JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Enzyme-Assisted Synthesis and Structural Characterization of the 3-, 8-, and 15-Glucuronides of Deoxynivalenol

Silvio Uhlig,* Lada Ivanova, and Christiane Kruse Fæste

Norwegian Veterinary Institute, P.O. Box 750 Sentrum, 0106 Oslo, Norway

Supporting Information

ABSTRACT: 4-Deoxynivalenol is one of the most prevalent mycotoxins in grain-based food and feed products worldwide. Conjugation of deoxynivalenol to glucuronic acid and elimination via the urine appears to be the major metabolism pathway, although with differing efficiency in different species. In order to make pure deoxynivalenol glucuronides for analytical methodologies available we intended to enzymatically synthesize glucuronides of deoxynivalenol using rat and human liver microsomes supplemented with uridine 5′-diphosphoglucuronic acid and alamethicin as detergent. Three glucuronides were isolated and purified using solid-phase extraction of microsomal incubations and subsequent semipreparative hydrophilic interaction chromatography. NMR spectra were obtained for all three compounds from solutions in methanol, showing that deoxynivalenol 3-*O*-*β*-D-glucuronide and deoxynivalenol 15-*O*-*β*-D-glucuronide were the major products from incubations of deoxynivalenol with rat and human liver microsomes, respectively. The NMR spectra of a third glucuronide showed replacement of the C-8 carbonyl by a ketal carbon. This glucuronide was finally identified as deoxynivalenol 8-*O*-*β*-D-glucuronide. The present study provides unequivocal structural evidence for three glucuronides of deoxynivalenol formed by liver enzymes.

KEYWORDS: *Deoxynivalenol, glucuronide, HILIC, microsomes, NMR*

INTRODUCTION

Glycosylation appears to be the major pathway for the detoxification of the *Fusarium* mycotoxin deoxynivalenol (**1**, Figure 1) in plants and animals. Metabolic conjugation

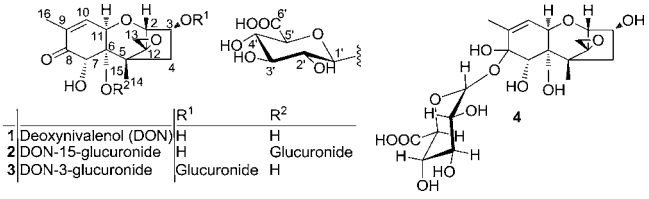

Figure 1. Structures of deoxynivalenol and its *O*-*β*-D-glucuronide conjugates isolated in this study after enzyme-assisted synthesis in rat and human microsomes.

reactions result in the formation of deoxynivalenol-3-*O*-*β*-D-glucoside in planta, while in animals one or several glucuronide conjugates are formed.[1−4] The exact nature of the glucuronide conjugates that are formed in different animal species including humans has so far not been determined, but literature data indicate the concurrent presence of several isomeric conjugates.[5−7] A recent study showed that up to three different glucuronic acid conjugates are formed with **1** in assays employing liver microsomes from humans and animals. In that study it was postulated that the three glucuronides were the 3-, 7-, and 15-*O*-glucuronides of **1**. However, the authors did not provide any spectroscopic data that confirmed glucuronidation of the hydroxyl groups at positions 7 and 15, while a synthetic standard was available for deoxynivalenol 3-*O*-*β*-D-glucuronide.[8] Thus, further research on the in vivo glucuronidation of **1** requires characterized analytical standards. Deoxynivalenol 3-*O*-*β*-D-glucuronide has recently been chemically synthesized using the Königs−Knorr procedure, and the same conjugate has been synthesized in an enzymatic assay using rat liver microsomes.[6,9] We detected up to three glucuronide conjugates of **1** in assays using liver microsomes from different animal species and recognized the need for structural characterization of individual analogues.[10] The objective of this study was therefore to isolate and purify the three glucuronide conjugates of **1** from liver microsome incubations and to elucidate their chemical structures.

MATERIALS AND METHODS

Chemicals and Reagents. Deoxynivalenol and deoxynivalenol-3-glucoside were obtained from Romer Laboratories (Tulln, Austria). Acetonitrile (Romil, Cambridge, UK or Rathburn, Walkerburn, Scotland), pyridine (Merck KGaA, Darmstadt, Germany), chloroform (Merck), and methanol (Romil) were of HPLC quality or analytical reagent grade. Glacial acetic acid and ammonium acetate were of pro analysi quality and purchased from Merck. Uridine 5′-diphosphoglucuronic acid, uridine 5′-diphospho-*N*-acetylglucosamine, and methoxyamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). UGT Reaction Mix Solution B containing 250 mM of Tris-HCl, 40 mM of $MgCl_2$, and 0.125 mg/mL of alamethicin in water was purchased from BD Biosciences (Woburn, MA). Human liver microsomes were purchased from Celsis In Vitro Technologies (Baltimore, MD) and consisted of a mixed gender pool of 50 donors resulting in preparation of average enzyme activities. Rat liver microsomes were also purchased from Celsis In Vitro Technologies and consisted of a pool of 20 male Wistar rats.

In-House Production of Rat Liver Microsomes. Four lactating HsdHan:Wistar rats of about 300 g body weight were guillotined, and

Received: November 2, 2012
Revised: February 1, 2013
Accepted: February 2, 2013
Published: February 2, 2013

their livers were immediately harvested and washed with ice-cold isotonic saline (pH 7.0). The animals were dams of which the progeny was included in an animal experiment at the National Institute of Public Health (Oslo, Norway). The keeping of dams for breeding purposes had been approved by the Norwegian Animal Research Authority. However, their sacrifice did not need additional approval because the animals were not part of any experiment itself. The organs were stored at −80 °C until further use. Microsomes were prepared from minced livers after manual tissue homogenization in 0.1 M potassium phosphate buffer (pH 7.5) in a Potter Elvehjem homogenizator by differential centrifugation. Cell debris was removed at 400*g* for 10 min, and cell nuclei and organelles were precipitated at 18 000*g* for 30 min at 4 °C. Finally, the endoplasmic reticulum was obtained using ultracentrifugation with swing-out rotor SW41Ti and polyallomer tubes at 240 000*g* for 1 h at 4 °C. Microsomes were resuspended from the precipitate by manual homogenization in 0.1 M potassium phosphate buffer (pH 7.5). The total protein content was determined by Lowry Protein Assay (Bio-Rad Laboratories, Hercules, CA), and the rat liver microsomes were stored at −80 °C until use in metabolism assays.

Optimization of Deoxynivalenol Glucuronidation in Microsome Assay. The assay was optimized with respect to the amount of microsomal protein as well as the type and concentration of the detergent. Preliminary experiments indicated that the conjugation reaction was linear up to 60 min and up to 2 mg of microsomal protein in a total volume of 0.5 mL. The optimized incubation mixture contained 0.15 mM deoxynivalenol, 7.4 mM uridine 5′-diphosphoglucuronic acid, 50 mM Tris-HCl buffer (pH 7.5), 8 mM $MgCl_2$, 0.3 mM uridine 5′-diphospho-*N*-acetylglucosamine, and 25 μg/mL alamethicin. After preincubation at 37 °C for 2 min, the reaction was initiated by adding **1** dissolved in acetonitrile. The fraction of acetonitrile in the microsomal incubation system did not exceed 0.9%. The incubation was performed at 37 °C for 60 min in a shaking water bath and terminated by placing the incubation tube on ice and immediate addition of acetonitrile (1:1). The incubation samples were centrifuged to precipitate protein for 5 min at 2000*g*. Incubations without **1** were used as vehicle controls for LC−MS background subtraction. For purification of glucuronides, the assay was linearly upscaled to a final volume of 2.5 mL without changing the amount of microsomal protein.

Solid-Phase Extraction (SPE) of Microsome Incubations. Incubation mixtures were evaporated to dryness, redissolved in 2 × 0.5 mL of water, and transferred to 500-mg Strata-X polymeric reversed phase columns (Phenomenex, Torrance, CA) that had been activated and conditioned with 4 mL of methanol and 4 mL of water. The columns were washed with 4 mL of water and dried under reduced pressure. Deoxynivalenol and its glucuronides were eluted with 4 mL of 3:2 (v/v) methanol:water. This fraction contained both **1** and its glucuronic acid conjugates. In cases where only the elution of glucuronides was desired, elution was performed with 4 mL of 1:4 (v/v) methanol:water.

Hydrophilic Interaction Chromatography−Mass Spectrometry (HILIC−MS). The fractions from the Strata-X cleanup were evaporated to dryness, and the residues were dissolved in 0.1 mL of 9:1 (v/v) acetonitrile:water, containing 20 mM ammonium acetate buffer (equal amounts of acid and base), filtered through 0.22 μm Spin-X Nylon filters (Costar, Corning, NY), and then transferred to HPLC vials. HILIC was performed on a TSKgel Amide-80 column (250 × 2.0 mm i.d., 5 μm; Tosoh Bioscience, Tokyo, Japan) fitted to a Finnigan Surveyor HPLC system equipped with autosampler (Thermo Fisher Scientific, Waltham, MA). Elution was at 0.2 mL/min. Mobile phase A was prepared by dissolving 10 mmol ammonium acetate in 10 mL of water, adding 10 mmol glacial acetic acid, and adding water to a final volume of 1 L. Mobile phase B was prepared by dissolving 10 mmol ammonium acetate and 10 mmol glacial acetic acid in 25 mL of water and adding acetonitrile to a final volume of 1 L. Separation was performed by eluting the column isocratically with 1:9 mobile phase A/B for 2 min and then changing the mobile phase composition linearly to 3:1 A/B over 22 min. The column was flushed with 45% A for 4 min and then eluted with 1:9 A/B for re-equilibration over 9 min.

The HPLC was interfaced to a Finnigan LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization interface (ESI). The MS was run in the full-scan mode in the mass range m/z 250−800. Simultaneous fragmentation of the two most intense ions was achieved using data-dependent scanning. Ions above an intensity threshold of 10^4 were isolated with a 2 m/z unit isolation width; the activation Q was set to 0.25, and the activation time was set to 30 ms for fragmentation with a relative fragmentation energy of 35 units. The ESI capillary voltage and tube lens offset were optimized by continuous infusion of a 5 μg/mL solution of **1** in methanol into a mobile phase composed of 1:1 A/B.

Production and Purification of Individual Deoxynivalenol Glucuronides. Five replicates (5 × 2.5 mL) from the upscaled enzyme assay using self-prepared rat liver microsomes or commercially available human liver microsomes were combined and evaporated to dryness using a rotary evaporator. The residues were dissolved in 4 × 5 mL of water and transferred to a separatory funnel. The aqueous solutions were washed twice with 20 mL of chloroform and then evaporated to dryness using a rotary evaporator. The residues were then dissolved in 2 × 3 mL of water and separated by SPE on preconditioned 500-mg Strata-X polymeric reversed phase columns as described above. Deoxynivalenol glucuronides were eluted using 4 mL of 1:4 (v/v) methanol:water. Eluates from 15 replicate incubations using rat liver microsomes and 10 replicate incubations using human liver microsomes were combined and evaporated to dryness. Final purification was achieved by semipreparative HILIC using a TSKgel Amide-80 column (300 mm ×7.8 mm i.d., 10 μm; Tosoh Bioscience) with a TSP model 4000 pump (Finnigan MAT Corp., now Thermo Fisher Scientific). The flow rate was 2.7 mL/min, and the column was eluted isocratically using 87:13 (v/v) acetonitrile/water (containing 20 mM acetate buffer). A portion of the eluent (approximately 10%) was continuously split into a LCQ Classic ion trap mass spectrometer (Finnigan MAT Corp.) to monitor the separation. The MS parameters were as follows: ESI negative mode; spray voltage, 4.5 kV; sheath gas flow rate, 60 arbitrary units; auxiliary gas flow rate, 5 arbitrary units; m/z range, 250−800. This procedure allowed for isolation and purification of glucuronides **2**−**4** in large enough quantities for structure determination by NMR spectroscopy.

High-Resolution MS (HRMS). An aliquot from incubation of **1** with rat liver microsomes was used to obtain HRMS data of glucuronides **2**−**4** using an Agilent 6520 Accurate Mass Q-TOF mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA) equipped with an electrospray interface, operated in negative ion mode, and coupled to an Agilent 1200 Series HPLC pump and autosampler (Table 1). HPLC conditions were identical to those described in the HILIC−MS section. The parameters of the electrospray interface were set as follows: capillary voltage, 3.5 kV; drying gas flow, 5 L/min; gas temperature, 325 °C; nebulizer pressure, 30 psi. The Q-TOF mass spectrometer was set to scan in the mass range m/z 100−1000 with a sampling rate of 1.4 spectra/s (4 GHz). The instrument was run with internal mass correction, and the fragmentor and skimmer voltage were 150 and 65 V, respectively.

Table 1. High-Resolution Mass Spectrometric Analyses of Deoxynivalenol Glucuronides as Their Deprotonated Molecular Ions (2, 3) or Dehydrated and Deprotonated Molecular Ions (4)

		m/z		
ions	elemental composition	found	calcd	error (ppm)
2	$C_{21}H_{27}O_{12}$	471.1505	471.1503	0.4
3	$C_{21}H_{27}O_{12}$	471.1505	471.1503	0.4
4	$C_{21}H_{27}O_{12}$	471.1510	471.1503	1.5

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra of glucuronides **2**−**4** (Figure 1) were obtained from solutions (70 μL) in methanol-d_4 (CD_3OD, 99.96 atom % D; Sigma-Aldrich) using 2.0 mm o.d. Norell tubes (Sigma-Aldrich) and a Bruker Match clamp holder (BrukerBioSpin, Silberstreifen, Germany). Additional

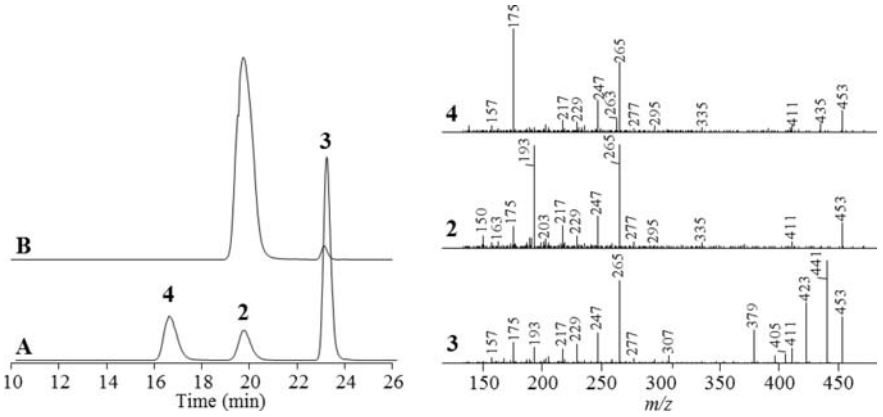

Figure 2. LC−MS chromatograms (m/z 471 trace) from rat (A) and human (B) microsome incubations and MS^2 spectra obtained from collision-induced fragmentation of $[M - H]^-$ of deoxynivalenol 15-*O*-*β*-D-glucuronide (**2**) and deoxynivalenol 3-*O*-*β*-D-glucuronide (**3**) and of $[M - H_2O - H]^-$ of deoxynivalenol 8-*O*-*β*-D-glucuronide (**4**).

NMR spectra of glucuronide **4** were obtained in DMSO-d_6 (99.96 atom % D; Sigma-Aldrich). The spectra were acquired on an Avance AVII 600 MHz NMR spectrometer (BrukerBioSpin) equipped with a 5 mm CP-TCI ($^1H/^{13}C$, $^{15}N-^2H$) triple-resonance inverse cryoprobe with a *Z*-gradient coil. NMR assignments were obtained from the examination of ^1H, JMOD (glucuronides **2** and **3** only), 1D-SELTOCSY, COSY, TOCSY, g-HSQC, g-HMBC, and NOESY NMR spectra (Table 1). The data were processed using Bruker TOPSPIN (version 2.1 pl4 or version 3) software. Chemical shifts, determined at 25 °C, are reported relative to internal CHD_2OD (3.34 ppm) and CD_3OD (49.86 ppm) or internal $CHD_2S(O)CD_3$ (2.54 ppm) and $CD_3S(O)CD_3$ (40.45 ppm).[11]

Derivatization. An aliquot from incubation of **1** with rat liver microsomes was evaporated to dryness and the residue redissolved in 200 μL of pyridine containing 1% of methoxyamine hydrochloride. The solution was placed in a heating cabinet at 40 °C for 4 days. As a negative control an aliquot from a rat liver microsome incubation in pyridine without addition of the derivatizing reagent was included. The solvent was evaporated under a gentle stream of nitrogen at 60 °C, and the residue was dissolved in 200 μL of methanol and analyzed by HILIC−MS.

Glucuronidase Treatment. Enzymatic hydrolysis was performed by treatment of an incubation sample with *β*-glucuronidase from *Escherichia coli* (Type IX A) (Sigma-Aldrich). An incubation sample (0.5 mL) was evaporated to dryness and the residue dissolved in 0.25 mL of PBS buffer (pH 7.4). Then, 0.25 mL of PBS buffer (pH 7.4) containing 4500 units of *β*-glucuronidase were added, and the mixture was incubated at 37 °C for 24 h. Control samples were diluted with PBS buffer (pH 7.4) only. After incubation, the sample was cleaned up by SPE and analyzed by HILIC−MS.

RESULTS AND DISCUSSION

Enzymatic Synthesis of Deoxynivalenol Glucuronides. Literature data showed that the presence of a detergent improved the accessibility of the membrane-bound UDP glucuronosyltransferases (uridine 5′-diphospho-glucuronosyltransferases, UGTs), likely by forming pores in the microsomal membranes.[12] In our study, microsomes were initially pretreated with digitonin (0.5 mg per mg of microsomal protein for 30 min at 0 °C);[13] however, the addition of alamethicin (25 μg/mL) resulted in a higher yield of deoxynivalenol glucuronides (data not shown). Furthermore, several experiments were performed in the presence of human UGT2B17 supersomes (BD Biosciences), with/without the presence of hepatic microsomes. Supersomes are microsomes derived from baculovirus-infected Sf9 insect cells expressing particular human UGT genes. Our experiments showed that the presence of hepatic microsomes was necessary in order to achieve deoxynivalenol glucuronidation and that the presence of UGT2B17 supersomes in the microsome assay did not result in a significant increase in the yield of deoxynivalenol glucuronides. Uridine-5′-diphospho-*N*-acetylglucosamine has been reported to be an important effector of UDP-glucuronosyltransferase and has therefore been used in order to maximize deoxynivalenol conjugation.[14] For production of deoxynivalenol glucuronides on a larger scale with the aim of isolating and purifying the conjugation products, the microsome assay was linearly upscaled to a final reaction volume of 2.5 mL.

Semipreparative Synthesis and Purification. The initial small-scale experiments in order to optimize glucuronidation of **1** were carried out using commercially available rat and human liver microsomes. However, rat liver microsomes were prepared in our laboratory for the production of deoxynivalenol glucuronides on a semipreparative scale. The relative ratio of individual glucuronide conjugates obtained in the rat liver microsome incubations was identical for both sources (data not shown). Glucuronide **3** was the principal glucuronide conjugate produced by rat liver microsomal glucuronyl transferases, while glucuronide **2** was the major product obtained with human liver microsomes (Figure 2). In order to get high enough quantities of **2** for structure determination, this glucuronide was therefore also purified from incubations with human liver microsomes and combined with **2** obtained from the rat liver microsome incubations. Glucuronide **4** was only produced in rat liver microsome incubations (Figure 2).

Since **1** is a polar and water-soluble sesquiterpenoid, reversed-phase chromatography was not suitable for retention and separation of individual deoxynivalenol glucuronides. We therefore explored several commercial polar sorbents for HILIC and used a commercially available deoxynivalenol calibrant as well as commercially available deoxynivalenol 3-*O*-*β*-D-glucoside in order to experiment with different column materials as well as mobile phase conditions. A TSKgel Amide-80 column was finally chosen, as it gave the most promising results with regard to retention and peak shape. Using this column, baseline separation of all three deoxynivalenol glucuronides was achieved both on analytical and semipreparative scale (Figure 2). For crude cleanup and enrichment of deoxynivalenol glucuronides, a polymeric, reversed-phase mixed-mode sorbent was found to be well-suited for retention of the conjugates from

Table 2. NMR Assignments for Glucuronide Conjugates of Deoxynivalenol Obtained by Enzyme-Assisted Synthesis Using Rat and Human Microsomes

	2[a] (CD_3OD)		**3**[a] (CD_3OD)		**4**[a] (CD_3OD)		**4**[b] (DMSO-d_6)	
atom	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C[c]	^1H	^{13}C[c]
2	3.56 (d, J = 4.5 Hz)	83.1	3.79 (d, J = 4.3 Hz)	82.9	3.47	83.5	3.35[e]	81.5
3	4.40 (ddd, J = 11.3, 4.6, 4.6 Hz)	70.6	4.62 (ddd, J = 11.3, 5.0, 4.5 Hz)	77.2	4.39 (ddd, J = 11.3, 5.2, 4.7 Hz)	70.7	4.22 (ddd, J = 15.1, 4.7, 4.0 Hz)	68.6
4	1.98 (dd, J = 15.4, 11.4 Hz); 2.46 (dd, J = 15.4, 4.3 Hz)	45.3	2.01 (dd, J = 15.0, 11.0 Hz); 2.62 (dd, J = 15.0, 4.2 Hz)	42.8	1.73 (dd, J = 14.6, 4.7 Hz); 1.98 (dd, 14.6, 11.2 Hz)	46.1	1.62 (m); 1.83 (dd, J = 13.8, 11.1 Hz)	45.4
5	–	48.2	–	47.8	–	45.8	–	44.4
6	–	54.5	–	54.6	–	55.2	–	53.5
7	4.86[e]	76.4	4.83 (s)	76.7	4.19 (s)	77.4	4.02 (d, J = 3.1 Hz)	75.3
8	–	203.7	–	202.6	–	110.2	–	109.0
9	–	137.5	–	137.8	–	143.7	–	141.7
10	6.63 (dd, J = 5.9, 1.6 Hz)	141.0	6.65 (dd, J = 5.9, 1.5 Hz)	140.5	5.57 (dd, J = 4.6, 1.6 Hz)	125.0	5.50 (dd, J = 4.7, 1.6 Hz)	124.3
11	5.02 (d, J = 5.8 Hz)	72.7	4.95 (d, J = 5.9 Hz)	72.5	4.72 (d, J = 4.3 Hz)	77.4	4.63 (d, J = 4.7 Hz)	75.4
12	–	67.5	–	67.4	–	68.3	–[d]	–[d]
13	3.10 (d, J = 4.6 Hz); 3.12 (d, J = 4.6 Hz)	48.9	3.10 (d, J = 4.4 Hz); 3.13 (d, J = 4.4 Hz)	49.0	3.00 (d, J = 4.4 Hz); 3.29 (d, J = 4.4 Hz)	48.5	–[d]	–[d]
14	1.14 (s)	15.6	1.15 (s)	15.7	1.18 (s)	16.1	1.09 (s)	15.9
15	3.62 (d, J = 10.4 Hz); 4.34 (d, J = 10.4 Hz)	70.9	3.75 (d, J = 11.9 Hz); 3.79 (d, J = 11.9 Hz)	62.9	3.44 (d, J = 8.9 Hz); 4.31 (d, J = 8.9 Hz)	69.0	3.29 (d, J = 8.8 Hz); 4.10 (d, J = 8.8 Hz)	67.2
16	1.86 (dd, J = 1.6, 0.7 Hz)	16.3	1.86 (dd, J = 1.5, 0.8 Hz)	16.2	1.89 (dd, J = 1.6, 1.5 Hz)	17.6	1.80 (dd, J = 1.6, 1.4 Hz)	17.3
1′	4.07 (d, J = 7.8 Hz)	105.7	4.47 (d, J = 7.9 Hz)	104.5	4.90[e]	98.9	4.44 (d, J = 7.7 Hz)	97.9
2′	3.10 (dd, J = 9.3, 7.8 Hz)	76.0	3.32[f]	75.6	3.40 (dd, J = 9.2, 8.2 Hz)	75.8	3.10[e]	74.0
3′	3.37 (dd, J = 9.3, 8.9 Hz)	78.4	3.45 (dd, J = 9.2, 9.1 Hz)	78.7	3.46 (m)	78.5	3.17[e]	73.7
4′	3.42 (dd, J = 9.6, 8.9 Hz)	74.4	3.53 (dd, J = 9.2, 8.7 Hz)	74.4	3.47 (m)	74.2	3.18[e]	77.6
5′	3.51 (d, J = 9.6 Hz)	76.9	3.68 (d, J = 8.7 Hz)	77.4	3.60 (d, J = 9.5 Hz)	76.6	3.16[e]	73.4
6′	–	177.5	–	176.2	–	177.0	–	172.3
3-OH							5.18 (d, J = 3.7 Hz)	–
7-OH							5.54 (d, J = 3.2 Hz)	–
2′-OH							5.65 (br s)	–
3′-OH							4.99 (br s)	–
4′-OH							2.90 (d, J = 4.4 Hz)	–

[a]Relative to internal CHD_2OD at 3.34 ppm and CD_3OD at 49.86 ppm.[10] [b]Relative to internal $CHD_2S(O)CD_3$ at 2.54 ppm and $CD_3S(O)CD_3$ at 40.45 ppm.[10] [c]Assignments based on g-HSQC and g-HMBC spectra. [d]Not observed. [e]^1H resonance hidden below water signal. [f]^1H resonance hidden below solvent signal.

aqueous solution. Because of the relatively poor absorption at UV wavelengths of deoxynivalenol and its conjugates, the final purification of individual glucuronides was monitored by splitting a portion of the mobile phase into an ion trap mass spectrometer. In this way, three isolates were obtained, allowing unequivocal structural characterization by NMR spectroscopy.

Enzymatic hydrolysis of the glucuronidation products with glucuronidase resulted in the recovery of **1** from glucuronides **2** and **3** but not from **4** (data not shown), indicating the presence of at least two isomeric glucuronides. All three glucuronidation products afforded primarily ions with m/z 471 upon LC−MS with electrospray ionization in the negative mode, believed to correspond to $[M - H]^-$. Mass spectra obtained from LC−MS^2 of **2**−**4** were generally similar. However, the MS^2 spectra from fragmentation of m/z 471, for glucuronide **3**, showed the presence of high intensity fragment ions from loss of 30 Da (m/z 441) (Figure 2). This fragment is likely to arise from cleavage of the CH_2OH moiety attached at C-6, but it could in principle also be due to cleavage of the epoxide.[15,16] A m/z 441 fragment was also among the major high-mass fragment ions for synthetic deoxynivalenol 3-*O*-β-D-glucuronide, which supported that **3** was the 3-glucuronide.[9]

NMR Assignments. Detailed analyses of one- and two-dimensional NMR data, including ^1H, JMOD, SELTOCSY, COSY, TOCSY, g-HSQC, g-HMBC, and NOESY spectra determined in CD_3OD, afforded complete ^1H and ^{13}C assignments for glucuronides **2**−**4** (Table 2). NMR assignments for **4** were also obtained in DMSO-d_6. Reference spectra were obtained for commercial **1** (2.5 mg) in CD_3OD, and the obtained assignments were compared with data from the literature.[13] Typically, $^1H-^1H$ correlations were established in COSY, TOCSY, and in some cases SELTOCSY experiments, and $^1H-^{13}C$ connectivities were identified in g-HSQC and g-HMBC experiments. Stereochemistries of structurally significant skeletal parts were established in NOESY experiments. Attachment points of the glucuronide residues in the three different conjugates were established via $^3J_{H-C}$ g-HMBC correlations from the glucuronide anomeric proton (4.09−4.90 ppm) across the *O*-glycosidic linkage to the corresponding carbon of the aglycon.

dx.doi.org/10.1021/jf304655d | J. Agric. Food Chem. 2013, 61, 2006−2012

The JMOD NMR spectra of deoxynivalenol 15-*O*-*β*-D-glucuronide (**2**) and deoxynivalenol 3-*O*-*β*-D-glucuronide (**3**) showed, in accord with the HR-MS data, the presence of 21 carbon atoms (2 methyl, 3 methylene, 10 methine, 4 quaternary, and 2 carbonyl) (Table 2, Figure 1). Notable features of the ^{1}H NMR spectrum of **2** and **3** included the presence of two methyl signals with nearly identical chemical shift for the two isomers (1.14/1.15 ppm and 1.86/1.86 ppm for **2**/**3**, respectively), five glucosyl proton signals (3.10−4.47 ppm), and an olefinic proton signal (6.62/6.63 ppm for **2**/**3**, respectively) (Table 2). The anomeric proton (H-1′) appeared as a doublet at 4.07 ppm (**2**) and 4.47 ppm (**3**) with a coupling constant of $^{3}J_{H-H}$ = 7.8−7.9 Hz. This coupling constant is consistent with a *β*-configuration of the glycosidic linkage, as the $^{3}J_{H-H}$ coupling constant for the corresponding *α*-anomeric proton would be expected to be significantly lower, typically between 1 and 4 Hz.[17] In addition, the anomeric proton H-1′ showed NOE correlations to H-3′ and H-5′, as expected for a *β*-configuration of the glycosidic linkage. Important entry points for the NMR assignment of the trichothecene aglycon were the methyl groups and the olefinic proton H-10. The methyl H-16 protons showed both $^{3}J_{H-H}$ (J = 1.5/1.6 Hz for **2**/**3**, respectively) and $^{5}J_{H-H}$ coupling to H-10 and H-11 (J = 0.8/0.7 Hz for **2**/**3**, respectively), while the carbonyl C-8 could be assigned via a $^{3}J_{H-C}$ correlation from H-16 in the g-HMBC spectrum. A methine proton (4.89 ppm for **2**, 4.83 ppm for **3**) belonging to a carbon with a chemical shift typical for oxygenated carbon atoms (76.4 ppm for **2**, 76.7 ppm for **3**) showed likewise a g-HMBC correlation to the same carbonyl signal and was attributable to CH-7 (Figure 3). H-7 showed all possible $^{2}J_{H-C}$ and $^{3}J_{H-C}$ correlations in the g-HMBC spectrum and was thus useful for the assignment of C-6, C-5, and CH_2-15. The diastereotopic proton pair of H-15 appeared at similar chemical shift in the case of glucuronide **3** (3.75/3.79 ppm). However, the glucuronidation of the C-15-hydroxyl in **2** resulted in a highly different chemical and, thus, magnetic environment for the two germinal protons, as shown by the significantly separated ^{1}H chemical shift values (3.62/4.34 ppm). The NMR assignments of CH-2 and -3 as well as of CH_2-4 and the epoxide moiety were facilitated by exploring the coupling environment of the methyl H-14 protons (Figure 3). The H-2, H-3, and H-4 protons could also be traced relatively easily from the COSY and TOCSY spectra and the corresponding carbons assigned from the g-HSQC spectra. The NMR assignment of the glucuronide moiety was achieved primarily on the basis of the COSY and g-HSQC spectra. The ^{13}C signal of the carboxyl-carbon (C-6′) was weak in the JMOD NMR spectra. However, unequivocal assignment was possible based on the $^{3}J_{H-C}$ and $^{2}J_{H-C}$ correlations from C-4′ and C-5′ in the g-HMBC spectra, respectively (Figure 3).

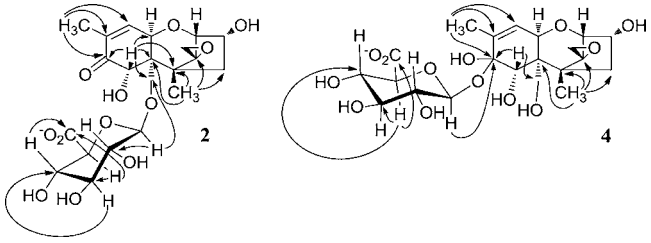

Figure 3. Selected correlations observed in the HMBC NMR spectrum of deoxynivalenol 15-*O*-*β*-D-glucuronide (**2**) and deoxynivalenol 8-*O*-*β*-D-glucuronide (**4**) in CD_3OD.

Glucuronide **4** was only produced by rat liver microsomes (Figure 1). Since **1** possesses one primary (C-15) and two secondary (C-3 and C-7) hydroxyl groups, it was obvious to assume that glucuronide **4** was the 7-*O*-glucuronide of **1**.[8] This hypothesis was supported by the MS and HRMS characteristics of the compound: Analogous to **2** and **3**, **4** afforded m/z 471 ions, believed to correspond to $[M - H]^-$ ions, upon electrospray ionization in the negative mode (Table 1). The careful evaluation of 1D and 2D NMR spectra for **4** showed, however, several notable differences compared to **2** and **3.** A carbonyl carbon corresponding to C-8 could not be observed in the NMR spectra of **4**. Instead, the protons corresponding to H-7, H-15, and H-16 at 4.19, 3.44/4.31, and 1.89 ppm, respectively, all showed correlations in the g-HMBC to a quaternary carbon at 110.2 ppm, typical for a dioxygenated carbon atom (Table 2, Figure 3). In addition, a methine resonance with a ^{1}H/^{13}C chemical shift of 4.90/98.9 ppm, was attributable to the anomeric proton and its associated carbon of the glucuronide moiety (CH-1′) and showed a clear $^{3}J_{H-C}$ correlation to the quaternary carbon at 110.2 ppm (Figure 3). The assignment of the remaining part of the trichothecene aglycon was relatively straightforward, as both the chemical shift of individual signals as well as coupling constants were similar to those observed for glucuronides **2** and **3**. An exception was the olefinic H-10 resonance, which had shifted by approximately 1 ppm to higher field (5.57 ppm) but otherwise showed identical coupling characteristics as observed for glucuronides **2** and **3**. These observations allowed us to conclude that **4** is consistent with a hemiketal deoxynivalenol 8-*O*-*β*-D-glucuronide (Figure 1). This assumption was supported by the following experiments: A mixture of all three glucuronides obtained from incubation of **1** with rat liver microsomes was reacted with methoxyamine hydrochloride. It had previously been shown that the 8-keto group in **1** readily forms an oxime as a product of this reaction.[18] In our experiment, both glucuronides **2** and **3** formed a product that had increased in molecular mass by 29 Da (analyzed by LC−MS) in accordance with formation of an oxime. However, glucuronide **4** did not react and was unable to form an oxime (Figure 4). Furthermore, ^{1}H NMR, COSY, TOCSY, g-HSQC,

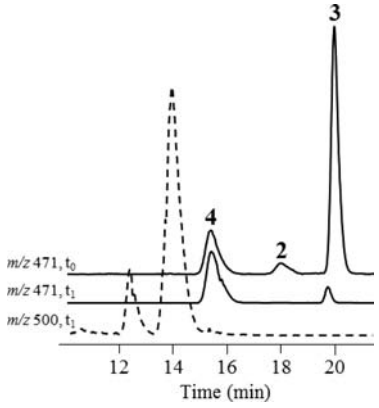

Figure 4. Reaction of deoxynivalenol glucuronides (m/z 471) with methoxyamine hydrochloride: deoxynivalenol 15-*O*-*β*-D-glucuronide (**2**) and deoxynivalenol 3-*O*-*β*-D-glucuronide (**3**) form C-8-oximes (m/z 500) while deoxynivalenol 8-*O*-*β*-D-glucuronide (**4**) does not react. The glucuronide mixture was allowed to react for 96 h (t_1) at 40 °C.

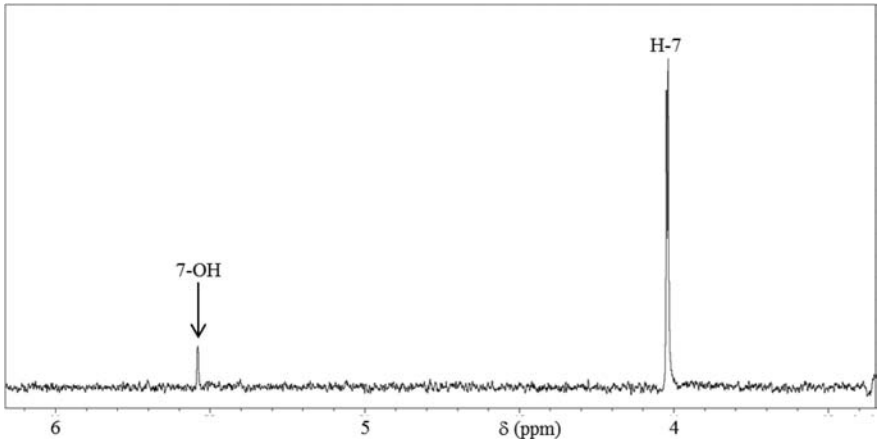

Figure 5. 1D-SELTOCSY of C-7-O*H* in **4** (in DMSO-d_6) confirming the presence of a hydroxyl group at C-7.

and g-HMBC as well as a series of SELTOCSY experiments were rerun with glucuronide **4** dissolved in DMSO-d_6 with the intention to visualize hydroxyl protons (Table 2). Several ^{1}H signals attributable to hydroxyls were observed and identified as such because they lacked $^1J_{H-C}$ correlations in the g-HSQC spectra and disappeared upon addition of a few microliters of D_2O. Two of the hydroxyl protons could be assigned to the trichothecene aglycon (5.18 and 5.54 ppm for C-3-O*H*, C-7-O*H*, respectively), while three of the hydroxyl protons could be assigned to the glucuronide moiety (5.65, 4.99, and 2.90 ppm for C-2′-O*H*, C-3′-O*H*, C-4′-O*H*, respectively). These assignments were based on correlations observed in the COSY and TOCSY spectra. 1D-SELTOCSY experiments confirmed that the ^{1}H NMR signals observed at 5.18 and 5.54 ppm in the NMR spectra of **4** in DMSO-d_6 were related to the hydroxyl protons at C-3 and C-7, respectively (Figure 5). Proton signals corresponding to hydroxyl groups at C-8, C-15, C-1′, and C-5′ were not observed, which could be a result of overlap with the water signal generated from residual moisture in the sample. ^{1}H NMR signals due to residual water are practically unavoidable at sample amounts below 100 μg. Even though the signal/noise ratio and quality of spectra can be improved by using different solvent suppression techniques, these techniques also suppress relevant ^{1}H NMR signals from protons that resonate at similar frequencies. It is not uncommon that hydroxyl protons resonate at a similar frequency as water. The anomeric proton (H-1′) in **4** appeared as a doublet at 4.44 ppm with a coupling constant of $^3J_{H-H}$ = 7.7 Hz in the ^{1}H NMR spectrum in DMSO-d_6, which is equivalent with a β-configuration of the glycosidic linkage as discussed above.

The NMR assignment, stereochemistry, and point of attachment of the glucuronide moiety in **4** were further verified from correlations observed in the NOESY NMR spectrum in CD_3OD (Figure 6). Thus, the axial orientations of H-3′ (3.46 ppm) and H-5′ (3.60 ppm) were confirmed by NOE correlations from H-1′ (4.90 ppm), implicating an equatorial orientation of the carboxyl group (Figure 6). H-1′ also exhibited NOE correlations to both H-7 (4.19 ppm) and H-16 (1.89 ppm) (Figure 6).

Re-examination of the LC−MS chromatograms of samples from the glucuronidase-treated, SPE-purified incubation mixture of **1** with rat microsomes did not reveal any ketal-deoxynivalenol that would be the expected product from enzymatic cleavage of the glucuronic acid moiety in **4**. Repetition of the experiment with an aliquot of the NMR

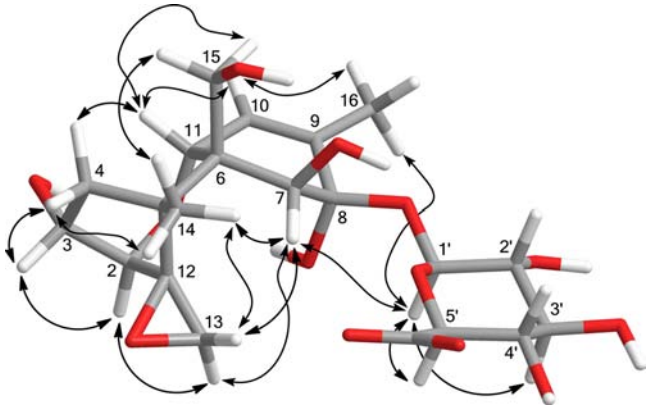

Figure 6. Calculated structure of deoxynivalenol 8-*O*-β-D-glucuronide (**4**) showing correlations observed in the NOESY NMR spectrum in CD_3OD.

sample of **4** confirmed that no deconjugation occurred after treatment with β-glucuronidase.

LC−MS using electrospray ionization in the negative mode affords m/z 471 ions for all three glucuronides, even though glucuronide **4** is expected to have a higher molecular mass compared to **2** and **3**. Our explanation for this oddity is that the ketal OH present at C-8 in **4** is readily lost as water during ionization. MS^2 fragmentation of m/z 471 gave, in addition to the above-mentioned dominating loss of 30 Da in the case of **2**, m/z 265 ions as major product for all three glucuronide conjugates, likely corresponding to $[\text{aglycon} - CH_2O - H]^-$ (Figure 2). The fragment ions with m/z 175, which were predominant in the MS^2 spectrum of **4**, appear to be due to a dehydrated glucuronic acid moiety ($[\text{GlcA} - H_2O - H]^-$) (Figure 2). Interestingly, fragment ions with m/z 193, likely corresponding to a deptrotonated glucuronic acid moiety ($[\text{GlcA} - H]^-$), were not present in the MS^2 spectrum of **4** but were dominant in the MS^2 spectrum of **2** and also present in the MS^2 spectrum of **3** (Figure 2). This supports that the m/z 471 base peak observed in the mass spectrum of **4** is the result of dehydration at C-8 (cleavage of the hemiketal-hydroxyl as water) because the remaining product contains a 7,8-double bond that makes cleavage of the glucuronide moiety on the aglycon side of the glycosidic linkage less likely.

In summary, our results indicate that, at least in vitro, glucuronyl transferases present in rat liver microsomes conjugate glucuronic acid primarily to the 3-position of **1**,

while glucuronyl transferases present in human liver microsomes conjugate glucuronic acid primarily to the 15-position of **1**. However, conjugation at the 8-position can also occur, while *O*-conjugation of the hydroxyl group at C-7, which has recently been postulated, is apparently not favored.

■ ASSOCIATED CONTENT

Supporting Information

Phase-sensitive $^{1}H-^{13}C$ HSQC spectrum, part of phase-sensitive $^{1}H-^{13}C$ HSQC spectrum superimposed with $^{1}H-^{13}C$ HMBC spectrum in order to visualize the point of attachment of the glucuronide moiety, and JMOD spectrum (CH_2, C positive, CH_3, CH negative), for deoxynivalenol 15-*O*-β-D-glucuronide; phase-sensitive $^{1}H-^{13}C$ HSQC spectrum and part of phase-sensitive $^{1}H-^{13}C$ HSQC spectrum superimposed with $^{1}H-^{13}C$ HMBC spectrum in order to visualize the point of attachment of the glucuronide moiety, for deoxynivalenol 8-*O*-β-D-glucuronide. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +47 23216264. Fax: +47 23216201. E-mail: silvio.uhlig@vetinst.no.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Christopher O. Miles (Norwegian Veterinary Institute) for useful comments and discussions during this work and Helene Rønning (Norwegian School of Veterinary Science) for assistance with HRMS. Frode Rise is acknowledged for providing access to the NMR center at the University of Oslo.

■ ABBREVIATIONS USED

SPE, solid-phase extraction; HILIC, hydrophilic interaction chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; UGT, uridine 5′-diphospho-glucuronosyltransferase; NMR, nuclear magnetic resonance; JMOD, *J*-modulated spin−echo; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; SELTOCSY, selective total correlation spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple-bond correlation; NOESY, nuclear overhauser spectroscopy.

■ REFERENCES

(1) Berthiller, F.; Dall'Asta, C.; Schuhmacher, R.; Lemmens, M.; Adam, G.; Krska, R. Masked mycotoxins: Determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography−tandem mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 3421−3425.

(2) Berthiller, F.; Krska, R.; Domig, K. J.; Kneifel, W.; Juge, N.; Schuhmacher, R.; Adam, G. Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicol. Lett.* **2011**, *206*, 264−267.

(3) Warth, B.; Sulyok, M.; Berthiller, F.; Schuhmacher, R.; Fruhmann, P.; Hametner, C.; Adam, G.; Frohlich, J.; Krska, R. Direct quantification of deoxynivalenol glucuronide in human urine as biomarker of exposure to the *Fusarium* mycotoxin deoxynivalenol. *Anal. Bioanal. Chem.* **2011**, *401*, 195−200.

(4) Wu, X. N.; Murphy, P.; Cunnick, J.; Hendrich, S. Synthesis and characterization of deoxynivalenol glucuronide: Its comparative immunotoxicity with deoxynivalenol. *Food Chem. Toxicol.* **2007**, *45*, 1846−1855.

(5) Lattanzio, V. M. T.; Solfrizzo, M.; De Girolamo, A.; Chulze, S. N.; Torres, A. M.; Visconti, A. LC−MS/MS characterization of the urinary excretion profile of the mycotoxin deoxynivalenol in human and rat. *J. Chromatogr. B* **2011**, *879*, 707−715.

(6) Versilovskis, A.; Geys, J.; Huybrechts, B.; Goossens, E.; De Saeger, S.; Callebaut, A. Simultaneous determination of masked forms of deoxynivalenol and zearalenone after oral dosing in rats by LC−MS/MS. *World Mycotoxin J.* **2012**, *5*, 303−318.

(7) Warth, B.; Sulyok, M.; Fruhmann, P.; Berthiller, F.; Schuhmacher, R.; Hametner, C.; Adam, G.; Frohlich, J.; Krska, R. Assessment of human deoxynivalenol exposure using an LC−MS/MS based biomarker method. *Toxicol. Lett.* **2012**, *211*, 85−90.

(8) Maul, R.; Warth, B.; Kant, J.-S.; Schebb, N. H.; Krska, R.; Koch, M.; Sulyok, M. Investigation of the hepatic glucuronidation pattern of the *Fusarium* mycotoxin deoxynivalenol in various species. *Chem. Res. Toxicol.* **2012**, *25*, 2715−2717.

(9) Fruhmann, P.; Warth, B.; Hametner, C.; Berthiller, F.; Horkel, E.; Adam, G.; Sulyok, M.; Krska, R.; Frohlich, J. Synthesis of deoxynivalenol-3-β-D-*O*-glucuronide for its use as biomarker for dietary deoxynivalenol exposure. *World Mycotoxin J.* **2012**, *5*, 127−132.

(10) Fæste, C. K.; Ivanova, L.; Uhlig, S. Species-specific in vitro glucuronidation of the mycotoxin deoxynivalenol. (http://issx.confex.com/issx/12euro/webprogram/Paper27544.html).

(11) Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. NMR chemical shifts of common laboratory solvents as trace impurities. *J. Org. Chem.* **1997**, *62*, 7512−7515.

(12) Fisher, M. B.; Campanale, K.; Ackermann, B. L.; Vandenbranden, M.; Wrighton, S. A. In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. *Drug Metab. Dispos.* **2000**, *28*, 560−566.

(13) D'Andrea, V.; Perez, L. M.; Pozzi, E. J. S. Inhibition of rat liver UDP-glucuronosyltransferase by silymarin and the metabolite silibinin-glucuronide. *Life Sci.* **2005**, *77*, 683−692.

(14) Zakim, D.; Vessey, D. A. The properties of uridine diphosphate glucuronyltransferase(s) which catalyse the synthesis of steroid glucuronides in microsomal fractions from guinea-pig liver. *Biochem. J.* **1976**, *157*, 667−673.

(15) Krska, R.; Szente, E.; Freudenschuss, M.; Hametner, C.; Zollner, P. Purity assessment of commercially available crystalline deoxynivalenol. *J. AOAC Int.* **2004**, *87*, 909−919.

(16) Liu, Z. Y.; Yu, C. H.; Wan, L. R.; Sun, Z. L. Fragmentation study of five trichothecenes using electrospray hybrid ion trap/time-of-flight mass spectrometry with accurate mass measurements. *Int. J. Mass Spectrom.* **2012**, *309*, 133−140.

(17) Agrawal, P. K. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry* **1992**, *31*, 3307−3330.

(18) Beyer, M.; Dänicke, S.; Rohweder, D.; Humpf, H.-U. Determination of deoxynivalenol-sulfonate (DONS) in cereals by hydrophilic interaction chromatography coupled to tandem mass spectrometry. *Mycotoxin Res.* **2010**, *26*, 109−117.