Isolation and Characterization of Two New Fusaric Acid Analogs from Fusarium moniliforme NRRL 13,163

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Fusarium moniliforme NRRL 13,163 produced two new fusaric acid analogs, a 10,11-dihydroxyfusaric acid and a diacid of fusaric acid in which the C-11 methyl was oxidized to a carboxyl. Several hundred milligrams of the 10,11-dihydroxyfusaric acid were routinely recovered from a kilogram of corn grit medium. It crystallized as white, irregularly shaped rectangles that melted at 153 to 154°C. The diacid analog of fusaric acid crystallized as white rods that melted at 210 to 211°C. Unlike the consistent recovery experienced with the 10,11-dihydroxyfusaric acid, the diacid analog proved difficult to purify after the initial discovery and was detectable in subsequent fermentations only by mass spectrometry.

Fusarium moniliforme Sheldon, a field fungus common on many crops, including corn, small grains, millet, sorghum, and citrus fruits (1, 9), produces a number of interesting, biologically active secondary metabolites. The more studied products of this species are the plant growth hormone gibberellin and its analogs (8), along with certain mycotoxins, moniliformin (3), fusarin C (20), fusaricin (7), and fusaric acid (17). Fusaric acid is not only moderately toxic to animals (intraperitoneal 50% lethal dose of 100 mg/kg of body weight in mice) (18), but also has antibiotic (4, 15), pharmacological (6, 11, 12), insecticidal (2), and phytotoxic properties (4, 15).

During a survey for antibiotics and mycotoxins produced by fusaria, several crystalline products appeared in fractions of the culture extract of F. moniliforme NRRL 13,163 separated by high-pressure liquid chromatography (HPLC). Two of the products had infrared (IR) spectra similar to the spectrum of fusaric acid. Mass spectrometric and nuclear magnetic resonance (NMR) spectra of the two products established their structures as 10,11-dihydroxyfusaric acid and a fusaric acid analog differing from fusaric acid by the oxidation of the C-11 methyl to a carboxyl [5-(3'-carboxypropyl)-2-pyridinecarboxylic acid: DAOF A] and a fusaric acid analog differing from fusaric acid by the oxidation of the C-11 methyl to a carboxyl [5-(3'-carboxypropyl)-2-pyridinecarboxylic acid: DAOFA].

MATERIALS AND METHODS

Microorganism. F. moniliforme NRRL 13,163 is maintained by the Agricultural Research Service Culture Collection (NRRL), Peoria, Ill. For this study, the strain was cultured on Bacto YM Agar (Difco Laboratories) slants and transferred weekly.

Production and concentration of fusaric acid analogs. A stock culture of F. moniliforme NRRL 13,163 was incubated at ambient temperature until sporulation (ca. 3 days) and then transferred to a refrigerator. Subcultures were made weekly. The conidia-bearing surface of a 7-day-old slant was scraped into 10 ml of water, and about 0.5 ml of the conidial suspension was added to each 50 g of white corn grits (WCG) in 300-ml Erlenmeyer flasks. Before autoclaving 15 ml of water was added to the WCG, and 10 ml of water was added after autoclaving. The inoculated medium was incubated for 3 days at ambient temperature followed by incubation for 18 days at 15°C. After incubation, 250 ml of methanol was added to each flask before the culture medium was macerated in a Waring blender jar. The extract was recovered by filtration, and the aqueous methanol was removed on a rotary evaporator. The residual gummy material was dissolved in water, and any water-insoluble solids were discarded. After reconcentration, the gummy material from 10 flasks was combined and dissolved in 500 ml of methanol, to which 300 ml of acetone was then added. The precipitate formed on the addition of the acetone was discarded and the solution was then dried. The acetone-methanol solubles were redissolved in 50 to 75 ml of water and were applied to a column (4.5 by 20 cm) of Silica Gel 60 silanized with dimethylsilane (RP-2, 70-230 mesh; EM Science). The column was eluted with water until the eluate no longer quenched the fluorescence in thin-layer chromatographic (TLC) plates containing indicator. After removal of water from the column eluate, about 20 to 30 g of solids was obtained from 0.5 kg of culture medium.

HPLC. Preparative HPLC separations of the fusaric acid analogs were carried out on a Prep LC/System 500 (Waters Associates, Milford, Mass.) fitted with a Prep PAK-500/C18 cartridge. Samples were eluted with water-acetonitrile (90:10) at 100 ml/min, and the eluant was monitored with a refractive index detector at a sensitivity of 20. Approximately 50 g (the residue from 1 kg of WCG medium) of solids from the silanized silica gel column was divided into two equal portions. Each fraction was dissolved in 100 ml water for preparative HPLC fractionation. After elution, DHFA and DAOFA were found primarily in the fractions collected between 1,500 and 1,900 ml and 2,350 and 2,850 ml, respectively. Fractions were evaporated to dryness and dissolved in warm methanol. On evaporation, crystals contaminated with yellow, water-soluble impurities were obtained.

Thin-layer chromatography. Crystalline fusaric acid, DHFA, and the diacid analog of fusaric acid, DAOFA, used as TLC standards were either purchased, as was fusaric acid (Aldrich Chemical Co.), or were purified by HPLC and recrystallization until they gave a single spot on TLC plates coated with 0.25-mm-thick Silica Gel 60 F-254 with fluorescent indicator (E. M. Merck).

TLC plates were developed either in isopropyl alcohol-ethyl acetate-water-acetic acid (4:0:3:8:2:0:0:2) (solution A)
or in isopropyl alcohol-butanol-water-ammonium hydroxide (6:0.2:0.1:5:0.5) (solution B). The compounds were identified either by quenching of the indicator when observed under shortwave UV light (254 nm) or by spraying with 50% sulfuric acid followed by charring at 130°C.

**Melting point determinations.** Melting points (uncorrected) were determined with a Fisher-Johns melting block apparatus.

**Crystallization.** Fractions obtained by HPLC containing DHFA or DAOFA crystallized from methanol solutions on evaporation. The crystals were washed with acetone and recrystallized by dissolving crude crystals in hot methanol until saturation was nearly reached. To the nearly saturated solution, an equal volume of acetone was added, and the solution was placed in a freezer.

**DHF A methyl ester.** DHFA (10 mg) dissolved in 1 ml of methanolic boron trifluoride (14%) was heated at 60°C for 1 h. The reaction mixture was concentrated at reduced pressure, taken up in dichloromethane-methanol (95:5), neutralized with NaHCO₃, and dried (Na₂SO₄). The organic solution was concentrated and chromatographed on a column of silica gel (5 g). Elution with dichloromethane-methanol (95:5) gave DHFA methyl ester: IR (KRS-5 plate) 1725 cm⁻¹. MS m/z 225.1009 (M⁻). C₁₃H₁₁NO₄ requires 225.1001.

**Infrared spectra.** IR spectra were recorded with a Perkin-Elmer 1320 spectrophotometer from KBr pellets.

**Mass spectrometry.** Low resolution mass spectra (MS) were obtained by chemical ionization with isobutane as the reagent gas in a Finnigan TSQ. High resolution electron impact mass spectra were determined with a Fisher-Johns melting block apparatus. In some instances assignments were verified by specific proton decoupling.

**Results and Discussion**

*F. moniliforme* NRRL 13,163 was isolated from corn and deposited in the Agricultural Research Service Culture Collection in 1971. This strain was reported to be toxic, and analyses of O.4L but in developing solution A and an E of 0.22 in developing solution B, and DAOFA had maxima at 225 nm (E = 1.85) and 268 nm (E = 1.32), and DAOFA had maxima at 225 nm (E = 7.756) and 268 nm (E = 5.089).

**TABLE 2.** ¹³C NMR data for fusaric acid and analogs

<table>
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<th>Carbon assignment</th>
<th>Chemical shifts (ppm) of the following compounda:</th>
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<th>3</th>
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<td>147.8s</td>
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<td>181.0s</td>
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a Chemical shifts (δ) are expressed in parts per million from internal sodium 3-trimethylsilylpropionate-2,2,3,3,δ₄₄ as the internal standard. Extensive decoupling was used to verify assignments. Spectra were recorded in D₂O on a Bruker WM-300 spectrometer.

b See Fig. 1 for structures. Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet.

c Carbon Chemical shifts (ppm) of the following compound:

<table>
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<th>Proton assignment</th>
<th>Chemical shifts (ppm) of the following compound</th>
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<tbody>
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</tr>
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<tr>
<td>4</td>
<td>8.54d (8.0, 1.5)</td>
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<td>11</td>
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d See Fig. 1 for structures. Abbreviations: d, doublet; dd, double doublet; bs, broad singlet; m, multiplet; t, triplet.
of the NMR data (Tables 1 and 2) of fusaric acid with the fusaric acid analogs support the described chemical characterizations of the analogs.

The IR spectrum of DHFA exhibited absorptions at 3,310 cm⁻¹ (hydroxyl), 1,640 cm⁻¹ (carboxylate ion), and 1,580 and 1,525 cm⁻¹ (aromatic ring). The abnormal band appearing at 1,640 cm⁻¹ instead of 1,705 cm⁻¹ likely is due to the formation of a zwitter ion with KBr in the presence of a small amount of moisture, as explained previously by Taylor (16). The MH⁺ ion at m/z 212 in chemical ionization MS indicated a compound with an odd number of nitrogen atoms and is consistent with a derivative of fusaric acid. This assignment was supported by the UV spectrum which exhibited a characteristic absorption at 268 nm (2, 5, 14). High-resolution MS of the methyl ester showed a molecular ion at m/z 225.1009 with an empirical formula of C₁₁H₁₅NO₄ (consistent with a dihydroxyfusaric acid derivative). The ¹H NMR spectrum showed two aliphatic methylene protons at chemical shifts (δ) 1.88: two benzylic protons at δ 3.03; a methylene group attached to oxygen at δ 3.57: a methine proton adjacent to oxygen at δ 3.73; and three aromatic protons at δ 8.31, δ 8.54, and δ 8.65. The ¹³C NMR spectrum exhibited two methylene carbons at δ 30.9 and δ 35.5, a methylene carbon bearing oxygen at δ 67.9, a methine carbon bearing oxygen at δ 73.4, five aromatic carbons at δ 128.8 to δ 146.9, and a carbonyl carbon at δ 166.8. The NMR data reduced the structural possibilities for the compound to either 10.11-dihydroxyfusaric acid or 9.11-dihydroxyfusaric acid. However, the 9.11-dihydroxyfusaric acid possibility was eliminated from consideration because the δ 3.58 signal was due to the AB portion of an ABC system and not of an ABCベンゾ system. That the primary alcohol methylene is the δ 3.57 signal and not the more complex pattern at δ 3.03 was established by selective heteronuclear decoupling experiments. Irradiation at δ 3.57 resulted in collapse of the triplet at δ 67.9 in the ¹³C spectrum to a singlet. Likewise, irradiation of the methylene proton at δ 3.03 resulted in collapse of the high-field methylene carbon triplet at δ 30.8 to a singlet. These data establish the structure of DHFA (Fig. 1, structure 1).

The IR of DAOFA showed, in addition to the absorptions exhibited by DHFA, a band at 1,710 cm⁻¹ (carbonyl). That it is a fusaric acid derivative was indicated by UV absorption at 268 nm and the even mass MH⁺ ion at m/z 210 by chemical ionization-MS. The ¹H NMR spectrum exhibited signals at δ 2.02, δ 2.45, and δ 2.96 as a multiplet and two triplets, respectively, for a -CH₂-CH₂-CH₂- grouping, plus three aromatic protons at δ 8.32, δ 8.54, and δ 8.63. The ¹³C NMR spectrum consisted of signals for three methylene carbons at δ 27.5, δ 33.8, and δ 35.7, five aromatic carbons at δ 129 to 150 and two carbonyl carbons at δ 166.3 and δ 181.0. These data establish the compound as DAOFA (Fig. 1, structure 2).

Fusaric acid (Fig. 1, structure 3), a compound with diverse biological activities, affects animals, insects, plants, and microbial cells. It was first isolated by Yabuta et al. in 1934 (21) and was subsequently implicated in wilt diseases of plants (4, 15). In plants, the presence of fusaric acid impairs water permeability and exaggerates transpiration, resulting in loss of turgor and ionic imbalance (15). Whether these new fusaric acid analogs contribute to phytotoxicity is not known, but results of preliminary tests in our laboratory indicate that they have no effect on the germination or development of grass, cucumber, or velvet leaf seeds at 50 μg/ml in water.

Hidaka et al. (6) have found fusaric acid to inhibit dopamine-β-hydroxylase, one of the enzymes involved in the biosynthesis of norepinephrine, and they showed that it had hypotensive effects in mice, rabbits, and dogs. These and other biological activities have created an interest in the pharmacological properties of fusaric acid and synthetic derivatives made from it (11, 12, 19). Before this report, DAOFA was identified from MS analyses by Miyazaki et al. (13) as a catabolic product in the urine of rats dosed with 5-α-chloro-α-butyl) picolinic acid. They were able to separate the urine products on an amberlite column and established the structure of DAOFA by the presence of a molecular ion of the dimethyl ester at m/z 237 and by comparison of a synthetic product with the one recovered from urine. The facts that DAOFA occurred in urine as a catabolite of chlorinated fusaric acid and that it also appeared in culture media that supported the growth of F. moniliforme suggest that fusaric acid undergoes biological oxidation with DAOFA as the likely end product. Pitel and Vining (14) were able to demonstrate that dehydrofusaric acid is interconvertible to either fusaric acid or 10-hydroxyfusaric acid, suggesting a biological oxidation of fusaric acid through dehydrofusaric acid. 10-hydroxyfusaric acid, DHFA, and DAOFA.

Because fusaric acid and numerous derivatives of fusaric
acid are being extensively studied for their efficacy as blockers of dopamine β-hydroxylase, these newly characterized fusaric acid analogs, DHFA and DAOFA, could be useful in evaluating the catabolism of fusaric acid in treated animals.

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LITERATURE CITED