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## Tricarballic ester formation during biosynthesis of fumonisin mycotoxins in *Fusarium verticillioides*

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Fumonisin is an agriculturally important mycotoxin produced by the maize pathogen *Fusarium verticillioides*. The chemical structure of fumonisin contains two tricarballic esters, which are rare structural moieties and important for toxicity. The mechanism for the tricarballic ester formation is not well understood. *FUM7* gene of *F. verticillioides* was predicted to encode a dehydrogenase/reductase, and when it was deleted, the mutant produced tetrahydro fumonisin (DH<sub>4</sub>-FB). MS and NMR analysis of DH<sub>4</sub>-FB<sub>1</sub> indicated that the esters consist of aconitate with a 3'-alkene function, rather than a 2'-alkene function. Interestingly, the purified DH<sub>4</sub>-FB<sub>1</sub> eventually yielded three chromatographic peaks in HPLC. However, MS revealed that the metabolites of the three peaks all had the same mass as the initial single-peak DH<sub>4</sub>-FB<sub>1</sub>. The results suggest that DH<sub>4</sub>-FB<sub>1</sub> can undergo spontaneous isomerization, probably including both *cis-trans* stereoisomerization and 3'- to 2'-ene regioisomerization. In addition, when *FUM7* was expressed in *Escherichia coli* and the resulting enzyme, Fum7p, was incubated with DH<sub>4</sub>-FB, no fumonisin with typical tricarballic esters was formed. Instead, new fumonisin analogs that probably contained isocitrate and/or oxalosuccinate esters were formed, which reveals new insight into fumonisin biosynthesis. Together, the data provided both genetic and biochemical evidence for the mechanism of tricarballic ester formation in fumonisin biosynthesis.

**Keywords:** fumonisins; mycotoxins; biosynthesis; *Fusarium verticillioides*

### 1. Introduction

Fumonisin is a group of mycotoxins produced by several agriculturally important fungi, including *Fusarium verticillioides* (synonym *F. moniliforme*, teleomorph *Gibberella moniliformis*, synonym *G. fujikuroi* mating population A), which is a common fungal contaminant of maize and maize-derived products worldwide (Marasas et al. 2004; Wang et al. 2006, 2008). The fungus can cause ear, stalk, and seedling rots of maize, and can also occur at a high frequency in healthy corn tissues (Miller 2001). Fumonisin contamination in maize has been associated with diseases in some livestock and with human health problems, including esophageal cancer and neural tube defects (Marasas et al. 2004).

At least 28 fumonisin analogs have been characterized (Rheeder et al. 2002). The B-series fumonisins, FB<sub>1</sub>, FB<sub>2</sub>, FB<sub>3</sub>, and FB<sub>4</sub>, are the predominant analogs produced by wild-type isolates of *F. verticillioides* (Figure 1, FB<sub>1</sub> structure shown) (Nelson et al. 1993). The metabolites contain a linear 18-carbon chain (C-3 to C-20) that is synthesized from acetate by the activity of an iterative polyketide

synthase (Fum1p) (Proctor et al. 1999; Zhu et al. 2007; Du et al. 2008). The amino group and C-1 and C-2 are derived from alanine (Branham and Plattner 1993; Blackwell et al. 1996; Gerber et al. 2009). The two methyl groups at C-12 and C-16 are derived from methionine (Plattner and Shackelford 1992). In FB<sub>1</sub>, the hydroxyl groups at C-5, C-10, C-14, and C-15 are derived from molecular oxygen and are formed following synthesis of the polyketide (Caldas et al. 1998; Ding et al. 2004; Proctor et al. 2006), while the C-3 hydroxyl originates from acetate and results from reduction of a 3-keto group (Butchko et al. 2003; Yi et al. 2005). The condensation between the 18-carbon chain and alanine involves a unique polyketide chain-releasing mechanism (Du et al. 2008; Gerber et al. 2009).

Another unusual modification during fumonisin biosynthesis is esterification of tricarballic moieties to the C-14 and C-15 hydroxyls. Tricarballic esters are rare structural features in natural products and are necessary for fumonisin toxicity (Yin et al. 1996; Humpf et al. 1998; Seefelder et al. 2003). The molecular mechanism by

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Yaoyao Li and Lili Lou contributed equally to this work.

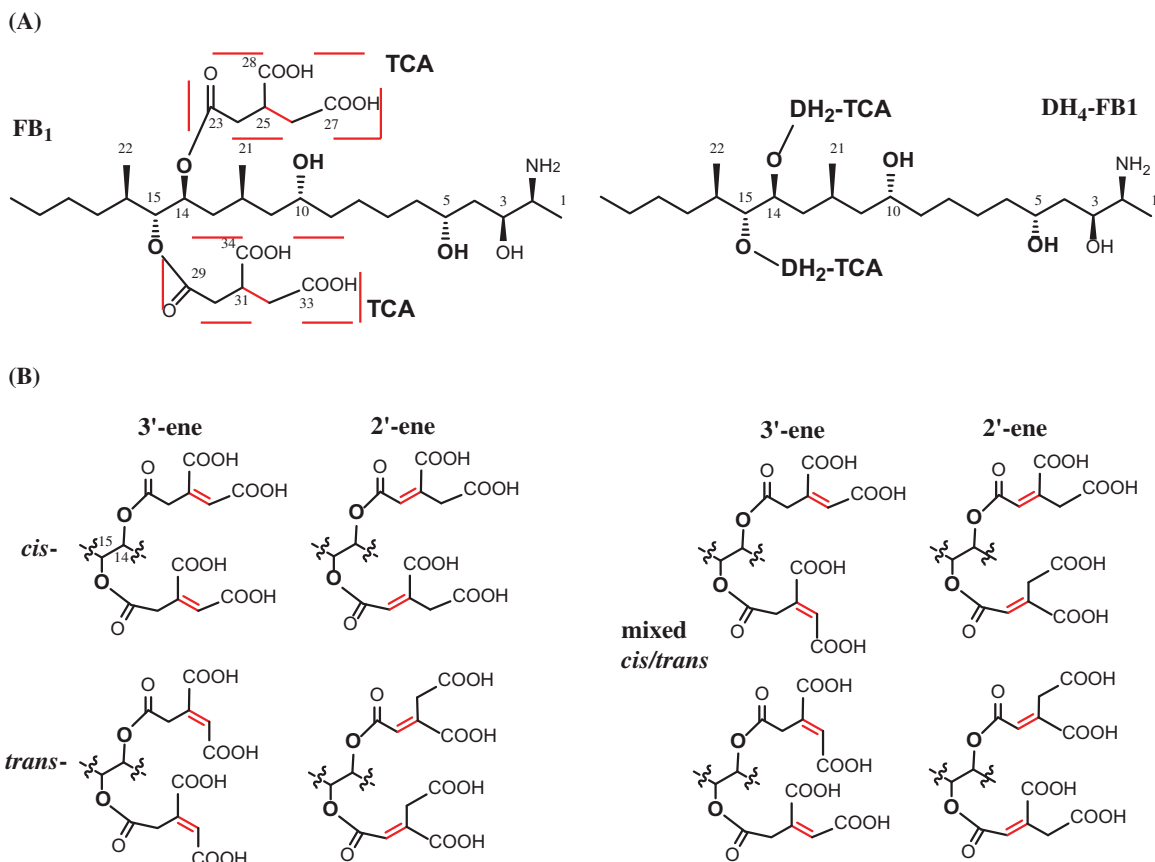


Figure 1. Chemical structure of fumonisins and analogs.  $\text{DH}_4\text{-FB}_1$ , tetradehydro fumonisins B<sub>1</sub>. (A) Structure of fumonisins B<sub>1</sub> and tetradehydro fumonisins B<sub>1</sub>. (B) Example of possible regio- and stereoisomers of  $\text{DH}_4\text{-FB}_1$ .

which fumonisin tricarballic esters are formed has not been fully elucidated. Previous studies using  $^{13}\text{C}$ -substrates suggested that the precursor for the tricarballic esters is an intermediate from the citric acid cycle (Blackwell et al. 1996). Data obtained from gene-deletion mutants of *F. verticillioides* suggest that four genes, *FUM7*, *FUM10*, *FUM11*, and *FUM14*, are required for the formation of esters (Butchko et al. 2006; Zaleta-Rivera et al. 2006). *FUM7* is predicted to encode an iron-containing dehydrogenases/reductases, similar to those that catalyze the reversible reduction of an alkene to an alkane (Seibert et al. 1998; Kallberg et al. 2002). *FUM7*-deletion mutants produce FB analogs with an alkene function in both esters (tetradehydro-FB,  $\text{DH}_4\text{-FB}$ ) (Figure 1) (Butchko et al. 2006). This suggests that the enzyme (Fum7p) encoded by *FUM7* is likely to catalyze reduction of the alkene. In order to determine the regio- and stereospecificity of this biosynthetic step, we prepared the predominant component ( $\text{DH}_4\text{-FB}_1$ ) and determined the positions of the carbon-carbon double bond in tricarballic ester. We also investigated the alkene reductase activity of Fum7p through heterologous expression.

## 2. Materials and methods

### 2.1. Materials and general methods

Chemicals were purchased from Fisher Scientific or Sigma. All oligonucleotide primers for PCR were synthesized by Integrated DNA Technologies (IDT; Coralville, IA). *Escherichia coli* strain DH5 $\alpha$  was used as the host for general plasmid DNA propagation, and cloning vectors were the pGEM-zf series from Promega (Madison, WI). Plasmid preparation and DNA extraction were carried out using Qiagen kits (Valencia, CA), and all other DNA manipulations were carried out according to standard methods (Sambrook et al. 1989).

### 2.2. Isolation and analysis of fumonisin analogs from *F. verticillioides*

Procedures for isolating the B-series fumonisins from *F. verticillioides* were essentially same as described previously (Bojja et al. 2004). For isolating and analyzing the tetradehydro tricarballic ester-containing analogs from the *FUM7* mutant, the procedure was modified as follows. Single

colonies of the fungus growing on YPD/hygromycin (300 µg/ml) agar medium were transferred to test tubes containing 3 ml YPD/hygromycin (150 µg/ml) liquid medium and allowed to grow in a shaker (60 rpm) at room temperature for 2 days. From the 3 ml culture, 100 µl was transferred to a flask containing 25 ml YPD/hygromycin (150 µg/ml). The culture was incubated with shaking at room temperature for another 2 days. The culture was transferred to a 50-ml tube and centrifuged at 2500 rpm for 20 min. The pellet was washed three times with sterile water and finally re-suspended in 10 ml sterile water. From the suspended solution, 500 µl was transferred to a new flask containing 10 g of autoclaved Cracked Maize Kernel (CMK) medium (Proctor et al. 2003). After 4 weeks of growth at room temperature in the dark, metabolites were extracted from the CMK cultures with 20 ml of 50% acetonitrile (CH<sub>3</sub>CN). An aliquot of the extracts was filtered and a sample of 50 µl was injected in HPLC-ELSD to analyze the metabolites, following the method described previously (Bojja et al. 2004).

To prepare DH<sub>4</sub>-FB<sub>1</sub> for spectroscopic studies, the crude extract was dried and partitioned into EtOAc-soluble and H<sub>2</sub>O-soluble extracts. The H<sub>2</sub>O extract was loaded onto a 10-g C18 cartridge and eluted with the stepwise gradient of 0, 15%, 30%, 50%, and 70% CH<sub>3</sub>CN in H<sub>2</sub>O (containing 0.025% TFA, 100 ml for each step). The 30% CH<sub>3</sub>CN fraction was loaded onto another 10-g C18 cartridge and eluted with the stepwise gradient of 15%, 25%, and 35% CH<sub>3</sub>CN in H<sub>2</sub>O (containing 0.025% TFA, 100 ml for each step). The 35% CH<sub>3</sub>CN fraction (280 mg) was subjected to a gel permeation chromatography (50 g Sephadex LH-20, in methanol) for further purification. Purified DH<sub>4</sub>-FB<sub>1</sub> was analyzed by ESI-MS on an API Qtrap 4000 and NMR spectra (<sup>1</sup>H- and <sup>13</sup>C-NMR and HMBC) on a Bruker DRX-500 spectrometer, at 500/125 MHz, respectively, in methanol-d<sub>6</sub>, δ in ppm relative to Me<sub>4</sub>Si, J in Hz.

### 2.3. FUM7 gene expression and enzyme purification

*FUM7* has no introns (Proctor et al. 2003) and was amplified directly from the genomic DNA of *F. verticillioides* by PCR using a forward primer of 5'-C TCT AGA CAT ATG AGT CTT GAT CAT CAC CAG-3' (*Nde*I site underlined) and a reverse primer of 5'-CCC AAG CTT CTA TGC TGC CAT GTA CAG-3' (*Hind*III site underlined). After the identity of the 1275 bp fragment was confirmed by DNA sequencing, it was cloned into pET28a for expression in *E. coli* BL21(DE3) and pMAL-c2 for expression in *E. coli* TB1 as specified by the manufacturers. Fum7p was highly expressed in both systems, but only the TB1/pMAL-c2 system yielded a soluble protein. The soluble fraction of the expressed protein was loaded onto an amylose resin column (New England BioLabs) for affinity purification of the protein. The purified protein was desalted by dialysis

against a buffer containing 50 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, and 15% glycerol. The resulting preparation was stored at -80°C until it was used in assays.

### 2.4. In vitro activity assay for Fum7p

The reactions contained 0.4 µM Fum7p, 2.8 mM DH<sub>4</sub>-FB, 1 mM NAD(P)H, 1 mM FeCl<sub>2</sub>, and 100 mM Tris-HCl, pH 7.8, in a total volume of 100 µl. The reactions were incubated at 37°C for 30 min and stopped by adding two volumes of ethanol at 4°C. After 30 min on ice, the precipitated proteins were removed by centrifugation at 13,200 rpm at 4°C for 20 min, and the supernatant was transferred to a new tube. A 100 µl fraction of supernatant was used for HPLC-ELSD and ESI-MS analysis.

## 3. Results and discussion

### 3.1. Position and configuration of the alkene in tricarballic esters of DH<sub>4</sub>-FB<sub>1</sub>

*FUM7* mutants of *F. verticillioides* produce fumonisins analogs, tetrahydro fumonisins (DH<sub>4</sub>-FBs), that are four mass units less ( $[M + H]^+$  *m/z* 718 and *m/z* 702) than FB<sub>1</sub> (*m/z* 722) or FB<sub>2/3</sub> (*m/z* 706) (Butchko et al. 2006). The difference in mass between DH<sub>4</sub>-FBs and FBs is due to an alkene function present in each of the two tricarballic esters of DH<sub>4</sub>-FBs. However, the position and configuration of the alkene function are not clear, as multiple regio- and stereoisomers of aconitate could account for the difference (Figure 1). We therefore set out to determine the structure of tricarballic esters in DH<sub>4</sub>-FB. After several purification steps, we were able to obtain DH<sub>4</sub>-FB<sub>1</sub> that yielded as a single chromatographic peak in HPLC analysis (Figure 2). MS analysis confirmed a  $[M + H]^+$  *m/z* 718.4 for the purified DH<sub>4</sub>-FB<sub>1</sub>, and <sup>1</sup>H-NMR analysis was consistent with one major compound. The position of the carbon-carbon double bond in the tricarballic esters of DH<sub>4</sub>-FB<sub>1</sub> was identified by spectroscopic analyses, including 1D- and 2D-NMR data (Table 1), and by comparison of these data to those previously published for fumonisins (Bezuidenhout et al. 1988). The selected HMBC correlations from H-15 to C-22 and C-29; H-30/24 to C-29/23 and C-34/28; and H-32/26 to C-30/24, C-33/27, and C-34/28 established the tricarballic ester on the C-15 hydroxyl with a double bond between C-31 and C-32 (3'-ene) and the tricarballic ester on the C-14 hydroxyl with a double bond between C-25 and C-26 (3'-ene) (Table 1 and Figure 1).

Interestingly, when the purified DH<sub>4</sub>-FB<sub>1</sub> was incubated at room temperature for 2 days, additional chromatographic peaks were observed in the sample in HPLC analysis. To exclude potential degradation products, we attempted to further separate the preparation using a gel

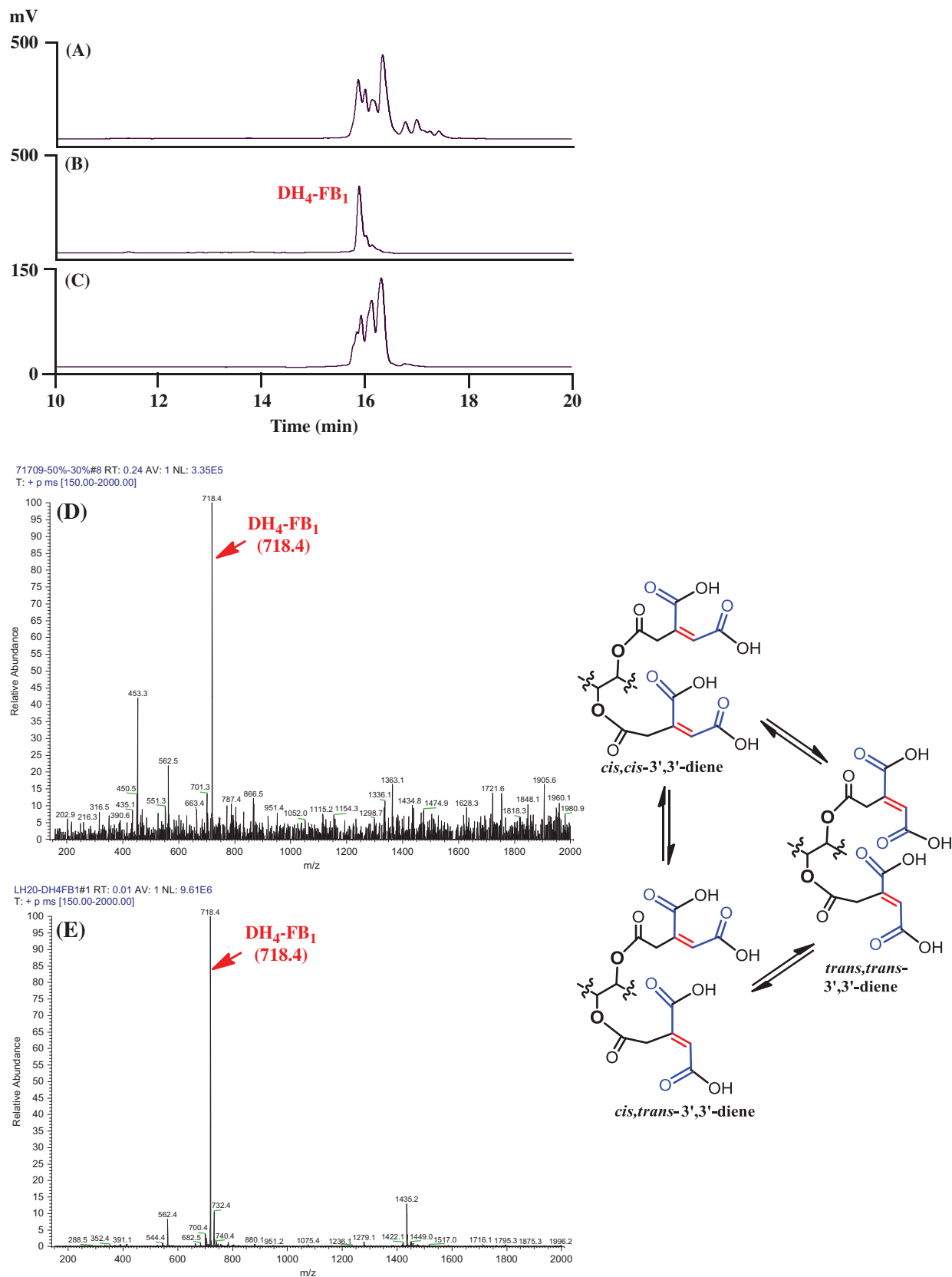
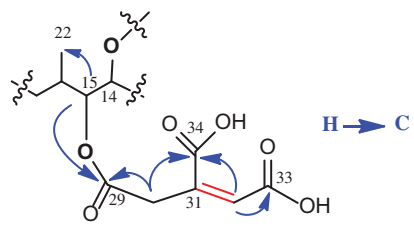


Figure 2. Isolation and analysis of  $\text{DH}_4\text{-FB}_1$  from the *FUM7* mutant. (A) HPLC analysis of crude extract; (B) HPLC analysis of the initially purified  $\text{DH}_4\text{-FB}_1$ ; (C) HPLC analysis of the isomerized  $\text{DH}_4\text{-FB}_1$ ; (D) MS analysis of the initially purified  $\text{DH}_4\text{-FB}_1$ ; and (E) MS analysis of the isomerized  $\text{DH}_4\text{-FB}_1$ . A proposed *cis-trans* stereoisomerization of  $\text{DH}_4\text{-FB}_1$  is included.

Table 1. Selected HMBC correlations (H→C) for DH<sub>4</sub>-FB<sub>1</sub> and <sup>1</sup>H-NMR (500 MHz) and <sup>13</sup>C-NMR (125 MHz) spectroscopic data (methanol-d<sub>4</sub>) for DH<sub>4</sub>-FB<sub>1</sub>. δ in ppm. J in Hz.



Position	δ <sub>(H)</sub> , (multiplicity, J in Hz)	δ <sub>(C)</sub>
14	5.10 (m)	71.8 (d)
15	4.91 (m)	78.2 (d)
22	0.93 (d, J = 6.9)	14.6 (q)
23/29 <sup>a</sup>	–	170.6 (s)
24/30 <sup>a</sup>	3.98 (m)	33.4 (t)
	3.93 (m)	
25/31 <sup>a</sup>	–	139.1 (s)
26/32 <sup>a</sup>	6.94 (s)	130.2 (d)
27/33 <sup>a</sup>	–	168.5 (s) <sup>b</sup>
28/34 <sup>a</sup>	–	168.4 (s) <sup>b</sup>

Notes: <sup>a</sup>The chemical shift for each of the pairs could not be assigned specifically, due to the weak and contaminated signals in NMR spectra. <sup>b</sup>May be interchanged.

permeation chromatography (Sephadex LH-20). Surprisingly, the DH<sub>4</sub>-FB<sub>1</sub> fraction continued to yield three major chromatographic peaks, instead of one peak, in HPLC analysis, but yielded only one signal [*M* + H]<sup>+</sup> *m/z* 718.4, in MS analysis (Figure 2). These results indicate that the three peaks in the preparation represent three isomers of DH<sub>4</sub>-FB<sub>1</sub>. One probable explanation for formation of the isomers is that the original preparation of purified single 3',3'-diene stereoisomer of DH<sub>4</sub>-FB<sub>1</sub> was converted to other *cis-trans* stereoisomers (Figure 2) and/or 2'-ene regioisomers during the 2-day incubation period. For the didehydrotricarballylate (aconitate) moieties of DH<sub>4</sub>-FB<sub>1</sub>, both regio- and stereoisomerization could occur either through a C-3' to C-4' bond rotation of the aconitate carbonium ion following a protonation of the double bond from an acid and then deprotonation, or through an allylic rearrangement by elimination of a proton at C-2' of the aconitate carbonium ion (Klinman and Rose 1971). These mechanisms are reasonable considering that all DH<sub>4</sub>-FB<sub>1</sub> solvents contained an acid (0.025% TFA). These results are also consistent with the previous study that observed broad NMR peaks for the tricarballylic carbons in DH<sub>4</sub>-FB (Butchko et al. 2006).

### 3.2. Activity of heterologously expressed Fum7p

Previous *in vivo* feeding experiments suggested that the tetrahydro fumonisins (DH<sub>4</sub>-FB) produced by *FUM7*-

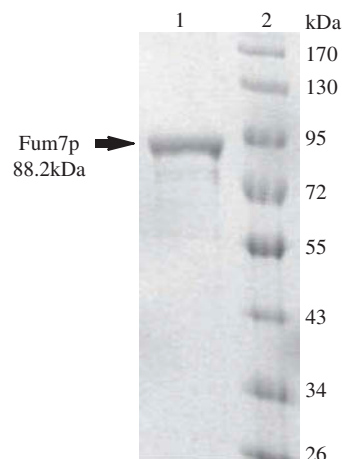


Figure 3. SDS-PAGE of Fum7p produced in *E. coli*. Lane 1, purified Fum7p; Lane 2, size markers.

deletion mutants of *F. verticillioides* are probably shunt metabolites of the biosynthetic pathway, because strains of the fungus with a wild-type *FUM7* were unable to convert the DH<sub>4</sub>-FB into FB (Butchko et al. 2006). To obtain direct evidence, we heterologously expressed *FUM7* and examined whether purified Fum7p could catalyze conversion of DH<sub>4</sub>-FB to FB *in vitro*. Fum7p produced in the TB2/pMALc2 expression system was partly soluble as a fusion protein with MBP. The protein purified on an amylose affinity column yielded approximately 88.2 kDa band on SDS-PAGE (Figure 3), which is consistent with the expected size for the fusion protein. Fum7p was incubated with purified DH<sub>4</sub>-FB in the presence of NADPH and Fe<sup>2+</sup>, which are cofactors usually required for activity of the class of dehydrogenases/reductases to which Fum7p is most similar (Seibert et al. 1998; Kallberg et al. 2002). The MS analysis of the reaction mixtures showed that fumonisins with typical tricarballylic esters were not produced and that the [*M* + H]<sup>+</sup> ions of 718.43 (DH<sub>4</sub>-FB<sub>1</sub>) and 702.42 (DH<sub>4</sub>-FB<sub>2/3</sub>) remained in the reaction mixtures, under the conditions tested (Figure 4). The results are consistent with the *in vivo* feeding experiments (Butchko et al. 2006) and suggest that free DH<sub>4</sub>-FB are not direct substrates of Fum7p. Interestingly, although fumonisins with typical tricarballylic esters were not detected, several novel metabolites were produced in the reactions. One (compound 1) of these had a molecular ion of 750.33, which is the same as that expected for an FB<sub>1</sub> analog with two oxalosuccinate esters (Figures 4 and 5). Another novel metabolite (compound 3) had a molecular ion of 754.33, which is consistent with an FB<sub>1</sub> analog with two isocitrate (or citrate) esters. Finally, a third metabolite (compound 2) with a molecular ion of 752.35, which is the same as that expected for an FB<sub>1</sub> analog with one isocitrate (or citrate) ester and one oxalosuccinate ester. These ions were not present in the initial DH<sub>4</sub>-FB isolated



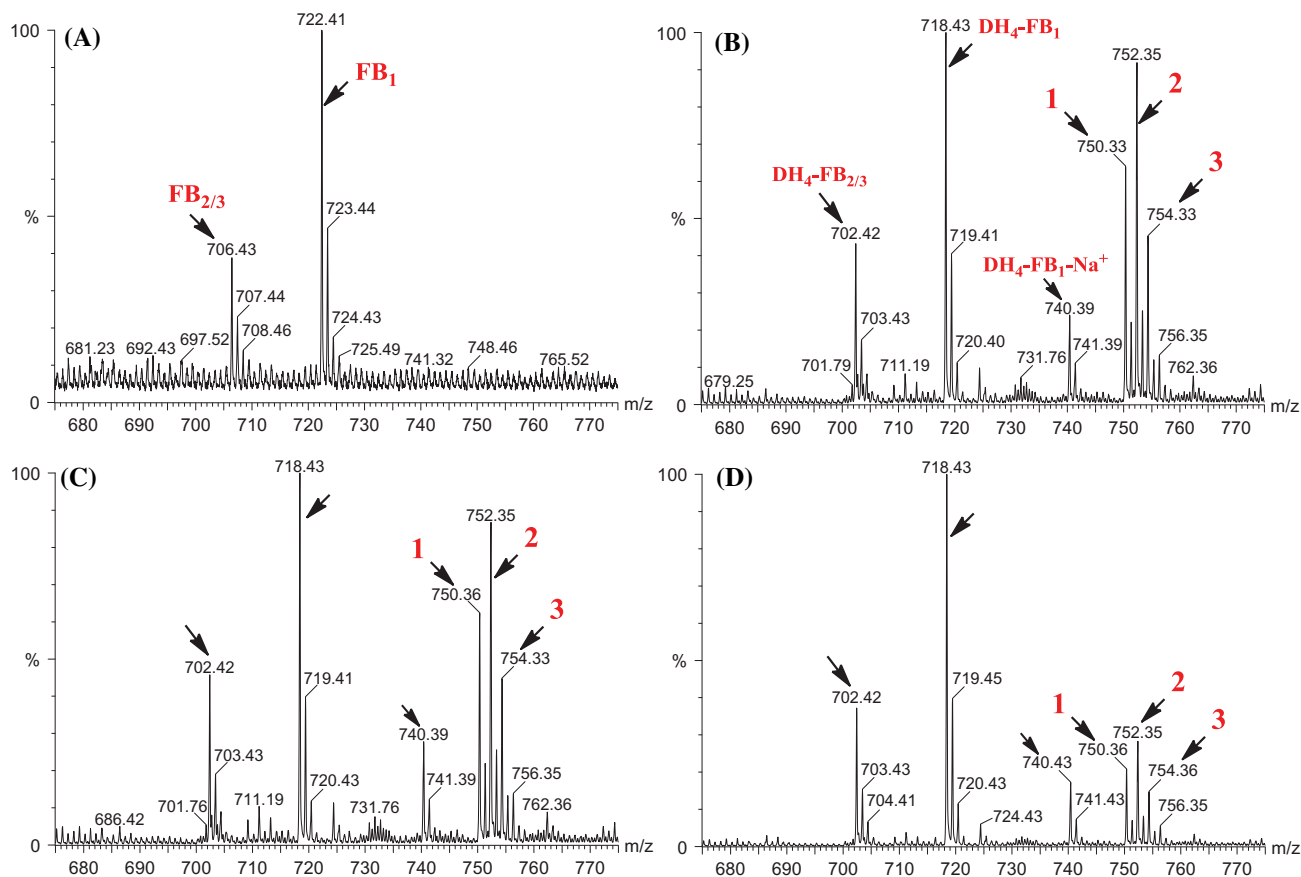


Figure 4. MS analysis of the reaction mixtures containing Fum7p and tetrahydro fumonisins. (A) Standard fumonisins; (B) Fum7p + DH<sub>4</sub>-FB + NADPH; (C) Fum7p + DH<sub>4</sub>-FB + NADPH + FeCl<sub>2</sub>; and (D) Fum7p + DH<sub>4</sub>-FB + NADH + FeCl<sub>2</sub>. The peaks corresponding to FB<sub>1</sub>, FB<sub>2/3</sub>, as well as the DH<sub>4</sub>-FB analogs are indicated with arrows. The proposed structure for compounds 1, 2, and 3 are shown in Figure 5. 1, fumonisins analog with two oxalosuccinate esters; 2, fumonisins analog with one oxalosuccinate ester and one isocitrate ester; and 3, fumonisins analog with two isocitrate esters.

from the *FUM7*-deleted mutant. Interestingly, these ions were present, but at a lower level, in the reaction mixture containing boiled Fum7p. When NADH was used in the place of NADPH, the intensity of the ions was significantly reduced (Figure 4). The results suggest that Fum7p promotes the conversion of DH<sub>4</sub>-FB into analogs with isocitrate (or citrate) and oxalosuccinate side chains. This conversion could be realized through a hydration of aconitate to generate isocitrate (or citrate), followed by a dehydrogenation of isocitrate to produce oxalosuccinate (Figure 5). The hydration could be a non-enzymatic step, whereas the dehydrogenation should be facilitated by the dehydrogenase activity of Fum7p.

### 3.3. Mechanism for the tricarballylic ester formation

Previous studies have shown that *FUM7*, *FUM10*, *FUM11*, and *FUM14* are involved in formation of the tricarballylic esters of fumonisins (Butchko et al. 2006; Zaleta-Rivera et al. 2006). Among the four genes, the function of *FUM14* has been demonstrated both *in vivo*

and *in vitro* (Butchko et al. 2006; Zaleta-Rivera et al. 2006). *FUM14* is predicted to encode an unusual non-ribosomal peptide synthetase (NRPS) containing two domains, peptidyl carrier protein (PCP) and condensation domain. Fum14p catalyzes the transfer of the activated tricarballylic acid to the hydroxyl groups on C-14 and C-15 to form the two ester bonds. *FUM10*-deletion mutants produce hydrolyzed FB<sub>3</sub> and FB<sub>4</sub> (HFB<sub>3</sub> and HFB<sub>4</sub>, respectively), analogs of FB<sub>3</sub> and FB<sub>4</sub> that lack the esters (Butchko et al. 2006). *FUM10* is predicted to encode an acyl-CoA synthetase or the adenylation domain of NRPS (Proctor et al. 2003). Therefore, *FUM10* is likely responsible for *in vivo* activation of the tricarballylic acid. *FUM11*-deletion mutants produce the wild-type complement of fumonisins as well as a significant amount of partially hydrolyzed fumonisins, fumonisins analogs that lack one of the ester functions (Butchko et al. 2006). *FUM11* is predicted to encode a tricarballylate transporter and is likely to function *in vivo* to supply the tricarballylic acid precursors for esterification (Proctor et al. 2003). Two lines of evidence support its involvement of *FUM7* in the



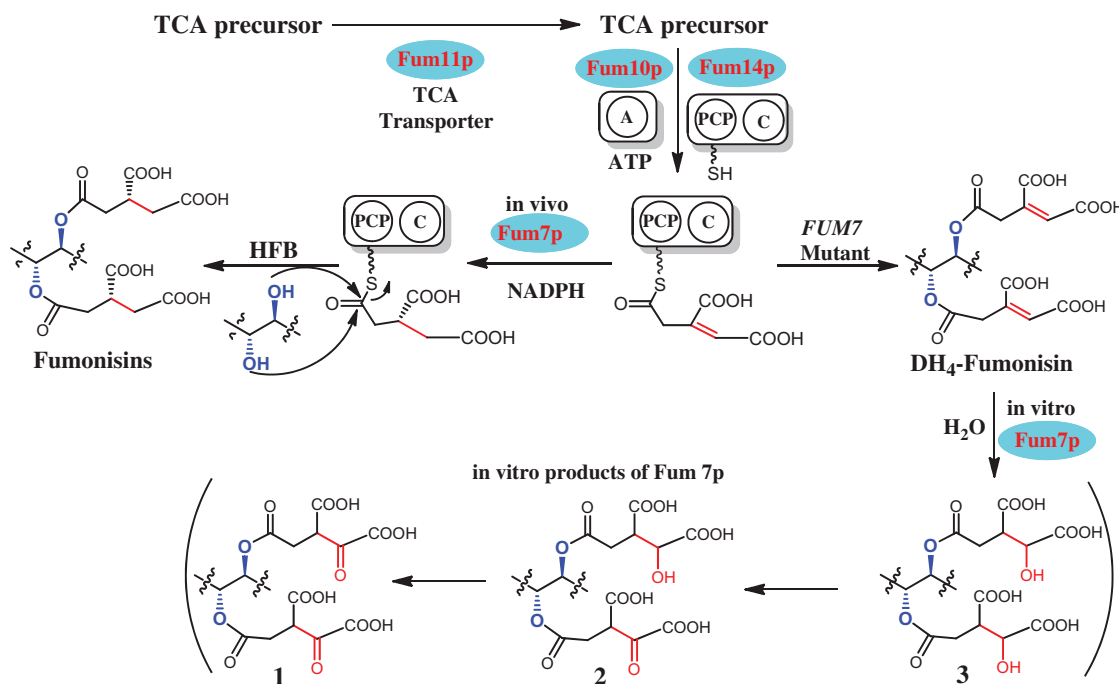


Figure 5. A proposed mechanism for *in vivo* formation of tricarballylic esters in fumonisin biosynthesis and the observed *in vitro* activity of Fum7p. TCA, tricarboxylic acid; A, adenylation domain encoded by *FUM10*; PCP, peptidyl carrier protein domain in the protein encoded by *FUM14*; C, condensation domain in the protein encoded by *FUM14*; and HFB, hydrolyzed fumonisins.

tricarballylic ester formation. First, *FUM7* mutants produce DH<sub>4</sub>-FBs with an alkene function in both esters rather than the wild-type complement of FBs (Butchko et al. 2006). Second, the deduced amino acid sequence of *FUM7* is most similar to a class of dehydrogenases/reductases that catalyze alkene-to-alkane reductions (Seibert et al. 1998; Kallberg et al. 2002). Given the evidence, it makes sense to propose that Fum7p catalyzes the hydrogenation of DH<sub>4</sub>-FB to form FB (Du et al. 2008; Huffman et al. 2010). However, the regio- and stereospecificity of the reduction was not clear. In addition, the timing of the reduction remained unknown, because the reduction could take place after the DH<sub>4</sub>-FB product has been released from the carrier domain (PCP) of Fum14p, or the reduction could take place while aconitate is still covalently linked to the PCP after the substrate has been activated by Fum10p and loaded to the PCP of Fum14p (Figure 5). In this study, we have determined the position of the carbon-carbon double bond in DH<sub>4</sub>-FB<sub>1</sub>, the predominant DH<sub>4</sub>-FB produced by *FUM7* mutants of *F. verticillioides*. In addition, our results indicate that alkene function in the tricarballylic esters of DH<sub>4</sub>-FB<sub>1</sub> undergo isomerization that leads to the formation of a mixture of regio- and stereoisomers. If this isomerization occurs *in vivo*, it seems probable that Fum7p would have a broad specificity to reduce the mixture into regular fumonisins with saturated tricarballylate esters, because wild-type *F. verticillioides* does not produce DH<sub>4</sub>-FBs. Furthermore,

the results of the heterologous expression of *FUM7* and assessment of *in vitro* activity of the resulting enzyme suggest that the Fum7p-catalyzed reduction reaction most likely takes place while the aconitate substrate is covalently linked to the PCP of Fum14p enzyme.

In conclusion, this work provides molecular insights into the biosynthetic mechanism of the rare structural moieties of fumonisins. Because the toxicity of DH<sub>4</sub>-FBs relative to FBs is not known and tricarballylic esterification is an essential step for the maturation of the mycotoxins, an understanding of the complexity of this process is useful for efforts in agriculture to reduce mycotoxin contamination.

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