Identification of the Calcium Salt of Epismilagenin β-D-Glucuronide in the Bile Crystals of Sheep Affected by *Panicum dichotomiflorum* and *Panicum schinzii* Toxicoses

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Crystals isolated from the bile of sheep affected by hepatogenous photosensitization after grazing *Panicum dichotomiflorum* or *Panicum schinzii* were soluble in acetic acid but essentially insoluble in common organic solvents. The main component of the crystals was identified by acidic and enzymatic hydrolysis, GC–MS, LSIMS, TLC, ^{1}H and ^{13}C NMR spectroscopy, and X-ray analysis as the calcium salt of epismilagenin β-D-glucuronide. The ^{13}C NMR spectrum was fully assigned, and the structure confirmed by comparison with model compounds (17-oxoetiocholan-3α-ol glucuronide and epismilagenin). Only saponins derived from diosgenin were detected in the foliage of *P. dichotomiflorum* and *P. schinzii*. A metabolic pathway for conversion of *P. dichotomiflorum* and *P. schinzii* saponins into the major bile crystal component is proposed.

INTRODUCTION

Several hepatogenous photosensitization diseases of ruminants throughout the world are characterized by the deposition of crystalloid material in and around the bile ducts. These diseases are closely associated with the consumption of certain plants, including *Panicum dichotomiflorum* (Holland et al., 1991), *Panicum schinzii* (Button et al., 1987), *Panicum coloratum* (Bridges et al., 1987), *Panicum miliaceum* (Clare, 1955), *Tribulus terrestris* (Coetzer et al., 1983; Kellerman et al., 1980), *Narthecium ossifragum* (Flåøyen et al., 1992), *Agave lecheguilla* (Matthews, 1937; Camp et al., 1988), and *Brachiaria decumbens* (Graydon et al., 1991). Many of these plants are known to contain saponins (Bridges et al., 1987).

Chemical analysis of the bile crystals has proved difficult due to their insolubility in common organic solvents. Camp et al. (1988) reported the presence of a steroidal sapogenin in the bile of a sheep fed *A. lecheguilla*, which was tentatively identified as smilagenin (**5**) or sarsasapogenin (the 25*S* isomer of **5**) by mass spectrometry. Lancaster et al. (1991) similarly detected a smilagenin-like compound in bile crystals from lambs grazing *P. schinzii*. In a recent detailed investigation, Holland et al. (1991) found that the bile crystals from sheep photosensitized through ingestion of *P. dichotomiflorum* consisted primarily of an acidic conjugate of the steroidal sapogenin epismilagenin (**6**) [(25*R*)-5β-spirostan-3α-ol], along with trace amounts of free **6** and a neutral conjugate of **6**.

In preliminary papers, we have reported the identity of the major compound from the bile crystals of *P. dichotomiflorum*- and *P. schinzii*-intoxicated sheep to be epismilagenin β-D-glucuronide (**1**) (Miles et al., 1991, 1992a). We now report in detail the structural elucidation of **1**, isolated as its calcium salt (**2**) from the bile crystals of sheep photosensitized while consuming *P. dichotomiflorum* or *P. schinzii*.

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MATERIALS AND METHODS

Chromatography. Thin-layer chromatography (TLC) was performed on silica gel plates (0.2 mm, E. Merck 5554) using the following solvent systems: A, chloroform–methanol 49:1; B, chloroform–methanol–water 65:35:10 (bottom layer); C, chloroform–methanol–water 65:35:10 (bottom layer) plus 1% v/v acetic acid. Plates were visualized by spraying with anisaldehyde reagent (Stahl, 1969) or 5% sulfuric acid in ethanol.

Mass Spectrometry. Liquid surface-assisted ionization mass spectrometry (LSIMS) was performed on a Kratos MS80 RFA instrument using a 7-keV Xe atom beam. The sample in acetic acid (1 μL) was mixed with the glycerol matrix (5 μL) on the probe tip. Gas chromatography–mass spectrometry (GC–MS) was carried out on a Hewlett-Packard 5980GC interfaced to an HP 5970 mass-selective detector using split–splitless injection on a 10 m × 0.25 mm FSOT column, 0.25 μm HP1 (Hewlett-Packard), with temperature programming (100 °C 0.5 min, 8 °C/min to 290 °C).

Nuclear Magnetic Resonance Spectroscopy. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC-300 instrument at 300 or 75 MHz, respectively, with either C_5D_5N or CD_3CO_2D as the solvent. Chemical shifts are reported relative to internal TMS. Two-dimensional NMR spectra were acquired in either absolute value mode (COSY, double quantum filtered COSY, and long-range ^{13}C–^{1}H correlated spectra) or in phase sensitive mode (HOHAHA and ^{13}C–^{1}H correlated spectra). NOE difference spectra were obtained using a preirradiation time of 4 s and a power level of 45 L.

Isolation of Bile Crystals. Bile crystals were isolated from photosensitized sheep fed *P. dichotomiflorum* as described previously (Miles et al., 1991). The concentration of *Pithomyces chartarum* spores present in the plant material was determined according to the method of Thornton and Sinclair (1960). Bile crystals were purified by dissolving them in acetic acid at room temperature and filtering. Removal of the solvent in vacuo (toluene azeotrope) gave **2** as a colorless solid.

Bile crystals from lambs photosensitized while grazing *P. schinzii* were obtained as described elsewhere (Lancaster et al., 1991). The crystals (14.3 mg) were washed for 30 min successively with methanol–water (1:1, 1 mL), methanol (1 mL), and chloroform (1 mL) at 100 °C in a sealed vial to remove small amounts of neutral saponins, sapogenins, and low-polarity contaminants. The bulk of the sample was unaffected by this treatment. Subsequent extraction with acetic acid (3 × 1 mL) at room temperature dissolved most of the sample, leaving a dark green residue. After

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filtration, the acetic acid was removed in vacuo (toluene azeotrope) to afford **2** (10.7 mg, 75%) as a colorless solid.

Hydrolysis of Bile Crystals. *Acidic Hydrolysis.* An aliquot of bile crystal extract in acetic acid (100 μL) was added to hydrochloric acid (1 mL; 1 M) and heated to 100 °C for 3 h in a sealed vial. The reaction mixture was cooled and then extracted with chloroform (0.5 mL) and the extract examined by TLC (eluent A) and GC–MS.

Enzymatic Hydrolysis. A powdered sample of the bile crystal extract was added to acetate buffer (2 mL; 0.2 M, pH 5.5) containing β-glucuronidase (ca. 5000 units) and stirred vigorously. Samples (1 mL) were taken after 1 and 3 days, centrifuged to remove suspended matter, and extracted with chloroform (0.5 mL). The extracts were examined by TLC (eluent A).

Isolation of Plant Sapogenins. Freeze-dried, ground *P. dichotomiflorum* or *P. schinzii* foliage (6 g) was extracted (Soxhlet apparatus) with dichloromethane for 6 h to remove pigments. The plant material was then air-dried, extracted by boiling in ethanol–water (17:3; 200 mL) for 3 h, and filtered and the solvent removed in vacuo. This extract was dissolved in water (100 mL) and washed with petroleum spirit (40–60 °C), then saturated with NaCl, and extracted with 1-butanol (3 × 50 mL). The butanol was evaporated in vacuo, the residue washed with dry acetone (50 mL), and the insoluble crude saponin recovered by filtration. A sample of crude saponin (0.5 g) was hydrolyzed for 1 h with 1 M HCl (2 mL) at 100 °C in a sealed vial. The hydrolysate was diluted with water (100 mL) and extracted with diethyl ether (50 mL). The ether extract was washed with 0.1 M NaOH (50 mL) and dried ($MgSO_4$) and the solvent removed in vacuo to leave the crude sapogenins (ca. 5 mg) as a cream-colored solid.

X-ray Analysis. Bile crystals (prior to treatment with acetic acid) obtained from sheep fed *P. dichotomiflorum* were examined by energy-dispersive X-ray analysis (EDXA) on a Cambridge 250 Mk II SEM fitted with a Link 860 energy-dispersive X-ray analyzer. Spectra both from a small spot on a single crystal and from a bulk sample of the crystals were identical. Bile crystals from sheep fed *P. schinzii* were purified by the extraction procedure described above prior to EDXA.

Chemicals. 17-Oxoetiocholan-3α-ol glucuronide (**3**), diosgenin (**4**), and β-glucuronidase (type B1 from bovine liver) were obtained from Sigma Chemical Co. Epismilagenin (**6**) was obtained from Steraloids Inc., and tigogenin, smilagenin (**5**), and sarsasapogenin were obtained from Upjohn Laboratories.

RESULTS AND DISCUSSION

The bile crystals from sheep consuming *P. dichotomiflorum* and *P. schinzii* proved to be identical and are therefore discussed together.

The isolated bile crystals were soluble in acetic acid, sparingly soluble in pyridine, and practically insoluble in water, acetone, chloroform, and methanol. These solubility properties, along with features of their infrared spectrum (Holland et al., 1991), suggested the presence of the salt of a carboxylic acid. We were subsequently able to confirm this by NMR spectroscopy (see below), and samples were therefore dissolved in acetic acid prior to TLC or LSIMS. The only element detected by EDXA of the *P. dichotomiflorum*- or *P. schinzii*-derived bile crystals was calcium; these crystals are therefore calcium salts. The purification procedure described for the *P. schinzii*-associated bile crystals took advantage of their insolubility in nonacidic organic solvents; saponin and sapogenin impurities were removed with neutral solvents prior to extraction with acetic acid.

TLC analysis of the bile crystals showed only one spot; the R_f values using eluents A, B, and C (0.0, 0.13, and 0.22 respectively) were indicative of an acidic saponin. Hydrolysis with acid gave a compound identical by TLC (eluent A) and GC–MS to **6**, easily distinguished from **4**, **5**, tigogenin, and sarsasapogenin.

LSIMS gave quasi-molecular ions at m/z 591 and 593 in the negative and positive ion modes, respectively. A fragment at m/z 417 in the positive ion spectrum [see Miles et al. (1992a)] corresponds to loss of a glycuronic acid from the quasi-molecular ion. The LSIMS is therefore consistent with a glucuronic acid conjugate of epismilagenin (molecular weight 592).

Table I. ^{13}C (and ^1H) NMR Chemical Shifts of Epismilagenin and the Aglycon Carbons of Some Glucuronides (δ) in C_5D_5N and CD_3CO_2D at 300 K

	C_5D_5N			CD_3COOD		
atom	**6**	**2**[a]	**3**	**6**	**1**	**3**
1	35.4	35.6	35.3	36.3 (1.82, 0.99)[b]	36.0	36.0
2	30.6	27.4	27.0	30.6 (1.39, 1.68)	27.4	27.5
3	71.9	78.7	78.2	73.0 (3.70)	80.2	80.2
4	36.5	34.6	34.5	36.6 (1.84, 1.53)	34.9	34.9
5	42.1	42.4	42.2	43.3 (1.40)	43.2	43.2
6	27.1	27.4	27.2	28.1 (1.28, 1.91)	28.0	27.8
7	26.7	27.1	25.4	27.7 (1.36, 1.46)	27.6	26.2
8	35.5	36.0	35.3	36.7 (1.63)	36.5	36.4
9	40.6	41.1	40.8	41.7 (1.48)	41.6	41.7
10	34.8	35.2	34.9	35.7	35.7	35.7
11	20.7	21.1	20.3	21.7 (1.43, 1.30)	21.5	20.9
12	40.3	40.3	36.0	41.2 (1.23, 1.74)	41.0	36.6
13	40.6	40.1	47.8	41.8	41.5	49.2
14	56.4	56.6	51.3	57.4 (1.23)	57.2	52.2
15	31.9	32.3	21.9	32.5 (1.99, 1.27)	32.3	22.6
16	80.9	80.9	32.2	82.3 (4.45)	82.2	32.5
17	63.3	63.5	219.8	63.4 (1.81)	63.1	225.5
18	16.8	16.5	13.8	17.4 (0.80)	17.3	14.1
19	23.6	23.6	23.3	23.9 (0.99)	23.8	23.7
20	41.7	42.2		42.8 (1.92)	42.7	
21	14.5	14.5		14.8 (0.78)	14.8	
22	109.3	109.2		110.8	110.8	
23	31.4	32.1		32.1 (1.60, 1.60)	32.0	
24	29.4	29.4		29.5 (1.59, 1.43)	29.5	
25	30.4	30.7		31.1 (1.60)	31.0	
26	66.9	67.1		67.7 (3.50, 3.66)	67.5	
27	17.2	17.2		16.9 (0.99)	16.8	

[a] At 363 K. [b] Methylene protons in the format (Hα, Hβ).

The ^1H NMR of the conjugate (**2**) in C_5D_5N was poorly resolved at 300 K, but warming to 363 K substantially sharpened the proton resonances. The ^1H NMR spectrum included two singlet and two doublet methyl group signals at chemical shifts close to those that we have recently observed (Holland et al., 1991) for **6**. The low-field region (3–5 ppm) included signals attributable to the five protons of a glycosidic uronide and to the H-16, H-3α, H-26α, and H-26β resonances of a spirostanol derivative (see Tables I and II). The uronide signals were sharpened (at 363 K) to a lesser extent than were the spirostanol resonances.

Thirty-three resonances appeared in the ^{13}C NMR spectrum of the solubilized bile crystals; 27 of these signals (see Table I) were attributable to a spirostanol sapogenin, while the remaining 6 signals (see Table II) were indicative of the presence of a uronic acid entity. A comparison of the chemical shifts observed for the spirostanol carbons of the bile uronide (**2**) (see Table I) with those reported in a recent review (Agrawal et al., 1985) for a variety of spirostanol derivatives established the uronide as a 3-O-substituted derivative of **6**. The downfield shift experienced by C-3 (6.8 ppm) and the upfield shifts experienced by C-2 and C-4 (3.2 and 1.9 ppm, respectively) of the uronide, relative to the corresponding resonances of **6**, are comparable to the substituent group effects noted by Agrawal et al. (1985) for spirostan-3β-ol β-D-glycosides. The identification of the parent spirostanol as **6** is consistent not only with TLC analysis of the hydrolysate but also with our earlier identification by NMR of **6** as the hydrolysis product of crystals isolated from the bile ducts of sheep affected by *P. dichotomiflorum* toxicosis (Holland et al., 1991).

The chemical shift of the carboxyl carbon in C_5D_5N (178.5 ppm) was suggestive of a carboxylate salt rather than a free carboxylic group such as occurs in model

Table II. ^1H and ^{13}C NMR Chemical Shifts (δ) in C_5D_5N and CD_3COOD at 300 K[a]

	C_5D_5N				CD_3COOD			
	2[b]		3		1		3	
atom	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
1′	102.6	5.25 (br d, 7.7)	102.6	5.48 (d, 7.7)	101.4	4.62 (d, 7.8)	101.8	4.61 (d, 7.8)
2′	74.7	4.30 (br t)	75.2	4.52 (~t, 8.1)	74.2	3.46 (dd, 7.8, 9.0)	74.2	3.45 (dd, 7.8, 9.1)
3′	78.1	4.40 (br m)	78.1	4.77 (~t, 9.0)	76.6	3.71 (~t, 9.1)	76.6	3.71 (~t, 9.1)
4′	73.4	4.48 (br m)	73.5	5.03 (~t, 9.3)	72.4	3.80 (~t, 9.3)	72.3	3.80 (~t, 9.2)
5′	76.9	4.62 (br m)	78.1	5.12 (d, 9.4)	75.1	4.01 (d, 9.5)	75.2	4.01 (d, 9.4)
6′	178.5		172.8		173.2		173.8	

[a] ^1H–^1H coupling constants in hertz and signal multiplicities are given in parentheses. [b] At 363 K.

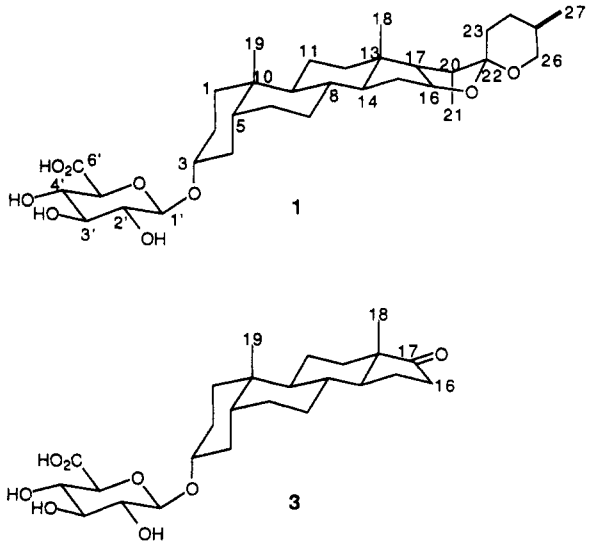

Figure 1. Structures of epismilagenin β-D-glucuronide (1) and 17-oxoetiocholan-3α-ol β-D-glucuronide (3).

compound 3 (172.8 ppm, see Table II). It therefore appeared likely that the material solubilized by treatment with acetic acid, and subsequently taken up in C_5D_5N, was the calcium salt of the uronide (2), possibly in equilibrium with the free acid (1).

This observation prompted the determination of the NMR spectra of the uronide in CD_3CO_2D, thereby ensuring that the free acid was the dominant species in solution. The chemical shifts and coupling constants for the uronide moiety are presented in Table II. Proton connectivities were established in two-dimensional COSY, double quantum filtered COSY, and HOHAHA experiments, while the magnitude of the coupling constants of the uronide protons (7.8–9.4 Hz) defined the orientations of H-1′, H-2′, H-3′, H-4′, and H-5′ as axial, identifying the uronide as a β-1-linked glucuronic acid entity.

In accord with this conclusion, and with the identification of the uronide as 1, irradiation of the H-1′ glucuronide resonance (4.62 ppm in CD_3CO_2D) resulted in NOE effects being observed for the glucuronide H-3′ (3.71 ppm), H-5′ (4.01 ppm), and epismilagenin H-3β (3.73 ppm) resonances, while irradiation of the glucuronide H-5′ resonance enhanced the H-3′ and H-1′ resonances.

The chemical shifts and coupling constants in CD_3CO_2D given in Tables I and II for 1 can be compared with those for 3, a model compound possessing a 3α-substituted ring A/B structure similar to that of 1, and for an authentic sample of 6. (Note: An attempt to obtain NMR spectra of 6 at 350 K resulted in the progressive exchange of deuterium onto C-23, as evidenced by the suppression of the corresponding carbon resonance and by the replacement of the original mass spectral molecular ion of m/z 416 with one of m/z 418.) The correspondence between the ^{13}C and ^1H NMR resonances of the glucuronide and A/B rings of 1 and 3 in CD_3CO_2D is remarkable; in general, shifts differ by less than 0.2 (^{13}C) or 0.02–0.05 (^1H) ppm. There is a similarly close correspondence between the ring C/D/E ^{13}C resonances of 1 and 6 (see Table I).

The complete assignment of the proton resonances of 6 in CD_3CO_2D (Table I) was achieved in a manner analogous to that described elsewhere for a number of triterpenoids (Wilkins et al., 1989a,b) and indole-diterpenes (Miles et al., 1992b). In summary, ^1H–^1H couplings were established in absolute value COSY and double quantum filtered COSY experiments, while ^{13}C and ^1H shifts were correlated in experiments optimized for the detection of ^1J (the resolution of the phase sensitive data was sufficient to distinguish axial and equatorial methylene protons) and for the detection of long-range (^2J and ^3J) couplings. Assignments were additionally corroborated in a series of NOE difference experiments.

Confirmation of the assigned structure was provided by hydrolysis of bile crystals with β-glucuronidase, to give epismilagenin (TLC). The structure of the *P. dichotomiflorum*- and *P. schinzii*-derived bile crystals is therefore established as 2.

Plants are the only known source of steroidal saponins and sapogenin derivatives, so the bile crystals presumably result from ruminal and/or tissue metabolism of ingested plant saponins. Analyses of the sapogenins from *P. dichotomiflorum* and *P. schinzii* foliage by ^1H and ^{13}C NMR spectroscopy, TLC (eluent A), and GC–MS revealed the presence of diosgenin (4) along with smaller amounts of (25*R*)-spirosta-3,5-diene arising (de Kock and Enslin, 1958) from dehydration of 4; no other spirostane derivatives were detected. The saponins present in *P. dichotomiflorum* and *P. schinzii* are therefore derived from diosgenin. The transformations required [for which there are precedents (Holland et al., 1991)] to change a diosgenin saponin into 2 are hydrolysis of the sugars, reduction of the 5–6 double bond, epimerization of 3β-OH to 3α-OH, and conjugation with glucuronic acid to form 1. In the presence of Ca^{2+}, the glucuronide may then form the insoluble calcium salt (2). This led us to propose the pathway shown in Figure 2 for the conversion of diosgenin into 2.

The fact that the bile crystals are saponin derivatives does not mean that plant saponins per se cause the hepatogenous photosensitization. Experimental reproduction of the bile crystal-associated photosensitizations alveld (Abdelkader et al., 1984) and geeldikkop (Kellerman et al., 1991) by administration of plant saponins required amounts of saponin much higher than would occur from normal consumption of the plant material. This, along with the sporadic nature of crystal-associated hepatogenous photosensitizations, has prompted the suggestion that the synergistic action of concurrently ingested hepatotoxins (e.g., mycotoxins) may initiate or exacerbate these diseases (Aas and Ulvund, 1989; Kellerman et al., 1980). The presence of *P. chartarum* spores [10^4 spores g^{-1} (wet weight) of grass], and therefore of the hepatotoxic

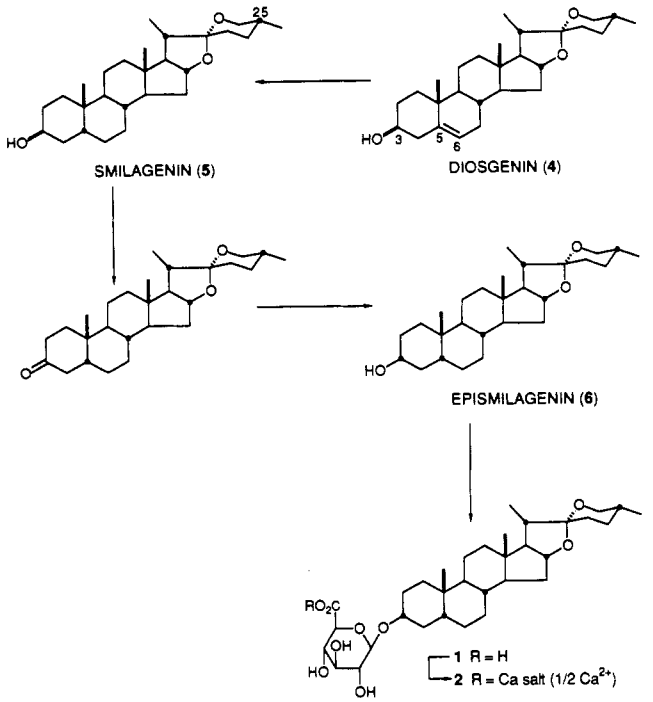

Figure 2. Possible metabolic transformations involved in the conversion of diosgenin (4) into 2.

mycotoxin sporidesmin, in the *P. dichotomiflorum* used in this work is consistent with this hypothesis. Alternatively, although the presence of bile crystals is characteristic of these diseases, the crystals themselves may not be involved in disease pathogenesis.

Our finding that steroidal saponins are associated with hepatogenous photosensitization of sheep is of added interest in view of a recent proposal to use the steroidal saponin tigogenin cellobioside (CP-88 818) as a hypocholesterolemic agent for humans (McCarthy, 1990).

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