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N-(1-Deoxy-D-fructos-1-yl) Fumonisin B_1, the Initial Reaction Product of Fumonisin B_1 and D-Glucose

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Incubation of fumonisin B_1 and D-glucose in aqueous solutions resulted in the formation of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 in addition to the previously reported *N*-(carboxymethyl) fumonisin B_1. *N*-(1-Deoxy-D-fructos-1-yl) fumonisin B_1 is the first stable product formed after the Amadori rearrangement of the Schiff base formed by the reaction of the primary amine of fumonisin B_1 and the aldehyde group of D-glucose. *N*-(1-Deoxy-D-fructos-1-yl) fumonisin B_1 was synthesized by reacting fumonisin B_1 with an excess of D-glucose in methanol and heating for 6 h at 64 °C. It was purified using C_{18} and strong cation exchange solid-phase extraction cartridges and characterized by nuclear magnetic resonance and liquid chromatography−mass spectrometry. Subsequently, *N*,*N*-dimethylformamide was found to be a better reaction solvent, requiring reaction for only 2−3 h at 64 °C and eliminating the formation of methyl esters. Alkaline hydrolysis of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 gave a mixture of hydrolyzed fumonisin B_1 and hydrolyzed *N*-(carboxymethyl) fumonisin B_1.

KEYWORDS: Fumonisins; *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1; Amadori rearrangement; LC−MS

INTRODUCTION

The fumonisins are a group of toxins that were first identified as being produced by *Fusarium verticillioides* Sacc. (Nirenberg) (synonym *F. moniliforme* Sheld.) isolated from corn (*1*). Fumonisins are commonly found in grains used for animal feed and can occur in processed corn products consumed by humans (*2*, *3*). Fumonisin B_1 is the predominant metabolite found in naturally contaminated corn and in purified form is known to cause a range of toxic responses in animals including equine leucoencephalomalacia, porcine pulmonary edema, and hepatosis and nephrotoxicity in rodents (*2*, *4*). Fumonisin B_1 has also been associated with high incidences of human esophageal cancer in South Africa (*5*) and China (*6*). The implications of the fumonisins in human and animal health have been reviewed (*4*, *7*). Fumonisin B_1 has been recently tested for carcinogenicity under the National Toxicological Program. This study demonstrated that fumonisin B_1 is a rodent carcinogen that induces renal tubule tumors in male F344 rats and hepatic tumors in female B6C3F1 mice (*8*).

There has been recent interest in fumonisin-related compounds that may be present in corn or are created during processing that could be a source of fumonisin toxicity and that would not be detected by the most commonly used analytical procedure. Typically, the fumonisins are cleaned up after extraction using either a strong anion exchange or a C_{18} cartridge, derivatized with *o*-phthaldialdehyde, and determined by liquid chromatography (LC) with fluorescent detection by comparison to authentic standards (*9*).

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One process that changes the fumonisins present in corn is nixtamalization. This is the traditional process of treating corn with calcium hydroxide and heat to produce masa used to make tortillas. The treatment of fumonisins with calcium hydroxide causes the hydrolysis of one or both of the tricarballylic acid groups that are esterified to the C-14 and C-15 hydroxy groups (**Figure 1**) in the intact molecules (*10*, *11*). In partially hydrolyzed fumonisins, only one of the tricarballylic acid groups has been removed. Partially hydrolyzed fumonisins exist as an equilibrium mixture of the C-14 and C-15 esters (*10*). Hydrolyzed fumonisins lack both groups. Hydrolyzed and partially hydrolyzed fumonisins retain the primary amine group needed to react with *o*-phthaldialdehyde and give a fluorescent derivative. After modifying the extraction and cleanup procedures, the amounts of fumonisin B_1 and hydrolyzed fumonisin B_1 in corn tortillas could be accurately measured (*12*).

A different process seems to be involved when fumonisin B_1 is heated with corn-based products. The recovery of spiked fumonisin B_1 decreases as the temperature or heating time is increased. Scott and Lawrence (*13*) reported that fumonisin B_1 and fumonisin B_2 were about 40% recovered when heated in corn meal at 190 °C, about 20−30% recovered when heated in moist corn meal at 190 °C, and not recovered from corn meal heated at 220 °C. Jackson et al. (*14*) studied the effects of baking and frying on the fumonisin B_1 content of corn-based foods. They concluded that under normal commercial frying and baking conditions, fumonisin B_1 losses are small. They also showed that at higher temperatures and longer cooking times there was a significant loss of fumonisin B_1 and that hydrolysis was not the mechanism of this loss. More recently, Katta et al. (*15*) showed that there were significant losses of fumonisin B_1 in

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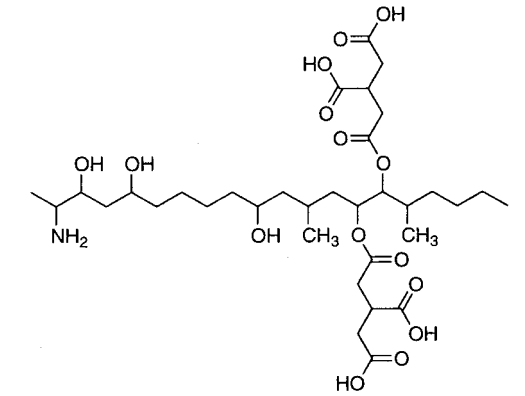

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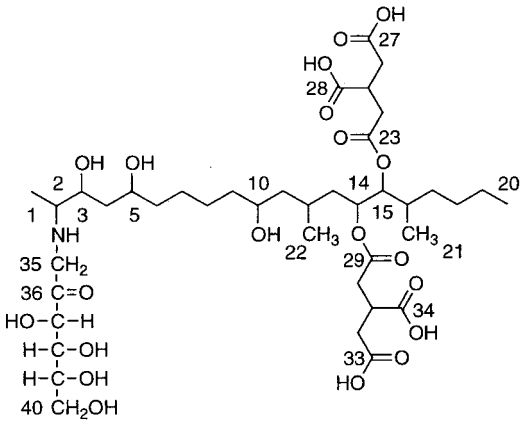

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Figure 1. Chemical structures of fumonisin B_1 (**1**) and **2**.

extrusion-cooked corn grits. They reported about 46−76% of the spiked fumonisin B_1 was lost when the grits were cooked at temperatures and screw speeds that resulted in acceptable product expansion and color.

Murphy et al. (*16*) have suggested that nonenzymatic browning could explain the increasing loss of fumonisin B_1 in samples with increasing heating times and temperatures based on the results from a model system where fumonisin B_1 was incubated with glucose or fructose (*17*). Fumonisins should be able to react with reducing sugars in a manner similar to the reaction of amino acids with reducing sugars involved in the first steps of nonenzymatic browning (*18*). The primary amine group of the fumonisin would react with the carbonyl group of a reducing sugar to give a Schiff base, which could then rearrange into a β-ketoamine. For aldoses, this is known as the Amadori rearrangement, and for ketoses, this is known as the Heyns rearrangement (*18*). The products of these reactions would no longer react with *o*-phthaldialdehyde to give a fluorescent derivative. Murphy et al. (*17*) reported on the disappearance of fumonisin B_1 with time when heated with an excess of fructose or glucose in a neutral phosphate buffer. The fumonisin B_1 remaining was quantified as the *o*-phthaldialdehyde derivative by high-performance liquid chromatography (HPLC). The fumonisin B_1−fructose adduct, in the form of the dried reaction mixture, has been used in feeding studies. The products formed were not characterized other than to report that all of the fumonisin B_1 could be recovered as hydrolyzed fumonisin B_1 after alkaline hydrolysis (*19*, *20*). The same group reported that their limited attempts to identify the products of the fumonisin B_1−fructose (and −glucose) model systems had so far been unsuccessful (*21*).

Howard et al. (*22*) studied the reaction of fumonisin B_1 when heated with aqueous solutions of reducing sugars and concluded that *N*-(carboxymethyl) fumonisin B_1 was the principal product. Their result for the reaction of fumonisin B_1 and D-glucose was consistent with the scheme that the Schiff base formed initially underwent the Amadori rearrangement to the β-ketoamine, which was then oxidized to *N*-(carboxymethyl) fumonisin B_1. On the basis of these observations, they concluded that under the conditions investigated, no stable intermediates of *N*-(carboxymethyl) fumonisin B_1 were accumulated and they also showed that the alkaline hydrolysis of *N*-(carboxymethyl) fumonisin B_1 gave the *N*-carboxymethyl derivative of hydrolyzed fumonisin B_1.

We now report that *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (**Figure 1**), the Amadori rearrangement product, is formed during the reaction of fumonisin B_1 with D-glucose in an aqueous solution. A simple synthesis and cleanup procedure is described for *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1.

MATERIALS AND METHODS

Materials. Methanol was HPLC grade, and sodium hydroxide and *N*,*N*-dimethyl formamide were certified A. C. S. (Fisher Scientific, Fair Lawn, NJ). Acetonitrile was HPLC grade, and potassium phosphate, monobasic, was reagent grade (EM Sciences, Gibbstown, NJ). Sodium borohydride was powder, 98% (Sigma-Aldrich, St. Louis, MO). The glucose used was Difco dextrose for microbiological culture media (Becton Dickinson, Sparks, MD). Ultrapure water was used for all experiments and for HPLC solvents (Simplicity Water Purification System, Millipore, Bedford, MA). Fumonisin B_1 (97% purity) was isolated from cultures of *F. verticillioides* grown on corn (*11*).

LC−Mass Spectrometry (MS) Analysis of Underivatized Fumonisins. A SpectraSYSTEM P4000 pump was coupled to a LCQ mass spectrometer via an electrospray interface (ESI) (Finnigan-MAT, San Jose, CA). An Inertsil ODS-3 15 cm × 3.0 mm i.d. column (MetaChem Technologies, Torrance, CA) was used, and the entire HPLC eluent was introduced into the detector. A 10 min linear gradient from 50/35/15 to 5/35/60 water/1% acetic acid in methanol/methanol was used, and the final composition was held for 20 min. The flow rate was 0.3 mL/min. Mass spectra were obtained by scanning from *m*/*z* 300 to 950. The ESI spray voltage was 4.5 kV, and the capillary temperature was 220 °C. The auxiliary gas flow was 65 arb.

High-Resolution Fast Atom Bombardment Mass Spectroscopy (HR FABMS). HR FABMS was performed on a Micromass 70-SE-4F mass spectrometer utilizing an 8 kV beam of neutral Xe. The sample was dissolved in a 3:1 mixture of dithiothreitol and dithioerythritol. The exact mass was determined by peak matching, and the elemental composition was calculated on a Micromass OPUS data system.

Reaction of Fumonisin B_1 with D-Glucose in Aqueous Phosphate. The procedure of Howard et al. (*22*) for the synthesis of *N*-(carboxymethyl) fumonisin B_1 was followed. Briefly, a solution of 3 mg (4.2 μmol) of fumonisin B_1, 54 mg (300 μmol) of D-glucose, and 2 mL of 50 mM KH_2PO_4, pH 7.5, was sealed in a 4.5 mL amber septum vial and heated for 17 h at 79 °C in a heating block. In the second experiment, 4 mL of the same solution was prepared. Three milliliters was sealed in a 4.5 mL vial and sparged with He for 10 min through a syringe needle inserted through the septum and into the liquid. A second needle above the liquid served as the vent. The remaining 1 mL was sealed in another vial, and both vials were heated at 79 °C for 17 h.

Reaction of Fumonisin B_1 with D-Glucose in Methanol. A mixture of 6 mg (8.3 μmol) of fumonisin B_1, 54 mg (300 μmol) of D-glucose, and 2 mL of methanol was sealed in a 4.5 mL amber septum vial and heated for 3 h at 64 °C in a heating block. The vial was removed, cooled under running water, and sampled before heating for an additional hour. It was again cooled and sampled. For each sample, 10 μL was removed and diluted to 1 mL with 10/90 CH_3CN/H_2O before analysis. The experiment was repeated using twice the amount of each component and sampled at 4, 6, 8, and 24 h.

Synthesis of *N*-(1-Deoxy-D-fructos-1-yl) Fumonisin B_1. Four 4.5 mL amber septum vials, each containing 12 mg (16.6 μmol) of fumonisin B_1, 108 mg (600 μmol) of D-glucose, and 4 mL of methanol, were sealed and heated at 64 °C for 6 h. The vials were removed briefly and shaken every 1−1.5 h. The glucose did not completely dissolve until the vials had been heated about 5 h. The contents of the vials were combined and diluted with 4 volumes of water (64 mL). The products were recovered from the diluted reaction mixture by passing it through a 2 g tC_{18} cartridge (Sep-Pak Vac 12 cm^3 (2 g) tC_{18} cartridge, Waters Corp., Milford, MA) that had been preconditioned with 20 mL of MeOH, followed by 20 mL of MeOH/H_2O (20/80). After the products were loaded, the cartridge was washed with 20 mL of MeOH/H_2O (20/80) and then 20 mL of CH_3CN/H_2O (10/90). The products were eluted with 20 mL of 50/50 CH_3CN/H_2O. The procedure was then repeated using 8 vials, and the products were recovered using a 2 g tC_{18} cartridge. The 50/50 eluates from the 4 and 8 vial preparations were combined. A total of 153 mg (212 μmol) of fumonisin B_1 were used for the 12 vials. Fumonisin B_1 and *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 were separated using a 10 g strong cation exchange (SCX) Mega Bond Elut, 40 μm, SCX cartridge (Varian Sample Preparation Products, Harbor City, CA). The cartridge was conditioned with 100 mL of MeOH followed by 100 mL of 50/50 CH_3CN/H_2O. The combined 50/50 eluates were loaded onto the SCX cartridge. The cartridge was eluted with the following solvents, and the fractions were collected and analyzed, 100 mL of 20/80 CH_3CN/H_2O; 100 mL of 1% HCOOH in 20/80 CH_3CN/H_2O; and 2 × 100 mL and then 6 × 50 mL of 5% HCOOH in 20/80 CH_3CN/H_2O. *N*-(1-Deoxy-D-fructos-1-yl) fumonisin B_1 started eluting in the first 100 mL portion of the latter solvent, while fumonisin B_1 did not start eluting until the second 50 mL portion. The first three portions were combined, diluted with an equal volume of water (250 mL), and loaded onto a 2 g tC_{18} cartridge that had been preconditioned with 20 mL of MeOH, followed by 20 mL of 10/90 CH_3CN/H_2O. The cartridge was washed with 20 mL of the latter solvent and eluted with 20 mL of 50/50 CH_3CN/H_2O. The 50/50 CH_3CN/H_2O eluate was freeze-dried to give 106 mg (120 μmol) of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1.

Synthesis of *N*-(1-Deoxy-D-fructos-1-yl) Fumonisin B_1 in *N*,*N*-Dimethyl Formamide. The reaction was carried out using the same amounts of fumonisin B_1 and glucose (3 mg/mL (4.2 μmol/mL) and 27 mg/mL (150 μmol/mL), respectively) as above but with the replacement of methanol by *N*,*N*-dimethyl formamide as the solvent. The mixture was shaken several minutes until the glucose dissolved and then either heated for 2−3 h at 64 °C or kept at room temperature for 6−8 days.

Incubation of Fumonisin B_1 with Formic Acid. Fumonisin B_1 (1.1 mg) was dissolved in 1 mL of 5% HCOOH in 20/80 CH_3CN/H_2O in a sealed vial and heated at 64 °C. The mixture was sampled after 1 h and then heated an additional 16 h and resampled. The mixture was diluted with 1 mL of water and loaded onto a 500 mg Sep-Pak Vac 3 cm^3 tC_{18} cartridge (Waters Corp., Milford, MA) that had been preconditioned with 5 mL of MeOH, followed by 5 mL of 10/90 CH_3CN/H_2O. The cartridge was washed with 5 mL of this solvent and eluted with 2.5 mL of 50/50 CH_3CN/H_2O. The latter eluate was heated in a sealed vial for 4 h and sampled every hour.

Reduction of *N*-(1-Deoxy-D-fructos-1-yl) Fumonisin B_1. *N*-(1-Deoxy-D-fructos-1-yl) fumonisin B_1 (10.5 mg) dissolved in 1 mL of water was added dropwise to 20.1 mg of sodium borohydride dissolved in 1 mL of water with gentle shaking between drops. The vial was rinsed with 1 mL of water, which was also added dropwise. The mixture was kept at room temperature for 2 h and then added to 5 mL of water, and then, 200 μL of acetic acid in 2 mL of water was added dropwise (final pH 3.5). This was loaded onto a 500 mg tC_{18} cartridge preconditioned with 5 mL of MeOH followed by 5 mL of H_2O. The cartridge was washed with 10 mL of water, then 5 mL of 10/90 CH_3CN/H_2O and eluted with 5 mL of 50/50 CH_3CN/H_2O. The latter eluate was freeze-dried after the addition of 1 mL of water.

Hydrolysis of *N*-(1-Deoxy-D-fructos-1-yl) Fumonisin B_1. Three solutions were prepared using concentrations similar to those used by Howard et al. (*22*) but with the replacement of fumonisin B_1 by *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1. Each solution contained 1.4 mg/mL (1.58 μmol/mL) *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 dissolved in water, 50 mM KH_2PO_4, pH 7.5, or 150 mM glucose (27 mg/mL) in 50 mM KH_2PO_4, pH 7.5. All three solutions were heated at 79 °C and sampled, as described above for the reaction of fumonisin B_1 with glucose in MeOH, at 0, 2, 4, and 22 h. For alkaline hydrolysis at 30 °C, two solutions containing either fumonisin B_1 or *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 were prepared by adding 100 mL of the appropriate 100 ng/mL stock solution to 800 mL of water and then adding 100 mL of 1 M sodium hydroxide. The samples were loaded into the autosampler, and alternate injections were made. The room temperature remained at about 30 °C during the experiment. For hydrolysis at 100 °C, 2 mL of 1 M sodium hydroxide was added to 1 mL of 100 ng/mL aqueous solutions of each compound. These were heated for 2 h in a heating block at 100 °C and then cooled in water and diluted 10-fold for analysis.

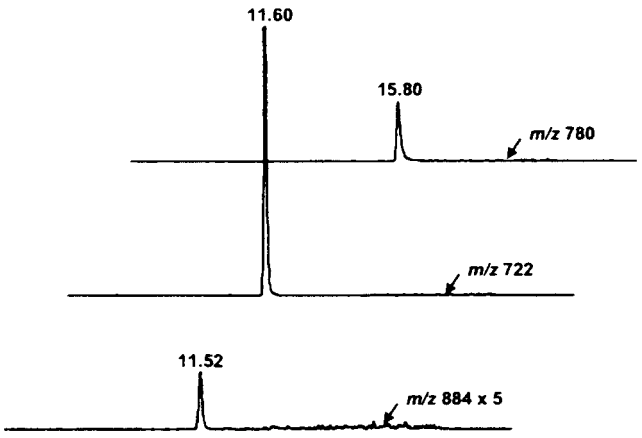

Figure 2. LC–MS analysis of the reaction mixture resulting from heating fumonisin B_1 and glucose in 50 mM KH_2PO_4, pH 7.5, for 17 h at 79 °C using the procedure of Howard et al. (*22*). The heights of the peaks in the ion chromatograms for *N*-(carboxymethyl) fumonisin B_1 (*m*/*z* 780) and *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 884 amplified 5-fold) are relative to the height of the fumonisin B_1 peak (*m*/*z* 722).

Nuclear Magnetic Resonance Spectrometry (NMR). Proton and ^{13}C NMR spectra of fumonisin B_1 and its derivatives were obtained in D_2O. A Bruker ARX 400 spectrometer (400 MHz) equipped with a 5 mm $^{13}C/^1H$ dual probe was used. Spectra are referenced to deuterated TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-$^2H_4$).

RESULTS AND DISCUSSION

The reaction mixture from the preparation of *N*-(carboxymethyl) fumonisin B_1 from fumonisin B_1 and D-glucose following the procedure of Howard et al. (*22*) was analyzed by LC−MS. A peak at *m*/*z* 884 was observed in addition to the expected peaks at *m*/*z* 722 and 780 for the protonated molecular ions of fumonisin B_1 and *N*-(carboxymethyl) fumonisin B_1, respectively (**Figure 2**). The area of the *m*/*z* 884 peak was about 6% of the area of the fumonisin B_1 peak (*m*/*z* 722) and eluted less than 0.1 min faster. *N*-(1-Deoxy-D-fructos-1-yl) fumonisin B_1, the Amadori rearrangement product from the reaction of fumonisin B_1 and D-glucose, would have a molecular mass of 883 Da (M + H, *m*/*z* 884).

Howard et al. (*22*) reported that when the reaction of fumonisin B_1 and D-glucose was carried out under argon there was no depletion of fumonisin B_1 and no formation of *N*-(carboxymethyl) fumonisin B_1. Our experiments showed that under helium, the area of the *N*-(carboxymethyl) fumonisin B_1 peak (*m*/*z* 780) was only 0.5% of the area of the remaining fumonisin B_1 peak (*m*/*z* 722) as compared to 26% when oxygen was not excluded. The area of the *m*/*z* 884 peak did not increase when compared to the area of the remaining fumonisin B_1 peak (*m*/*z* 722) when the reaction was carried out under helium (5.9%) as compared to air (5.8%). When *N*-(1-deoxy-D-fructos-1-yl)

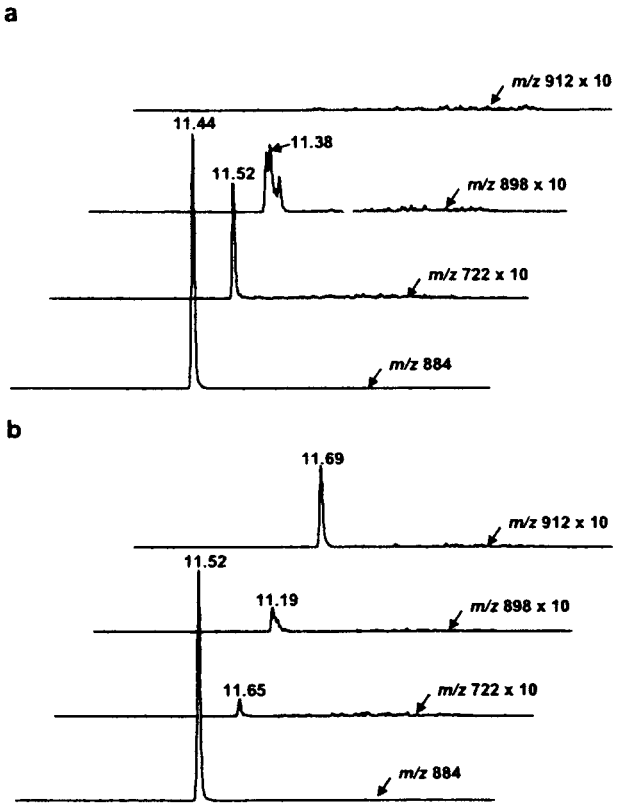

Figure 3. LC–MS analysis of the reaction mixture resulting from heating fumonisin B_1 and glucose in methanol for 6 h at 64 °C (**a**) and of the purified *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 after cleanup using a SCX cartridge (**b**). The heights of the peaks in the ion chromatograms for fumonisin B_1 (*m*/*z* 722), methyl *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 898), and the putative 3-*O*-formyl *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 912), all three amplified 10-fold, are relative to the height of the *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 884) peak.

fumonisin B_1 was heated in air under the conditions used by Howard et al. (*22*), it was oxidized to *N*-(carboxymethyl) fumonisin B_1, but it also reverted to fumonisin B_1. This suggests that fumonisin B_1 and glucose exist in equilibrium with *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 under these conditions and that the rate of oxidation to *N*-(carboxymethyl) fumonisin B_1 is much slower than the rates involved in maintaining the equilibrium.

The most common method of preparing the Amadori rearrangement products of amino acids is to reflux the amino acid in the presence of excess glucose in anhydrous methanol for several hours (*18*, *23*). When fumonisin B_1 was heated with excess glucose in methanol for 6 h at 64 °C, most of the fumonisin B_1 was converted to the *m*/*z* 884 product (**Figure 3a**). The main side reaction was methylation of the acid groups to give the presumed monomethyl esters (*m*/*z* 898) of the compound with *m*/*z* 884. There was also some hydrolysis to the partially hydrolyzed forms of this product. At 6 h, the optimum reaction time, the areas of the fumonisin B_1 peak (*m*/*z* 722) and the methylated *m*/*z* 884 peak were 4 and 7% of the area of the *m*/*z* 884 peak, respectively. The glucose did not completely dissolve until the mixture had been heated for about 5 h, while heating for longer periods only increased the degree of methylation and hydrolysis.

The putative *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 884) was difficult to separate from fumonisin B_1, but about 71% of it could be eluted from a SCX cartridge before fumonisin B_1 started to elute. The yield of purified *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 884) from fumonisin B_1 was 57%, while 80% was converted to *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 using this procedure. The HR FABMS data (*m*/*z* 884.4488) for this material agree with the expected value (*m*/*z* 884.4491, $[M + H]^+$) for the proposed molecular formula ($C_{40}H_{69}NO_{20}$). From the ion chromatograms (**Figure 3b**), the freeze-dried *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 contained less than 0.5% fumonisin B_1 or partially hydrolyzed *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 and only about 2% of the mono methyl esters of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1. An additional compound with *m*/*z* 912 was present in the purified material that was not present in the initial reaction mixture. On the basis of the *m*/*z* value, this could have been a dimethyl ester, but instead, it appears to be an artifact caused by the formylation of the 3-OH group of the adduct because formic acid was used during cleanup of the reaction mixture. When *N*-acetyl fumonisin B_1 (fumonisin A_1) is incubated in a weak solution of acetic acid at elevated temperatures, some of the acetyl groups migrate to the 3- and 5-OH groups, with about three times as much of the 3-*O*-acetyl fumonisin B_1 formed as the 5-*O*-acetyl fumonisin B_1. When the 3- and 5-*O*-acetylated forms are allowed to stand in a neutral solution, the acetyl group migrates back to the nitrogen giving fumonisin A_1 (*24*). A similar reaction probably gave rise to the *m*/*z* 912 compound during the delay between the separation on the SCX cartridge and the analysis of the fractions. During that 3 day period, they were kept in 5% formic acid solution and the nitrogen could have been formylated, with the subsequent transfer of the formyl group to the 3-OH group. To confirm this, fumonisin B_1 was incubated in the eluting solvent and sampled after 1 h and after heating overnight. After 1 h, about 2% of the fumonisin B_1 was formylated giving two peaks (*m*/*z* 750) with the larger one three times greater. After 17 h, about 7% of the fumonisin B_1 was formylated. This was the same pattern observed for the rearrangement of fumonisin A_1 to 3- and 5-*O*-acetyl fumonisin B_1 under acidic conditions (*24*). When this mixture was recovered and incubated in a neutral solution, the formyl groups did not migrate to the nitrogen as was observed with the acetyl groups but remained on the 3- and 5-OH groups. The *O*-formylated fumonisins seem to be the stable forms in neutral aqueous solvent systems, while for the corresponding acetyl derivatives, the *N*-acetyl form is the stable form under these conditions. The mass spectra of the formylated compounds also show predominantly the protonated molecular ion and little sodiated molecular ion, which is characteristic of fumonisins where the nitrogen is free to be protonated in solution. For *N*-acetylated fumonisins, the sodiated molecular ion is almost as intense as the protonated molecular ion. Thus, the artifact observed in the purified *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 appears to be the 3-*O*-formyl derivative of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1. Recovery of the fractions containing *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 using a tC_{18} cartridge immediately after separation from the remaining fumonisin B_1 using a SCX cartridge should prevent the artifact from forming.

A standard solution of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 was prepared and gave a linear response between 2 and 50 ng when analyzed. Although *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 is hydrolyzed in aqueous solutions, at room temperature, hydrolysis is slow enough that working standards can be prepared weekly from frozen stock solutions. This standard was compared to a fumonisin B_1 standard to calculate the response factors. On a weight basis, the response for *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 as compared to fumonisin B_1 is 0.55, while on a molar basis, the response was 0.67. Using this

response factor, the purified fraction was 94% *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1, about 4% of the 3-*O*-formyl derivative, about 2% of the monomethyl esters, and about 0.5% each of fumonisin B_1 and partially hydrolyzed fumonisin B_1.

^{13}C NMR was used to confirm the structure of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1. The ^{13}C spectra of fumonisin B_1 and *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 were compared and C-2 was shifted downfield from 52.39 to 59.70 ppm. This is similar to the downfield shift (about 7 ppm) observed for C-2 of amino acids when the 1-deoxyfructos-1-yl group is a substituent of an amine (*25*). In solution, the 1-deoxyfructos-1-yl group of Amadori rearrangement products exists as a mixture of the α- or *β*-pyranose forms and the α- or *β*-furanose forms as well as a small amount of the open chain form. The *β*-pyranose form is the predominant form in solution. Thus, each of the carbons of the sugar displays four different resonances in the ^{13}C spectra. The open chain form is usually too small to observe except in concentrated solutions (*25*, *26*). The ^{13}C spectra of the fructosyl group of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 are similar to those observed for the fructosyl groups of the Amadori rearrangement products of amino acids (*25*) and of a series of *ω*-aminoalkanoic acids (*26*). The resonances for C-35, C-36, and C-40, originating from C-1, C-2, and C-6 of glucose, respectively, are easily assigned. C-35 (50.10, 48.62, 47.78, and 46.36 ppm) and C-40 (63.93, 62.87, 61.71, and 60.93 ppm) are the only sugar methylene carbons and are revealed by the DEPT experiment. The C-36 resonances (101.86, 98.76, 95.09, and 94.55 ppm) are shifted downfield into the same region where the resonances of the anomeric carbons of fructosyl derivatives of amino acids and *ω*-aminoalkanoic acids are found. The C-37, C-38, and C-39 resonances fall between 65 and 85 ppm along with the C-3, C-5, C-10, C-14, and C-15 resonances of fumonisin B_1 and are difficult to assign because of overlap and variable intensities.

N-(1-Deoxy-D-fructos-1-yl) fumonisin B_1 was reduced with sodium borohydride to simplify the ^{13}C spectra. When the glucose adduct with valine is reduced with sodium borohydride, it gives a mixture of the glucitol and mannitol derivatives of valine (*27*). The reduction of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 should give a mixture of the *N*-(1-deoxyglucit-1-yl) fumonisin B_1 (**3**) and *N*-(1-deoxymannit-1-yl) fumonisin B_1 (**4**). LC−MS showed that the reduction was complete. The *m*/*z* 884 peak was replaced by a *m*/*z* 886 peak eluting at the same retention time. The ^{13}C spectra of the reduced sample are shown in **Table 1**. There are now two C-2 resonances caused by C-2 being coupled to C-35 of either the 1-deoxyglucityl or the 1-deoxymannityl group. Comparison with the published spectra of glucamine (1-amino-1-deoxyglucitol) (*28*) and of glucitol and mannitol (*29*) allowed C-35 to C-40 to be assigned. The C-36 resonances show the expected large upfield shift. This shows that the Amadori rearrangement product, *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (**2**) (**Figure 1**), was formed by the reaction of glucose and fumonisin B_1 and agrees with the results of Howard et al. (*22*), who showed, using ^{13}C labeled glucose, that the carbons of the carboxymethyl group of *N*-(carboxymethyl) fumonisin B_1 are the C-1 and C-2 carbons of glucose.

To prevent the formation of the methyl esters as side products during the formation of the glucose adduct, *N*,*N*-dimethyl formamide was used as the solvent instead of methanol. **Figure 4** shows the reaction mixture after 2 h at 64 °C. The conversion of fumonisin B_1 to the glucose adduct is similar to that seen after 6 h in methanol (**Figure 3a**), and the formation of the methyl esters has been avoided. The increased reaction rate is probably due to the higher concentration of glucose in solution.

Table 1. ^{13}C NMR Spectra of **3** and **4** Formed by the Reduction of **2**[a]

carbon	3	4	carbon	3	4
1	13.7		21	14.9	
2	59.4	58.7	22	19.9	
3	65.9		23	173.0	
4	39.6		24	35.9	
5	67.1		25	38.5	
6	37.1		26	36.3	
7	25.1		27	178.6	
8	24.9		28	177.8	
9	37.1		29	172.9	
10	68.4		30	35.9	
11	42.5		31	38.3	
12	25.1		32	36.5	
13	34.7		33	176.9	
14	71.9		34	176.3	
15	77.8		35	46.5	47.0
16	33.3		36	71.3	68.7
17	31.6		37	70.8	68.9
18	28.1		38	70.8	68.2
19	22.4		39	70.8	70.5
20	13.7		40	62.6	63.0

[a] Only the values for **4** that differ from **3** are listed.

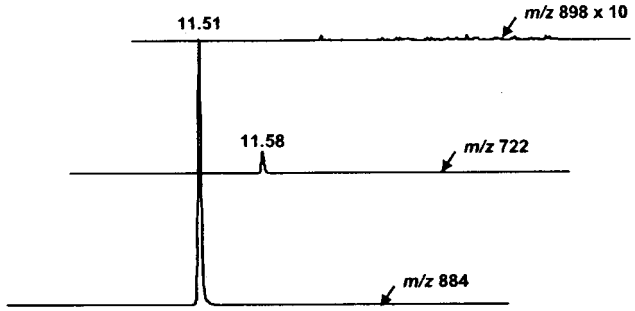

Figure 4. LC–MS analysis of the reaction mixture resulting from heating fumonisin B_1 and glucose in *N*,*N*-dimethyl formamide for 2 h at 64 °C. The heights of the peaks in the ion chromatograms for fumonisin B_1 (*m*/*z* 722) and methyl *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 898 amplified 10-fold) are relative to the height of the *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 884) peak.

Table 2. Hydrolysis of *N*-(1-Deoxy-D-fructos-1-yl) Fumonisin B_1 in Three Different Solutions after 22 h at 79 °C[a]

m/*z*	884	726	722	564	780
water	178	18	219	22	5
50 mM KH_2PO_4, pH 7.5	2	0	215	5	60
150 mM glucose in 50 mM KH_2PO_4, pH 7.5	6	0	110	2	71

[a] The values are the areas of the peaks from the ion chromatograms of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 884), partially hydrolyzed *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*m*/*z* 726), fumonisin B_1 (*m*/*z* 722), partially hydrolyzed fumonisin B_1 (*m*/*z* 564), or *N*-(carboxymethyl) fumonisin B_1 (*m*/*z* 780).

The glucose dissolved in *N*,*N*-dimethyl formamide when the reaction mixture was shaken for several minutes at room temperature, whereas with methanol as the solvent, the glucose did not completely dissolve until the mixture had been heated for about 5 h. If the reaction mixture in *N*,*N*-dimethyl formamide was not heated but allowed to stand at room temperature, similar conversions were obtained after 6−8 days.

Table 2 shows the result of hydrolyzing the same amount of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 for 22 h under three different conditions at 79 °C, the temperature used by Howard et al. (*22*) for the formation of *N*-(carboxymethyl) fumonisin B_1. In water, about half of the *N*-(1-deoxy-D-fructos-1-yl)

Table 3. Hydrolysis of *N*-(1-Deoxy-D-fructos-1-yl) Fumonisin B_1 in 100 mM NaOH at 30 °C[a]

	N-(1-df) FB_1			FB_1			*N*-(cm) FB_1		
m/z	884	726	568	722	564	406	780	622	464
starting	517	2							
106 min	156	46	14	174	44	6	24	9	5
180 min	56	30	18	189	88	23	26	22	12
252 min	22	14	15	178	111	46	30	29	26
1355 min	1	0	0	18	23	228	0	7	95

[a] The values are the areas of the peaks from the ion chromatograms of *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (*N*-(1-df) FB_1, *m/z* 884) and hydrolyzed (*m/z* 568) or partially hydrolyzed *N*-(1-df) FB_1 (*m/z* 726), fumonisin B_1 (FB_1, *m/z* 722) and hydrolyzed (*m/z* 406) or partially hydrolyzed FB_1 (*m/z* 564), or *N*-(carboxymethyl) fumonisin B_1 (*N*-(cm) FB_1, *m/z* 780) and hydrolyzed (*m/z* 464) or partially hydrolyzed *N*-(cm) FB_1 (*m/z* 622).

fumonisin B_1 was hydrolyzed to give mainly fumonisin B_1 and smaller amounts of the partially hydrolyzed forms but little *N*-(carboxymethyl) fumonisin B_1. The observed products give roughly quantitative recovery of the starting material. In 50 mM phosphate at pH 7.5, the pH and phosphate concentration used by Howard et al. (*22*), all of the *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 was hydrolyzed. The same amount of fumonisin B_1 as in the water hydrolysis was formed, and under conditions favoring the oxidation of the sugar group, *N*-(carboxymethyl) fumonisin B_1 was produced. In the third hydrolysis, 150 mM glucose was added, in addition to the phosphate, to give the conditions used by Howard et al. (*22*) to convert fumonisin B_1 to *N*-(carboxymethyl) fumonisin B_1. A slightly larger amount of *N*-(carboxymethyl) fumonisin B_1 was formed than was with phosphate alone, but only half of the amount of fumonisin B_1 was recovered. As the reaction proceeded, some of the fumonisin B_1 released by hydrolysis probably reacted with the glucose to form additional *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1, which was then oxidized to *N*-(carboxymethyl) fumonisin B_1.

In other experiments where the reaction mixture was heated for longer periods or at higher temperatures, the detected reaction products were insufficient to account for all of the starting material. No additional peaks were observed to account for the missing material nor were any observed under negative ionization or if the mass range was extended to 2000 amu. The unknown products were either outside the range of our chromatographic conditions or, more likely, do not ionize well under electrospray. When fumonisin B_1 and glucose in methanol were heated for 24 h, only about half of the starting material could be accounted for by the observed products. When Howard et al. (*22*) increased the temperature of the reaction from 78 to 94 °C, the amounts of both fumonisin B_1 and *N*-(carboxymethyl) fumonisin B_1 decreased relative to the amounts at 78 °C and additional peaks that eluted after *N*-(carboxymethyl) fumonisin B_1 were observed, using an evaporative light scattering detector. At higher temperature and for longer reaction times, glucose is probably converted into more reactive compounds that can then react with fumonisin B_1 or *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 to give more complex products similar to those observed for the reactions of sugars with simple amino acids (*18*).

The results of hydrolyzing *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 with 100 mM sodium hydroxide at 30 °C are shown in **Table 3**. The hydrolysis of the *N*-(1-deoxy-D-fructos-1-yl) group is more rapid than its oxidation to the *N*-carboxymethyl group or the hydrolysis of the esterified tricarballylic acid groups. After they were kept overnight, hydrolyzed fumonisin B_1 and hydrolyzed *N*-(carboxymethyl) fumonisin B_1, in a ratio of 2:1, were the main products. The areas for fumonisin B_1 (*m/z* 722) and partially hydrolyzed fumonisin B_1 (*m/z* 564) remaining were about 10% of the area of the hydrolyzed fumonisin (*m/z* 406). Fumonisin B_1 hydrolyzed at the same time under the same conditions and formed only hydrolyzed and partially hydrolyzed fumonisin B_1, with the area for fumonisin B_1 and partially hydrolyzed fumonisin B_1 about 10% of the area of the hydrolyzed fumonisin B_1 peak. When *N*-(1-deoxy-D-fructos-1-yl) fumonisin B_1 was hydrolyzed with 667 mM sodium hydroxide for 2 h at 100 °C, conditions that give complete hydrolysis of fumonisin B_1, hydrolyzed fumonisin B_1 and hydrolyzed *N*-(carboxymethyl) fumonisin B_1 were produced in a ratio of 5:1. The higher ratio of hydrolyzed fumonisin B_1 to hydrolyzed *N*-(carboxymethyl) fumonisin B_1 probably arises because the *N*-(1-deoxy-D-fructos-1-yl) group is eliminated more rapidly and has less time to undergo oxidation than at the lower temperature.

N-(1-Deoxy-D-fructos-1-yl) fumonisin B_1 is formed as the initial stable product of the reaction of fumonisin B_1 with glucose in nonaqueous systems. In aqueous systems, it exists at low concentrations in equilibrium with fumonisin B_1 and glucose. Under the proper conditions, it will be oxidized leading to the accumulation of *N*-(carboxymethyl) fumonisin B_1 (*22*). Under different conditions, it can probably be converted to other, as yet unidentified, products by reactions analogous to those of sugars and amino acids (*18*).

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Received for review July 23, 2001. Revised manuscript received December 13, 2001. Accepted December 14, 2001. The 70-SE-4F mass spectrometer was purchased in part with funds from the National Institute of General Medical Sciences (GM 27029). Fumonisins are cancer promoters, interfere with lipid metabolism, and are toxic to several animal species and should be handled with appropriate care. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the products, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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