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Preparative Enzymatic Synthesis of Glucuronides of Zearalenone and Five of Its Metabolites

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The resorcylic acid lactones zearalenone (**1**), α-zearalenol (**2**), β-zearalenol (**3**), α-zearalanol (zeranol) (**4**), β-zearalanol (taleranol) (**5**), and zearalanone (**6**) were converted to their glucuronides on a preparative scale in good yields. Reactions were conducted with bovine uridine 5′-diphosphoglucuronyl transferase (UDPGT) as catalyst and uridine 5′-diphosphoglucuronic acid (UDPGA) as cofactor. The glucuronides were isolated by column chromatography and characterized by NMR spectroscopy and mass spectrometry. Although the principal products were 4-*O*-glucuronides (i.e., linkage through a phenolic hydroxyl), significant quantities of the 6′-*O*-glucuronides (i.e., linkage through the aliphatic hydroxyl) of alcohols **2**, **4**, and **5** were also isolated. In the case of **3**, the 2-*O*-glucuronide was isolated as the minor product. Overall isolated yields of glucuronides, performed on a 20−50 mg scale, were typically ca. 80% based on the resorcylic acid lactone starting material. LC-UV-MS^2 analysis of purified specimens revealed MS^2 fragmentations useful for defining the point of attachment of the glucuronide moiety to the zearalenone nucleus.

KEYWORDS: Zearalenone; zearalenol; zearalanol; zeranol; taleranol; glucuronide; synthesis

INTRODUCTION

Livestock are often exposed to the β-resorcylic acid lactone zearalenone (**1**) through ingestion of pasture or feeds containing grain previously subject to infection by *Fusarium* fungi (*1–5*). Stock in some countries are also sometimes treated with the related resorcylic acid lactone α-zearalanol (zeranol) (**4**), a growth promotant produced from **1** (*6*). Sheep and cattle metabolize **1** to a mixture of resorcylic acid lactones **1**−**6** (**Figure 1**) (*7, 8*), and cattle metabolize **4** to a mixture of **4**−**6** (*9–11*). Recently, an array of oxidized metabolites was detected during incubation of **1** with rat liver microsomes (*12*), suggesting that oxidative metabolism of resorcylic acid lactones may also be important in vivo. Resorcylic acid lactones **1**−**6** are converted to sulfate and glucuronide conjugates in many animal species (*13–19*), but detailed metabolic studies have been hindered by the lack of authentic specimens of the glucuronides. Synthesis of zearalenone 4-*O*-β-D-glucoside (**1d**) (**Figure 2**), via the Koenigs−Knorr procedure, has been reported (*20, 21*). However, although this glucoside synthesis proceeded smoothly in our hands, the analogous reaction to produce zearalenone 4-*O*-β-D-glucuronide (**1a**) via the methyl ester was unsuccessful under a variety of coupling conditions (unpublished observations). The reported efficient preparative-scale enzymatic synthesis of glucuronides of an alcohol and a phenol (*22, 23*) prompted us to apply this approach to the synthesis of resorcylic acid lactone glucuronides. Optimization of this procedure led to synthesis of zearalenone 4-*O*-β-D-glucuronide (**1a**) (**Figure 3**), and its application to resorcylic acid lactones **1**−**6** yielded the glucuronides in moderate-to-high yields.

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MATERIALS AND METHODS

General. Bovine liver uridine 5′-diphosphoglucuronyl transferase (UDPGT) and bovine β-glucuronidase (type B-1) were from Sigma Chemicals (St. Louis, MO), and uridine 5′-diphosphoglucuronic acid (UDPGA) was from Boehringer Mannheim (Mannheim, Germany). Buffers, solvents, and general chemicals were obtained from local suppliers. LiChroprep RP-18 silica gel (particle size = 25−40 μm) and aluminum-backed silica gel $60F_{254}$ TLC plates were obtained from Merck (Darmstadt, Germany), and resorcylic acid lactones were visualized after TLC by their absorbance or fluorescence during irradiation at 254 or 366 nm, respectively. Zearalenone (**1**), zearalanone

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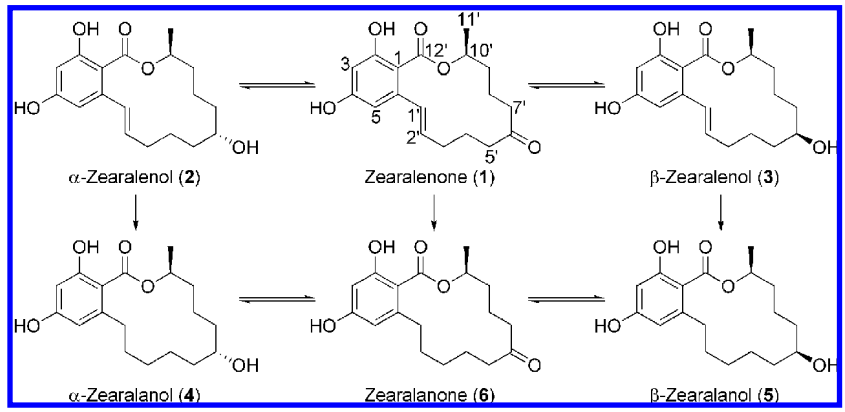


Figure 1. Structures of resorcylic acid lactones and the proposed metabolism of zearalenone in cattle and sheep (*7*). Double arrows indicate readily reversible oxidation/reduction of the oxygen atom at C-6′.

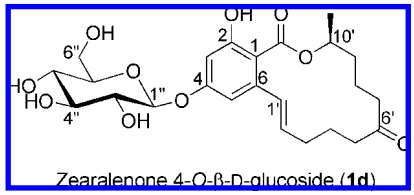


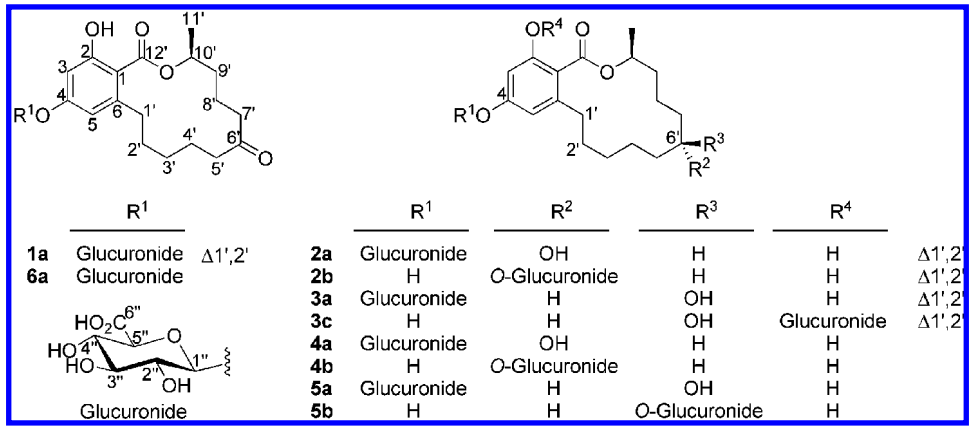
Figure 2. Structure of zearalenone 4-*O*-*β*-D-glucoside (**1d**), prepared from **1** by the Koenigs−Knorr procedure. Atom numbering is the same as for the glucuronide derivatives (**Figure 3**).

(**6**), α-zearalanol (**4**), and *β*-zearalanol (**5**) were from International Minerals & Chemical Corp., Terre Haute, IN. Chemical manipulations of **1**−**3** were performed with protection from sunlight to prevent isomerization to the corresponding cis-isomers (*7*). α-Zearalenol (**2**) and *β*-zearalenol (**3**) were prepared by reduction of **1** with $NaBH_4$ followed by separation of the isomeric alcohols by reversed-phase flash chromatography. The calculated structure of **1a** was obtained using Chem3D Ultra 8.0 (CambridgeSoft, Cambridge, MA) by attaching a glucuronide unit to the 4-position of the X-ray crystal structure of **1** (*24*) and minimizing the resulting structure. Zearalenone 4-*O*-*β*-D-glucoside (**1d**) was prepared by following minor modifications to a published procedure (*21*).

Optimization of Zearalenone Glucuronide Synthesis. Reaction conditions were based on those developed for benzyl alcohol and 4-methylphenol (*23*). The substrate (**1**) in acetonitrile (1−8 *μ*L, 200 mM) was added to a solution of $CaCl_2$ (6 mM), UDPGA, dithiothreitol (1 mM), BSA (3% w/v), and UDPGT (4 mg) in Tris buffer (200 *μ*L, 50 mM, pH 8.0) at 30 °C. Reactions were terminated after 24 h by the addition of acetonitrile (200 *μ*L), precipitated protein was removed by centrifugation at 13000 rpm for 10 min in a Micro Centaur microfuge (MSE, London, U.K.), and the supernatants were assayed for **1** and **1a** by gradient HPLC. Substrate (1−8 mM) and cofactor (UDPGA) (1−16 mM) concentrations were varied to determine optimal values for synthesis (**Figure 4**).

HPLC. Supernatant (20 *μ*L) was analyzed by analytical gradient HPLC on a 250 × 4.6 mm i.d., 5 *μ*m, Nucleosil C18 column (Phenomenex, Torrance, CA) fitted to a Gilson (Middleton, WI) dual-pump HPLC system equipped with an autoinjector and a Gilson 160 UV−visible diode array detector. Elution was at 0.7 mL/min with 0.01 M phosphoric acid/acetonitrile ramped from 65:35 (**1** and **6**) or 70:30 (**2**−**5**) to 80:20 over 20 (**1** and **6**) or 25 (**2**−**5**) min. Eluting compounds were detected by UV absorbance at 274 nm (**1** and **6**) and 260 nm (**2**−**5**). Preparative HPLC purification was performed on a Waters 600 system equipped with a Waters 486 UV detector and fitted with a 250 × 21.2 mm i.d. Zorbax ODS column eluted with 70:30:0.7 water/MeCN/HOAc at 10 mL/min.

Preparative-Scale Glucuronide Synthesis. The optimized analytical-scale reaction was scaled up to larger reaction volume, with substrate (**1**, **2**, **3**, **4**, **5**, or **6**) at 8 mM and UDPGA at 16 mM (**Table 1**). After 24 h, EDTA was added to the reaction mixture (to give a concentration of 10 mM) to prevent formation of intractable calcium salts of the glucuronides, and the pH was adjusted to 3.5 with 0.1 M HCl. The reaction mixture was diluted with 3 volumes of MeCN and the resulting proteinaceous precipitate removed by filtration through filter aid. The precipitate was redissolved in water, reprecipitated with 3 volumes of MeCN, and refiltered. The filtrates were combined, and the solvent was removed in vacuo. The residue was dissolved in water (5 mL), filtered, and applied to a 35 × 1.25 cm i.d. column of reversed-phase silica gel (LiChroprep RP-18). The column was eluted with water (100 mL) followed by water-MeOH (1:1). Fractions (3 mL) were collected and pooled as indicated by TLC (elution with ethyl acetate/methanol/



	R^1			R^1	R^2	R^3	R^4	
1a	Glucuronide	Δ1′,2′	**2a**	Glucuronide	OH	H	H	Δ1′,2′
6a	Glucuronide		**2b**	H	*O*-Glucuronide	H	H	Δ1′,2′
			3a	Glucuronide	H	OH	H	Δ1′,2′
			3c	H	H	OH	Glucuronide	Δ1′,2′
			4a	Glucuronide	OH	H	H	
			4b	H	*O*-Glucuronide	H	H	
			5a	Glucuronide	H	OH	H	
			5b	H	H	*O*-Glucuronide	H	

Figure 3. Structures of glucuronide derivatives of zearalenone metabolites **1**−**6** isolated in this study after enzymatic synthesis. In the text, glucuronides of compounds **1**−**6** with glucuronic acid attached via C-4 are designated **a** (e.g., **1a** is zearalenone 4-*O*-*β*-D-glucuronide), whereas compounds with glucuronic acid attached via C-6′ are designated **b** (e.g., **2b** is α-zearalenol 6′-*O*-*β*-D-glucuronide) and compounds with glucuronic acid conjugated via C-2 are designated **c** (e.g., **3c** is *β*-zearalenol 2-*O*-*β*-D-glucuronide).

Table 1. Reaction Conditions, Analytical HPLC Retention Times, and Yields during Preparative Enzymatic Glucuronidations of **1**−**6**

substrate[a]	t_R[b]	wt[c]	solv[d]	vol[e]	Rn vol[f]	prod[a]	yield[g]	t_R[b]	prod[a]	yield[g]	t_R[b]
1	23.9	38	CH_3CN	0.6	16	**1a**	50.0 (85%)	12.3			
2	18.5	19.3	DMSO	0.1	8	**2a**	18.1 (61%)	10.2	**2b**	4.0 (13%)	11.8
3	16.4	19.6	DMSO	0.1	8	**3a**	18.5 (61%)	9.4	**3c**	3.0 (10%)	11.0
4	18.2	19.6	DMSO	0.1	8	**4a**	3.5 (12%)	10.3	**4b**	3.1 (10%)	11.8
5	16.3	20.3	DMSO	0.1	8	**5a**	19.5 (62%)	9.2	**5b**	3.7 (12%)	10.4
6	21.2	20.2	DMSO	0.1	8	**6a**	25.7 (82%)	10.8			

[a] Substrate or product (**Figure 1**). [b] Retention time (min) on gradient HPLC. [c] Weight (mg) of substrate. [d] Cosolvent used to introduce the substrate. [e] Volume (mL) of cosolvent used to introduce the substrate. [f] Volume (mL) of buffered enzymatic reaction mixture. [g] Isolated yield (mg) of product (free acid form) based on the substrate.

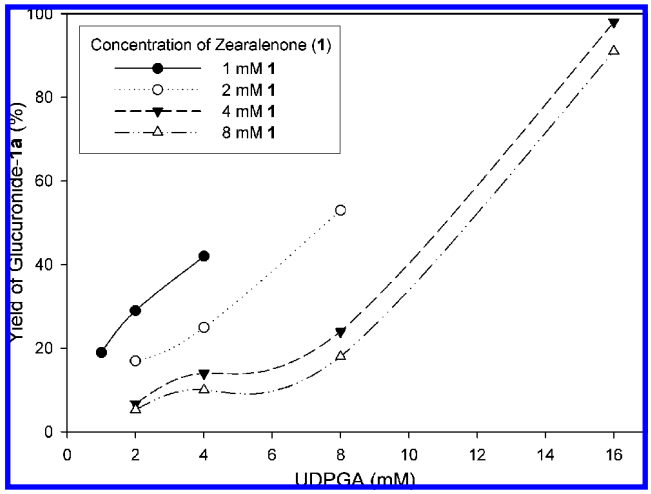


Figure 4. Effect of substrate [zearalenone (**1**)] and cofactor (UDPGA) concentrations on the yield of zearalenone 4-*O*-β-D-glucuronide (**1a**), as measured by HPLC.

acetic acid, 67:30:3), and solvent removed in vacuo. Final purification was achieved by preparative HPLC, and the products were weighed to determine yields. Compounds were then dissolved in MeOH and passed through a 5 × 5 mm column of Chelex-100, Na^+ form, ion-exchange resin (Sigma-Aldrich, St. Louis, MO) with rinsing and then concentrated under a stream of nitrogen to remove any residual calcium ions. This treatment afforded the compounds as colorless powders, probably in their sodium salt forms, for NMR analysis. After NMR analysis, specimens of the purified glucuronides were analyzed by LC-UV-MS^2 in ESI negative ion mode using a generic method developed for analysis of phenolic compounds (*25*).

HR-MS. High-resolution mass spectrometry (HR-MS) (**Table 2**) was performed in negative and positive ion modes on a Bruker Daltonics MicrOTOF spectrometer. The samples (sodium salt form) were dissolved in MeOH and infused via a syringe pump at 4 μL/min. Cluster ions from sodium formate (2 mM) were used for mass calibration. Mass spectra were acquired with a time-of-flight analyzer over *m*/*z* 500−1500. Capillary voltage and skimmer cone voltage were set at −90 and −24 V for negative ion and at 80 and 24 V for positive ion spectra.

NMR Spectroscopy. NMR spectra of all compounds were obtained from solutions in CD_3OD (99.8+ atom % D; Aldrich) using a Bruker DRX 400 MHz spectrometer fitted with a 5 mm dual, gradient-shielded, inverse probe. NMR assignments (**Table 3**) were obtained from examination of ^1H, ^{13}C, DEPT135, 1D-SELTOCSY, COSY, TOCSY, g-HSQC, g-HMBC, NOESY, and ROESY NMR spectra. Where required, additional HSQC−TOCSY spectra were obtained with mixing times of 30, 50, 80, and 160 ms. Chemical shifts, determined at 30 °C, are reported relative to internal CHD_2OD (3.31 ppm) and CD_3OD (49.0 ppm).

Glucuronidase Treatment. To confirm that the multiple reaction products from alcohols **2** and **3** were not due to isomerization at C-1′−C-2′ (*7*) or at the 6′-hydroxyl group, samples of each reaction mixture (100 μL) were worked up as for HPLC analysis and the solvent was removed under a stream of nitrogen. The residue was dissolved in succinate buffer (200 μL, 100 mM, pH 5.0) containing β-glucuronidase (2 mg), the mixture incubated at 37 °C overnight, and the solution analyzed by HPLC.

Table 2. High-Resolution Mass Spectrometric Analyses for Isolated Glucuronides of **1**−**6** in Their Sodium Salt Forms

	negative mode ($[M - H]^-$)			positive mode ($[M + Na]^+$)		
	m/*z* found	*m*/*z* calcd	error (ppm)	*m*/*z* found	*m*/*z* calcd	error (ppm)
1a	493.1735	493.1715	−4.2	517.1677	517.1680	0.7
2a	495.1880	495.1872	−1.3	519.1857	519.1837	−4.0
2b	495.1894	495.1872	−4.4	519.1832[a]	519.1837	0.9
3a	495.1884	495.1872	−2.4	519.1843	519.1837	−1.2
3c	495.1882	495.1872	−2.1	519.1850[a]	519.1837	−2.6
4a	497.2019	497.2028	−2.5	521.1982[a]	521.1993	2.2
4b	497.2041	497.2028	−2.5	521.1983[a]	521.1993	1.9
5a	497.2048	497.2028	−4.1	521.2016	521.1993	−4.4
5b	497.2040	497.2028	−2.3	521.1996[a]	521.1993	−0.5
6a	495.1875	495.1872	−0.6	519.1821	519.1837	3.1

[a] Also present was a major peak with the correct *m*/*z* value for the disodium adduct.

RESULTS AND DISCUSSION

Optimization of Zearalenone Glucuronide Synthesis. Optimization experiments gave the best results with at least a 2-fold excess of UDPGA and a high zearalenone concentration (**Figure 4**). The highest yield of **1a** (98%) was obtained with 4 mM zearalenone and 16 mM UDPGA. Although doubling the zearalenone concentration reduced the yield to 91%, this halved the reaction volume and the consumption of expensive cofactor. All of the preparative reactions were therefore performed with 8 mM substrate and 16 mM UDPGA.

Preparative Synthesis of Resorcylic Acid Lactone Glucuronides. Zearalenone glucuronide (**1a**) was prepared on a large scale (**Table 1**) and the pure material isolated in high yield (85%) by reverse-phase flash chromatography as a crystalline solid. Ketone **1** and its glucuronide (**1a**) were fluorescent and had very similar UV absorbance spectra, indicating that the chromophore had not been significantly altered. The HR-MS (**Table 2**) and LC-MS^2 spectra were also consistent with **1a**.

Because alcohols **2** and **3** were poorly soluble in acetonitrile, the procedure was modified such that the substrate (ca. 20 mg) in DMSO (100 μL) was added to buffer (8 mL). HPLC analysis revealed that alcohol **2** was almost quantitatively converted into a major and a minor product. Hydrolysis of the products with glucuronidase was found to proceed cleanly to **2** (HPLC analysis), indicating the presence of a pair of isomeric glucuronides. The products were isolated as for zearalenone (**1**). Partial separation of the two products was achieved by flash chromatography, with final purification by preparative HPLC to give a more polar (**2a**) and a less polar (**2b**) glucuronide in yields of 61 and 13%, respectively (**Table 1**). The UV spectra of both glucuronides were very similar to that of the substrate (**2**). This procedure was applied to alcohols **3**−**5** and ketone **6**, with satisfactory results (**Table 1**).

Table 3. NMR Assignments for Sodium Salts of Glucuronides of Zearalenone and Related Metabolites in CD_3OD[a]

	1a		1d		2a		2b		3a		3c		4a		4b		5a		5b		6a	
atom	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	107.6		107.2		106.6		104.0		112.7		117.5		110.7		107.2		114.8		109.7		108.7	
2	165.0		165.2		165.9		166.5		160.9		156.5		163.7		165.5		160.2		162.4[b]		165.4	
3	104.1	6.54	104.1	6.54	104.0	6.52	102.9	6.22	103.9	6.52	103.5	6.63	103.1	6.51	101.9	6.16	102.9	6.47	101.7	6.15	103.1	6.47
4	162.7		162.9		162.7		163.8		161.6		160.4		162.5		163.4		161.2		162.5[b]		162.6	
5	109.6	6.68	109.5	6.69	110.3	6.66	109.8	6.38	107.8	6.76	106.9	6.67	111.9	6.51	111.5	6.21	110.7	6.51	110.4	6.23	112.4	6.47
6	143.9		144.1		144.9		145.3		141.6		138.3		147.7		149.0		145.8		147.4		148.8	
1′	133.6	6.95	133.7	6.95	133.5	7.11	133.9	7.13	130.9	6.62	128.8	6.34	36.3	2.55, 3.06	37.0	2.46, 3.19	34.4	2.67, 2.75	34.7	2.65, 2.91	38.1	2.43, 3.05
2′	134.0	5.81	134.1	5.84	135.2	5.78	134.4	5.73	134.2	6.07	134.4	6.09	31.9	1.37, 1.75	32.3	1.30, 1.77	31.6	1.57	32.0	1.53, 1.61	32.6	1.26, 1.70
3′	32.2	2.18, 2.30	32.2	2.18, 2.32	31.5	2.32	31.7	2.35	31.8	2.28	31.3	2.27	28.2	1.39, 1.53	28.4	1.45, 1.53	28.6	1.44	28.9	1.40, 1.46	28.6	1.26, 1.45
4′	22.1	1.61, 2.01	22.1	1.60, 2.03	23.6	1.54, 1.87	23.7	1.53, 1.98	23.7	1.63, 1.74	23.8	1.55, 1.77	23.6	1.39, 1.56	24.3	1.42, 1.63	23.1	1.40	23.0	1.38, 1.48	23.6	1.41, 2.02
5′	37.6	2.35, 2.78	37.7	2.31, 2.80	32.7	1.18, 1.68	31.4	1.41, 1.67	32.9	1.27, 1.71	33.2	1.23, 1.66	32.9	1.37, 1.66	30.3	1.55, 1.68	33.0	1.47, 1.54	28.9	1.66	37.8	2.15, 2.90
6′	213.8		213.8		67.3	3.75	78.2	3.84	69.4	3.69	69.3	3.64	69.8	3.75	78.7	3.90	70.8	3.73	78.6	3.92	215.0	
7′	43.9	2.17, 2.62	43.9	2.16, 2.63	37.4	1.51	35.6	1.50, 1.97	37.2	1.43, 1.64	37.5	1.42, 1.61	36.0	1.50, 1.62	34.7	1.70, 1.77	35.4	1.47, 1.69	31.5	1.60, 1.76	44.7	2.15, 2.67
8′	23.1	1.77	23.1	1.77	22.7	1.43	22.8	1.36, 1.42	20.4	1.40, 1.54	20.2	1.33, 1.51	22.4	1.49	22.5	1.50	19.6	1.42, 1.50	19.6	1.43, 1.58	23.2	1.78, 1.89
9′	35.9	1.64	35.9	1.64	36.1	1.61, 1.86	36.0	1.64, 1.90	35.6	1.60, 1.86	35.7	1.51, 1.83	36.2	1.62, 1.82	36.2	1.65, 1.83	36.2	1.72	36.6	1.73	36.1	1.53, 1.61
10′	74.7	5.07	74.7	5.05	75.2	5.00	75.0	4.96	73.9	5.20	72.8	5.32	74.0	5.20	74.3	5.18	73.2	5.21	73.3	5.19	74.0	5.21
11′	20.8	1.38	20.9	1.38	21.3	1.40	21.3	1.39	19.2	1.33	19.5	1.33	21.3	1.35	21.4	1.34	20.3	1.34	20.5	1.34	21.2	1.34
12′	172.2		172.3		172.7		173.1		171.4		169.8		172.3		172.9		171.4		172.2		172.4	
1″	101.5	5.05	101.4	4.99	101.3	5.07	105.7	4.40	102.1	4.99	102.8	4.93	101.6	4.96	104.1	4.38	101.9	4.96	102.5	4.34	101.3	5.04
2″	74.5	3.51	74.8	3.46	74.5	3.51	75.4	3.22	74.5	3.51	74.6	3.45	74.6	3.50	75.2	3.19	74.5	3.49	75.1	3.19	74.5	3.50
3″	77.4	3.51	78.0	3.47	77.3	3.51	78.0	3.40	77.3	3.51	77.9	3.46	77.7	3.50	77.8	3.38	77.4	3.49	78.0	3.39	77.3	3.50
4″	73.0	3.60	71.4	3.37	72.9	3.63	73.6	3.48	73.0	3.61	73.1	3.57	73.4	3.53	73.6	3.48	73.1	3.60	73.8	3.45	72.9	3.61
5″	76.5	3.97	78.4	3.47	76.5	4.00	76.7	3.65	76.6	3.98	76.7	3.86	76.6	3.82	76.5	3.64	76.6	3.93	76.5	3.55	76.6	4.00
6″	172.9		62.6	3.69, 3.90	172.4		175.7		172.5		173.6		175.2		175.4		173.1		176.4		172.4	

[a] Relative to internal CHD_2OD at 3.31 ppm, and CD_3OD at 49.0 ppm. [b] Assignments interchangeable.

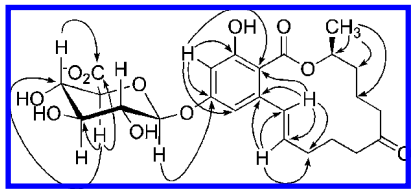


Figure 5. Selected correlations observed in the HMBC NMR spectrum of zearalenone 4-*O*-β-D-glucuronide (**1a**) sodium salt in CD_3OD.

NMR Assignments. Detailed analyses of one- and two-dimensional NMR data, including ^1H, ^{13}C, DEPT135, SELTOCSY, COSY, TOCSY, g-HSQC, g-HMBC, HSQC–TOCSY, SELNOESY, and NOESY spectra determined in CD_3OD, afforded complete ^1H and ^{13}C assignments for glucoside **1d** and the series of resorcylic acid lactone glucuronide derivatives of **1**−**6** (**Table 3**). Typically, $^1H-^1H$ connectivities were established in COSY and TOCSY experiments, and $^1H-^{13}C$ connectivities were identified in g-HSQC and g-HMBC experiments. Stereochemistries of structurally significant skeletal portions were established in SELNOESY and NOESY experiments. Points of attachment of the glucuronide residues were routinely established via $^3J_{H-C}$ g-HMBC correlations from the glucuronide anomeric proton (ca. 4.30−5.10 ppm) across the O-glycosidic linkage to one of the phenolic carbons (C-2 or C-4 at ca. 156.0−166.0 ppm) or to C-6′ of the macrocyclic ring (ca. 78.5 ppm).

As a representative example of structural assignment, the ^{13}C and DEPT135 NMR spectra of zearalenone 4-*O*-β-D-glucuronide (**1a**), in accord with the HR-MS data, revealed the presence of 24 carbon resonances (1 methyl, 6 methylene, 10 methine, and 7 quaternary). A complete assignment of ^1H and ^{13}C NMR signals for zearalenone 4-*O*-β-D-glucuronide (**1a**) is presented in **Table 3**. NMR spectroscopic assignments for **1a** closely paralleled those for zearalenone 4-*O*-β-D-glucoside (**1d**) in CD_3OD (**Table 1**), although some of our assignments for the macrocyclic resonances of **1d** differed from those reported by Kamimura (*26*).

Notable features of the ^1H NMR spectrum of zearalenone 4-*O*-β-D-glucuronide (**1a**) included the presence of one methyl signal at 1.38 ppm (d, J = 6.2 Hz, H-11′), five glucosyl proton signals in the region of 3.51−5.05 ppm, two aromatic proton signals at 6.54 ppm (d, J = 2.6 Hz, H-3) and 6.68 ppm (d, J = 2.6 Hz, H-5), and trans-coupled olefinic proton signals at 6.95 ppm (br d, J = 15.4 Hz, H-1′) and 5.81 ppm (ddd, J = 4.7, 9.6, 15.4 Hz, H-2′). The H-1″ NMR signal of the anomeric proton appeared as a doublet at 5.05 ppm (J = 7.7 Hz). This coupling demonstrated that H-1″ was 1,2-trans-diaxially orientated with respect to H-2″ (*27*) and showed the glycosidic linkage was therefore β-(equatorially) orientated.

H-5″ (3.97 ppm) exhibited a single COSY correlation to the H-4″ proton (3.60 ppm), which correlated to C-4″ (73.0 ppm) in the g-HSQC spectrum. The H-2″ and H-3″ glucuronide protons exhibited unresolved resonances at 3.51 ppm, which correlated to carbons at 74.5 and 77.4 ppm in the g-HSQC spectrum. These were unambiguously assigned via a g-HMBC correlation from H-5″ (3.97 ppm) to the C-3″ carbon resonance at 77.5 ppm. A g-HMBC correlation from the anomeric proton (H-1″, 5.05 ppm) to the carbon resonance at 85.1 ppm (C-4) confirmed the presence of an O-glycosodic linkage at C-4 of zearalenone. Selected HMBC correlations are presented in **Figure 5**.

COSY correlations from the H-4′ protons (1.61 and 2.01 ppm) to protons at 2.35 and 2.78 ppm identified the H-5′ protons, which in turn correlated to a carbon resonance at 37.6 ppm (C-5′) in the g-HSQC spectrum. Similarly, H-7′ proton resonances (2.17 and 2.62 ppm) were identified via COSY correlations from the H-8′ protons (1.77 ppm, 2H). H-7′ in turn correlated to a carbon resonance at 43.9 ppm (C-7′) in the g-HSQC spectrum.

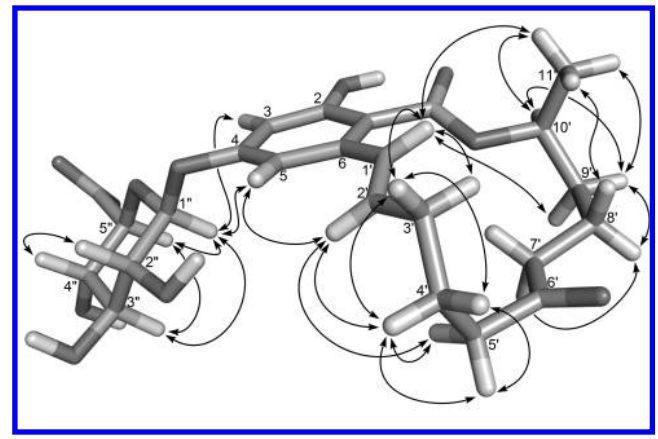


Figure 6. Calculated structure of zearalenone 4-*O*-β-D-glucuronide (**1a**) sodium salt, showing selected correlations observed in the NOESY NMR spectrum in CD_3OD.

NMR assignments, stereochemistry, and point of attachment were verified from correlations observed in the NOESY NMR spectrum (**Figure 6**). Thus, the axial orientations of H-5″ (and, therefore, the equatorial orientation of the glucuronide C-6″ carboxylic acid group) and H-3″ were confirmed by NOE correlations from H-1″ (5.05 ppm) to both H-3″ (3.51 ppm) and H-5″ (3.97 ppm, d, J = 9.8 Hz). H-1″ also exhibited NOE correlations to both H-3 (6.54 ppm) and H-5 (6.68 ppm) protons, indicating free rotation about the O-glycosidic linkage. An NOE correlation from H-5 (6.68 ppm) to H-2′ (5.81 ppm) and the absence of a corresponding NOE correlation to H-1′ confirmed the trans-coupled nature of the H-1′/H-2′ olefinic double bond, as opposed to a cis-coupled olefinic double bond.

Problems arose in assigning resonances of some of the macrocyclic ring atoms in the 4-*O*- and 6′-*O*-glucuronides of the saturated α- and β-alcohols (**4a**, **4b**, **5a**, and **5b**) due to considerable overlap of proton resonances. This problem was overcome by use of HSQC−TOCSY experiments, which allow the stepwise tracking of protonated carbon correlations ($^1J_{H-CH}-^4J_{H-CH}$) through the use of a range of mixing times. The carrier signal is proton-coupling-dependent, and only correlations to protonated carbons are observed. Mixing times of 15−30 ms tended to give $^1J_{H-CH}$ (HSQC) and $^2J_{H-CH}$ correlations, whereas 50−80 ms mixing times also gave rise to $^3J_{H-CH}$ and $^4J_{H-CH}$ correlations.

Application of this technique is exemplified by α-zearalanol 6′-*O*-β-D-glucuronide (**4b**), in which unequivocal assignments for atoms 4′, 5′, and 7′ were required from the $^{13}C/^1H$ pairs: 24.3 ppm/1.42, 1.63 ppm; 30.3 ppm/1.55, 1.68 ppm; and 34.7 ppm/1.71, 1.77 ppm. From a series of HSQC−TOCSY experiments, with mixing times of 15, 30, and 50 ms, respectively, stepwise J_{H-CH} correlations from the well-defined H-10′ (5.18 ppm) and H-1′ (2.46, 3.19) resonances for the macrocyclic ring system were mapped out. For example, J_{H-CH} correlations were observed from H-10′ (5.18 ppm) to C-10′ (74.3 ppm, $^1J_{H-CH}$), C-9′ (36.2) and C-11′ (21.4 ppm) (both $^2J_{H-CH}$), C-8′ (22.5 ppm, $^3J_{H-CH}$), and C-7′ (34.7 ppm, $^4J_{H-CH}$). Similarly, H-1 (2.46, 3.19 ppm) correlated to C-1′ (37.0, $^1J_{H-CH}$), C-2′ (32.3 ppm, $^2J_{H-CH}$), C-3′ (28.4 ppm, $^3J_{H-CH}$), and C-4′ (24.3 ppm, $^4J_{H-CH}$). The remaining 5′-resonances were assigned via $^2J_{H-CH}$ HSQC−TOCSY

correlations from H-6′ (3.90 ppm) to C-6′ (78.7 ppm, $^1J_{H-CH}$), C-5′ (30.3 ppm, $^2J_{H-CH}$), and C-7′ (34.7 ppm, $^2J_{H-CH}$). The other saturated alcohol derivatives were assigned or had their assignments confirmed in this manner using HSQC−TOCSY experiments.

Formation of β-zearalenol 2-*O*-β-D-glucuronide (**3c**) was unanticipated, and prior to NMR characterization the fraction was believed to contain β-zearalenol 6′-*O*-β-D-glucuronide (**3b**). However, a g-HMBC correlation was observed from the H-1″ anomeric proton (4.93 ppm) to a carbon resonance at 156.5 ppm (C-2). Furthermore, H-3 (6.63 ppm) and H-5 (6.67 ppm) showed common g-HMBC correlations to 160.4 ppm (C-4), but only H-3 (6.63 ppm) showed a g-HMBC correlation to 156.5 ppm (C-2), thus, defining the point of attachment at C-2 on the aromatic ring. In addition, the H-1″ anomeric proton (4.93 ppm) showed an NOE correlation to H-3 (6.63 ppm) but not to H-5 (6.67 ppm). In contrast, the 4-substituted resorcylic acid lactone glucuronides all exhibited NOE correlations from the H-1″ anomeric proton to both H-3 and H-5 (**Figure 6**). β-Zearalenol 2-*O*-β-D-glucuronide (**3c**) eluted earlier than other analogues under the reverse-phase conditions employed, consistent with the presence of two non-intramolecularly hydrogen bonded hydroxyl groups at both the C-4 and C-6′ positions.

MS^2 fragmentation of 4-*O*-glucuronides during negative ion LC-MS^2 analyses gave $[aglycone - H]^-$ as the main daughter ion, together with a weak fragment corresponding to $[M - H - aglycone]^-$ (*m*/*z* 175). No significant $[M - H - CO_2]^-$ daughter ion was detected. In contrast, the main daughter from the 6′-*O*-glucuronides was attributable to $[M - H - CO_2]^-$, and only low-intensity daughter ions were present corresponding to $[aglycone - H]^-$ and $[M - H - aglycone]^-$. The MS^2 spectrum for the isolated 2-*O*-glucuronide (**3c**) was intermediate between these, with $[aglycone - H]^-$ as the dominant daughter ion but with moderate intensity $[M - H - CO_2]^-$ and $[M - H - aglycone]^-$ daughter ions. 6′-*O*-Glucuronides eluted slightly later than their corresponding 4-*O*-glucuronides, whereas the 2-*O*-glucuronide (**3c**) eluted earlier. Although the 4-*O*-glucuronide was the most abundant metabolite for each of the zearalenone analogues studied, examination of LC-MS^2 chromatograms of the purified compounds (Supporting Information) revealed low-intensity peaks with retention times and daughter ions suggesting the presence of 2-*O*- and 6′-*O*-glucuronides as minor contaminants. This suggests that the 2-, 4-, and 6′-positions of zearalenone analogues can all potentially be conjugated to glucuronic acid in the bovine liver, with the proportion of glucuronidation at each position being structure-dependent. Biologically mediated conjugation of glucosides to the 4- and 2-positions of **1**−**3** has been documented in plants and fungi (*26, 28–32*). These studies, together with the findings reported here, suggest that enzyme-catalyzed glycosylation of resorcylic acid lactones is widespread and can occur with low regioselectivity.

In summary, the results indicate that, at least in vitro, bovine liver glucuronyl transferases conjugate glucuronic acid principally to the 4-position of zearalenone analogues. However, derivatization of the 2- and 6′-positions can also occur, and to a significant degree for some analogues. It remains to be seen whether this also holds true for in vivo metabolism and whether this phenomenon is species-dependent. Glucuronidation of hydroxylated metabolites of **1**, such as those reported by Pfeiffer et al. (*12*), can also be expected. The synthetic method reported here will enable production of analytical standards and analogues for toxicological evaluation and structure−activity studies. Optimization of the chromatographic conditions should permit separation and direct detection and quantitation of the glucuronides of **1**−**6** by LC-MS analysis.

ABBREVIATIONS USED

BSA, bovine serum albumin; UDPGA, uridine 5′-diphosphoglucuronic acid; UDPGT, 5′-diphosphoglucuronyl transferase.

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Supporting Information Available: Full NMR assignment table for each compound including coupling constants, plus LC-UV-MS^2 chromatograms and extracted spectra, for each sample. This information is available free of charge via the Internet at http://pubs.acs.org.

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