Structure, Synthesis, and Biosynthesis of Fumonisin B_1 and Related Compounds

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The absolute stereochemical description of fumonisin B_1 (FB_1) and presumably of its congeners is now secure. In this article I summarize studies leading to this conclusion and outline the biosynthetic and synthetic studies of FB_1. *Key words:* absolute configuration, fumonisin biosynthesis, fumonisin B_1, fumonisins, fumonisin synthesis, mycotoxins. — *Environ Health Perspect* 109(suppl 2):245–249 (2001).
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Fumonisin B_1 (FB_1) (Structure 1) is the primary mycotoxin produced by *Fusarium verticillioides* Nirenberg (= *Fusarium moniliforme* Sheldon). Its structure and biosynthesis have been the subject of extensive studies by our group [(*1–5*) and references therein] and others (*6–8*).

Along with FB_1, a large number of related congeners have been reported, as summarized in Structure 2.

Biosynthesis of FB_1

Fumonisins are structurally similar to the sphingoid base backbone of sphingolipids, prompting the idea that they may be biosynthetically related (*9,10*). The biosynthesis of sphingosine proceeds via the condensation of palmitoyl coenzyme A (CoA) with serine. Past studies have shown the advantage of incorporating specifically enriched ^{13}carbon (C)-acetate into fungal cultures at the appropriate time of biosynthesis of a particular metabolite as a preliminary step to preparing radiolabeled (^{14}C) compounds for toxicologic research (*11,12*). The ^{13}C studies permit determination of the most cost-effective method to manufacture highly enriched ^{14}C-FB_1 labeled in multiple locations for use in radiotracer studies. An earlier study has shown that the ^{13}C-acetate is incorporated into the fumonisin backbone in a manner more suggestive of polyketide biosynthesis than of lipid biosynthesis, but the analyses were performed on impure FB_1 (*13*).

In our laboratories, we incorporated specifically enriched ^{13}C-acetate (^{13}C-1 and ^{13}C-2) as well as ^{13}C-3 L-alanine, ^{13}C-5 L-glutamic acid, ^{13}C-3 L-serine, and ^{13}C-CH_3 L-methionine into 50 mL cultures of *F. moniliforme* as previously described (*11,12*). The precursors were added as small aliquots spread over a 24 hr in a manner designed to coordinate with the onset of rapid fumonisin production. FB_1 was purified from the fungal extracts in a manner similar to that used for the 10 L cultures, only on a smaller scale.

The pattern of acetate incorporation illustrated in Structure 3 is consistent with the head-to-tail pattern from the condensation of acetyl CoA units expected from both lipid and polyketide biosynthesis. However, the pattern alone cannot distinguish between the two pathways. The fact that substantial enrichment has occurred after initial lipid synthesis has occurred in cultures that are not carbon-compromised favors the polyketide pathway. In addition, the hydroxyl functions at C-3, C-5, and C-15 are in the correct position for oxidation from polyketide carbonyl groups, and the polyketide model is more flexible in patterns of substitution. The large number of functionalities would be difficult to evoke from stearic or oleic CoA.

The label from C-3 of alanine was incorporated into C-1 of fumonisin in agreement with previous studies (*14*). However, alanine was also catabolized as evidenced by the labeling of the even positions of the backbone and the ester functions, although to a lesser extent. ^{13}C-3 serine showed poor incorporation, producing some increase in the intensities of the resonances caused by C-1 and the backbone carbons but also by the C-21 and C-22 methyl groups. Why these methyl groups were enriched by serine but not alanine is unclear, but may indicate the greater extent of catabolism of serine and the more direct incorporation of alanine into fumonisin. Under no conditions was C-2 enriched, indicating that "scrambling of labeled atoms" was minimal. The S-CH_3 group of methionine was incorporated uniquely and efficiently into the C-21 and C-22 methyl functions, as predicted by previous studies using deuterium enrichment and mass spectrometric analysis (*10*). Plattner and Branham (*15*) have used this efficient incorporation of methionine to produce stable, highly enriched deuterated FB_1 (FB_1–d_6) to be used as an analytic standard for accurate quantitation of fumonisins by gas chromatography/mass spectography or fast atom bombardment/mass spectography techniques. Glutamic acid was incorporated least efficiently into FB_1, but showed a unique enrichment of the secondary carboxyl functions of both side chains at C-28 and C-34. When combined with the data from acetate enrichment, this indicates that the precursor to esterification involves a condensation between α-keto glutarate (the deaminated precursor of glutamate) and a second acetyl CoA unit. Enrichment in the side chains is less than in the backbone of the fumonisin (approximately a factor of 2) and is unevenly distributed—the two carbons at the esterified end receiving somewhat less of the label than the other carbons—for both of the tricarballylic groups. This suggests that a 4-carbon unit is formed first, likely from the Krebs acid cycle, and a third acetate unit is added later. The precursor to the esterification step must therefore be unsymmetrical, because symmetry would induce an even labeling pattern.

The condensation of alanine with an 18-C polyketide chain confirms previous studies on *Alternaria alternata f.* sp. *lycopersici* (AAL) toxin, where alanine was shown to be directly incorporated into the C-1 and C-2 positions (*16*). The array of functionalities is consistent with the carbonyl derivation of the hydroxyl groups at C-3, C-5, and C-15, subsequent hydroxylation at C-10 and C-14, methionine-derived methylation at C-12 and C-16, followed by esterification by a precursor yet to be defined. Less incorporation of acetate into the side chains suggests that this precursor is formed earlier or later than the fumonisin backbone, and that esterification occurs after the C-14 and C-15 hydroxyl groups are formed.

Caldas and co-workers (*17*) have demonstrated that the oxygen atoms in fumonisin

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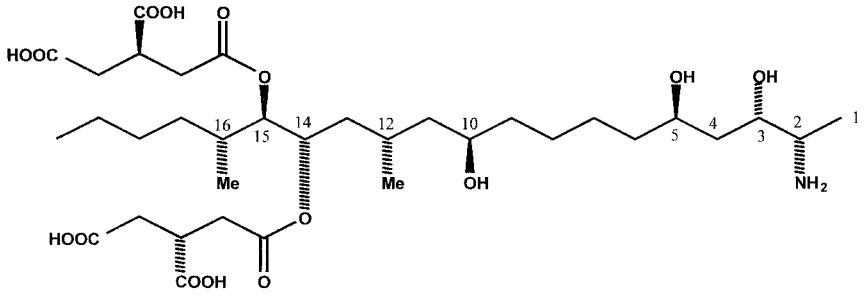
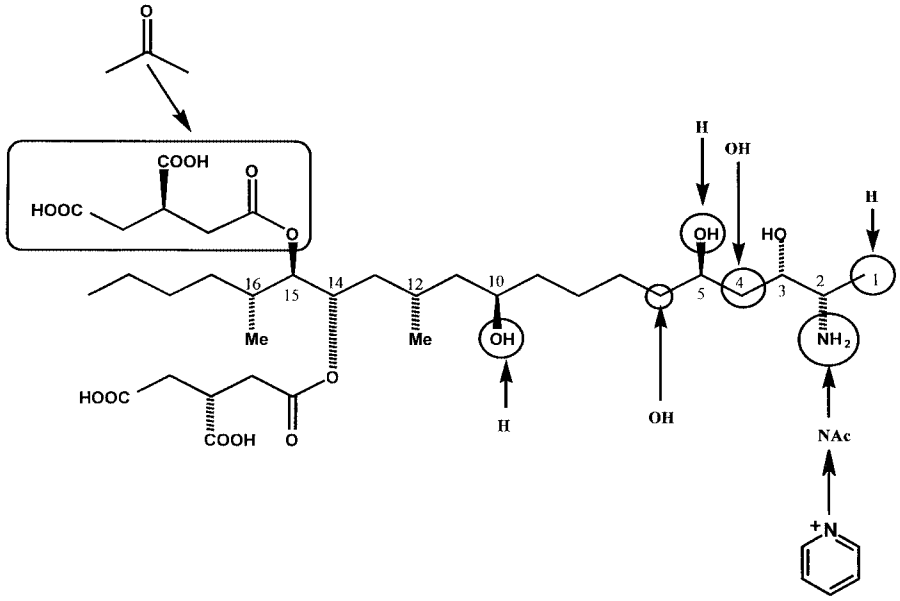
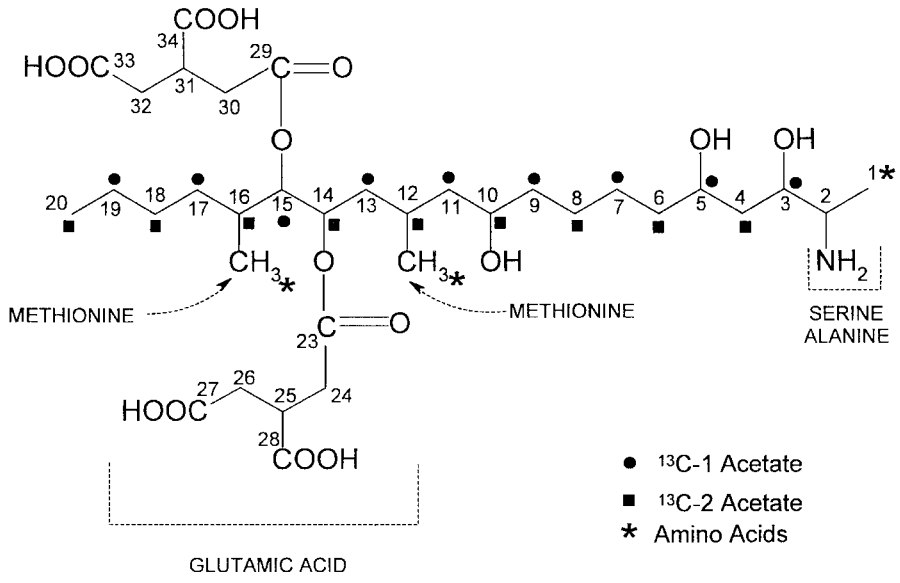


Structure 1. FB_1.



Structure 2. Variety of FB_1-related congeners.



Structure 3. Incorporation of acetate in FB_1.

are derived from molecular oxygen for the backbone and from water for the tricarballylic acids that esterify positions 15 and 16 (Structure 1). These observations are entirely consistent with the biosynthesis proposals described.

Stereochemistry of FB_1

Examination of the structure of FB_1 shows that the molecule contains 10 stereocenters, providing a possible 1,024 different stereochemical structures (*1*). The presence of a single unique high-resolution nuclear magnetic resonance (^{1}H NMR) spectrum for FB_1 derived from different species of *Fusarium* and isolated by different methods suggests that only one of these 1,024 structures describes FB_1. Because fumonisin does not crystallize, several groups have been involved in determining the stereochemistry through the synthesis of derivatives and NMR analysis thereof (*1–8*). To date, all have come to the same conclusions using different derivatization schemes.

Hoping to obtain a crystalline compound for X-ray analysis, we prepared several derivatives containing "bulky" functions and semirigid units in our laboratories. All of the derivatives failed to crystallize, but their spectral properties revealed the relative stereochemistry for portions of the FB_1 backbone. The synthetic strategy is summarized in Figure 1. Generally the derivatives were formed from tetramethyl FB_1 [$FB_1(CH_3)_4$] where the carboxylic groups have been methylated so that organic solvents could be used in the purification steps and so as not to involve the tricarballylic ester functions. $FB_1(CH_3)_4$ (Structure 1 with COOMe for COOH) was converted to the 2,3-carbamate (*2*) using phosgene and triethylamine in benzene. The 3,5-carbonate (*5*) was prepared using phosgene in pyridine after protecting the amide function with *N-p*-bromobenzoate. We used these two compounds to determine the relative stereochemistry of the C-1 to C-5 fragment of FB_1 as shown in Structure 1, using coupling constants and nuclear Overhauser effects (nOe). A parallel study (*7*) confirmed these results.

Compound 7 was obtained by taking advantage of the adventitious formation of chloro-compound 6 (see Figure 1), which on treatment with potassium hydroxide in ethanol provided the pyran 7.

Analysis of nOe data defines the relative configuration of the 6-membered ring of Structure 7 as shown in Figure 2. Enchancements were observed between H-10, H-12, and H-15, but not between H-10 and H-14, thus indicating these protons to be *trans*. We determined the relative stereochemistry of the C-10 to C-16 moiety by comparing the NMR parameters of FB_1 to those of the 10,14-cyclic ether derivative of

N-p-bromobenzoate FB_1 aminopentanol (Structure 7).

With the relative configuration of two portions of the molecule thus defined, the absolute stereochemistry at any one position must be determined to define the absolute stereochemistry at each asymmetric center. Mosher's method (*18*) was applied directly to FB_1 to form the α-methoxy-α-trifluoromethylphenylacetyl (MPTA) amide derivatives in a method similar to that of Hoye et al. (*8*), except that in this case positions C-3 and C-5 were underivatized. $FB_1(CH_3)_4$ was treated with MPTA chloride in tetrahydrofuran (THF) for 30 min and the amides purified on silica using 10% methanol in chloroform. The *S*-amide was prepared using the *R*-MPTA chloride, and the *R*-amide was prepared using the *S*-MPTA chloride. NMR analysis and comparison of the magnitude and sign of the differences in the proton chemical shifts between the two amides (*18*) determined that the absolute configuration at C-2 was *S* (as shown in Structure 1), identical to that of the naturally occuring amino acids. L-alanine is incorporated with retention of configuration into FB_1.

The resultant absolute configuration of the FB_1 backbone is therefore as shown in Structure 1. The connection between C-10 and C-5 was provided by the hexanoic acid derivative of Hoye et al. (*8*). This also agrees with the studies of Harmange et al. on FB_2 (*19*) and shows that the fumonisins have the same absolute configuration as the AAL toxins as determined by Boyle et al. (*20*). The configuration of the side chains was suggested to be *S* (*21*) and more recently argued to be *R* in studies on FB_2 by Kishi and colleagues (*22,23*).

Hartl and Humpf have confirmed the absolute configuration of the amino terminus (C-1 → C-5) using the circular dichroism exciton method (*24*).

The disagreement that has surfaced over the stereochemistry of the two tricarballylic acid (TCA) esters present at positions C-14 and C-15 in this and related molecules led us to examine an independent route to determine the stereochemistry of the side chain acids.

We began by stabilizing the asymmetric centers in the TCA units of FB_1 by borane reduction of the free carboxyl groups, as was also done by Shier et al. (*21*). In practice, this required solubilization of FB_1 in THF, which was accomplished by conversion to the *N*-acetyl-*O*-triacetate *bis*-anhydride Structure 8a, using acetic anhydride. This was followed by partial hydrolysis in aqueous THF at room temperature to the acid Structure 8B, a compound that is the triacetate of the naturally occurring fumonisin A_1, (FA_1) first described by Bezuidenhout et al. (*6*). Reduction of Structure 8b using excess THF/BH_3, in THF

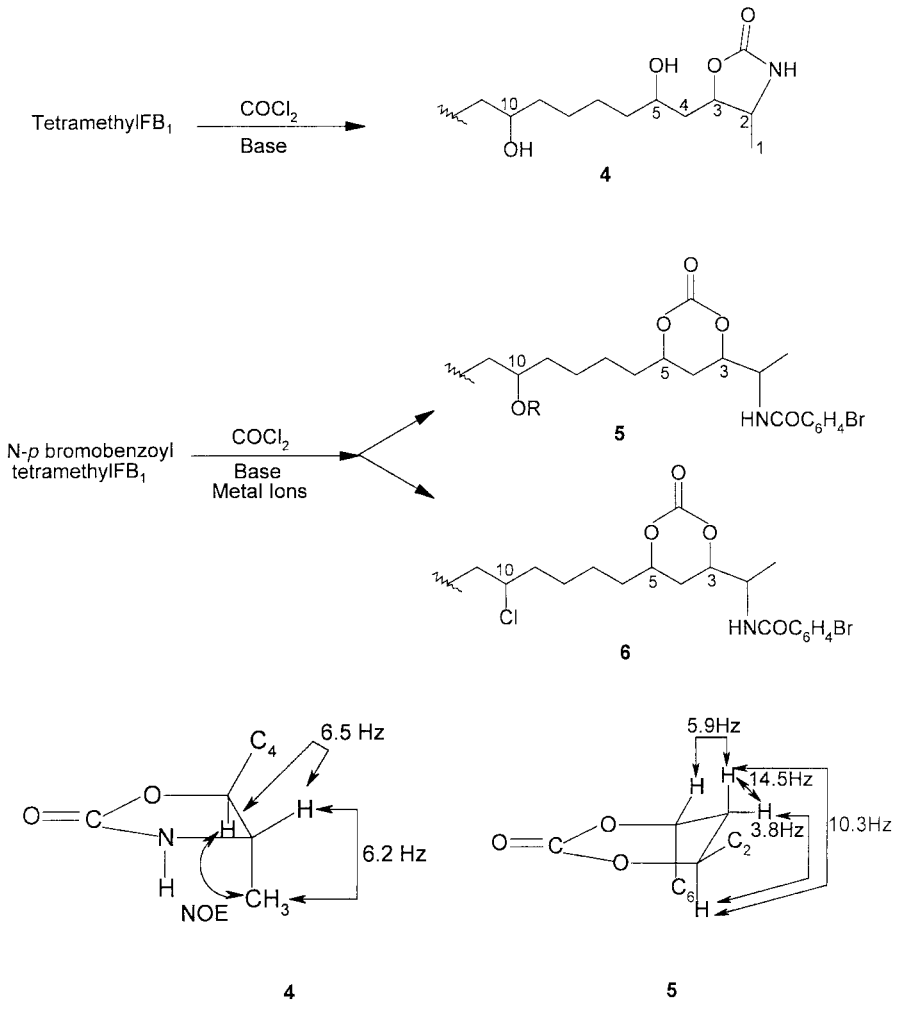


Figure 1. Synthesis of cyclic derivations of FB_1 tetramethyl ester.

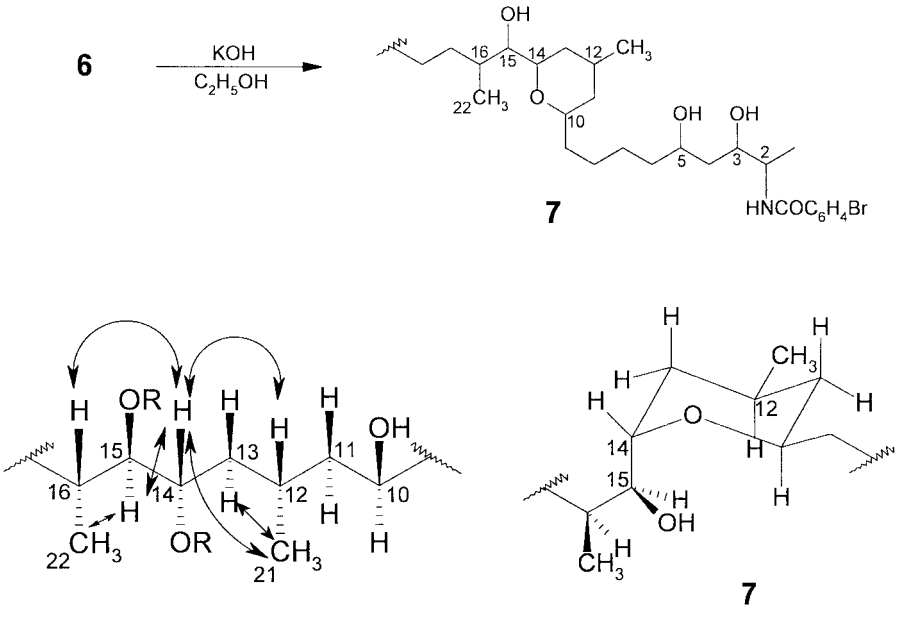
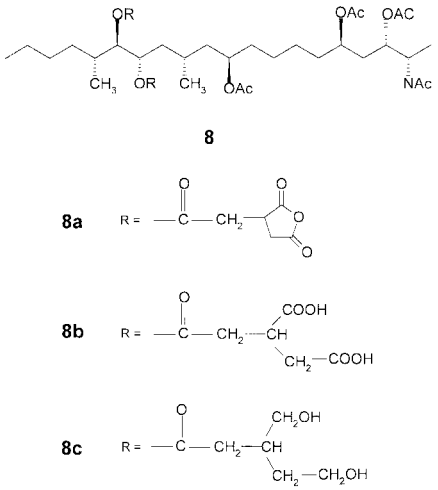


Figure 2. NMR analysis of cyclic derivatives of FB_1.

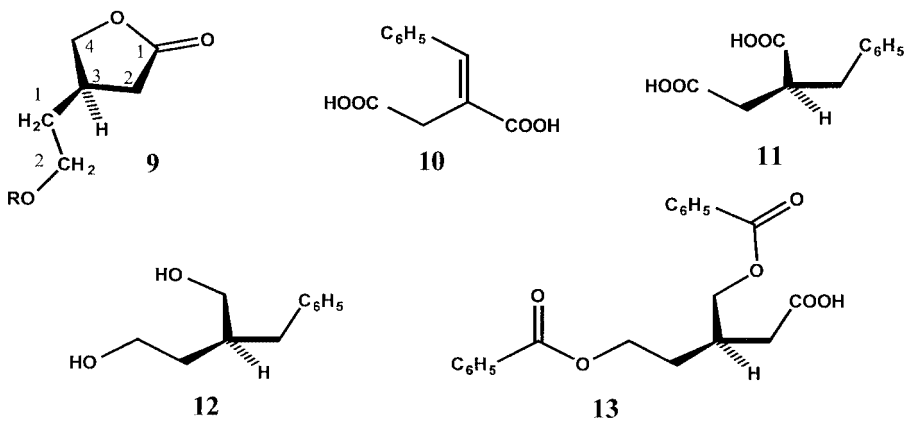


Structure 8. Derivatives of FB_1 required to determine side chain stereochemistry.

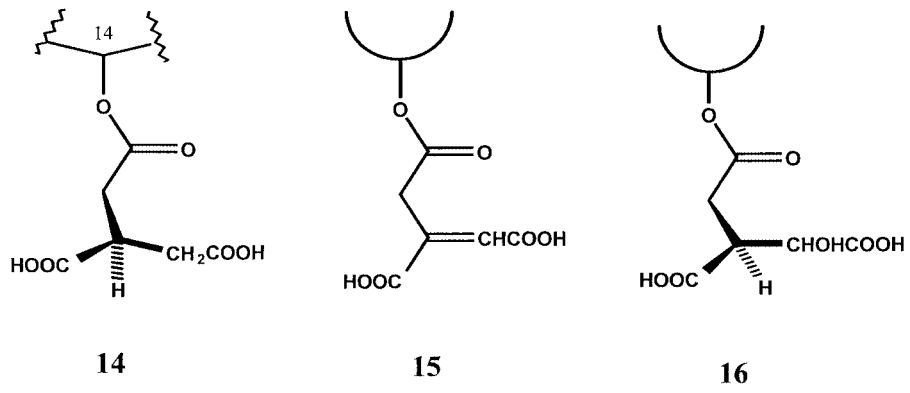
gave the *N*-acetyl triacetyl tetraol Structure 8c, which is identical to compound 1C described by Shier et al. (*21*). Complete hydrolysis of Structure 8c using potassium hydroxide in aqueous methanol, followed by acidification gave a mixture rich in hydroxy γ-lactone (Structure 9) (R = H). Benzoylation of this mixture and separation on SiO_2 using 1:1 ethyl acetate/hexane produced the benzoyloxy γ-lactone Structure 9 (R = COC_6H_5).

An authentic sample of optically active Structure 9 (R = COC_6H_5) was prepared from *E*-phenylitoconic acid (Structure 10) by asymmetric reduction (*25*) to S(–) benzylsuccinic acid Structure 11 [$(\alpha)_D^{25}$-27° (c 1.5, ethyl acetate)]. The absolute stereochemistry assigned to this (*25,26*) was confirmed by X-ray crystallography. Conversion of Structure 11 to the diol Structure 12 using borane-THF, then benzoylation and oxidation gave the dibenzoyloxy acid Structure 13. Alkaline hydrolysis followed by acidification gave the *R*(–) hydroxy γ-lactone Structure 9 (R = H) identified in all respects to the product obtained from FB_1.

It follows that both the TCA units in FB_1 have the R configuration illustrated in Structure 14. Thus, our conclusions agree with those of Boyle and Kishi (*22*). Our detailed NMR assignments for the carbons and hydrogens of the TCA units in FB_1 (as well as FB_2 and FB_3) (*2,18*) agree well with those in the literature (*13*), making it improbable that two optical isomers of FB_1 have been isolated. It is evident both from our work and that of Boyle and Kishi (*22*) that only one configuration exists for the TCA units at both the C-14 and C-15 positions in the backbone for all the fumonisins isolated to date. Moreover, it is interesting to observe that no fumonisin has yet been isolated with TCA units at any other position on the backbone, implying a biosynthetic preference for those



Structures 9–13. Synthetic compounds prepared to study side chain stereochemistry.



Structures 14–16. Possible biosynthetic origins of the side chains of FB_1.

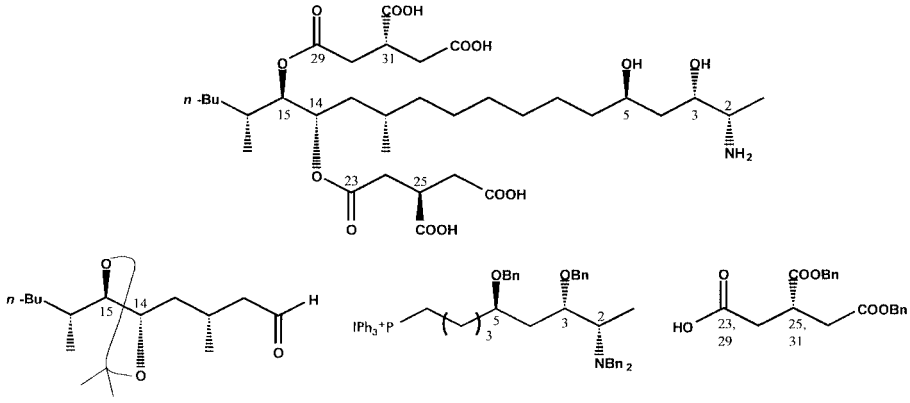


Figure 3. Basic strategy behind the two fumonisin syntheses; subunits shown below the target structures.

sites. Comparison of the present results with previous biosynthetic studies (*5,12*) raise an intriguing question with respect to the biosynthetic origin of these TCA units. Studies using ^{13}C-enriched glutamate have shown that the secondary carboxyl functions (C-28 and C-34) are derived from C-5 of L-glutamic acid, whereas studies with ^{13}C-enriched acetate have shown that the unesterified four-carbon unit of the TCA unit (C-25, C-26, C-27, C-28, and C-31, C-32, C-33, C-34) is formed before the addition of a third acetate unit (leading to C-23, C-24 and C-29, C-30). The specific incorporation of glutamic acid suggests that the TCA units are derived from the Krebs acid cycle.

These results can be explained by three possible mechanisms: *a*) simple chiral esterification using TCA itself; *b*) esterification with cis-aconitate as in Structure 15, followed by chiral reduction of the double bonds; or *c*) esterification with the chiral intermediate 2R-3S isocitrate to give Structure 16, followed by deoxygenation at C-24 and C-30. In the latter case, the *R* configuration of the TCA units would arise, consistent with the

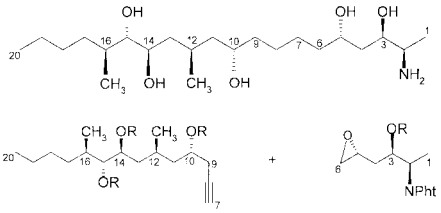


Figure 4. Basic strategy behind the two fumonisin syntheses; subunits shown below the target structures.

results presented here. Obviously more work is needed to address this question.

We therefore confirm that the absolute stereochemical description of FB_1 is that shown in Structure 1.

Synthetic Approaches to FB_1

One report discusses the stereoselective synthesis of the fumonisin skeleton (*27*), and another presents a related synthesis of AAL-toxin TA_1 that possess many of features present in the fumonisin (*28*). These elegant stereoselective syntheses open the route of the synthesis of previously undescribed analogs of the fumonisin susceptible to a study of their structure/activity relationships. This basic strategy behind the two fumonisin syntheses is outlined in Figures 3 and 4, where the subunits that were assembled during the synthetic routes are shown below the target structures, with indications of the eventual carbon sites.

Summary

The studies described in this brief review demonstrate the absolute stereochemistry of the fumonisins, provide extensive information on their biosynthesis, and reveal that the total synthesis of these molecules is achievable. The determination of the absolute stereochemistry of FB_1 has been achieved, providing a sound basis for a deeper understanding of the structural basis for the biologic effects of this class of mycotoxins.

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