Identification of a New Series of Fumonisins Containing 3-Hydroxypyridine

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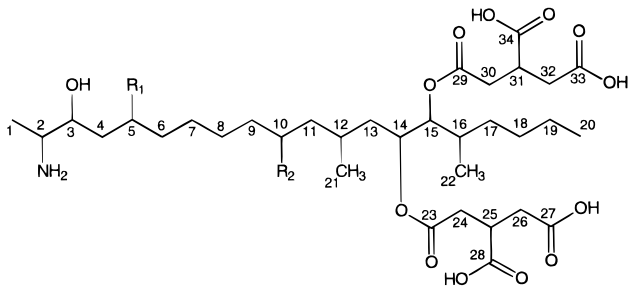
Received March 11, 1996[⊗]

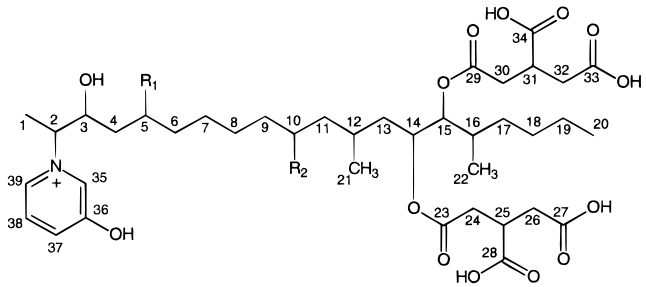
A new series of fumonisins, designated FP_{1-3}, were isolated from a culture of *Fusarium moniliforme* (M-2285) grown on solid corn. The new compounds contain a 3-hydroxypyridinium moiety at the C-2 position of the eicosane backbone instead of the amine found in the B series of fumonisins. The new fumonisins were characterized by UV, LC−MS-MS, ^{1}H NMR, and ^{13}C NMR. LC−MS analysis of culture extracts indicates that the new fumonisins can occur at levels up to approximately one-third the amount of their amine-containing analogues (FB_1, FB_2, and FB_3).

The fungal toxins known as fumonisins are a recently identified group of toxins characterized by having an aminopolyol eicosane backbone and two tricarballylic acid esters.[1] These toxins are produced by several species of *Fusaria*, most notably *F. moniliforme* and *F. proliferatum*. The most commonly occurring fumonisins B_1(**1**), B_2, and B_3 (FB_{1-3}) are found in corn and corn products[2,3] and have been directly associated with a variety of animal illnesses, including porcine pulmonary edema,[4] hepatotoxicity in rats,[5] and a fatal neurotoxic syndrome in horses known as leukoencephalomalacia.[6] In addition to inducing sickness in animals, ingestion of corn contaminated with high levels (1−10 ppm) of the B series of fumonisins has been associated with human esophogeal cancer.[7]

Several toxicological studies on purified forms of the B series of fumonisins have been performed and, while there is little doubt that the B series has a toxic effect, levels needed to induce toxicity are often more than 100 times higher than the levels found in feed naturally contaminated with fumonisins.[8,9] This observation has resulted in the search for more toxic forms of fumonisins and the characterization of a number of minor fumonisin metabolites occurring in cultures of *F. moniliforme* and *F. proliferatum*. These metabolites include a trihydroxy eicosane form of the B series known as FB_4,[10] *N*-acetyl amides of the B series,[10] FC_1, which lacks a terminal methyl group,[11] and a keto amide fumonisin.[12] All of these minor components occur in naturally contaminated corn at very low levels (<5% of total fumonisins). In this paper we report the isolation and characterization of a new series of fumonisins designated FP_{1-3} (**2**−**4**), in which the amine found at the C-2 position of the B series is replaced with an *N*-linked 3-hydroxypyridinium moiety. In addition to structural differences, the P series of fumonisins, unlike many previously identified fumonisins, can occur at levels up to 30% of FB_1 when grown on solid corn cultures. This new class of fumonisins is also of interest because of the known toxic effects of long alkyl-chain pyridinium compounds.[13]



FB_1 (**1**)



	R_1	R_2
FP_1 (**2**)	OH	OH
FP_2 (**3**)	OH	H
FP_3 (**4**)	H	OH

LC−electrospray (ESI)/MS analysis of extracts from *F. moniliforme* cultures indicated the presence of several new fumonisins, one of which, compound **2**, occurred at 30% of the amount of FB_1 present in the extract. The new fumonisins were prepared from cultures of *F. moniliforme* by semi-preparative HPLC and analytical HPLC. This procedure yielded approximately 9 mg of FP_1 (**2**), 3 mg of FP_2 (**3**), and 1.8 mg of FP_3 (**4**). These compounds were characterized by UV absorption, HR-LSIMS−MS, ESI−MS−MS, ^{1}H NMR, and ^{13}C NMR. UV spectra of the new compounds all show a characteristic UV maximum at 289 nm under acidic conditions (0.1 N HCl) with a shift to 322 nm under basic (0.1 N NaOH) conditions. The 33-nm shift in absorbance maxima under basic conditions is characteristic of 3-hydroxy-*N*-alkylpyridinium compounds and is not observed with the other possible hydroxypyridine isomers.[14,15]

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[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1996.

S0163-3864(96)00349-7

Table 1. ^{13}C NMR and Selected ^{1}H Data[a] for Fumonisins (2−4)

position	FP_1 (**2**)	FP_2 (**3**)	FP_3 (**4**)
1	18.2 (1.71 d) [7]	18.2 (1.70 d) [7]	18.1 (1.70 d) [7]
2	74.6 (4.64 dq) [7]	74.6 (4.64 dq) [7]	74.4 (4.59 dq) [7]
3	71.5 (4.10 ddd) [10,7,3]	71.6 (4.10 ddd) [10,7,3]	74.0 (3.85 ddd) [10,7,3]
4	42.2 (1.3, 1.55 m)	42.1 (1.3, 1.56 m)	34.9 (1.3−1.5)
5	68.6 (3.8 m)	68.7 (3.8 m)	26.3 (1.3−1.5)
6	39.3 (1.3−1.55)	39.2 (1.3−1.5)	30.6[b] (1.3−1.5)
7	26.7	26.6	30.5[b]
8	26.8	30.6[b]	26.8
9	39.2	30.7[b]	39.3
10	69.9 (3.6 m)	27.6 (1.3−1.5)	69.9 (3.6 m)
11	44.6	36.1[c]	44.5
12	26.9	30.1	26.9
13	36.0	36.0[c]	36.0
14	73.1 (5.14 ddd) [11,3,2]	73.0 (5.17 ddd) [11,3,2]	73.1 (5.14 ddd) [11,3,2]
15	78.8 (4.96 dd) [9,3]	78.9 (4.92 dd) [9,3]	78.7 (4.96 dd) [9,3]
16	34.9	34.9	34.9
17	33.1	33.0	33.1
18	29.6	29.7	29.6
19	23.9	23.9	23.9
20	14.4	14.4	14.4
21	20.6	20.9	20.6
22	15.9	16.0	15.9
23	173.2	173.1	173.2
24	36.4	36.4	36.4
25	38.6	38.6	38.6
26	36.7	36.5	36.5
27	175.2	175.1	175.2
28	177.0	176.8	177.1
29	173.0	173.0	173.0
30	36.1	36.3	36.4
31	38.5	38.5	38.6
32	36.6	36.4	36.5
33	175.0	175.0	175.0
34	176.6	176.6	176.7
35	133.5 (8.50 dd) [2,1]	133.5 (8.49 dd) [2,1]	133.4 (8.49 dd) [2,1]
36	158.8	158.8	158.8
37	132.8 (7.94 ddd) [9,2,1]	132.8 (7.94 ddd) [9,2,1]	132.8 (7.93 ddd) [9,2,1]
38	129.3 (7.88 dd) [9,6]	129.3 (7.87 dd) [9,6]	129.3 (7.87 dd) [9,6]
39	136.2 (8.48 dt) [6,1]	136.2 (8.47 dt) [6,1]	136.0 (8.45 dt) [6,1]

[a] Proton data are in parenthesis with *J* values in brackets; values not reported are essentially the same as for the B series. [b,c] Values may be interchanged.

A molecular weight of 800 was determined for compound **2**, and a molecular weight of 784 was determined for compounds **3** and **4**. Hydrolysis of compounds **2**−**4** in 2 N KOH, with subsequent purification by HPLC and HRMS of the UV absorbing hydrolysis products resulted in an observed mass of 484 for the hydrolysis product of compound **2** and an observed mass of 468 for the hydrolysis products of compounds **3** and **4**. The loss of 316 mass units from each of the new compounds upon treatment with base is consistent with the hydrolysis of two tricarballylic acid groups and is the same loss that is observed when any of the B series of fumonisins are treated with base.[8] MS−MS analysis of compounds **2**−**4** showed a similar fragmentation pattern as the B series of fumonisins,[16] except the masses are offset 78 amu, implying a similarity in structure. The base peak in the MS−MS spectrum of compounds **2**−**4** is *m*/*z* 96. This ion is consistent with a hydroxypyridinium ion and is a characteristic fragment ion in the MS−MS spectra of alkylpyridinium compounds.[17] HRMS analysis and LRMS−MS of both the intact and hydrolyzed compounds indicated that the increase in mass was a modification to the eicosane backbone and not to the tricarballylic acid esters. Additionally, the MS and UV data suggest that the difference between the B series of fumonisins and the new compounds is replacement of the C-2 amine with a 3-hydroxypyridinium group.

Confirmation of the proposed structures of the new compounds and assignment of the proton and carbon resonances were done by 1D and 2D ^{1}H- and ^{13}C-NMR experiments. The ^{1}H- and ^{13}C-NMR spectra (Table 1) of compounds **2**−**4** were very similar to those reported for the B series of fumonisins,[18] with the notable exception of the aromatic resonances and a significant downfield shift of C-2 (Δ 19.1 ppm) and H-2 (Δ 1.5 ppm). Analogous to their B series analogues, hydroxyl groups were determined to be missing from C-10 in FP_2 and C-5 in FP_3, on the basis of the following experimental evidence. Compared to FP_1, which possesses hydroxyl groups in both positions, the carbon NMR signal of C-10 was shifted 42.3 ppm upfield in FP_2, while the chemical shifts of carbons 1−7 were identical to those of FP_1. Similarly, the resonance of C-5 was shifted 42.4 ppm upfield in FP_3, while the chemical shifts of carbons 8−13 and 21 were now identical to those of FP_1. Moreover, ^{13}C-NMR signal assignments for FP_2 and FP_3 were almost identical to those for FB_2 and FB_3, respectively, which are known to lack hydroxyl groups in the same positions.

The presence of a 3-hydroxypyridinium moiety was confirmed by five signals appropriate for sp^2-hybridized carbons in the ^{13}C-NMR spectrum and the similarity of these signals with those reported for *N*-methyl-3-hydroxypyridinium iodide.[19] In addition, inspection of the ^{1}H-NMR spectrum revealed that only one of the four aromatic protons (H-35 at 8.5 ppm) exhibited no *ortho* couplings. This indicated that the hydroxyl group was situated *meta* to the pyridinium nitrogen at C-36. The absence of ^{13}C-NMR signals at 50−55 ppm and the presence of an additional resonance in the 70-ppm range

(74.6) suggested that the pyridinium group was attached at C-2. The proton attached to C-2 was identified as a 6.6-Hz quintet by virtue of its identical coupling to H-3 and methyl-1. An HMQC experiment established its direct connectivity to the carbon resonance at 74.6 ppm as well as those of protons 35 and 39 to their respective carbons. Further proof that the 3-hydroxypyridinium group was attached to C-2 was provided by an HMBC experiment that revealed the following three-bond proton−carbon connectivities: (a) H-2 (4.64 ppm) to C-35 and C-39 and (b) C-2 (74.6 ppm) to H-35 and H-39. The HMBC experiment also confirmed attachment of the hydroxypyridinium moiety to the C-2 position via the nitrogen and not the oxygen atom.

Experimental Section

General Experimental Procedures. All reagents used were HPLC grade. UV spectra were recorded on a Beckman DU-7 spectrophotometer. A Hewlett-Packard Model 1050 HPLC pump and diode array detector were used for all HPLC procedures. LRESI−MS and ESI−MS−MS spectra were recorded on a Finnigan Model TSQ-7000. High resolution/liquid secondary ion mass spectrometry (HRLSIMS)−MS were obtained on a Fisons Autospec Q. ^{1}H- and ^{13}C-NMR spectra were obtained using a Varian VXR-400S spectrometer equipped with a Nalorac Z SPEC MD400-3 microprobe. HMBC and HMQC experiments were also performed on this instrument. All samples were run in CD_3OD. Chemical shifts were referenced to CD_3OD at 3.30 ppm for ^{1}H and 49 ppm for ^{13}C-NMR spectra.

Fungal Culture Conditions. Solid cultures of *F. moniliforme* M-2285, obtained from the the Fusarium Research Center, Pennsylvania State University, were grown on cracked corn as previously described.[20] Levels of production of **1** were approximately 3 mg/g solid culture material.

Isolation. Solid culture material (20 g) was extracted with 20 mL of MeCN−H_2O solution (75:25) and filtered though a Buchner funnel. The filtrate was passed through a Sep-Pak C-18 column (1 g) and the eluent evaporated to dryness. The residue was then taken up in 100 mL of a MeCN−H_2O solution (25:75). This solution was divided into four equal parts and applied to four Sep-Pak C-18 (1 g) cartridges. The columns were washed with a MeCN−H_2O solution (75:25), and the fumonisin-containing fraction, as determined by LC−MS, was eluted with MeCN−H_2O (50:50). The eluent was concentrated and then fractionated on a YMC Inc, J-sphere L80 ODS (10 × 250 mm) column using a MeCN−H_2O gradient containing 0.1% formic acid. The fractions containing **2**−**4** were further fractionated on a YMC Inc., Cyano (10 × 250 mm) column using the same conditions as the first column. This procedure produced 9 mg of **2** and 3 mg of **3** at 98% purity by LC−MS. Compound **4** was subjected to additional fractionation on a Polymer Labs poly(sytrene/divinylbenzene) PLRPS column (4.6 × 250 mm) using a MeCN−H_2O gradient containing 0.1% TFA. This procedure yielded 1.8 mg of compound **4** of 95% purity by LC−MS.

Bis(1,2,3-propanetricarboxylic acid), 1,1′-[1-(12-(3-hydroxy-1-pyridinyl)-4,9,11-trihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl] ester (2): white solid, 9 mg; ^{1}H and ^{13}C NMR ($^{12}CD_3OD$), see Table 1; UV λ_{max} (aqueous 0.1 N HCl) 288 nm (1320), ν_{max} (aqueous 0.1 N NaOH) 322 (1170); HRLSIMS−MS, *m/z* $[M]^+$ 800.4070, $C_{39}H_{62}NO_{16}$ requires 800.4069; LRESI−MS−MS, *m/z* 800 (75), 624 (10), 448 (85), 353 (8), 335 (15), 245 (120), 96 (100); hydrolysis of 500 μg (**2**) in 2 N KOH yielded one UV peak (289 nm) by HPLC, HRLSIMS−MS *m/z* $[M]^+$ 484.3640, $C_{27}H_{50}NO_6$ requires 484.3638.

Bis(1,2,3-propanetricarboxylic acid), 1,1′-[1-(12-(3-hydroxy-1-pyridinyl)-9,11-dihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl] ester (3): white solid, 3 mg; ^{1}H and ^{13}C NMR ($^{12}CD_3OD$), see Table 1; UV λ_{max} (aqueous 0.1N HCl) 288 nm (1370), ν_{max} (aqueous 0.1 N NaOH) 320 (1191); HRLSIMS−MS, *m/z* $[M]^+$ 784.4123, $C_{39}H_{62}NO_{15}$ requires 784.4119; LRESI−MS−MS, *m/z* 784 (90), 608 (32), 432 (100), 337 (12), 319 (8), 203 (12), 96 (100); hydrolysis of 500 μg (**3**) in 2 N KOH yielded one UV peak (289 nm) by HPLC, HRLSIMS−MS *m/z* $[M]^+$ 468.3679, $C_{27}H_{50}NO_5$ requires 468.3689.

Bis(1,2,3-propanetricarboxylic acid) 1,1′-[1-(12-(3-hydroxy-1-pyridinyl)-4,11-dihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl] ester (4): white solid, 1.8 mg; ^{1}H and ^{13}C NMR ($^{12}CD_3OD$), see Table 1; UV λ_{max} (aqueous 0.1N HCl) 288 nm (1324), ν_{max} (aqueous 0.1 N NaOH) 322 (1183); HRLSIMS−MS, *m/z* $[M]^+$ 784.4123, $C_{39}H_{62}NO_{15}$ requires 784.4119; LRESI−MS−MS, *m/z* 784 (90), 608 (32), 432 (100), 337 (12), 319 (8), 203 (12), 96 (100); hydrolysis of 500 μg (**4**) in 2 N KOH yielded one UV peak (289 nm) by HPLC, HRLSIMS−MS *m/z* $[M]^+$ 468.3684, $C_{27}H_{50}NO_5$ requires 468.3689.

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NP960349T