was resolved into two bands at R_f 0.33 and 0.28 when developed in chloroform–absolute ethanol (95:5, v/v). Both components yielded mass spectra identical with respect to fragmentation pattern. The component at R_f 0.28 was designated as F-5-1 and that at R_f 0.33 as F-5-2. Amounts of F-5-1 and F-5-2 after separation were <0.4 and <2.0 mg, respectively.

The high-resolution mass spectra established the elemental composition of $C_{18}H_{22}O_6$ and molecular weight as 334 for both compounds compared to $C_{18}H_{22}O_5$ and 318 for zearalenone.

The trimethylsilyl ethers of F-5-1 and F-5-2 were inseparable by gas–liquid chromatography on 3% OV-1, OV-17, or QF-1 columns. However, they gave identical mass spectra with molecular ion at m/e^+ 550 compared to a molecular ion at m/e^+ 462 for zearalenone and at m/e^+

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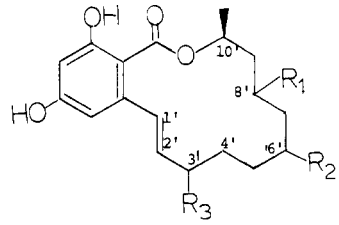


	R_1	R_2	R_3
Zearalenone	H	=O	H
8'-Hydroxyzearalenone	OH	=O	H
6',8'-Dihydroxyzearalene	OH	OH	H
3'-Hydroxyzearalenone	H	=O	OH

Figure 1. Structures of zearalenones described in the text.

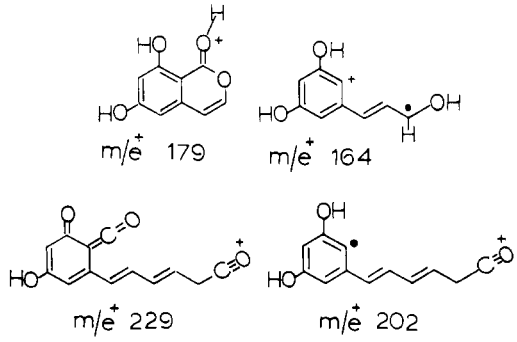


Figure 2. Fragments observed in the high-resolution mass spectra of 3'-hydroxyzearalenones.

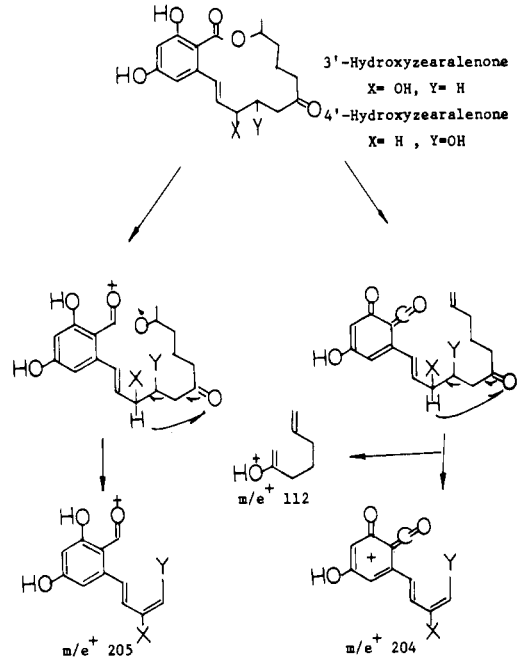
550 for F-5-3 and F-5-4. The ultraviolet spectra of F-5-1 and F-5-2 (λ_{max} 236, 274, 316 nm) were identical with those of zearalenone, F-5-3, and F-5-4 with respect to the position of absorption maxima. Further, the high-resolution mass spectra of F-5-2 and F-5-1 indicated a significant loss of H_2O from the molecular ion, M^+ = 334. These observations are consistent with hydroxyzearalenones.

Mass spectra of F-5-1 and F-5-2 at 70 eV were compared with those of zearalenone, F-5-3, and F-5-4. Mass spectra of F-5-1 and F-5-2 showed a major fragment at m/e^+ 316 due to the loss of H_2O from the molecular ion at m/e^+ 334. The other fragments observed at m/e^+ 219, 205, 204, 202, 201, 112, and 97 are important in the mass spectra of F-5-1 and F-5-2 but relatively unimportant in those of F-5-3 and F-5-4. The formation of these fragments, as depicted in Schemes I and II, can be explained by assuming that F-5-1 and F-5-2 are diastereomers of either 3'-hydroxyzearalenone or 4'-hydroxyzearalenone (see Figure 1 for structures).

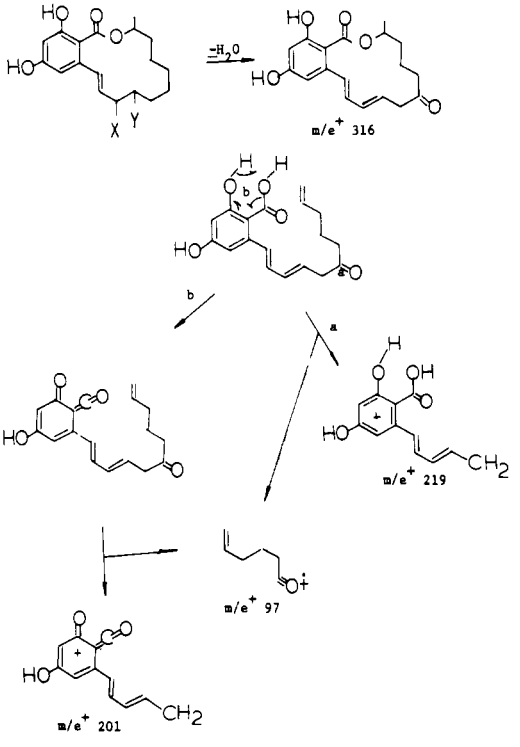
However, the significant fragments observed at m/e^+ 179 and 164 (Figure 2) are highly indicative of 3'-hydroxy substitution since the fragment at m/e^+ 164 would not have formed from 4'-hydroxyzearalenone. Fragments m/e^+ 112 and 97 substantiate the presence of carbonyl at C-6' (Jackson et al., 1974). Further, the fragments m/e^+ 229 and 202 provided additional evidence for the 6'-ketone function. The high-resolution mass spectroscopy of F-5-2 and F-5-1 was in agreement with the assignment of peaks of these fragments.

The ^{1}H-NMR spectrum of F-5-2 (Figure 3a) showed the typical features of zearalenone system (Bolliger and Tamm, 1972; Pathre and Mirocha, 1976). The aromatic protons at C-3 and C-5 of the macrolide constituting an AB system (J_{AB} = 2.0–2.5 Hz) were observed at 6.45 and 6.6 ppm, respectively. The doublet at 1.38 ppm (3 H, J = 16 Hz) was due to the methyl protons at C-11'. A doublet of doublets centered at about 5.7 ppm (1 H) was assigned to the proton at C-2' which was found to be strongly coupled to the protons at C-1' and C-3'. Irradiation of the doublet

Scheme I



Scheme II



of doublets at 5.7 ppm resulted in collapse of the doublet at 7.22 ppm and the multiplet (1 H) at 4.25 ppm to a singlet and double doublets (J = 10.8, 5 Hz), respectively (Figure 3b). Decoupling the multiplet at 4.25 resulted in the collapse of C-2' resonances to a clean doublet with J = 16 Hz (Figure 3c). The multiplet at 4.25 ppm therefore was assigned to the proton at C-3' bearing an hydroxyl group. The decoupling of the 3'-proton caused a relatively minor change in the broad resonance near 1.5 ppm. This broad resonance was assigned to methylene protons at C-4' and C-9' since the multiplets at 4.95 (1 H, C-10') and 4.3 ppm were reduced to a quartet (J = 6 Hz) and a broad doublet (J = 8.8 Hz), respectively, when the protons at 1.5 ppm were irradiated (Figure 3e); these methylene protons

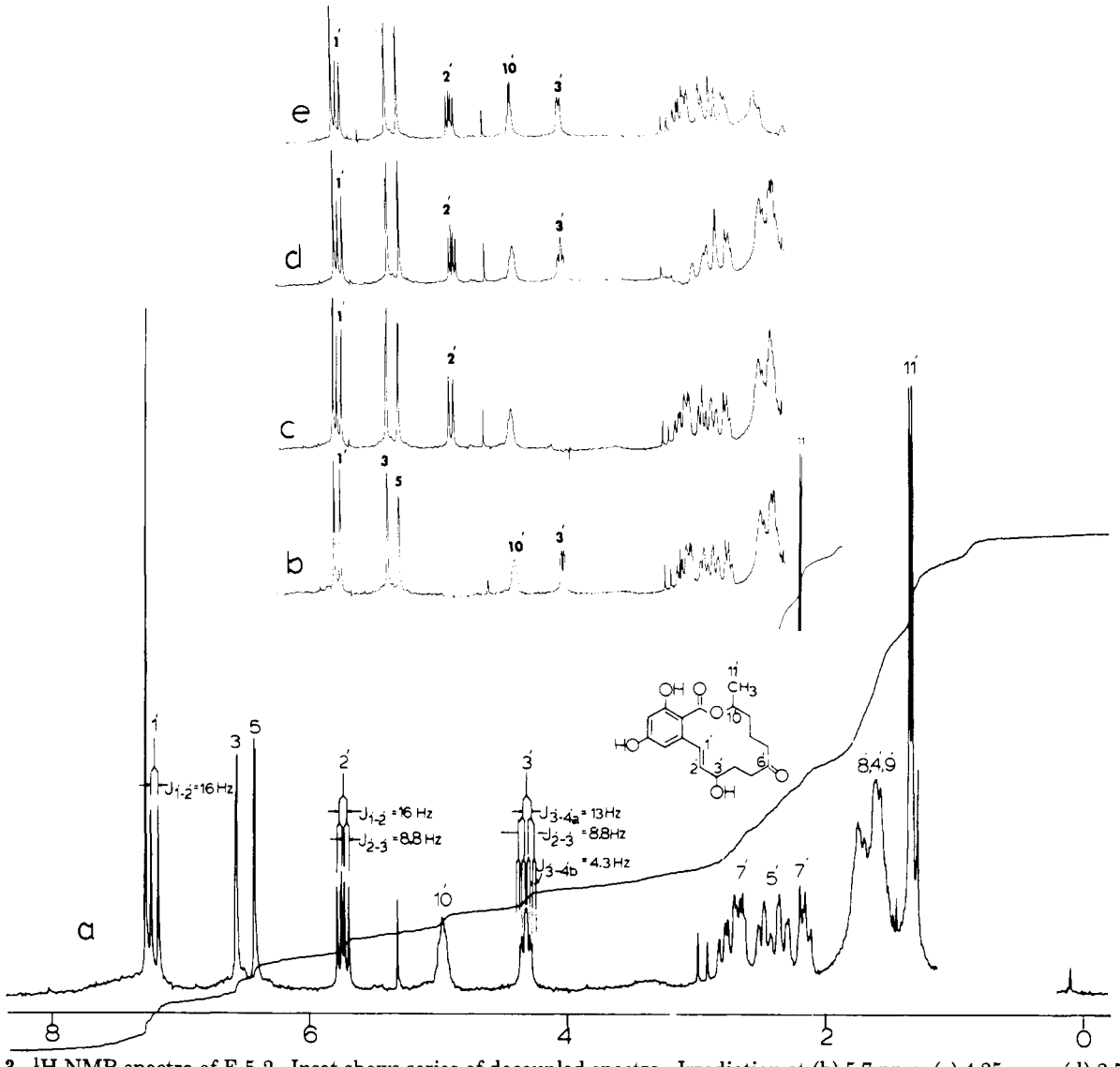


Figure 3. ^1H NMR spectra of F-5-2. Inset shows series of decoupled spectra. Irradiation at (b) 5.7 ppm, (c) 4.25 ppm, (d) 2.5 ppm, and (e) 1.5 ppm. The spectra were recorded on Bruker HFX-270 Superconducting NMR spectrometer and were run in deuteriochloroform ($CDCl_3$).

were also coupled to the protons observed in the 2–3-ppm region. The complex resonance pattern in this region (2–3 ppm) is attributed to methylene protons adjacent to the carbonyl at C-6′. Further irradiation at 2.5 ppm produced a change in the multiplet of the 3′-proton (Figure 3d). This change, due to removal of long range couplings, was found to be dependent on the position and the power of irradiation frequency. These observations are consistent with the structure of F-5-2 as 3′-hydroxyzearalenone.

Examination of the rice cultures at various times indicated that 3′-hydroxyzearalenone could not be detected during the period when the concentration of zearalenone was increasing rapidly. However, it was detected only after the rate of production of zearalenone in cultures slowed down, i.e., after 20 days. The concentration of 3′-hydroxyzearalenones steadily increased during 20–30 days. It appears that 3′-hydroxyzearalenones are the product of zearalenone metabolism by *Fusarium* and might have gone undetected during the study of zearalenone metabolism by Steele et al. (1976). Steele et al. (1976) noted that the amounts of 8′-hydroxyzearalenones formed by *F. roseum* were dependent upon the amount of zearalenone remaining in the cultures. With sufficient statistical evidence, they argued that zearalenone gives rise to 8′-hydroxyzearalenones which in turn serves as a precursor of 6′,8′-dihydroxyzearalene. Steele et al. (1976) found that the correlation of 8′-hydroxyzearalene with zearalenone was 0.90, whereas that between 8′-hydroxyzearalenones and 6′,8′-hydroxyzearalenones was 0.72. Although the 8′-hydroxyzearalenone was the immediate precursor of 6′,8′-hydroxyzearalene, the correlation was low compared to those indicating the most immediate precursor-product relationship. Steele et al. (1976) obtained their analytical data by gas chromatography/mass spectroscopy utilizing a column packed with 3% OV-1 on Gas-Chrom Q. We found that 8′-hydroxyzearalenones and 3′-hydroxyzearalenones could not be separated on OV-1 column, and it is possible that the total concentration of 8′-hydroxyzearalenone measured by Steele et al. (1976) was the sum of concentrations of 8′-hydroxyzearalenone and 3′-hydroxyzearalenone, which might have contributed to the lowering of the correlation coefficient between the 8′-hydroxy and 6′,8′-hydroxy derivative.

EXPERIMENTAL SECTION

Production and Isolation of F-5-3, F-5-4, F-5-2 and F-5-1. An isolate of *Fusarium roseum* was grown on dehulled Uncle Ben's parboiled rice, which was autoclaved

with 60% (fresh weight basis) water. The seeded rice culture medium was kept for 7 days at 25 °C and 30 days at 12 °C. At the end of this period, the cultures were dried and ground to a fine mesh.

The dry culture (2.5 kg) was moistened with 30% water (20 mL of water/100 g of dry cultures); about 3 L of ethyl acetate was added to this moistened culture and kept at 5 °C for 24 h and filtered. The residue was once more extracted with 2 L of fresh ethyl acetate. The combined ethyl acetate was concentrated to a gum, which was redissolved in 600 mL of acetonitrile and partitioned against 600 mL of petroleum ether (bp 60–70 °C); the petroleum ether was discarded. The acetonitrile layer was concentrated and chromatographed on 800 g of silica gel (Davison 923, 100 mesh, activated at least 1 h at 110 °C, slurry packed in dichloromethane, eluting solvent: 20% ethyl acetate in dichloromethane → 100% ethyl acetate). The fractions were collected in 300-mL portions and were examined for zearalenone and its derivatives by TLC (Jackson et al., 1976). The fractions eluted with 2 L of 20% ethyl acetate contained mainly zearalenone. The fractions eluted with 3 L of ethyl acetate were enriched in F-5-4 and F-5-3 and those eluted with 3 L of 100% ethyl acetate had highly polar fluorescent components besides F-5-4 and F-5-3.

The fraction enriched in F-5-4 and F-5-3 was chromatographed on five preparative TLC plates developed three times in chloroform–absolute ethanol (97:3). The bands corresponding to F-5-4 and F-5-3 were removed and eluted with acetone; these components were successively rechromatographed on TLC to give 40 mg of F-5-4 (mp 168–168.5 °C) and 30 mg F-5-3 (mp 201–202 °C). Both compounds were identical with the authentic samples as determined by TLC, GC/MS, UV, and mass spectra.

The fraction containing components more polar than F-5-3 were chromatographed on four preparative TLC plates and were developed four times in chloroform–absolute ethanol (97:3). A fluorescent band which appeared just below F-5-3 was eluted with acetone; the evaporation of acetone gave approximately 40 mg of a yellow viscous residue. This residue was rechromatographed on preparative TLC plate to give approximately 15 mg of the material, which had ^{1}H NMR and UV spectrum similar to zearalenone. This material was further purified by TLC to yield 6 mg of pure sample. A ^{13}C NMR spectrum obtained on this compound was similar to that of F-5-3 and F-5-4. The compound was further resolved into two components at R_f 0.28 and 0.33, which were designated as F-5-1 (<1 mg) and F-5-2 (<2 mg: mp 168–169.5 °C). Anal. Calcd. for $C_{18}H_{22}O_5$: (F-5-1) 334.1415; found 334.1426; (F-5-2) 334.1415, found 334.1416. UV spectrum F-5-2 in MEOH: 235 (21036), 274 (9155), 336 (4493).

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Effect of Processing on the Amino Acid Composition and Nitrosamine Formation in Pork Belly Adipose Tissue

Ann M. Spinelli-Gugger,* Leon Lakritz, and Aaron E. Wasserman

The curing process does not alter the concentration of free amino acids in the adipose tissue of pork bellies. Frying of the adipose tissue leads to a decrease in the level of free amino acids; greater reductions were seen in the processed vs. untreated tissue. Analysis for nitrosamines in the fried samples indicate that dimethylnitrosamine and nitrosopyrrolidine formation is independent of free amino acid concentration. A study of the lean, adipose and intact tissue of country cured bacon indicates that frying generates up to a tenfold increase in free amino acids in the lean and intact tissue, while comparatively negligible changes are noted in the adipose.

The potential human health hazard posed by nitrosamines (NAs) was recognized as early as 1954 by Barnes and Magee. NAs are produced by the acid-catalyzed reaction of nitrite or nitrogen oxides with certain nitrogen-containing compounds. Amino acids, amines, and amides are examples of these compounds that are present as natural constituents of meats and other foodstuffs (Lijinsky et al., 1970; Ender and Ceh, 1971; Bills et al., 1973; Huxel et al., 1974). The formation of NAs has been reported in model systems in which amino acids are heated with sodium nitrite at elevated temperatures (~170 °C) similar to those attained when frying bacon (Ender and Ceh, 1971; Bills et al., 1973; Huxel et al., 1974; Gray and Dugan, 1973; Coleman, 1978).

Traditionally, nitrite and to a lesser extent nitrate have been used to prepare cured meats that are shelf-stable and possess desirable color and flavor characteristics. Although NAs are not found consistently in all cured meat products

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