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Bound Fumonisin B_1: Analysis of Fumonisin-B_1 Glyco and Amino Acid Conjugates by Liquid Chromatography−Electrospray Ionization−Tandem Mass Spectrometry

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To study the formation of fumonisin artifacts and the binding of fumonisins to matrix components (e.g., saccharides and proteins) in thermal-treated food, model experiments were performed. Fumonisin B_1 and hydrolyzed fumonisin B_1 were incubated with α-D-glucose and sucrose (mono- and disaccharide models), with methyl α-D-glucopyranoside (starch model), and with the amino acid derivatives *N*-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester (protein models). The reaction products formed were analyzed by liquid chromatography−electrospray ionization−tandem mass spectrometry. The incubation of D-glucose with fumonisin B_1 or hydrolyzed fumonisin B_1 resulted in the formation of Amadori rearrangement products. Whereas conjugates were found following the reaction of sucrose, methyl α-D-glucopyranoside, and the amino acid derivatives with fumonisin B_1, the heating with hydrolyzed fumonisin B_1 yielded no artifacts. For structural determination, the stable reaction product formed by heating of methyl α-D-glucopyranoside (as starch model) with fumonisin B_1 was purified and identified by nuclear magnetic resonance spectroscopy as the diester of the fumonisin tricarballylic acid side chains with methyl α-D-glucopyranoside. These model experiments demonstrate that fumonisins are able to bind to polysaccharides and proteins via their two tricarballylic acid side chains.

KEYWORDS: Fumonisin B_1; hydrolyzed fumonisin B_1; artifacts; binding; matrix components; LC-ESI-MS/MS; model experiments; thermally treated food

INTRODUCTION

The fumonisins are a group of structurally related toxic metabolites of *Fusarium verticillioides* (formerly *Fusarium moniliforme*), one of the most common molds that is associated with corn and corn-based foods and feeds worldwide (*1*). Fumonisin B_1 **1** (**Figure 1**), the predominant metabolite, is known to cause a range of species-specific toxic responses, including leucoencephalomalacia in horses, pulmonary edema in swine, and hepatosis and nephrotoxicity in rodents (*1*−*3*). The question of whether these toxins show a positive correlation to human esophageal cancer rates in South Africa (*4*) and China (*5*) remains unanswered. However, recently, a National Toxicology Program (NTP) long-term feeding study provided clear evidence for the carcinogenic activities of fumonisin B_1 in female mice and male rats (*6*). Fumonisin B_1 is known to intervene in the sphingolipid metabolism by inhibition of ceramide synthase, leading to elevated sphinganine levels (*7*). This is believed to be the key event in the toxicogenesis of fumonisins; thus, the majority of the current studies focus on the correlation between a high sphinganine content in cells and carcinogenicity induced by fumonisin B_1 (*8*, *9*).

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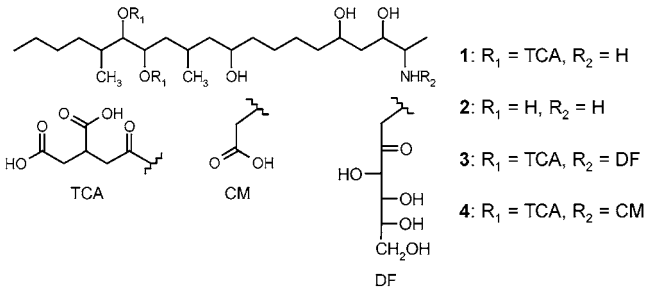

Figure 1. Structures of fumonisin B_1 **1**, hydrolyzed fumonisin B_1 **2**, *N*-(1-deoxy-D-fructos-1-yl)fumonisin B_1 **3**, and *N*-(carboxymethyl)fumonisin B_1 **4**.

Fumonisins can be found in corn and processed corn-based food products. Although fumonisins are generally heat stable, it is well known that processing decreases fumonisin concentrations, probably by changing their chemical structures (*10*−*16*). Our own data show that about 70−90% of spiked fumonisin B_1 was lost when corn grits were manufactured into extrusion products (*10*); this was recently confirmed by Cortez-Rocha et al. (*11*) by extruding alkali-processed corn. Voss and Sanders et al. (*12*, *13*) reported that fumonisin B_1 concentrations were reduced significantly in masa, baked chips, and fried chips

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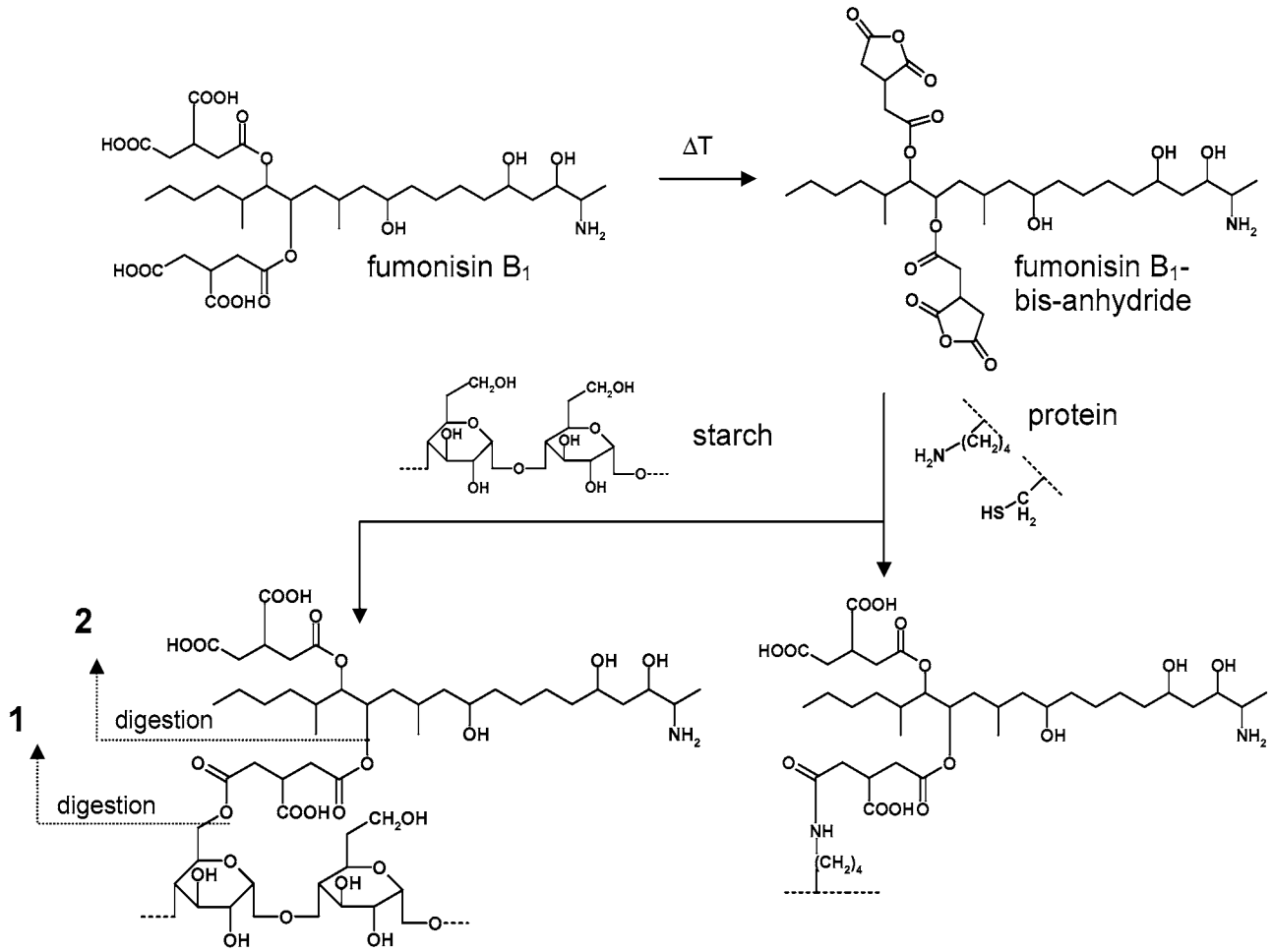

Figure 2. Postulated binding of fumonisin B_1 **1** to protein and starch during heating and its release in the gastrointestinal tract (*22*).

compared to their respective raw corn samples. The effects of baking and frying were studied by Jackson et al. (*14*) and Bullerman et al. (*15*), who concluded that high temperatures and long cooking times lead to remarkable reduction of fumonisin B_1 contents. Recently, Kim et al. described the occurrence of hidden (protein-bound) fumonisins in corn flakes (*16*).

Fumonisin B_1 **1** is characterized by the presence of two tricarballylic acid (TCA) groups esterified to a 20-carbon backbone (**Figure 1**). Alkaline treatment causes the removal of one or both of the TCA groups, yielding the partial hydrolyzed and/or hydrolyzed fumonisin B_1 **2** (**Figure 1**) (*17*). Nixtamalization, the traditional treatment of corn with calcium hydroxide to produce masa (tortilla flour), can convert fumonisin B_1 to hydrolyzed fumonisin B_1. The removal of the TCA side chains was thought to be a method for detoxifying fumonisin-contaminated corn; however, in a short-term liver cancer initiation/promotion model, hydrolyzed fumonisin B_1 revealed greater cytotoxicity than fumonisin B_1 (*18*). The study demonstrated the importance of knowledge about the reaction and stability of fumonisins during food processing. In addition to hydrolyzed fumonisin B_1, there exist some other derivatives which are formed during the processing of corn. Fumonisins are able to react with reducing sugars in a manner similar to nonenzymatic browning (*19*). The primary amine group of fumonisins reacts with the carbonyl group of reducing sugars, forming a Schiff base which converts into further products. Recently, Poling at al. (*20*) described *N*-(1-deoxy-D-fructos-1-yl)fumonisin B_1 **3** (**Figure 1**) as the first stable product formed after the Amadori rearrangement of the Schiff base. Previously, Howard et al. (*21*) concluded that *N*-(carboxymethyl)fumonisin B_1 **4** (**Figure 1**) was the principal reaction product when fumonisin B_1 was heated with aqueous solutions of reducing sugars.

Besides the amine group, fumonisins possess two TCA side chains as functional groups which probably can react with food components. Shier at al. (*22*−*24*) partially characterized the covalent binding of radiolabeled fumonisin B_1 to corn proteins and starch. For linkage they proposed that binding of the toxin occurs by first activating the toxin molecule by loss of water from the side chain to produce an anhydride, which could then react with free functional groups of starch and protein like hydroxy, amine, or thiol groups (**Figure 2**). The exact nature of the linkage is still not clear; however, it is of concern because it would be expected that digestion of "bound" fumonisin in the gastrointestinal tract would release either intact fumonisins or hydrolyzed fumonisins (**Figure 2**). To get more information about the binding of fumonisins via their TCA side chains to saccharides, polysaccharides, and proteins after thermal treatment, we performed model experiments. Therefore, we heated fumonisin **1** and **2** with α-D-glucose and sucrose (mono- and disaccharide models), with methyl α-D-glucopyranoside (starch model), and with the amino acid derivatives *N*-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester (protein model). These amino acid derivatives served as protein model compounds in our studies. The products which were formed upon heating of **1** or **2** with the above-mentioned model compounds

were characterized by liquid chromatography−electrospray ionization−tandem mass spectrometry (LC-ESI-MS/MS) experiments.

MATERIALS AND METHODS

Materials. Fumonisin B_1 and *N*-α-acetyl-L-lysine methyl ester hydrochloride were purchased from Calbiochem-Novabiochem (Bad Soden, Germany). L-Cysteine methyl ester was obtained from Sigma-Aldrich (Steinheim, Germany). Methyl α-D-glucopyranoside was from Fluka (Deisenhofen, Germany). Hydrolyzed fumonisin B_1 was produced from fumonisin B_1 according to the method of Hopmans et al. (*25*). Water, acetonitrile, and methanol, all of HPLC grade, and trifluoroacetic acid (TCA) were from Merck (Darmstadt, Germany). All other chemicals, of analytical purity, were obtained from Fluka or Sigma-Aldrich. C18 solid-phase cartridges (500 mg, 3 mL) were from Separtis (Grenzach-Wyhlen, Germany)

LC-ESI-MS/MS Analysis. LC-ESI-MS/MS analyses were conducted in the positive-ion mode on a TSQ 7000 tandem mass spectrometer system equipped with electrospray ionization (ESI) as the interface (Finnigan MAT, Bremen, Germany). Chromatographic separation was performed by using an Applied Biosystems 140b LC pump (Bai, Bensheim, Germany). For sample injection, a SunChrom Triathlon autosampler (SunChrom, Friedrichsdorf, Germany) was used. Data acquisition and mass spectrometric evaluation were carried out with ICIS 8.1 software (Finnigan MAT).

For LC-ESI-MS/MS, chromatographic separations were carried out on a 150- × 2.1-mm-i.d., 5-μm Symmetry C18 column (Waters, Milford, MA) using a binary gradient. Solvent A was 0.05% TFA in water (v/v), and solvent B was 0.05% TCA in acetonitrile. The LC program consisted of a linear gradient starting from 30% B to 45% B in 15 min, followed by a linear gradient to 99% B in 2 min. The column was washed for 3 min with 100% solvent B after each injection and equilibrated for 5 min at the starting conditions. The flow rate was set to 200 μL/min, and the injection volume was 10 μL. For pneumatically assisted electrospray ionization, the spray capillary voltage was set to 3.5 kV, and the temperature of the heated capillary, acting simultaneously as a repeller electrode (20 V), was 210 °C. Nitrogen served both as sheath gas (70 psi) and as auxiliary gas (10 units). The mass spectrometer was operated in the full-scan mode, detecting positive ions. The mass range was dependent on the individual heating experiment. Basically, two mass ranges were used, *m/z* 300−900 and *m/z* 900−1600, each with a total scan duration of 1.0 s. The MS/MS experiments were performed at a collision energy of 20−65 eV, with argon (2.00 Pa) serving as collision gas.

Exact Mass. Exact mass measurements were performed on a Quattro-LC instrument modified for higher sampling rates (Micromass, Manchester, UK).

NMR Spectroscopy. NMR spectra were acquired with a Bruker DMX 600 spectrometer; the chemical shifts were calibrated by utilizing the solvent signal (MeCN, 1.95 ppm for ^{1}H NMR, 1.3 ppm for ^{13}C NMR; MeOH, 3.5 ppm for ^{1}H NMR, 49.3 ppm for ^{13}C NMR) as reference. Chemical shifts are given on the δ-scale. In the 1D and 2D NMR experiments, pulse programs were taken from the Bruker software library. For structure elucidation and NMR signal assignment, ^{1}H, ^{13}C, DEPT, ^{1}H,^{1}H COSY, HMQC, and HMBC spectra were recorded.

Synthesis of BOC-L-Cysteine Methyl Ester. L-Cysteine methyl ester (74 μmol) was dissolved in a 10% solution of triethylamine in methanol (50 μL) and further diluted with methanol (500 μL), and then di-*tert*-butyl dicarbonate ($(BOC)_2O$, 148 μmol) was added with vigorous stirring. The mixture was then heated to 40 °C for 15 min. Stirring was continued at room temperature for 30 min, the solvent was evaporated under a nitrogen stream, and the residue was stirred for 10 min with ice-cold dilute hydrochloric acid, pH 2.15 (500 μL). The solution was extracted with ethyl acetate (5 × 500 μL), the organic layer was dried over sodium sulfate, and the solvent was removed by evaporation. Yield: 10.7 mg (62%). ESI-MS: *m/z* 235 $[M + H]^+$. ESI-MS/MS (−20 eV): *m/z* 135 $[M + H - BOC]^+$.

Synthesis of *N*-Acetyl-tri-*O*-acetylfumonisin B_1 Bis-anhydride and *N*-Acetyl-tri-*O*-acetylfumonisin B_1. To a solution of fumonisin B_1 (7 μM) in 200 μL of pyridine was added 200 μL of acetic anhydride. The mixture was stirred at room temperature for 16 h. The solvent was evaporated under a nitrogen stream. Yield: 5.9 mg (98%) of *N*-acetyl-tri-*O*-acetylfumonisin B_1 bis-anhydride (acetylated fumonisin B_1 bis-anhydride). ESI-MS: *m/z* 854 $[M + H]^+$.

For the synthesis of *N*-acetyl-tri-*O*-acetylfumonisin B_1, the TCA groups, which were converted into anhydrides, had to be released. *N*-Acetyl-tri-*O*-acetylfumonisin B_1 bis-anhydride was stirred in a mixture of tetrahydrofuran and water (1:1, v/v) at room temperature for 12 h before evaporation of the solvent. Yield: 6 mg (98%) of *N*-acetyl-tri-*O*-acetylfumonisin B_1 (acetylated fumonisin B_1). ESI-MS: *m/z* 890 $[M + H]^+$. A detailed description of this reaction, including structures, can be found in ref (*26*).

Synthesis of *N*-(1-Deoxy-D-fructos-1-yl)fumonisin B_1. The synthesis of *N*-(1-deoxy-D-fructos-1-yl)fumonisin B_1 was based on the procedure of Poling et al. (*20*). The identity was confirmed by ESI-MS, ESI-MS/MS, and NMR spectroscopy. The NMR data were in agreement with the data published by Poling et al. (*20*). ESI-MS: *m/z* 884 $[M + H]^+$. ESI-MS/MS (−45 eV): *m/z* 866 $[M + H - H_2O]^+$, 514 $[M + H - 2TCA - H_2O]^+$, 442 $[M + H - 2TCA - 5H_2O]^+$, 334 $[M + H - 2TCA - Glc - Rest - 2H_2O]^+$. Exact mass: *m/z* $[M + H]^+$ calcd for $C_{40}H_{70}NO_{20}$, 884.4491; found, 884.4551.

Model Experiments. *General Remarks.* All model experiments were performed by heating the reactants without solvent. To get a homogeneous mixture, aliquots of stock solutions of the reactants (1−100 mg/mL in water) were mixed in a 1.5-mL or 4.5-mL glass vial and sonicated, and then the solvent was removed under a stream of nitrogen before heating.

*Reaction of **1** and **2** with α-D-Glucose.* In a 1.5-mL septum vial, a mixture of 0.1 mg of fumonisin B_1 (0.14 μmol) or hydrolyzed fumonisin B_1 (0.25 μmol) and 1 mg of α-D-glucose (5.6 μmol) was heated in a heating block for 3 min at 160 °C without solvent. The reaction mixture was dissolved with 100 μL of water/acetonitrile (70:30, v/v) and used for LC-MS/MS measurements.

*Reaction of **1** and **2** with Sucrose and Methyl a-D-Glucopyranoside.* In a 1.5-mL septum vial, a mixture of 0.1 mg of fumonisin B_1 (0.14 μmol) or hydrolyzed fumonisin B_1 (0.25 μmol) and 1 mg of sucrose (2.9 μmol) or methyl α-D-glucopyranoside (5.2 μmol) was heated in a heating block for 40 min at 150 °C without solvent. The reaction mixture was dissolved with 100 μL of water/acetonitrile (70:30, v/v) and used for LC-MS/MS measurements.

*Reaction of **1** and **2** with Amino Acid Derivatives.* In a 1.5-mL septum vial, a mixture of 0.1 mg of fumonisin B_1 (0.14 μmol) or hydrolyzed fumonisin B_1 (0.25 μmol) and 1 mg of *N*-α-acetyl-L-lysine methyl ester (4.9 μmol) or BOC-L-cysteine methyl ester (4.2 μmol) was heated in a heating block for 40 min at 105 °C without solvent. The reaction mixture was dissolved with 100 μL of water/acetonitrile (70:30, v/v) and used for LC-MS/MS measurements.

Reaction of Acetylated Fumonisin B_1 Bis-anhydride or Acetylated Fumonisin B_1 with Methyl α-D-Glucopyranoside. In a 1.5-mL septum vial, a mixture of 0.1 mg of *N*-acetyl-tri-*O*-acetylfumonisin B_1 bis-anhydride (0.14 μmol) or *N*-acetyl-tri-*O*-acetylfumonisin B_1 (0.14 μmol) and 1 mg of methyl α-D-glucopyranoside (5.2 μmol) was heated in a heating block for 40 min at 150 °C without solvent. The reaction mixture was dissolved with 100 μL of water/acetonitrile (70:30, v/v) and used for LC-MS/MS measurements.

*Synthesis of Acetylated Fumonisin B_1−Di-(methyl α-D-glucopyranoside) **5**.* In a 4.5-mL vial, a mixture of 5 mg of *N*-acetyl-tri-*O*-acetylfumonisin B_1 bis-anhydride (7 μmol) and 50 mg of methyl α-D-glucopyranoside (260 μmol) was heated in a heating block for 60 min at 150 °C without solvent. The reaction mixture was dissolved with 1.5 mL of water (pH 3.5) and divided into two samples of 750 μL each. The main reaction product was then recovered from the reaction mixtures by passage through C18 cartridges. Each cartridge had been preconditioned with 2 mL of methanol (MeOH) followed by 2 mL of water, adjusted to pH 3.5 with 0.1 M hydrochloric acid. After the mixtures were loaded, the cartridges were washed with 3 mL of H_2O, pH 3.5, 1 mL of H_2O, pH 3.5/MeOH (80:20), followed by 1 mL of H_2O, pH 3.5/MeOH (50:50). The product was eluted with 2 mL of H_2O, pH 3.5/ MeOH (30:70). The eluate was freeze-dried to give 4.8 mg (yield 64%) of *N*-acetyl-tri-*O*-acetylfumonisin B_1−di-(methyl α-D-glucopyranoside) **5** (**Figure 6**, below). ESI-MS: *m/z* 1242.6 $[M + H]^+$,

m/z 1264.6 $[M + Na]^+$. Exact mass: *m/z* $[M + Na]^+$, calcd for $C_{56}H_{91}NNaO_{29}$, 1264.5574; found, 1264.5606. ^1H NMR (600 MHz, CD_3OD, ppm, *J* in Hz): δ 0.91 (d, $J = 6.9$, 3H), 0.92 (t, $J = 7.2$, 3H), 0.96 (d, $J = 6.5$, 3H), 1.07 (d, $J = 6.9$, 3H) 1.18−1.75 (m, 20H), 1.78 (m, 2H), 1.94 (s, 3H), 2.00 (s, 3H), 2.02 (s, 3H), 2.03 (s, 3H), 2.48−2.86 (m, 4H), 3.19 (q, 4H), 3.28−3.31 (m, 1H) 3.38−3.42 (m, 1H), 3.60 (m, 1H), 3.70 (m, 1H), 3.92 (s, 3H), 4.20−4.28 (m, 1H), 4.35−4.42 (m, 1H), 4,08 (m, 1H), 4.65 (d, $J = 3.33$, 1H), 5.00 (m, 1H). ^{13}C NMR (600 MHz, CD_3OD, ppm): δ 14.4 (C20), 16.0 (C21), 17.4 (C1), 20.9 (C22), 21.2 (acetyl a,c,d), 22.6 (acetyl b), 23.9 (C19), 26.0 (C7), 26.2 (C8), 27.2 (C12), 29.7 (C18), 33.1 (C17), 34.9 (C16), 35.7 (C6/9/13), 36.5 (C2′/2″/4′/4″), 36.8 (C4), 39.2 (C3′/3″), 41.6 (C11), 48.5 (C2), 55.8 (C7*), 62.4 (C6*), 71.0 (C5*), 71.2 (C5), 71.8 (C4*), 73.0 (C14), 73.2 (C3/10), 73.4 (C2*), 75.1 (C3*), 78.8 (C15), 101.3 (C1*), 172.4 (acetyl c), 172.7 (acetyl a), 172.8 (acetyl b), 173.0 (acetyl d), 173.4 (C5′/5″), 173.8 (C1′/1″), 177.9 (C6′/6″) (for numbering and indexing, see **Figure 6**).

RESULTS AND DISCUSSION

The fate of fumonisin B_1 **1** that disappears during thermal food processing is not known. Our own data showed that about 70−90% of spiked fumonisin B_1 was lost when corn grits were manufactured into extrusion products (*10*). Further, we demonstrated that the *N*-(carboxymethyl)fumonisin B_1 **4** concentrations in the fumonisin B_1-spiked extrusion products were very low, even when free D-glucose was added. From these data, we concluded that the formation of **4** cannot explain the loss of fumonisin B_1. These results are in agreement with literature data. Shier et al. (*22*−*24*) showed that, by heating of corn meal spiked with [^{3}H]fumonisin B_1, only a small percentage of fumonisin B_1 was converted to more polar products, such as would be expected from the Maillard-type reaction. They also demonstrated in the same study that the largest fraction of radiolabeled fumonisin B_1 becomes covalently bound to protein probably via the TCA side chains; however, the nature of the linkage is not known yet. Heating of purified starch also resulted in binding of the toxin, but as in the case of proteins, the question of the type of binding still remains unanswered. The binding of fumonisins to matrix components is a food safety concern, as the bound fumonisin B_1 would be expected to release either **1** or **2** in the gastrointestinal tract. Thus, in the case of bound fumonisins, the real exposure of consumers of thermally treated corn products to fumonisins could be much higher than previously supposed, since present fumonisin extraction and measurement methods do not consider bound toxins. First attempts have been made to analyze bound forms of fumonisins in corn products. Kim et al. (*16*) found 2.6 times more fumonisin B_1 in corn flakes after extraction with sodium dodecyl sulfate and hydrolysis with potassium hydroxide. From this background, the aim of our current study was to get more detailed information about the binding of fumonisins to saccharides and protein. Therefore, we performed model experiments by heating fumonisin B_1 and hydrolyzed fumonisin B_1 with α-D-glucose and sucrose (mono- and disaccharide models), with methyl α-D-glucopyranoside (starch model), and with the amino acid derivatives *N*-α-acetyl-L-lysine methyl ester and BOC-L-cysteine methyl ester (protein model). In methyl α-D-glucopyranoside (and in starch), the anomeric carbon is protected and cannot react with fumonisin B_1 in a Maillard-type reaction. Therefore, it is a useful model compound for starch. In the case of the two amino acid derivatives, the α-amine group and the carboxyl group are protected so that only the amino acid side chains could react with fumonisin B_1. To simulate food processing conditions, the studies were carried out by dry heating without solvent.

Reaction of Fumonisins with α-D-Glucose. In the case of the incubation of fumonisin B_1 **1** with α-D-glucose, besides **1** (*m/z* 722, $[M + H]^+$), one major compound with *m/z* of 884 was detected when samples were analyzed by LC-MS/MS (**Figure 3**). The compound was isolated and identified as *N*-(1-deoxy-D-fructos-1-yl)fumonisin B_1 **3** by comparison with a synthesized reference compound (*20*). The product ion spectrum of **3** (**Figure 4**), obtained by collision-induced dissociation of the protonated molecule using argon as collision gas, revealed signals at *m/z* 514, 496, 478, 460, 442, and 424, generated by the loss of the two TCA groups and two to six molecules of water. The other signals at *m/z* 352 and 334 are characteristic for the fragmentation of fumonisins and are generated by the loss of the two TCA groups, the sugar molecule, and one or two molecules of water. Heating of α-D-glucose with hydrolyzed fumonisin B_1 **2** under the same conditions resulted in the formation of a similar compound with *m/z* 568. Hydrolyzed fumonisin B_1 lacks the TCA side chains, so it can be concluded that the compound at *m/z* 568 was formed by a Maillard-type reaction of the primary amine and the aldehyde group of D-glucose.

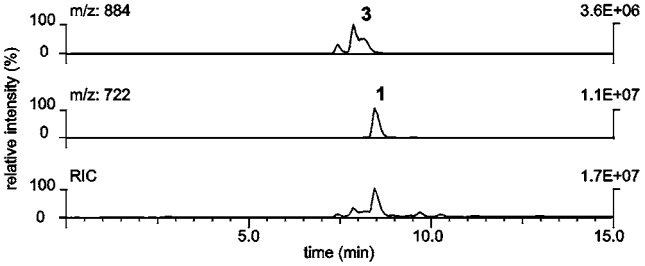

Figure 3. LC-ESI-MS analysis of the reaction mixture resulting from heating α-D-glucose and fumonisin B_1 **1** at 160 °C for 3 min (**3**, *N*-(1-deoxy-D-fructos-1-yl)fumonisin B_1; TIC, total ion chromatogram).

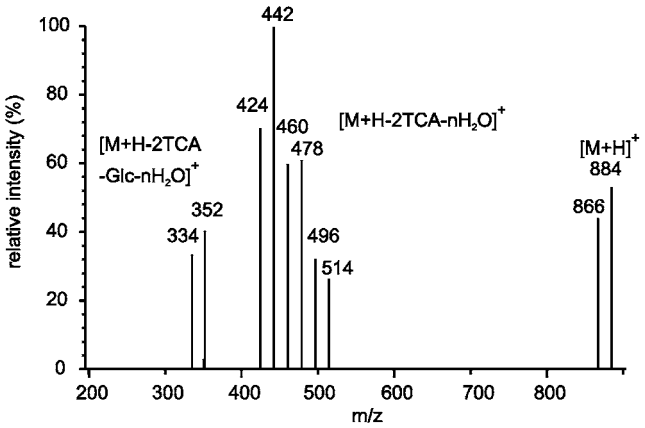

Figure 4. Product ion spectra of *N*-(1-deoxy-D-fructos-1-yl)fumonisin B_1 **3** obtained by collision-induced dissociation (CID) (−40 eV, 2.0 mTorr argon) of the precursor ion, *m/z* 884, $[M + H]^+$.

Reaction of Fumonisins with Sucrose and Methyl α-D-Glucopyranoside. Whereas heating of **1** and **2** with α-D-glucose induced the formation of these two compounds in remarkable amounts, only low concentrations of fumonisin B_1 conjugates were formed when **1** was heated at 150 °C for 40 min with sucrose or methyl α-D-glucopyranoside. In the case of sucrose, there was a significant signal for a protonated molecule, $[M + H]^+$, at *m/z* 1047, and in the case of methyl α-D-glucopyranoside, there were two $[M + H]^+$ signals at *m/z* 898 and 1074. The unknown compounds were formed in approximately 5−10% yield, calculated from the LC-MS peak areas compared to **1**, and eluted less than 2 min faster. These three compounds indicate a covalent binding of one molecule of sucrose (molecular mass 1046 Da) or one (molecular mass 897 Da) or two

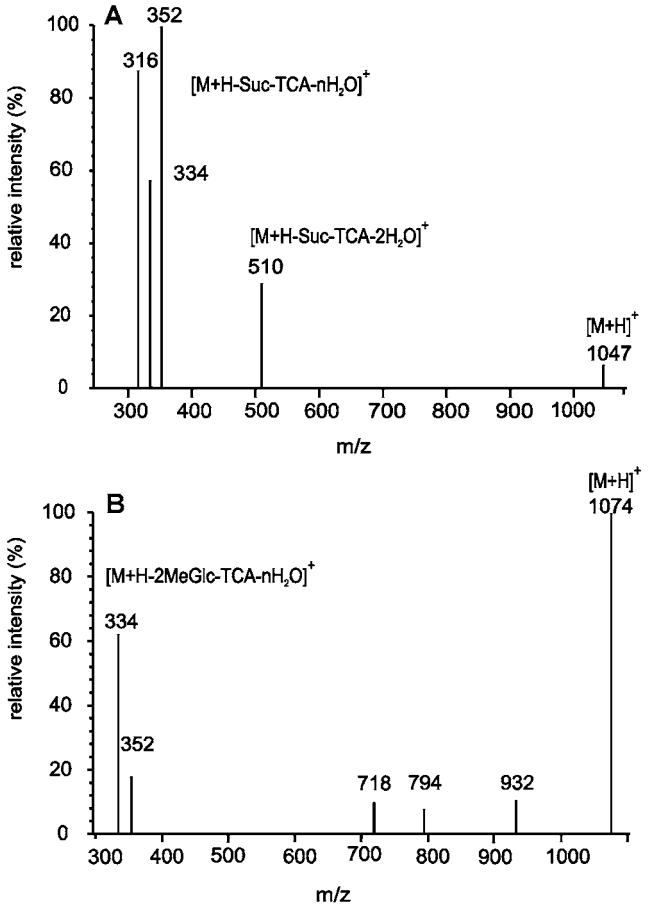

Figure 5. Product ion spectra of (A) the precursor ion, *m/z* 1047, $[M + H]^+$, in the reaction mixture resulting from heating sucrose and fumonisin B_1 at 150 °C for 40 min, obtained by collision-induced dissociation (CID) (–50 eV, 2.0 mTorr argon), and of (B) the precursor ion, *m/z* 1047, $[M + H]^+$, in the reaction mixture resulting from heating methyl α-D-glucopyranoside and fumonisin B_1 at 150 °C for 40 min, obtained by collision-induced dissociation (CID) (–65 eV, 2.0 mTorr argon).

(molecular mass 1073 Da) molecules of methyl α-D-glucopyranoside to fumonisin B_1. The product ion spectra of all three compounds showed the same fragmentation pattern. **Figure 5** represents the product ion spectra of the $[M + H]^+$ ions, *m/z* 1047 and 1074. Both compounds revealed the typical fragment ions of the fumonisin B_1 backbone at *m/z* 352 and 334, generated by the loss of the two TCA side chains, the saccharide, and one or two molecules of water. Sucrose and methyl α-D-glucopyranoside are both nonreducing sugars, and thus covalent binding to fumonisin B_1 via the amine group in a Maillard-type reaction is not possible. The product ion spectra, and the fact that in both sugars the anomeric carbon atom is protected, suggested that the sugars were bound via the TCA side chains to **1**. This assumption was confirmed by the observation that, after heating **2** with sucrose or methyl α-D-glucopyranoside under the same conditions, no reaction products were detectable. Therefore, there was no binding of sucrose or methyl α-D-glucopyranoside in the case of **2**. It is known that heating of **1** can lead to the formation of a fumonisin B_1 bis-anhydride by an intramolecular loss of water from the TCA side chains (*22*−*24*). Since anhydrides are considered to be very reactive, the side-chain anhydride moiety of **1** would be expected to react readily with nucleophilic moieties of saccharides or proteins. Particularly, hydroxy groups of (poly)saccharides, or thiol groups of cysteine and ϵ-amine groups of lysine residues, could react to form ester and thioester or an amide linkage between

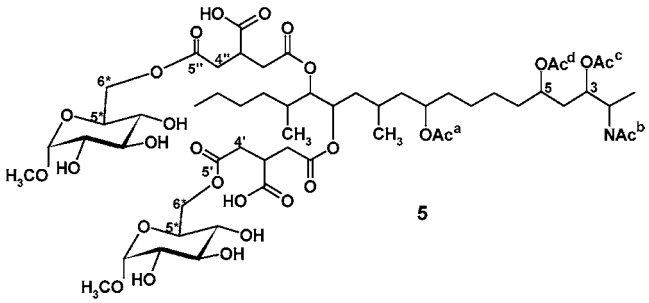

Figure 6. Structure of the isolated main compound **5** resulting from heating acetylated fumonisin B_1 or acetylated fumonisin B_1 bis-anhydride with methyl α-D-glucopyranoside. The molecule demonstrates the binding of fumonisin B_1 via the TCA side chains to saccharides during thermal treatment.

the toxin and (poly)saccharides or proteins. We focused our studies on the covalent binding of fumonisin B_1 to starch, which is the main constituent of corn meal. In methyl α-D-glucopyranoside and in starch, the anomeric carbon atom is protected and cannot react with **1** in a Maillard-type reaction. Therefore, methyl α-D-glucopyranoside is a useful model compound for starch. To get more information about the binding of **1** to methyl α-D-glucopyranoside, we performed further model reactions. In the first step, we synthesized a fumonisin B_1 bis-anhydride by stirring **1** with acetic anhydride. During this reaction, a complete acetylation of the molecule also occurred, and we finally obtained the *N*-acetyl-tri-*O*-acetyl bis-anhydride of fumonisin B_1 (*26*). Since the amine and hydroxy groups are protected, only the TCA side chains could react with methyl α-D-glucopyranoside. When this acetylated fumonisin B_1 bis-anhydride was heated with methyl α-D-glucopyranoside, a new compound with a $[M + H]^+$ signal at *m/z* 1242 could be detected. Subsequent fragmentation of the molecular ion yielded characteristic product ions at *m/z* 352 and 334, indicating a fumonisin backbone. The same compound could be detected when acetylated fumonisin B_1 (*N*-acetyl-tri-*O*-acetylfumonisin B_1) was heated with methyl α-D-glucopyranoside, but the yield was lower. The reaction product with *m/z* 1242 was isolated by using solid-phase extraction on an RP18 column and used for 1D and 2D NMR studies. The isolated compound **5** was identified as the diester of the fumonisin TCA side chains with methyl α-D-glucopyranoside (**Figure 6**). Intensive HMBC connectives between the protons H-6* (δ 4.2−4.4 ppm) of methyl α-D-glucopyranoside and the carbonyl carbons (C-5′/5″, δ 173.4 ppm) proved the linkage between the acid and the sugar moieties at those positions. The ^{13}C spectra of fumonisin B_1, methyl α-D-glucopyranoside, and the new compound at *m/z* 1242 were compared. We observed a slight upfield shift of C-4′/4″ from δ 37.78/37.51 ppm to δ 36.64/36.53 ppm, indicating that the two molecules of methyl α-D-glucopyranoside were bound via C-5'/5'' of fumonisin B_1. The linkage at position 6* of the glucose caused a strong upfield shift of C-5* (from δ 73.52 to δ 71.04 ppm) and a strong downfield shift of C-6* (from δ 62.67 to δ 64.98 ppm), which is in agreement with literature data (*27*, *28*). Thus, we could determine the final structure of the unknown compound **5** with *m/z* 1242 to be the diester of the acetylated fumonisin with two molecules of methyl α-D-glucopyranoside bound to the TCA side chains (**Figure 6**). As mentioned above, methyl α-D-glucopyranoside is very similar to starch, so we can conclude that binding to starch could occur in the same way in heat-treated food.

Reaction of Fumonisins with Amino Acid Derivatives. After we proved the binding of fumonisin B_1 **1** via the TCA side chains to saccharides, we studied the binding of **1** to amino

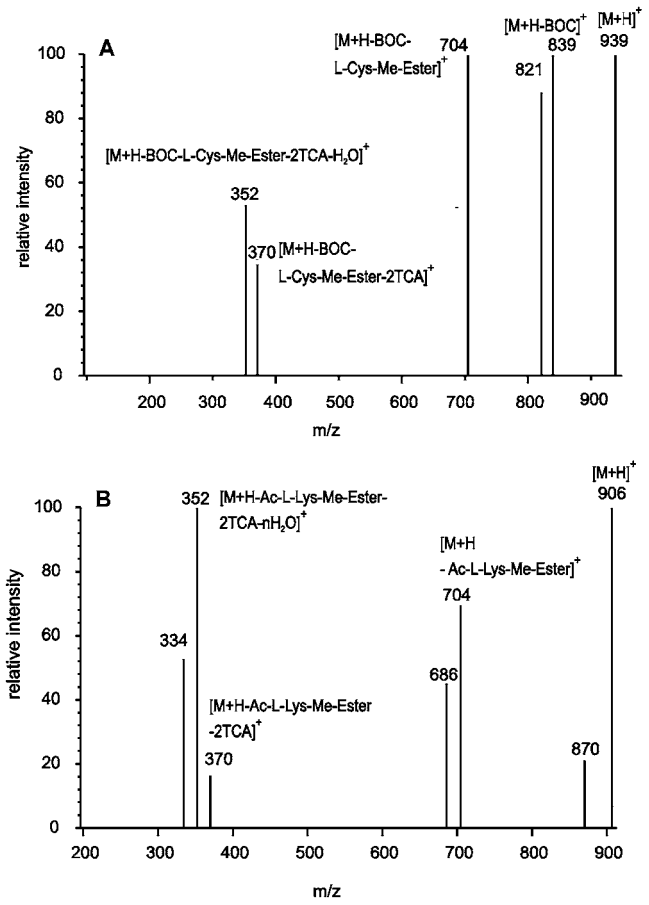

Figure 7. Product ion spectra of (A) the precursor ion, *m/z* 939, $[M + H]^+$, in the reaction mixture resulting from heating BOC-L-cysteine-methyl ester and fumonisin B_1 at 105 °C for 40 min, obtained by collision-induced dissociation (CID) (–35 eV, 2.0 mTorr argon), and of (B) the precursor ion, *m/z* 906, $[M + H]^+$, in the reaction mixture resulting from heating *N*-α-acetyl-L-lysine methyl ester and fumonisin B_1 at 105 °C for 40 min obtained by collision-induced dissociation (CID) (–40 eV, 2.0 mTorr argon).

acids, which were protected at the α-amine group and at the carboxyl group and served as protein models. For our studies, we used the two amino acids, L-lysine and L-cysteine. The heating with **1** resulted in the formation of $[M + H]^+$ signals at *m/z* 906 (heating with *N*-α-acetyl-L-lysine methyl ester) and at *m/z* 939 (heating with BOC-L-cysteine methyl ester). A conjugate with an amide linkage between a TCA moiety and the γ-amine group of *N*-α-acetyl-L-lysine methyl ester would have a molecular mass of 905 Da ($[M + H]^+$, *m/z* 906), and a conjugate with a linkage between the thiol group of BOC-L-cysteine methyl ester would have a mass of 938 Da ($[M + H]^+$, *m/z* 939). The fragmentation of the molecular ions (**Figure 7**) yielded characteristic product ions at *m/z* 370 and 352, indicating a fumonisin backbone. The fragments at *m/z* 704 result from the elimination of the *N*-α-acetyl-L-lysine methyl ester or the BOC-L-cysteine methyl ester from the fumonisin B_1 conjugate. In the case of the incubation of **1** with BOC-L-cysteine methyl ester, the signal at *m/z* 839 was generated by the loss of the BOC protecting group. Under the conditions we used (40 min, 105 °C), we did not observe conjugates with two (or more) amino acid derivatives bound to **1**. Since incubation with **2** did not lead to comparable products, we conclude that, during thermal treatment, **1** can also bind to amino acids and proteins via the TCA side chains. Since binding to amino acid derivatives occurred only in lower yield, compared to that of the methyl α-D-glucopyranoside−fumonisine B_1 conjugate **5**, we did not isolate the formed products.

In our studies, we demonstrated that fumonisin B_1 is able to bind to starch and proteins after thermal treatment, as was shown with methyl α-D-glucopyranoside and protected amino acids as model compounds. Although it is difficult to estimate the amount of bound fumonisins in corn-based food products, we can conclude from our results that the binding to starch, shown with methyl α-D-glucopyranoside, occurs in much higher yield compared to the binding to proteins, shown with amino acid derivatives. The binding of fumonisins to matrix components is primarily influenced by the fumonisin B_1 concentration, the heating temperature, the heating time, and also the water content. Thus, further investigations are necessary to estimate the amount of "bound" fumonisins in thermally treated corn-based food. Since the analysis of bound forms of fumonisins is difficult, we also suggest an indirect method, as already proposed in the literature. Hydrolysis of corn-based food samples with sodium hydroxide would release TCA-bound forms of fumonisins, which could then be analyzed as hydrolyzed fumonisins (*10*, *16*, *29*). However, one has to take into account that fumonisins bound via the amine group are usually not released with this method. Since the hydrolysis of TCA-bound fumonisins could also occur in the gastrointestinal tract, the bioavailability and the toxicity of bound forms are important points which have to be studied in the future.

SAFETY

Fumonisins are potential carcinogens and should be handled with care.

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