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Isolation and NMR Characterization of Fumonisin B₂ and a New Fumonisin B₆ from *Aspergillus niger*MARIA MÅNSSON,[†] MARIE LOUISE KLEJNSTRUP,[†] RICHARD K. PHIPPS,[†]
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A new fumonisin, fumonisin B₆ (**1**), has been isolated by cation-exchange and reverse-phase chromatography, together with fumonisin B₂ (**2**), from stationary cultures of the fungus *Aspergillus niger* NRRL 326. Analysis of mass spectrometric and NMR data determined that FB₆ is a positional isomer of FB₁ and iso-FB₁, having hydroxyl functions at C3, C4, and C5. Analysis of the NMR data for FB₂ showed very similar chemical shift values when compared to an authentic *Fusarium* FB₂ standard, strongly indicating identical molecules despite that an absolute stereochemical assignment of FB₂ from *A. niger* was not possible.

KEYWORDS: Fumonisin; *Aspergillus niger*; mycotoxins; food safety; NMR

INTRODUCTION

Recently, our group was the first to report the production of the mycotoxin fumonisin B₂ (FB₂) from *Aspergillus niger* NRRL 3122, using HPLC–MS (*1*). The finding did not come as a great surprise, since others (*2, 3*) had already reported a fumonisin-like gene cluster in the *A. niger* genome of two different strains of the species. Later, we also reported FB₄ from *A. niger*, generally produced in amounts around 10–25% of the FB₂ (*4, 5*). Until these findings, fumonisin production has only been previously reported in certain species within the *Gibberella fujikuroi* and *Fusarium oxysporum* species complexes, which are common fungal contaminants of maize-based foods and feeds (*6–9*). The first fumonisins were described by Gelderblom et al. (*9*) from cultures of *F. verticillioides*. Since then, more than 25 fumonisins have been identified by NMR (*6*) and a further 25 have been putatively identified by LC–MSⁿ (*10*). The most abundant and most toxic fumonisins are the B-series analogues (*11–13*), which contain a terminal 2-amino-3-hydroxy motif on an eicosane backbone and two hydroxy groups esterified with tricarboxylic acids (TCA). In most *Fusaria*, FB₁ is predominant, usually representing some 70% of the total fumonisin content. FB₂ and FB₃ usually account for up to 15–25% and 3–8%, respectively, while FB₄ is normally present in insignificant amounts (*14–16*).

The recent documentation of fumonisin B-series production by *A. niger* has raised the question of whether fumonisins might be an overlooked health risk due to the ubiquitous presence of *A. niger* on a wide range of food stuffs not usually contaminated with *Fusarium* species (*17*). This includes grapes and thereby raisins and wine, peanuts, coffee, tea, and several other products (*1*). Moreover, one could be concerned that industrial products such

as enzymes and fine chemicals could be contaminated with traces of fumonisins because of the wide application of *A. niger* as “workhorse” in industrial fermentations (*18–21*).

Our recent LC–HRMS based investigations of fumonisin production by *A. niger* and other black *Aspergilli* suggested that besides FB₂ and FB₄ *A. niger* can also produce an additional third fumonisin with the same elemental composition as FB₁ and iso-FB₁ but with a different retention time than the two latter compounds. The present study reports the isolation and characterization of this novel compound, which we named fumonisin B₆. In addition, we isolated and characterized FB₂ from *A. niger* by NMR in order to further validate that FB₂ from *A. niger* is indeed identical to that of FB₂ previously reported from several *Fusarium* species.

MATERIALS AND METHODS

Fungal Incubation. In a 10 L glass vessel, 2 L of still culture of 55% rice meal and 45% corn steep liquor (RC) medium was prepared according to Bullermann (*22*) but with a reduced amount of agar (1.5 g/L). It was inoculated with a spore suspension from 7 d old ex-type cultures of *A. niger* NRRL 326, ex-tannin-gallic acid fermentation (= IBT 27876 = ATCC 16888 = CBS 554.65) from CYA. After incubation for 14 d in darkness at 25 °C, the mycelia and media were homogenized with 2 L of MeCN/H₂O (50/50, v/v) and shaken overnight. After filtration the extract was concentrated on a rotary evaporator and freeze-dried.

Preparative Chromatography. The raw extract, 9.3 g, was initially subjected to flash chromatography on a 135 g, 50 μm C₁₈ material (Phenomenex, Torrance, CA) which was eluted with H₂O–MeOH mixtures in steps of 10%, starting from 10% MeOH. The 70% MeOH fraction (163 mg) was further enriched for fumonisins on a 1000 mg, 33 μm Strata X-C mixed-mode RP-cation-exchange material (Phenomenex), which was washed with MeCN and eluted with MeCN–H₂O (containing 4% NH₄OH) in steps of 20% from 4% NH₄OH. The fumonisin-containing fraction (20% MeCN, 19 mg), was finally subjected

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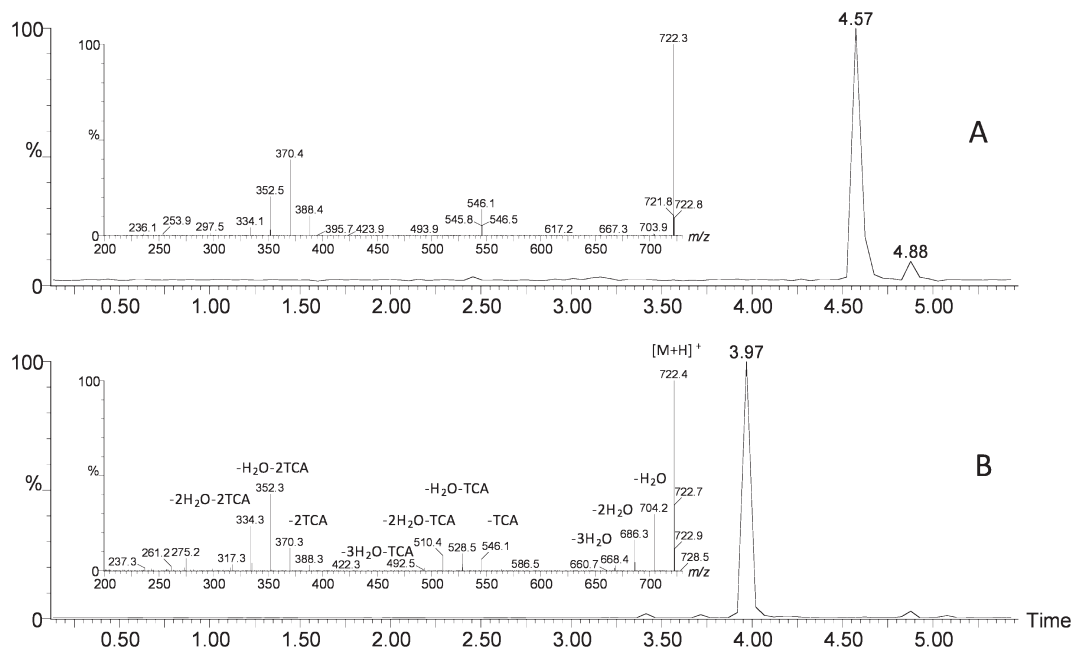


Figure 1. Daughter-ion chromatogram (m/z 722) showing FB_6 (4.57 min) and FB_1 (retention time = 3.97 min) and the corresponding daughter-ion spectra (25 eV) from the compound produced by *A. niger* (FB_6) sample (A) and an authentic FB_1 standard (B).

to RP separation on a 250 mm \times 10 mm i.d., 5 μm Luna C_{18} (II) column (Phenomenex) using an MeCN– H_2O gradient (starting at 30% MeCN + 50 ppm TFA increasing to 70% over 15 min). Fractions were collected automatically every 20 s and pooled for each chromatographic run after analysis of the content of each fraction by LC–MS had shown which fractions contained fumonisins B_6 (1.8 mg) and fumonisins B_2 (4.2 mg).

Analytical LC–MS. LC–DAD–HRMS was performed as described by Frisvad et al. (1) or by LC–tandem MS (MS/MS) on an Agilent 1100 liquid chromatograph (Waldbronn, Germany) coupled to a Quattro Ultima triple mass spectrometer (Micromass, Manchester, U.K.) with an ESI source. The separation was performed on a 50 mm \times 2 mm i.d., 3 μm Gemini C_6 -phenyl (Phenomenex) column fitted with a security guard system using a linear gradient starting from 20% MeCN in H_2O (both with 20 mM formic acid) to 55% MeCN for 6 min at a flow rate of 300 $\mu\text{L}/\text{min}$. MS/MS was performed using nitrogen as collision gas and making daughter ion scans of the protonated molecular ions (m/z 722 and 706), using fragmentation potentials from 15 to 50 V. Solvents were HPLC grade, and all other chemicals were analytical grade unless otherwise stated. Fumonisin standards (FB_1 and FB_2 mixture 50 $\mu\text{g}/\text{mL}$) used for MS analysis were acquired from Biopure (Tulln, Austria). FB_3 was from Chiron (Trondheim, Norway). FB_2 used for NMR analysis was from Alexis Biochemicals (Lausen, Switzerland).

NMR Spectroscopy. NMR spectra were recorded on a Bruker Avance 800 MHz spectrometer with a 5 mm TCI cryoprobe at the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules. The 2D DQF-COSY, NOESY, gHSQC, gH2BC, and gHMBC spectra were acquired using standard pulse sequences. The NMR data used for the structural assignment of the fumonisins were acquired in CD_3OD (δ_{H} 3.31 ppm and δ_{C} 49.15 ppm).

RESULTS AND DISCUSSION

In the current study, the ex-type culture of *A. niger* was used, as it has been found to be a strong producer strain of fumonisins (1). With no published protocols for purification of fumonisins from *A. niger*, we chose to use mixed-mode RP cation-exchange for purification. This was done because *A. niger* produces high amounts of acidic naphtho- γ -pyrones and organic acids, which would likely also bind to a strong anion-exchanger (SAX), which is the usual way to purify fumonisins (23).

Analytical LC–MS was used to compare the retention times and daughter-ion spectra of the purified *A. niger* fumonisin

analogues to those of authentic standards of FB_1 , FB_2 , and FB_3 . The daughter-ion spectra of the FB_2 standard and FB_2 from *A. niger*, eluting at the same time, were identical and consistent with previously reported ESI-MS/MS data (24–26), with losses of H_2O and TCA groups from the alkyl backbone dominating the spectra. This confirmed the presence of FB_2 or an isomer thereof in the *A. niger* extract. LC–HRMS analyses also showed a peak with the same elemental composition as FB_1 , however, eluting slightly later (Figure 1). LC–MS/MS also confirmed this and showed that it eluted 0.6 min after the authentic standard of FB_1 and 0.15 min before FB_2 on the C_6 -phenyl column. Significant differences in the daughter-ion spectra clearly showed that *A. niger* produces an FB_1 analogue and not FB_1 (Figure 1). The same was observed in the crude *A. niger* extract; thus, the FB_1 analogue was not an artifact of the purification process but proved to be the novel analogue fumonisin B_6 (FB_6). FB_6 displayed significantly less abundant losses of H_2O than FB_1 , which indicates the presence of adjacent hydroxyl groups blocking the hydrogens from detaching, thereby allowing the H_2O loss. This is in agreement with our previous findings during our studies of the fragmentation patterns of 474 microbial metabolites using LC–MS ESI $^+$ and ESI $^-$ MS (27), where we have never observed H_2O losses unless a β -hydrogen could be lost because of double bond formation.

NMR Based Structural Investigations. To further validate that the FB_2 purified from *A. niger* is identical to that produced by *Fusarium*, 1D and 2D homo- and heteronuclear NMR spectra of the purified *A. niger* fumonisin FB_2 were carefully inspected and compared to those of an authentic standard of FB_2 . The NMR data obtained in methanol- d_4 displayed comparable chemical shifts for the 34 expected ^{13}C NMR chemical shift values except for the resonances originating from the two TCA side chains and their sites of attachment to the fumonisin backbone. In this region, deviations in the ^{13}C NMR chemical shifts of between -1.8 to $+0.3$ ppm were observed. These results could be explained by a difference in the structural fold of the FB_2 between the two samples, likely to be caused by differences in pH and thereby overall level of protonation of the four carboxylic acid functionalities. The fact that FB_2 does have a unique globular

Table 1. NMR Data for Fumonisin B₆, B₁, and Iso-B₁

ID	fumonisin B ₆				fumonisin B ₁ (16)		fumonisin iso-B ₁ (29)	
	H (ppm)	C (ppm)	HMBC ^c	H2BC ^c	H (ppm)	C (ppm)	H (ppm)	C (ppm)
1	1.34 (d, 7.0)	16.3	2, 3	2	1.27 (d, 6.7)	16.0	1.30 (d, 6.8)	14.2
2	3.67 (qd, 2.0, 7.0)	50.0	1, 3	1	3.14 (dq, 6.7, 6.8)	53.7	3.43 (dq, 4.3)	51.8
3	3.64 (dd, 2.0, 7.1)	71.8	1, 4		3.74 (ddd, 9.6, 6.8, 3.2)	70.3	3.55 (dd, 4.0)	73.2
4	3.46 (dd, 1.8, 7.1)	74.9	2, 5		1.55 (m)	41.7	3.65 (m)	72.2
5	3.71 (ddd, 1.8, 5.1, 8)	71.4			3.84 (m)	68.4	1.45	34.2
							1.55	
6	1.53 (m)	34.8			1.40–1.60	39.1	1.34 (br m)	30.6
	1.59 (m)							
7 ^b	1.26–1.37	30.6	4, 8		1.30–1.50	26.6	1.38	26.3
							1.54	
8 ^b	1.26–1.37	30.6			1.30–1.50	26.7	1.32	26.5
							1.52	
9 ^b	1.26–1.37	30.6	10		1.40–1.60	39.0	1.4	38.9
10	1.25	27.5	9		3.62 (m)	69.8	3.62 (m)	70.0
11	1.04 (m)	36.1			1.15 (m)	44.6	1.08	44.5
	1.40 (m)				1.45 (m)		1.5	
12	1.50 (m)	29.9	15	21	1.81 (m)	26.9	1.83 (m)	27.0
13	1.43 (m)	36.1	14, 16, 21	12	1.55 (m)	36.6	1.45	37.0
	1.62 (m)			14	1.70 (m)		1.66	
14	5.18 (ddd, ~3, ~4, 10.9) ^a	72.8			5.16 (ddd, 10.8, 3.7, 2.4)	72.8	5.16 (ddd, 11, 3, 4)	72.7
15	4.92 (dd, 3.5, 8.3)	78.7	13, 14, 16, 17, 22, 29		4.94 (dd, 8.1, 3.7)	78.7	4.94 (dd, 4, 8)	78.9
16	1.70 (m)	34.7	15	15, 22	1.70 (m)	34.8	1.70 (m)	34.9
17	1.09 (m)	32.9	16, 22		1.07 (m)	32.9	1.08	32.9
	1.44 (m)				1.44(m)		1.43	
18	1.18 (m)	29.5			1.18 (m)	29.5	1.18	29.8
	1.13 (m)				1.33 (m)		1.33	
19	1.31 (m)	23.7	17, 20	20	1.10 (m)	23.9	1.22	23.9
	1.26 (m)				1.40 (m)		1.38	
20	0.89 (t, 7.0)	14.2	18, 19		0.89 (t, 7.1)	14.4	0.89 (t, 7)	14.4
21	0.92 (d, 6.7)	20.8	12, 13	12	0.96 (d, 6.7)	20.8	0.96 (d, 6.7)	20.8
22	0.94 (d, 6.9)	15.7	14, 16	16	0.94 (d, 6.8)	16.0	0.93 (d, 6.8)	16.1
23		172.9				173.5		173.5
24	2.51 (dd, 6.7, 17.0)	36.3	23, 25, 26, 28		2.45 (dd, 16.7, 7.4)	37.1	2.45	37.3
	2.72 (dd, 7.2, 17.0)		25, 26, 27		2.71 (dd, 16.7, 7.4)		2.71	
25	3.15 (m)	38.7	23, 24, 28		3.10–3.20 (m)	39.6	3.14	39.7
26	2.55 (dd, 5.9, 17.0)	36.3	24, 25, 27		2.50 (dd, 16.5, 5.2)	37.8	2.48	38.0
	2.68 (dd, 7.5, 16.8)		25, 27		2.66 (dd, 16.5, 8.3)		2.64	
27		175.4				177.4		177.2
28		177.1				178.5		178.7
29		172.9				173.0		173.2
30	2.64 (dd, 6.3, 16.7)	36.3	29, 31, 32, 34		2.60 (dd, 16.6, 6.4)	36.9	2.59	37.0
	2.80 (dd, 7.2, 16.9)		29, 31, 32, 34		2.79 (dd, 16.6, 7.2)		2.79	
31	3.16 (m)	38.7	29, 30, 34		3.10–3.20 (m)	39.7	3.14	39.6
32	2.58 (dd, 6.2, 16.9)	36.1	30, 31, 33		2.52 (dd, 16.5, 6.0)	37.5	2.51	37.7
	2.74 (dd, 7.2, 16.9)		31, 33		2.72 (16.5, 7.5)		2.7	
33		175.2				177.0		176.7
34		176.8				178.0		178.2

^a Only approximate *J* coupling constants (in Hz) due to overlap in the region. ^b Ambiguous assignment due to total overlap in the region. ^c HMBC and H2BC connectivities are from the assigned proton to the indicated carbon atom(s).

folded structure has previously been addressed by Beier and Stanker (28). In order to ensure that the two samples had been subjected to the same purification protocol, the standard FB₂ sample was run over a cation exchange column, in the same way as the last purification step for the isolated *A. niger* FB₂ sample. This proved to be very important, since the spectra obtained for FB₂ from *Fusarium* after this procedure were now identical to those from *A. niger* FB₂, confirming that only minor changes in the sample preparation (e.g., pH) can have a large effect on the structural fold of the fumonisin molecules and thereby spectroscopic output. Comparisons of the *J* coupling constants for the protons in the amino terminal part of the FB₂ (C2–C5) and the attachments of the TCA side chains (C14 and C15), from *A. niger* and *Fusarium*, showed that they are of the same size, proving that the relative stereochemistry for these parts of the FB₂ backbone is

the same for *Aspergillus* and *Fusarium*. On the other hand the stereochemistry at positions C12 and C16 could not be elucidated on the basis of the data available because of chemical shift overlaps. The optical rotation of FB₂ from *A. niger* ([α]_D²⁰ –11.36 (*c* 1 mg/mL, MeOH)) was similar to that known from *Fusarium*; however, with several stereocenters in the molecule this is not a direct proof of identical stereochemistry for FB₂ originating from the two different genera.

Altogether, the presented data point toward identical stereochemistry of the two FB₂ molecules; however, a genetic comparison of the fumonisin gene cluster in the two different genera revealed several differences in the placement and orientation of the different involved synthase genes (2). This could lead to structural differences in the polyketide synthases and thereby the final stereochemistry of fumonisins produced by *A. niger*,

	M _r	R ₁	R ₂	R ₃
FB ₁	721.3884	H	OH	OH
Iso-FB ₁	721.3884	OH	H	OH
FB ₂ (2)	705.3935	H	OH	H
FB ₃	705.3935	H	H	OH
FB ₄	689.3986	H	H	H
FB ₆ (1)	721.3884	OH	OH	H

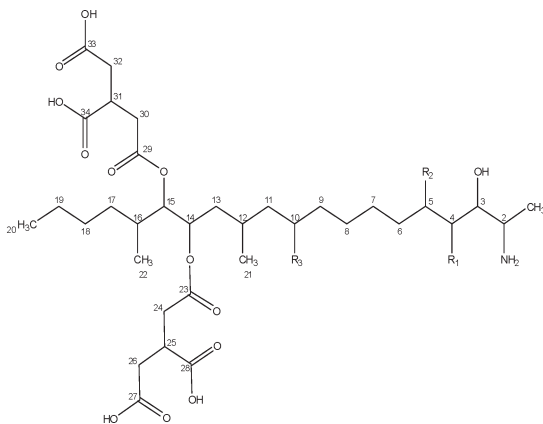


Figure 2. Fumonisin structures and numbering scheme.

which is why we conclude that further structural studies such as X-ray analysis need to be performed to establish the absolute stereochemistry of FB₂ from *A. niger*.

NMR studies of FB₆ substantiated the MS analyses, indicating a new fumonisin of the B type. Unambiguous assignments of the proton and carbon resonances of FB₆ could be obtained for most of the resonances. However, spectral overlap hampered some specific assignments in the alkane-like part of the molecule. The assignment was compared to literature values for fumonisins (16, 29). A series of 2D DQF-COSY, HSQC, HMBC, and H2BC experiments established partial assignment of the backbone as well as the TCA side chains present in the structure. Thirty-four carbon resonances could be identified comprising four CH₃ groups, 14 CH₂ groups, 10 CH groups, and 6 carboxyl carbon resonances (Table 1). The two TCA side chains bound to the backbone structure could be assigned as two separate spin systems C23–C28 and C29–C34 (Figure 2) with only minor differences in their chemical shifts. The attachment of the C29–C34 TCA side chain to the backbone structure could be confirmed by the presence of an HMBC correlation between H15 and C29, whereas the position of the C23–C28 TCA side chain is based on the chemical shift of H14/C14 and comparison with the assignment obtained for FB₁ and iso-FB₁. Unambiguous assignments of the fragments C1–C6 and C10–C20 were obtained. In Table 1, a comparison of the chemical shifts of FB₆ to FB₁ and iso-FB₁ can be seen. Comparing the resonance assignment of FB₆ to FB₁ and iso-FB₁ showed only minor deviations between the structures except at the positions of OH attachment in the backbone structure. Where FB₁ and iso-FB₁ have OH groups attached to C3, C5, and C10 and to C3, C4, and C10, respectively, the new fumonisin FB₆ has OH groups at C3, C4, and C5 (Figure 2). The chemical shift values of 1.25 ppm for H10 and 27.5 ppm for C10, DQF-COSY connectivities from H 11 to H 10 and HMBC correlations from H 9 to C10 and from H 10 to C9, confirm that no OH group is bound at this position due to the large upfield shift of the resonances when compared to FB₁, where H 10 and C10 resonate at 3.62 and 69.8 ppm, respectively. In the amino terminal portion of the backbone structure, DQF-COSY connectivities can be traced along the chain from H3 at 3.64 ppm, H4 at 3.46 ppm, and H5 at 3.71 ppm to H6 at 1.53/1.59 ppm. In addition, HMBC correlations can be seen from H4 to C2/C5 and from H3 to C4, confirming the presence of the third OH group at C4 in the structure. The chemical shift obtained for H4/C4 of 3.46 ppm/74.9 ppm compared to 3.65 ppm/72.2 ppm as seen in iso-FB₁ and equivalently for H5/C5 of 3.71 ppm/71.4 ppm compared to 3.84 ppm/68.4 ppm for FB₁ is in agreement with the proposed structure for FB₆.

Altogether, the analysis of these data proves FB₆, with hydroxyl functionalities at C3, C4, and C5, to be a structural isomer of FB₁ and iso-FB₁. As can be seen in Figure 2 FB₆ and iso-FB₁ have a hydroxyl at C4, which is rare for the B-series fumonisins and only previously seen for iso-FB₁ (29). As is the case for FB₂, the absolute stereochemistry of FB₆ still needs to be resolved, which is work that is ongoing in our laboratory. Analysis of several *A. niger* strains in our laboratory (results not shown) has shown that the frequencies of production of fumonisins in *A. niger* on average are 100% (FB₂), 10–25% (FB₄), and 5–10% (FB₆). Testing of the toxicological properties of not only FB₂ and FB₆ but also FB₄, including investigations of the different conformations that we have seen for these *A. niger* fumonisins, is also in progress in our laboratory.

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