Notes

Isolation of a New Fumonisin from *Fusarium moniliforme* Grown in Liquid Culture

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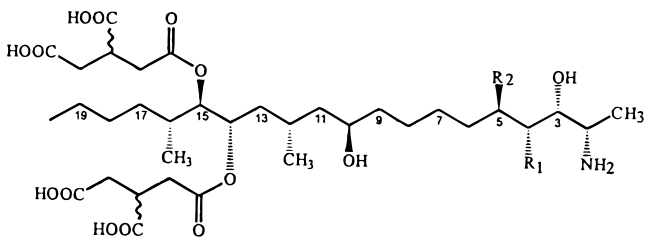
A new fumonisin, iso-fumonisin B_1 (iso-FB_1, **1**), has been isolated from liquid cultures of the fungus *Fusarium moniliforme* (Sheldon) NRRL 13616. On the basis of its spectroscopic data, its structure has been determined to differ from that of fumonisin B_1 only in the presence of a hydroxyl function at C-4 instead of C-5.

Fusarium moniliforme (Sheldon) is one of the most common fungi found on corn throughout the world.[1] A group of mycotoxins, called fumonisins, have been isolated from cultures of this fungus[2] and have been subsequently found as natural contaminants in corn and other grains.[3] The fumonisins are a group of structurally related toxins shown to be the causative agents of animal diseases such as leukoencephalomalacia in horses and pulmonary edema in swine. Fumonisins have also proven to be hepatocarcinogenic in rats.[4] As well, increased incidence of esophageal cancer in humans has been correlated with ingestion of fumonisin-contaminated food products in certain areas of the world where moldy corn is frequently consumed.[5] Because of the frequency and widespread contamination of corn with *F. moniliforme* and the proven toxicity of the fumonisins identified to date, interest in these compounds continues to grow.

Because there can be more toxicity associated with impure mixtures of fumonisin material than can be attributed to the known fumonisins,[6] it is thought that there are other toxic compounds present. For this reason, the search for novel metabolites related to fumonisins is important.[7−9] These unknown compounds have proven to be difficult to isolate for several reasons: they are present in much smaller quantities than FB_1, FB_2, or FB_3; they tend to elute very close together under similar high performance liquid chromatography (HPLC) conditions; and they do not have chromophores. Hence, separation is carried out without visualization, and each fraction must be analyzed after it is eluted by HPLC.

Although the three most abundant fumonisins isolated from cultures or natural samples of *F. moniliforme* are always FB_1, FB_2, and FB_3, an analysis of the *o*-phthaldialdehyde-(OPA) derivatized fraction of a mixture of "fumonisin-like" compounds reveals many smaller peaks. During the course of our investigation of these smaller peaks, one peak was always present and larger than the others (excluding FB_1, FB_2, and FB_3) under the analytical conditions employed. This compound, which we have named iso-fumonisin B_1 (iso-FB_1, **1**), is a new fumonisin differing from fumonisin B_1 (FB_1, **2**) in the placement of one hydroxyl group. The isolation and full characterization of this new mycotoxin are reported here.

A mixture of fumonisins isolated from the culture filtrate of *F. moniliforme* NRRL 13616 was chromatographed by preparative HPLC, and many fractions were collected. All fractions were individually analyzed by derivatization with OPA followed by analytical HPLC with fluorescence detection. Several fractions had as their main component a strong peak, **1**, which eluted 1.4 min after an FB_1 standard under the described conditions. In addition, one fraction contained this peak alone. The fractions that contained only the single peak of interest from each of the preparative HPLC runs were combined. The purity of this sample was confirmed by OPA analysis. Comparison to an $FB_1/FB_2/FB_3$ mixture and co-injection with FB_1 confirmed that it was none of these previously known compounds.



1 R_1 = OH, R_2=H
2 R_1 = H, R_2=OH

Structure **1** was assigned to this compound, designated iso-FB_1, based on the following analysis. Fast atom bombardment mass spectrometry (FABMS) data

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Table 1. NMR Assignments of Iso-FB_1 (1) in CD_3OD (δ, ppm, J, Hz)

position	^{13}C	^1H
1	14.2	1.30 (d, $J_{1,2} = 6.8$)
2	51.8	3.43 (dq, $J_{2,3} = 4.3$)
3	73.2	3.55 (dd, $J_{3,4} = 4.0$)
4	72.2	3.65 (m, $J_{4,5} = 12.6, 4.5$)
5	34.2	1.45; 1.55
6	30.6	1.34 (br m)
7	26.3	1.38; 1.54
8	26.5	1.32; 1.52
9	38.9	1.40 (sym AB)
10	70.0	3.62 (m)
11	44.5	1.08; 1.50
12	27.0	1.83 (m)
13	37.0	1.45; 1.66
14	72.7	5.16 (ddd, $J_{14,13} = 11.3, 3.1$; $J_{14,15} = 3.7$)
15	78.9	4.94 (dd, $J_{14,15} = 3.7$; $J_{15,16} = 8.0$)
16	34.9	1.70 (m)
17	32.9	1.08; 1.43
18	29.8	1.18; 1.33
19	23.9	1.22; 1.38
20	14.4	0.89 (t, $J_{20,19} = 7.1$)
21	20.8	0.96 (d, $J_{21,12} = 6.7$)
22	16.1	0.93 (d, $J_{22,16} = 6.8$)
23	173.5	
24	37.3	2.45; 2.71
25	39.7	3.14
26	38.0	2.48; 2.64
27	177.2	
28	178.7	
29	173.2	
30	37.0	2.59; 2.79
31	39.6	3.14
32	37.7	2.51; 2.70
33	176.7	
34	178.2	

showed that **1** had a molecular weight of 721 amu, identical with that of FB_1. As suspected from the mass spectrometric analysis, NMR investigations confirmed that iso-FB_1 and FB_1 had similar structures. The only noteworthy differences occurred in the chemical shifts associated with the C-1 to C-6 region of the molecule[10] (Table 1).

Analysis of the couplings present in the ^1H COSY spectrum of **1** showed only small variations from that of FB_1. The large doublet associated with H-1 had shifted to δ 1.30 from δ 1.27, H-2 had shifted from δ 3.14 to δ 3.43, and H-3 was observed at δ 3.55 (from δ 3.74 in FB_1), indicating the presence of only minor variations to the structure at the amino terminus. The signal associated with H-4, however, had shifted from δ 1.55 (CH_2) to δ 3.65 (secondary alcohol), and H-5 had become a CH_2 at δ 1.45, from δ 3.84 in FB_1. The signal associated with H-10 was observed with the same chemical shift and couplings as seen for H-10 in the proton spectrum of FB_1. These data indicated that compound **1** was a structural isomer of FB_1, with hydroxyl functions on C-3, C-4, and C-10 rather than on C-3, C-5, and C-10 as in FB_1. The remainder of the ^1H-NMR spectrum was identical with that of FB_1.

The proposed structure, **1**, was further supported by the ^{13}C signals, assigned by correlation to their respective protons. Again, the ^{13}C spectrum was identical with that of FB_1 from C-7 to C-20, including the ester functions. C-1 and C-2 were found at δ 14.2 and δ 51.8, respectively, differing slightly from the corresponding values of δ 16.0 and δ 53.7 reported for FB_1. C-4 showed a very large downfield shift from δ 41.7 ppm to δ 72.2, as would be expected, while C-3 occurred at δ 73.2 (from δ 70.3 in FB_1) and C-5, now a methylene, occurred at δ 34.2 (from δ 68.4). C-6 showed a concomitant and smaller upfield shift from δ 39.1 to δ 30.6.

The presence of positive NOEs observed between H-1 and H-2, between H-2 and H-3 and H-4, but not between H-3 and H-4, as well as consideration of the bond angles suggested by the J values, indicated the relative stereochemistry of H-4 to be cis with respect to H-3. Assuming that the absolute stereochemistry of **1** at the amino function was the same as that of FB_1,[11] the absolute stereochemistry of this metabolite was as shown in **1**.

Comparison of this metabolite to the FC series of fumonisins determined by Seo et al.,[9] in which the terminal methyl group is absent, suggests that **1** is an analogue of hydroxy-FC_1. From a biosynthetic standpoint, a comparison of the FB series (here as well as in others' work[7−9]) suggests that the primary sites of substitution are an amino function at C-2 (C-1 of the FC series, biosynthetically derived from serine [11]) and the hydroxyls at C-3, C-14, and C-15 with a more random pattern of hydroxylation at positions 4, 5, and 10.

A peak with the same retention time as iso-FB_1 (**1**) was observed in a crude culture filtrate analyzed by HPLC after OPA derivatization. Addition of isolated iso-FB_1 to this culture filtrate produced an increase in the size of this peak. This is positive evidence that **1** is a genuine metabolite of *F. moniliforme* and not an artifact of the isolation procedure.

In summary, iso-FB_1 (**1**) has been isolated from cultures of *F. moniliforme* and characterized for the first time, as consistently the fourth most abundant fumonisin present in these cultures.

Experimental Section

General Experimental Procedures. Detection of the derivatized fumonisins was achieved with a Shimadzu RF-551 fluorescence detector set at 335 nm (excitation) and 440 nm (emission). The ^1H- and ^{13}C-NMR spectra of **1** were recorded in MeOH-d_4 (CD_3OD) at 500.13 and 125.18 MHz, respectively, on a Bruker AM500 operating at 303 K, using a 5-mm normal geometry $^1H-^{13}C$ probe. Chemical shifts were referenced to CD_3OD at 3.3 and 49.0 ppm, respectively. Structural determinations were made using a combination of homonuclear (COSY) and heteronuclear (HMQC) correlation spectroscopy as well as ^{13}C multiplicity (DEPT) and NOE difference spectroscopy. Mass spectra were recorded on a Kratos concept model IIH machine with an acceleration voltage of 8 kV and a fast atom beam of 12 to 15 kV. The analysis was carried out in a glycerol matrix.

Organism. The culture of *Fusarium moniliforme* (Sheldon) was a subculture of an isolate originally obtained from corn in Illinois and assigned the number NRRL 13616 by the Northern Regional Research Laboratory (NRRL) (USDA−ARS, 1815 N. University St., Peoria, IL 61604).

Isolation of iso-FB_1. Liquid cultures of *F. moniliforme* NRRL 13616 were grown in a stirred jar fermenter, and a crude mixture of fumonisins was obtained under the conditions described in Miller et al.[12] Briefly,

four gravity-fed DEAE Sephadex A-25 (Pharmacia LKB) (20 g) anion-exchange columns were prepared. The four columns were then charged with a formate counterion and rinsed with distilled, deionized H_2O until the pH of the eluent was above 3. The culture filtrate was then loaded onto the columns, which were run simultaneously. The fractions collected from the columns were analyzed by TLC on Si gel. The fumonisin-containing fractions were combined, concentrated under vacuum, and lyophilized.

This crude solid was further cleaned up on an open Si gel column. Again, fractions were collected, analyzed by TLC, and the fumonisin-containing fractions were combined and dried. The crude solid was then dissolved in 28:72 MeCN−H_2O and chromatographed on a 300 × 30 mm i.d. C-18 preparative HPLC column packed with 50 μm, 120 Å ODS material. The solvent was driven through the column at a flow rate of 20 mL/min using a solvent gradient (MeCN−H_2O) in order to remove the majority of the FB_1. The TLC analysis was repeated on these fractions. The fractions containing mainly FB_1 were removed, and the remaining fractions were combined, concentrated under vacuum, and lyophilized to yield the crude fumonisin mixture.

Although most of the FB_1 (ca. 90%) had been removed from the fumonisin mixture, it was still present in greater concentration than any other compound. The crude fumonisin sample was then run through the same preparative HPLC system that was used to remove FB_1 using a slightly modified gradient (starting at 28% MeCN and held at 100% MeCN for 13 min rather than 5), which removed the residual FB_1 and fractionated the remainder of the sample.

All of the fractions obtained from the preparative HPLC were individually derivatized with OPA and analyzed by analytical HPLC as recommended in the AOAC−IUPAC collaborative study.[13] The only difference was the use of a 250 × 4.6 mm i.d. C-8 reversed-phase column packed with 5 μm, 80 Å spherisorb rather than the 150 × 4.6 mm i.d., C-18 column packed with 5 μm ODS material. The procedure for isolating iso-FB1 was carried out multiple times, and the product from all runs was combined in order to obtain a sufficient amount of sample for characterization.

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References and Notes

(1) Marasas W. F. O.; Nelson P. E.; Toussoun T. A. *Toxigenic Fusarium Species: Identity and Mycotoxicology*; The Pennsylvania State University: University Park, PA, 1984.
(2) Bezuidenhout, S. C.; Gelderblom, W. C. A.; Gorst-Allman, C. P.; Horak, R. M.; Marasas, W. F. O.; Spiteller, G.; Vleggaar, R. *J. Chem. Soc., Chem. Commun*. **1988**, 743−745.
(3) Shephard, G. S.; Thiel, P. G., Stockenstrom, S.; Sydenham, E. W. *J. AOAC Int*. **1996**, *79*, 671−687.
(4) Norred, W. P.; Voss, K. P. *J. Food Prot*. **1994**, *57*, 522−527.
(5) Rheeder, J. P.; Marasas, W. F. O.; Thiel, P. G.; Sydenham, E. W.; Shephard, G. S.; van Schalkwyk, D. J. *Phytopathology* **1992**, *82*, 353−357.
(6) Thiel, P. G.; Marasas, W. F. O.; Sydenham, E. W.; Shephard, G. S.; Gelderblom, W. C. A. *Mycopathologia* **1992**, *117*, 3−9.
(7) Gelderblom, W. C. A.; Marasas, W. F. O.; Vleggaar, R.; Thiel, P. G.; Cawood, M. E. *Mycopathologia* **1992**, *117*, 11−16.
(8) Musser, S. M.; Eppley, R. M.; Mazzola, E. P.; Hadden, C. E.; Shockhor, J. P.; Crouch, R. C.; Martin, G. E. *J. Nat. Prod*. **1995**, *58*, 1392−1397.
(9) Seo, J.; Kim, J.; Lee, Y. *J. Nat. Prod*. **1996**, *59*, 1003−1005.
(10) Savard, M. E.; Blackwell, B. A. In *Mycotoxins In Grain*; Miller, J. D., Trenholm, H. L., Ed. Eagan: St. Paul, MN, 1994; Chapter 4, pp 59−260.
(11) Blackwell, B. A.; Edwards, O. E.; Fruchier, A.; ApSimon, J. W.; Miller, J. D. In *Fumonisins in Food*; Jackson, L., et al., Eds. Plenum: New York, 1996, Chapter 7, pp 75−91.
(12) Miller, J. D.; Savard, M. E.; Rapior, S. *Natural Toxins* **1994**, *2*, 354−359.
(13) Sydenham, E. W.; Shephard, G. S.; Thiel, P. G., Stockenstrom, S.; Snijman, P. W.; van Schalkwyk, D. J. *J. AOAC Int*. **1996**, *79*, 688−696.

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