

Structure and Natural Occurrence of Stereoisomers of the Fumonisin B Series Mycotoxins

WENTZEL C. A. GELDERBLOM,^{†,‡} VIKASH SEWRAM,[†] GORDON S. SHEPHARD,[†]
 PETRA W. SNIJMAN,[†] KENNY TENZA,[§] LIANA VAN DER WESTHUIZEN,[†] AND
 ROBERT VLEGGAR^{*,§}

Programme on Mycotoxins and Experimental Carcinogenesis, Medical Research Council, P.O. Box 19070, Tygerberg 7505, Department of Biochemistry, University of Stellenbosch, Stellenbosch 7600, and Department of Chemistry, University of Pretoria, Pretoria 0002, South Africa

¹H and ¹³C NMR spectroscopy of both fumonisin B₃ and B₄, as well as high-performance liquid chromatography (HPLC) analysis of samples of fumonisin B₃ used as standards, showed in each case the presence of two stereoisomers, which could not be separated by preparative chromatography. The 2,3-*anti* relative configuration for the two minor stereoisomers of fumonisin B₃ and B₄ was deduced from the NMR data, and their 2*S*,3*R* absolute configurations were established by application of Mosher's method using the fumonisin B₃ sample. Samples of fumonisin B₃ and B₄ can contain between 10 and 40% of fumonisin B compounds of the 3-*epi* series. The 3-*epi*-FB₃, determined by HPLC with fluorescence detection of the *o*-phthaldialdehyde derivative and confirmed by liquid chromatography–tandem mass spectrometry, was found to occur naturally in a range of maize samples at levels much lower than FB₃ (<20%). The identification of members of the 3-*epi*-fumonisin B series provides insight into the order and selectivity of steps in fumonisin biosynthesis.

KEYWORDS: Fumonisin; corn; NMR; LC-MS; biosynthesis

INTRODUCTION

The fumonisins of the B series, for example, B_{1–4} (**1–4**) (see **Figure 1**), are a family of structurally related mycotoxins first isolated from cultures of *Fusarium verticillioides* (strain MRC 826) (1, 2). These secondary metabolites are common contaminants of corn throughout the world (3) and the causative agents of equine leucoencephalomalacia (4) and porcine pulmonary edema (5). The carcinogenic nature of these mycotoxins in rodent studies (6, 7), their association with human esophageal cancer (8), and the recent demonstration of their possible role in neural tube defects (9, 10) have heightened international concern over their natural occurrence. The International Agency for Research on Cancer has recently declared FB₁, the most abundant of the fumonisins, to be a group 2B carcinogen (i.e., possibly carcinogenic to humans) (11). Since the initial discovery of FB₁ (**1**) and FB₂ (**2**) in 1988, the number of known fumonisin analogues has greatly increased and a recent review of this family of compounds listed 28 members (12). The fumonisins are generally divided into the A, B, C, and P series. The significance of many of these analogues as natural contaminants of food is uncertain, and reports of fumonisin analysis are frequently restricted to FB₁ (**1**) and FB₂ (**2**), although some

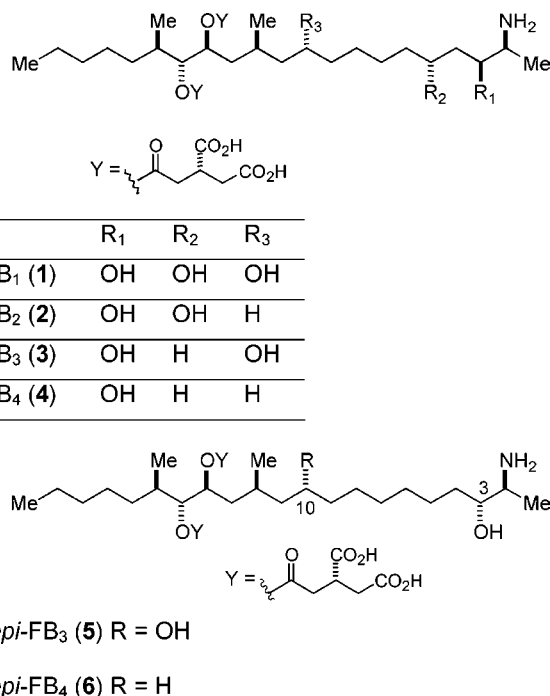


Figure 1. Chemical structures of the fumonisins.

reports include FB₃ (**3**). The necessity for including FB₃ in total fumonisin analysis has recently been highlighted by the inclusion

* To whom correspondence should be addressed. Tel: +27 12 420-3095. Fax: +27 12 362-5297. E-mail: robert.vleggaar@up.ac.za.

[†] Medical Research Council.

[‡] University of Stellenbosch.

[§] University of Pretoria.

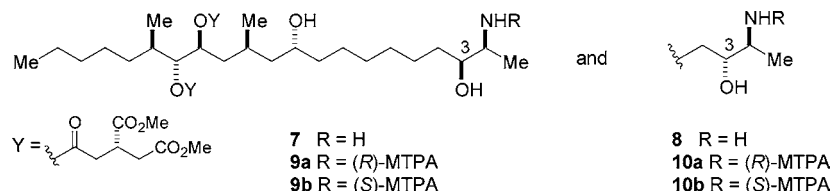


Figure 2. Chemical structures of FB₃ and 3-*epi*-FB₃ tetramethyl esters and their MTPA derivatives.

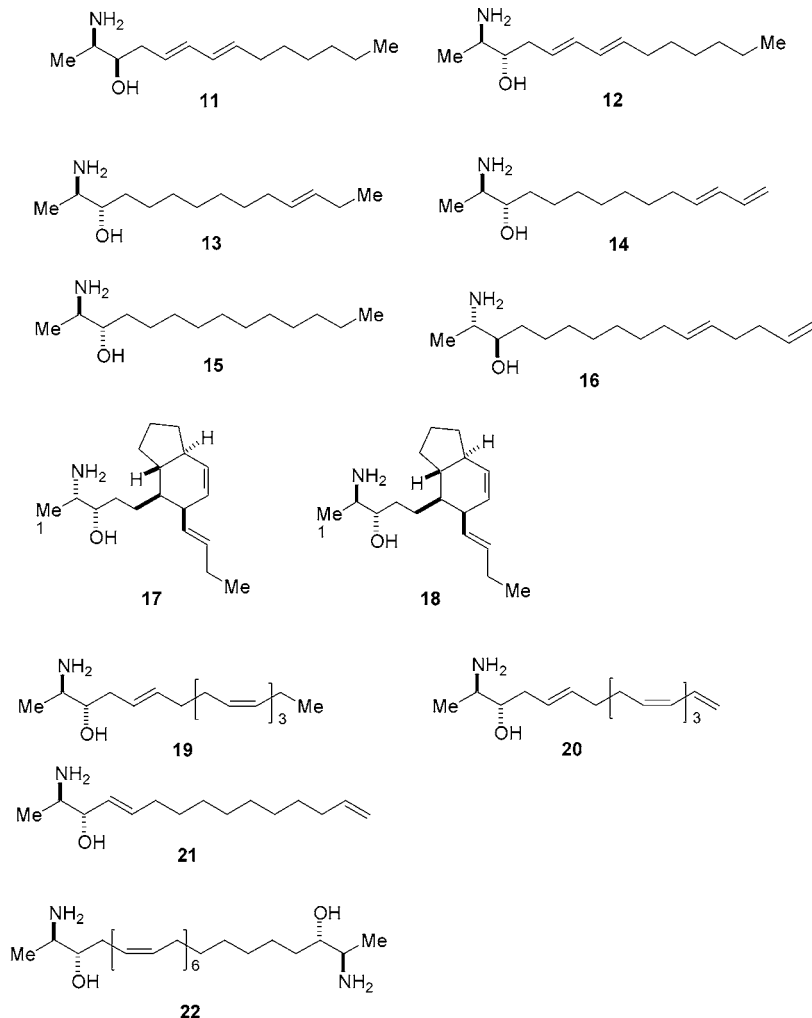


Figure 3. Chemical structures of natural products with the terminal 2-amino-3-hydroxy motif.

of all three analogues in the setting of Food and Drug Administration guidelines for industry in the United States (13) as well as in the provisional maximum tolerable daily intake determined in the recent risk assessment of fumonisins conducted by the Joint FAO/WHO Expert Committee on Food Additives (14).

Analytical standards of FB₁ (1), FB₂ (2), and FB₃ (3) have been prepared by scientists at the MRC, Tygerberg, South Africa, on a commercial basis since the early 1990s. Analysis of fumonisin B₃ samples obtained from different batches of *F. verticillioides* (MRC 826) has recently shown the presence of 10–40% of a stereoisomer of FB₃ that elutes immediately prior to FB₃ in the analytical reversed-phase high-performance liquid chromatography (HPLC) chromatogram of the *o*-phthalaldehyde (OPA)-derivatized standards. In this paper, we report on the identification and absolute configuration of the stereoisomers 3-*epi*-FB₃ (5) and 3-*epi*-FB₄ (6) (Figure 1) present in samples of FB₃ and FB₄, respectively, and the natural occurrence of 3-*epi*-FB₃ (5).

MATERIALS AND METHODS

HPLC–Tandem Mass Spectrometry (MS-MS) Analyses of Fumonisin Standards. The fumonisin standards (FB₁, FB₂, FB₃, and FB₄) were isolated from corn cultures of *F. verticillioides* as described by the method of Cawood et al. (2). The isolated FB₃ standard and the dried residues of corn sample extracts, prepared as described below for determination by fluorescence detection, were dissolved in acetonitrile–water–formic acid (10:90:0.1). The two stereoisomers of FB₃ were separated by binary gradient reversed-phase HPLC on a Luna C₁₈ column (Phenomenex, Torrance, CA). The individual elution solvents were mixtures of water–acetonitrile–formic acid in the ratios (90:10:0.1; solvent A) and (10:90:0.1; solvent B), respectively. The mobile phase was pumped at a flow rate of 0.7 mL/min. The initial composition of 80% solvent A was adjusted linearly to 75% solvent A over 35 min. The fumonisins were detected by mass spectrometry using a Finnigan MAT (San Jose, CA) LCQ ion trap instrument with positive ion electrospray (ES) ionization. The HPLC eluate was passed directly into the MS at a source voltage of 4.5 kV and a capillary voltage of 40 V. The heated capillary temperature was maintained at 220 °C, and the sheath to auxiliary gas ratio was set at 4:1. Fumonisin were

Table 1. NMR Data for the Normal and 3-*epi* Series of Fumonisin B₃ and B₄^a

atom	FB ₃ (3)		3- <i>epi</i> -FB ₃ (5)	
	δ _C	δ _H	δ _C	δ _H
1	15.61 Q	1.113 d (J 6.7)	12.01 Q	1.054 d (J 6.7)
2	51.36 D	2.956 qd (J 6.7, 6.8)	50.63 D	3.125 qd (J 6.8, 3.1)
3	71.08 D	3.336 m	69.65 D	3.568 m
4	32.90 T		32.46 T	

atom	FB ₄ (4)		3- <i>epi</i> -FB ₄ (6)	
	δ _C	δ _H	δ _C	δ _H
1	15.42 Q	1.120 d (J 6.6)	11.93 Q	1.060 d (J 6.7)
2	51.23 D	2.957 qd (J 6.7, 6.7)	50.56 D	3.120 qd (J 6.8, 3.1)
3	70.96 D	3.33 m	69.57 D	3.58 m
4	32.75 T		32.37 T	

^a Solvent DMSO-*d*₆. NMR analyses were performed on mixtures of stereoisomers of FB₃ and FB₄.

Table 2. ¹H NMR Data for the MTPA Amides (δ, CDCl₃)

compound	atom	(<i>S</i>)-MTPA (b)	(<i>R</i>)-MTPA (a)	Δδ (δ _S - δ _R)
9	H(1)	1.110	1.179	-0.069
	H(3) ^a	3.49	3.47	+0.02
10	H(1)	1.025	1.095	-0.070
	H(3) ^a	3.60	3.54	+0.06

^a Chemical shift values obtained from COSY and HETCOR data.

monitored by full-scan MS-MS between *m/z* 330 and *m/z* 730. A collision energy of 32% was used to fragment the molecular ions, and the resultant product ions were monitored as diagnostic indicators for the presence of the toxins.

NMR Analysis. NMR spectra were recorded on a Bruker Avance-500DRX spectrometer operating at 500 MHz for ¹H and 125 MHz for ¹³C nuclei using standard Bruker pulse sequences. Spectra acquired for DMSO-*d*₆ solutions were referenced to the signals at δ_H 2.49 and δ_C 39.50 and for spectra in CDCl₃ to the signals at δ_H 7.24 and δ_C 77.00.

Determination of Fumonisin Analogues by HPLC with Fluorescence Detection. Determination of fumonisin analogues in corn was conducted by the method of Sydenham et al. (15). Briefly, corn samples were homogenized in methanol–water (3:1), centrifuged, and filtered. An aliquot of the clear extract was applied to a strong anion exchange solid-phase extraction cartridge (Varian, Harbor City, CA) and washed with methanol, and the fumonisins were eluted with 1% acetic acid in methanol. The purified extracts were dried down under nitrogen at 60 °C. Immediately prior to analysis, the residues were redissolved in methanol and an aliquot was derivatized with OPA. The fumonisin analogues were separated and detected by reversed-phase HPLC (Ultracarb 5 ODS column; Phenomenex) with fluorescence detection (Figure 4). The mobile phase was pumped at 1 mL/min and consisted of methanol–sodium dihydrogen phosphate (0.1 M, pH 3.3) (77:23) as generally used for fumonisin determination.

Preparation of the FB₃ Derivatives. Fumonisin B₃ Tetramethyl Ester. A solution of FB₃ standard (549 mg) containing a mixture of FB₃ (3) and 3-*epi*-FB₃ (5) was treated with an excess of a diethyl ether solution of diazomethane (prepared from 4.28 g of Diazald). After 10 min, the excess diazomethane was evaporated in a stream of nitrogen and the residue was dried in vacuo to give the tetramethyl esters **7** and **8** (590 mg, 99%) (Figure 2).

Preparation of the Mosher Amide Derivatives. Oxalyl chloride (220 mg, 1.74 mmol) was added to a solution of (*R*)-(-)-MTPA (e.e. ≥99%, 82 mg, 0.35 mmol) and dimethyl formamide (DMF) (25.4 mg, 0.35 mmol) in hexane (5 mL) at room temperature. A white precipitate formed immediately. After 60 min, the mixture was passed through a small cotton plug to filter off the formed DMF–Cl. The filtrate was concentrated under reduced pressure to yield the (*S*)-MTPA chloride.

Table 3. Fumonisin Levels in Samples of Corn and Commercial Corn Meal

sample	fumonisin levels (μg/kg) ^a					% <i>epi</i> /FB ₃
	FB ₁	FB ₂	FB ₃	<i>epi</i> -FB ₃	total	
commercial corn meal	380	100	30	2	512	6.7
	625	215	60	5	905	8.3
	240	70	15	2	327	13
	265	80	25	4	374	16
Brazilian corn	1960	720	370	45	3095	12
	4515	255	665	95	5530	14
	3635	900	320	60	4915	19
	2405	1985	400	75	4865	19
Transkei home-grown, good corn	2350	540	200	35	3125	18
	1370	465	120	20	1975	17
	2980	780	215	45	4020	21
Transkei home-grown, moldy corn	26240	10070	2490	200	39000	8.1
	2665	830	165	25	3685	15
	12480	6860	1740	285	21365	16
	5035	2050	495	75	7655	15
	5365	2025	445	30	7865	6.7

^a FB₄ was not detected.

A solution of the tetramethyl esters **7** and **8** (265 mg, 0.35 mmol), Et₃N (352 mg, 3.48 mmol), and DMAP (5 mg) in CH₂Cl₂ (5 mL) was added to a solution of the (*S*)-MTPA chloride in CH₂Cl₂ (2 mL) solution. The mixture was stirred for 30 min and then quenched by addition of water (2 mL). The organic solution was washed with 0.5 M HCl, followed by saturated NaHCO₃ solution and water. The CH₂-Cl₂ solution was dried (Na₂SO₄), filtered, and evaporated. The product was purified by column chromatography on silica gel with EtOAc–hexane (3:2) as the eluent to give the (*R*)-Mosher amide derivative (180 mg, 59%), an oil, as a mixture of two diastereomers (**9a** and **10a**) (d.r. 82:18); *R_f* 0.34.

The same protocol was followed but using (*S*)-(+)-MTPA (e.e. ≥99%), to convert a sample of the tetramethyl esters **7** and **8** (265 mg, 0.34 mmol) to a mixture of the two diastereomeric (*S*)-Mosher amide derivatives (**9b** and **10b**) (214 mg, 63%) (d.r. 82:18) (Figure 2).

RESULTS AND DISCUSSION

The terminal 2-amino-3-hydroxy motif of the fumonisin C₂₀ backbone is also present in a number of marine natural products (see Figure 3). In 1989, Gulavita and Scheuer (16) isolated two epimeric aliphatic amino alcohols from a Papua New Guinea sponge, *Xestospongia* sp., and proposed their structures as (2*S*,3*S*,5*E*,7*E*)- and (2*S*,3*R*,5*E*,7*E*)-2-aminotetradeca-5,7-dien-3-ol, *ent*-(**11**) and *ent*-(**12**), respectively. The relative stereochemistry followed from nuclear Overhauser effect studies on the oxazolidinone derivative and the absolute configuration at C(2) by degradation of the diacetyl derivatives to alanine and HPLC analysis of the derivative formed with 1-fluoro-2,4-dinitrophen-5-yl-(2*S*)-alanine amide. Mori and Matsuda (17) reported the total synthesis of both enantiomers of **11** and **12** and assigned the enantiomeric stereochemistry to the natural products isolated by Gulavita and Scheuer (16), since the 2*R* stereoisomers showed the same sign of optical rotation as the natural products. The stereochemistry of the xestoaminols A (**13**), B (**14**), and C (**15**) isolated by Jiménez and Crews (18) from two different sponges of the genus *Xestospongia* was determined by Garrido et al. (19) in their study on the obscuraminols, for example, obscuraminol C (**16**) obtained from the tunicate *Pseudodistoma obscurum*. Sata and Fusetani (20) isolated two new cytotoxic stereoisomeric 2,3-amino alcohols, amaminol A (**17**) and B (**18**), from an unidentified tunicate of the family Polyclinidae. The stereochemical relationship between these two compounds, as in the case of the amino alcohols **11** and **12** isolated by Gulavita and Scheuer (16), is of particular

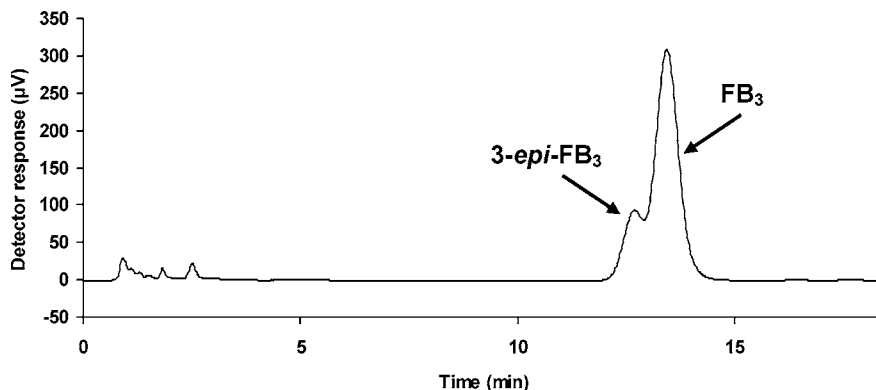


Figure 4. HPLC chromatogram with fluorescence detection of the OPA derivatives of the isolated FB_3 standard showing the relative areas of the two stereoisomers.

significance to the structure elucidation of the minor metabolites of the fumonisins. A detailed analysis of the ^1H NMR spectra of **17** and **18** disclosed that the relative stereochemistry of the C(2) and C(3) stereogenic centers differed as indicated by the coupling constant between H(2) and H(3) [$J_{2,3}$ 3.1 Hz for (**17**) vs 7.3 Hz for (**18**)]. The Mosher ester analysis (21–23) indicated that the two compounds had the same 3*S* absolute configuration and were epimeric at C(2). This is in direct contrast to the results obtained by Gulavita and Scheuer (16) on the amino alcohols **11** and **12**, which are C(3) epimers. The absolute configuration of the crucigasterins **19**–**21**, isolated by Jarez et al. (24) from a Mediterranean tunicate *Pseudodistoma crucigaster*, was assigned by chemical degradation to (3*S*,4*R*)-3-hydroxy-4-aminopentanoic acid. The *anti* stereochemistry of the 2-amino-3-hydroxy group of the crucigasterins is reflected by the 3.0 Hz coupling constant for the C(2) and C(3) protons. Analysis of the ^{13}C NMR data reported by Gulavita and Scheuer (16) and Mori and Matsuda (17) in the work on the amino alcohols **11** and **12** and by Sata and Fusitani (20) on the amaminols **17** and **18** showed characteristic chemical shift values for the methyl group of the *syn*- and *anti*-2,3-amino alcohols. Thus, the methyl group, C(1), in the diacetyl derivative of **11** appeared at δ_{C} 18.49 (*syn*) whereas in the diacetyl derivative of **12** it appeared at δ_{C} 14.93 (*anti*). The same trend is observed for the amaminols: δ_{C} 16.0 for the *syn* compound (**11**) and δ_{C} 12.1 for the *anti* compound (**12**). The *anti*-amino alcohol (**22**) (**25**) showed the methyl groups at δ_{C} 12.1 and 11.9, whereas in the *syn*-amino alcohols such as fumonisin B₃ (**3**) and B₄ (**4**) the methyl group appears at δ_{C} 15.61 and 15.42. It is thus evident that the ^{13}C chemical shift values for the methyl group, C(1), define the relative stereochemistry of the 2,3-amino alcohol moiety in these compounds.

Fast atom bombardment and ES mass spectrometry of FB_3 samples, which exhibited two peaks in the HPLC analysis, showed the $[\text{M} + \text{H}]^+$ ion at 706 and established the molecular formula $\text{C}_{34}\text{H}_{59}\text{NO}_{14}$ for each of the two compounds. The ^1H and ^{13}C NMR spectra showed in all cases the presence of a minor component 3-*epi*- FB_3 (**5**). The use of two-dimensional correlation spectroscopy (COSY) and heteronuclear correlation (HETCOR) experiments established that the discernible signals of the minor component in the ^1H and ^{13}C spectra represent the C(1)–C(4) unit of the backbone. The coupling constant of 6.8 Hz between the C(2) and the C(3) protons of FB_3 is characteristic of the *syn*-2,3-amino alcohol in these metabolites. The *anti* stereochemistry for the 2,3-amino alcohol unit of the minor metabolite 3-*epi*- FB_3 (**5**) followed from the 3.1 Hz coupling observed for the C(2) and C(3) protons (Table 1). The ^{13}C chemical shifts for the methyl group in fumonisin B₃ (δ_{C}

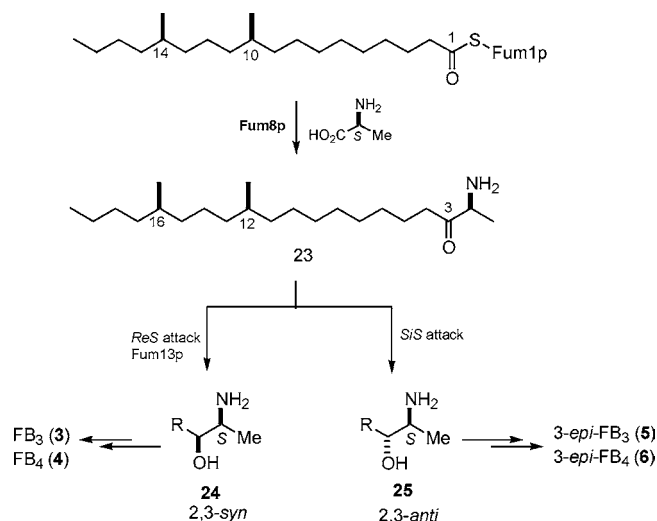


Figure 5. Biosynthetic formation of the fumonisins and the 3-*epi* stereoisomers.

15.61Q) and the minor component 3-*epi*- FB_3 (**5**) (δ_{C} 12.01Q) confirmed the *anti*-2,3 stereochemistry of the latter.

The ^1H and ^{13}C NMR spectra of samples of fumonisin B₄ (**4**) isolated over the years sometimes also showed the presence of up to 40% of a minor metabolite 3-*epi*- FB_4 (**6**) with the 2,3-*anti* stereochemistry (Table 1). The *anti* stereochemistry of 3-*epi*- FB_4 (**6**) followed once again from the coupling constant of 3.1 Hz for the C(2) and C(3) protons and the ^{13}C chemical shift value of 11.93 for the methyl group, C(1) (see Table 1). In contrast, the ^1H and ^{13}C NMR spectra of samples of FB_1 (**1**) and FB_2 (**2**) prepared by us have always lacked the signals for the corresponding 3-*epi* stereoisomers with the *anti*-2,3 stereochemistry.

The absolute configuration of the C(2) and C(3) stereogenic centers of FB_{1-4} has been established as (2*S*,3*S*) (26–32), and consequently, the minor metabolites 3-*epi*- FB_3 (**5**) and 3-*epi*- FB_4 (**6**) must either have the (2*S*,3*R*) or (2*R*,3*S*) configuration. The C(2) absolute configuration for the FB_3 minor metabolite, 3-*epi*- FB_3 (**5**), was determined using Mosher methodology (21–23). A sample of FB_3 (**3**) [containing 18% of the minor metabolite 3-*epi*- FB_3 (**5**)] was converted to the tetramethyl ester derivatives **7** and **8** (minor) by treatment with an excess of diazomethane. The amino group was then converted to the (*R*)- and (*S*)-MTPA amides (**7a,b** and **8a,b**) (minor), respectively, by reaction with the (*S*)- and (*R*)-MTPA chlorides [prepared from the (*R*)- and (*S*)-MTPA acid, respectively, following the protocol developed by Ward and Rhee (33)]. The $\Delta\delta$ ($\delta_{\text{S}} - \delta_{\text{R}}$) values observed for H(1) (−0.069 ppm) and H(3) (+0.02

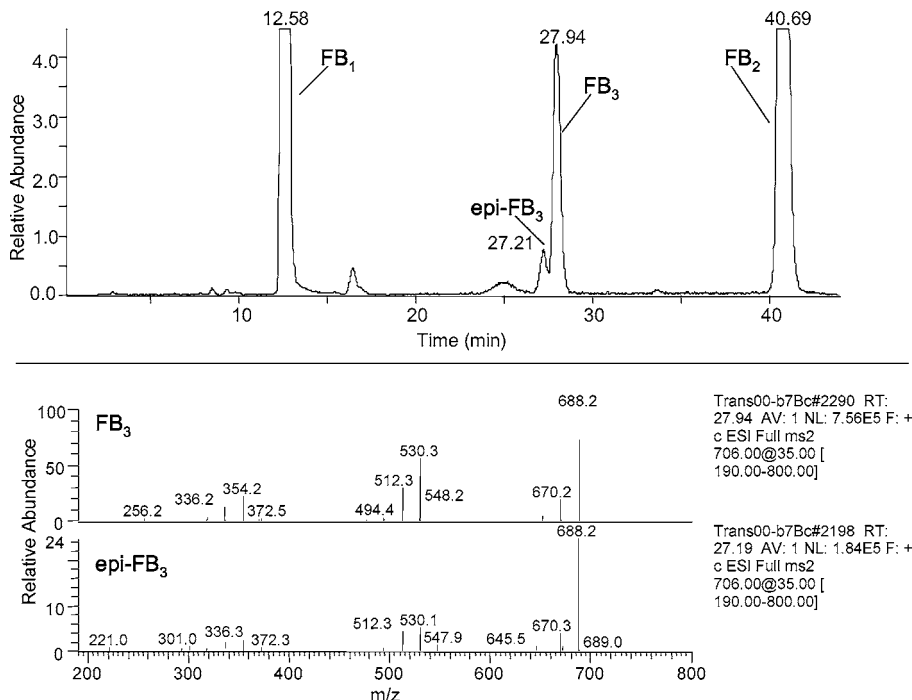


Figure 6. Total ion chromatogram of a Transkeian moldy corn sample and mass spectra of the protonated FB_3 and 3-*epi*- FB_3 analogues.

ppm) of the Mosher amide derivatives (**9**) (see **Table 2**) are in agreement with the 2*S* absolute configuration of the fumonisins. The fact that similar values are observed for the corresponding protons of the Mosher amide derivatives (**10**) implies that the minor metabolite 3-*epi*- FB_3 (**5**) present in samples of FB_3 (**3**) also has the 2*S* absolute configuration and therefore on the basis of its 2,3-*anti* relative stereochemistry, the 3*R* absolute configuration. As a consequence of this result, the assignment of the 2*S*,3*R* absolute configuration to the minor metabolite 3-*epi*- FB_4 (**6**) present in samples of fumonisin B₄ (**4**) is reasonable.

Biosynthetic studies on the fumonisins using [1- ^{13}C]-, [2- ^{13}C]-, and [1,2- $^{13}\text{C}_2$]acetate, [methyl- ^{13}C]-(*2S*)-methionine, and [3- ^{13}C]-(*2S*)-alanine precursors established that C(3)–C(20) are derived from acetate and C(1), C(2), and the 2-NH₂ group are derived from alanine. The C(12) and C(16) methyl groups are derived from methionine (34–38). An acetate-derived carbonyl group is the source of the C(3) hydroxyl group, whereas the C(5), C(10), C(14), and C(15) hydroxyl groups are likely derived from molecular oxygen (39). The citric acid cycle is believed to be the source of the tricarballic acid moiety (37). Recently, a 15-gene cluster (*FUM1*–*FUM3*, *FUM6*–*FUM8*, *FUM10*, *FUM11*, and *FUM13*–*FUM19*) required for the biosynthesis of the fumonisins in *F. verticilloides* was cloned and characterized (40–42). The data obtained from direct studies of these biosynthetic genes by genetic and biochemical approaches (40–48) provided the basis for a biosynthetic pathway (46–48). The biosynthesis starts with the formation of a dimethylated, saturated C₁₈ polyketide chain catalyzed by the Fum1p polyketide synthase (see **Figure 5**). The carbon–carbon bond formation between the (*2S*)-alanine and the enzyme-bound C₁₈ polyketide chain by the pyridoxal-phosphate-dependent aminoacyl transferase enzyme Fum8p, encoded by the *FUM8* gene (42, 43), involves the loss of CO₂ to form the 3-keto intermediate (**23**) with overall retention of the C(2) configuration. Stereospecific reduction of the 3-keto intermediate at the *ReS* face of the carbonyl group by ketoreductase Fum13p results in the formation of the 3*S* hydroxyl group and the *syn*-2,3 amino alcohol motif (**24**) (43, 45, 47). This reduction is analogous to that of 3-ketosphinganine to sphinganine (43) except that the reduction

of the carbonyl group with 3-ketosphinganine reductase must occur at the *Si* face to generate the 3*R* hydroxyl group and the *anti*-2,3 amino alcohol of sphinganine.

The formation of 3-*epi*- FB_3 (**5**) with its 3*R* hydroxyl group and thus the 2,3-*anti* stereochemistry is postulated to occur by reduction at the *SiS* face of the carbonyl group of the 3-keto intermediate (**23**) to give the intermediate (**25**) in the fumonisin biosynthesis by a 3-ketosphinganine type reductase. The presence of such a 3-ketosphinganine type reductase has been proposed to account for the low levels of FB_{1-4} production in *FUM13* deletion mutants (43).

Evidence has been presented that the reduction of the 3-keto group is an early step in the biosynthetic pathway, whereas the introduction of the C(5) hydroxyl group by a 2-ketoglutarate-dependent dioxygenase Fum3p, encoded by the *FUM3* gene, is the last step (43, 47, 48). On the basis of this proposed biosynthetic pathway, the presence of 3-*epi*- FB_3 (**5**) and 3-*epi*- FB_4 (**6**) should lead to the formation of the 3-*epi* stereoisomers of FB_1 and FB_2 by the Fum3p enzyme. This is not the case as these 3-*epi* stereoisomers of FB_1 and FB_2 have never been detected by us. This failure of Fum3p to convert 3-*epi*- FB_3 (**5**) and 3-*epi*- FB_4 (**6**) to 3-*epi*- FB_1 and 3-*epi*- FB_2 , respectively, suggests that the metabolites with the 3*R* hydroxyl group, that is, the 2,3-*anti* stereochemistry, are not recognized by Fum3p and thus do not serve as substrates.

The newly described 3-*epi*- FB_3 (**5**) was identified from the FB_3 standard isolated in the PROMEC Unit from various batches of corn patty culture material prepared from *F. verticilloides* MRC 826. In an attempt to avoid the presence of this analogue in the analytical standard, a range of other *F. verticilloides* strains, including MRC 8041 that only produces FB_3 , have been cultured. The 3-*epi*- FB_3 (**5**) has been found to occur in all of these at levels ranging from 21 to 42% of the level of the normal FB_3 . In a limited comparison, two strains grown in liquid medium showed lower levels (around 7–8%) of the *epi* stereoisomer. Of greater concern is the potential natural occurrence of this new fumonisin analogue. A range of corn samples from various sources have recently been analyzed by HPLC with fluorescence detection. 3-*epi*- FB_3 (**5**) was identified by its

retention time as a small peak eluting from the HPLC column immediately prior to normal FB₃. **Table 3** shows the results of the analytical determination of FB₃ (**3**) and 3-*epi*-FB₃ (**5**) for 16 samples of corn and commercial corn meal. The presence of the stereoisomer was confirmed by LC-MS-MS analysis and the presence of the characteristic fragmentation pattern for fumonisins. **Figure 6** shows the total ion chromatogram for a sample of Transkeian corn, as well as a comparison of the mass spectra for FB₃ (**3**) and for the peak corresponding to 3-*epi*-FB₃ (**5**). The similarity of the spectra and the presence of the fragment ions corresponding to [M + H - H₂O]⁺ (*m/z* 688), [M + H - 2H₂O]⁺ (*m/z* 670), [M + H - TCA]⁺ (*m/z* 530), [M + H - H₂O - TCA]⁺ (*m/z* 512), [M + H - 2TCA]⁺ (*m/z* 354), and [M + H - H₂O - 2TCA]⁺ (*m/z* 336) provides unequivocal identification of the compounds. The level of 3-*epi*-FB₃ (**5**) in these samples ranged from 6.7 to 21% of the level of FB₃ and contributed only marginally to the total level of fumonisins. On the basis of these data and the fact that it also only occurs at lower levels than FB₃ in culture, it can be concluded that, in general, the influence of 3-*epi*-FB₃ on fumonisin exposure will be small.

The use of an isolated FB₃ analytical standard for chromatographic analysis is problematic due to the presence of the two stereoisomers. Nevertheless, because of their chemical similarity and similar retention times on a reversed-phase HPLC column, the assumption of the same chromatographic response factors for the two isomers would appear to be reasonable and allows accurate analysis to be performed. The response factor calculated for the combination of the two isomers in the standard chromatogram is then applied separately to each isomer peak in the chromatogram of the sample.

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Received for review January 9, 2007. Revised manuscript received March 22, 2007. Accepted March 22, 2007. Financial support from the University of Pretoria and the National Research Foundation, Pretoria, as well as the Wellcome Trust toward the purchase of the LC-MS system is gratefully acknowledged.

JF070061H