Isolation and Structure Elucidation of Dichotomin, a Furostanol Saponin Implicated in Hepatogenous Photosensitization of Sheep Grazing *Panicum dichotomiflorum*

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Dichotomin, a furostanol saponin derived from diosgenin, has been isolated from *Panicum dichotomiflorum* and its structure determined by mass spectrometry, one- and two-dimensional NMR techniques, and acidic and enzymatic hydrolyses to known compounds. The ^1H and ^{13}C NMR resonances for dichotomin (**1a**) were assigned by analogy with those for its 26-deglucosoylated spirostanol derivative (**2**), which were unambiguously assigned in a series of one- and two-dimensional NMR experiments. Revisions to the published ^{13}C NMR assignments of **2** are presented. The role of plant steroidal saponins in ovine photosensitization diseases is discussed.

INTRODUCTION

A number of plants worldwide, including *Agave lechegeuilla*, *Brachiaria decumbens*, *Narthecium ossifragum*, *Panicum coloratum*, *Panicum dichotomiflorum*, *Panicum miliaceum*, *Panicum schinzii*, and *Tribulus terrestris* are associated with occasional outbreaks of hepatogenous photosensitization of ruminants characterized by the presence of birefringent crystals in the liver and bile ducts (Miles et al., 1992b). The presence of steroidal saponins in some of these plants has been implicated in such diseases (Bridges et al., 1987), but until recently definitive evidence on this point was lacking. Our finding that the bile crystals from outbreaks of hepatogenous photosensitizations in sheep grazing two species of *Panicum* (Miles et al., 1991, 1992a,b) are composed principally of the calcium salt of epismilagenin β-D-glucuronide caused us to examine *P. dichotomiflorum* for saponins. The finding of glycosides of diosgenin (Holland et al., 1991; Miles et al., 1992b) in *P. dichotomiflorum* prompted a more detailed investigation of the saponins of this grass.

EXPERIMENTAL PROCEDURES

Chromatography. Thin-layer chromatography (TLC) was performed on silica gel plates (E. Merck 5554) using the following solvent systems: A, chloroform–methanol–water 65:35:10 (bottom layer); B, chloroform–methanol 49:1. Plates were visualized by spraying with the anisaldehyde reagent of Stahl (1969). Flash chromatography (Still et al., 1978) was performed on silica gel (E. Merck 9385) using eluent A.

Mass Spectrometry. Liquid surface-assisted ionization mass spectrometry (LSIMS) was performed on a Kratos MS80 RFA instrument using a 7-keV Xe atom beam and a glycerol matrix. Gas chromatography–mass spectrometry (GC–MS) was carried out as described elsewhere (Miles et al., 1992b).

Nuclear Magnetic Resonance Spectroscopy. ^1H and ^{13}C NMR spectra were determined at 300.13 and 75.47 MHz, respectively, on a Bruker AC-300 instrument. Chemical shifts in C_5D_5N are reported relative to internal TMS. ^{13}C NMR signal multiplicities (s, d, t, or q) were determined using the DEPT sequence. NOE difference experiments were performed using the NOEDIFF (singlet-like signals) and NOEMULT (multiplet signals) sequences with irradiation power levels of 40L and 45L, respectively. Two-dimensional COSY, long-range COSY, and *J*-resolved spectra were determined in absolute value mode; NOESY, HOHAHA, and ^{13}C–^1H correlated spectra were determined in phase-sensitive mode.

Isolation of Dichotomin (1a). The *P. dichotomiflorum* was hand-picked in March 1989 from a predominantly *P. dichotomiflorum* pasture upon which an outbreak of hepatogenous photosensitization of goats had just occurred. The grass was air-dried and then ground. The extraction method is based on method B of Ceh and Hauge (1981). Plant material (50 g) was refluxed for 30 min with dichloromethane (350 mL) and then gravity filtered, and the filtrate was discarded. The extracted grass was then air-dried, refluxed in ethanol–water 17:3 (500 mL) for 3 h, and filtered. The filtrate was concentrated in vacuo, the volume was made up to 50 mL with water, and the solution was saturated with sodium chloride. Saponins were extracted with water-saturated 1-butanol (3 × 50 mL), and the solvent was removed in vacuo. The residue was taken up in methanol (20 mL) and gravity filtered into acetone (200 mL). The resultant red-brown precipitate of crude saponin (330 mg, 0.7%) was recovered by filtration.

A sample of the crude saponin (61.7 mg) was purified by flash chromatography to afford a pure sample (20.2 mg) of the major saponin, **1a**, which we name dichotomin [(25*R*)-furost-5-ene-3β,22α,26-triol 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyl 26-*O*-β-D-glucoside] (see text for LSIMS and Tables I, II, and IV for ^1H and ^{13}C NMR data).

Acidic Hydrolysis of Saponins. The saponin was added to methanol–hydrochloric acid (1 M) (1:2; 2 mL) and heated to 50 °C for 12 h in a sealed vial. After extraction with dichloromethane (2 mL), the hydrolysis products were examined by TLC (eluent B) and GC–MS.

Enzymatic Hydrolysis of Dichotomin (1a) to 2. A sample of furostanol **1a** (13.1 mg) was added to β-glucosidase (10 mg) in acetate buffer (1 mL; pH 5.5), and the mixture was incubated at 37 °C for 3 h before removal of the solvent in vacuo (toluene azeotrope). Examination of the product by TLC (eluent A) revealed that **1a** had been converted to a more mobile saponin. Purification by flash chromatography afforded spirostanol **2** [(25*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside] as a cream-colored solid (3.3 mg), mp 191–196 °C (see text for LSIMS and Tables I–III for ^1H and ^{13}C NMR data).

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Sugar Analysis. Saponin 2 (ca. 10 mg) in water (20 mL) and concentrated H_2SO_4 (0.5 mL) was refluxed for 1 h. The reaction mixture was cooled, neutralized with solid $CaCO_3$, and filtered. A portion of the filtrate (10 mL) was freeze-dried and derivatized for 16 h with TriSil (0.4 mL). The trimethylsilylated sugars were then analyzed by GC-FID on an HP-1 methyl silicone gum capillary column (25 m × 0.32 mm) (Hewlett-Packard) with the temperature from 150 to 220 °C at 3 °C min^{-1} (10-min hold at 220 °C) and He as carrier gas (1.8 mL min^{-1}).

Chemicals. Diosgenin, α-L-rhamnose, and almond β-glucosidase (EC 3.2.1.21) (4.8 units mg^{-1}) were obtained from Sigma Chemical Co. TriSil was from Pearce Lab Supply.

RESULTS AND DISCUSSION

Acid hydrolysis of the crude saponin extract yielded two spirostanes. One of the spirostanes was identified as diosgenin [(25*R*)-spirost-5-en-3β-ol] by comparison (TLC in eluent B, GC–MS) with an authentic standard. The other compound exhibited properties consistent with dehydrated diosgenin: R_f 0.68 upon TLC (eluent B); M^+ *m/z* 396 (GC–MS). Dehydration of diosgenin saponins during acid hydrolysis, to produce (25*R*)-spirosta-3,5-diene, has been reported previously (de Kock and Enslin, 1958). The saponins present in *P. dichotomiflorum* are therefore derived from diosgenin (Holland et al., 1991).

Analysis of the acid hydrolysate of **2** for sugars revealed the presence of only rhamnose and glucose. Since **2** was derived from **1a** by treatment with β-glucosidase, it follows that rhamnose and glucose are also the only sugars present in **1a**.

The negative ion LSIMS of dichotomin included a pseudomolecular ion at *m/z* 1193 (loss of H^+ from a compound of molecular weight 1194). Fragment ions at *m/z* 1047, 901, 755, and 593 correspond to successive loss of three rhamnose units and one glucose unit. The ion at *m/z* 593 corresponds to 413 + 18 + 162 Da (diosgenin + water + glucose − H^+). The ^{13}C NMR spectrum of dichotomin comprised 7 methyl, 12 methylene, 34 methine, and 4 quaternary carbons (a total of 57 carbon signals), while ^1H NMR revealed the presence of 2 tertiary and 5 secondary methyl groups and an olefinic proton (see Tables I and II).

The positive and negative LSIMS of the enzymatic hydrolysis product (**2**) from dichotomin gave pseudomolecular ions at *m/z* 1015 and 1013, respectively, indicating that **2** resulted from loss of glucose and water moieties from dichotomin. The positive LSIMS included fragment ions at *m/z* 869, 723, 577, and 415 corresponding to successive losses of three rhamnose units followed by loss of a glucose unit to leave a protonated diosgenin unit (*m/z* 415). It follows from the foregoing mass spectral data, and the identification of diosgenin as the parent sapogenin, that the glucose unit of **2** must be attached at the 3-position of the sapogenin. The ^{13}C NMR spectrum of **2** comprised 7 methyl, 11 methylene, 29 methine, and 4 quaternary carbons (a total of 51 carbon signals), while the ^1H NMR spectrum included an olefinic proton (5.34 ppm) and a broad multiplet (3.87 ppm) attributable, respectively, to the H-6 and H-3α resonances of a diosgenin saponin (see Tables I and II).

These characteristics suggested that dichotomin was a 26-(β-D-glucosyloxy)-furost-5-ene-3β,22α-diol derivative, which upon enzymatic hydrolysis of the C-26 glucosidic linkage undergoes cyclization (with elimination of H_2O) to the corresponding spirosten-3β-ol derivative.

A comparison of the ^{13}C NMR signals observed for the enzymatic hydrolysis product with those presented in a recent review (Agrawal et al., 1985) for a variety of saponins indicated it to be identical to spirostenol **2**, obtained by Miyamura et al. (1982) after emulsin-catalyzed hydrolysis of **1b** (isolated from the rhizomes of *Paris polyphylla* Sm.); thus, dichotomin is the 22-hydroxy analogue (**1a**) of **1b**.

Table I. ^{13}C and ^1H NMR Chemical Shifts (δ) of Aglycon Atoms[a] in C_5D_5N

	diosgenin		2		1a	
	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
C-1	38.0	1.11, 1.81	37.3	0.98, 1.75	37.5	
C-2	32.4	1.77, 2.07	30.0	1.87, 2.08	30.2	
C-3	71.4	3.82	78.0	3.87	78.2	3.86
C-4	34.6	2.57–2.60	38.8	2.73–2.78	38.9	2.69, 2.78
C-5	142.2		140.6		140.8	
C-6	121.0	5.38	121.6	5.34	121.9	5.34
C-7	32.7	1.53, 1.93	32.1	1.46, 1.88	32.4[b]	
C-8	32.0	1.57	31.5	1.59	31.6	
C-9	50.8	0.95	50.1	0.90	50.3	
C-10	37.3		37.0		37.3	
C-11	21.4	1.45–1.50	20.9	1.45, 1.45	21.1	
C-12	40.2	1.14, 1.73	39.7	1.12, 1.70	39.9	
C-13	40.7		40.3		40.8	
C-14	57.0	1.08	56.5	1.06	56.6	
C-15	32.6	2.02, 1.43	32.0	2.02, 1.46	32.1[b]	2.00, 1.45
C-16	81.3	4.53	80.9	4.55	81.1	4.55
C-17	63.2	1.81	62.7	1.80	63.6	1.80
C-18	16.5	0.85	16.2	0.84	16.4	0.90
C-19	19.8	1.04	19.2	1.06	19.4	1.03
C-20	42.2	1.95	41.8	1.96	40.6	
C-21	15.1	1.13	14.9	1.14	16.4	0.98
C-22	109.4		109.1		110.8	
C-23	32.0	1.55–1.69	31.7	1.57–1.67	37.1	
C-24	29.5	1.56, 1.56	29.1	1.58, 1.58	28.3	
C-25	30.8	1.57	30.4	1.55	34.2	1.60
C-26	67.1	3.48, 3.55	66.7	3.51, 3.58	75.3	3.60, 3.93
C-27	17.4	0.69	17.2	0.70	17.4	0.71

[a] Methylene protons in the format Hα, Hβ. [b] Assignments interchangeable.

Table II. ^{13}C and ^1H NMR Chemical Shifts (δ) of Glycoside Atoms in C_5D_5N

	2		1a	
	^{13}C	^1H	^{13}C	^1H
C-3 glucose				
C-1′	100.1	4.95	100.3	4.91
C-2′	77.8	4.21	78.2	4.15
C-3′	77.5	4.22	77.6	4.15
C-4′	77.6	4.40	78.0	4.32
C-5′	76.8	3.62	76.9	3.60
C-6′	61.0	4.05, 4.20	61.2	4.04, 4.16
rhamnose A				
C-1″	102.0	6.39	102.2	6.27
C-2″	72.3	4.85	72.4	4.80
C-3″	72.7	4.63	72.8	4.59
C-4″	73.9	4.37	74.0	4.33
C-5″	69.4	4.94	69.7	4.86
C-6″	18.5	1.78	18.6	1.72
rhamnose B				
C-1‴	102.1	5.83	102.2	5.72
C-2‴	72.7	4.56	72.8	4.52
C-3‴	73.2	4.56	73.1	4.48
C-4‴	80.2	4.44	80.3	4.36
C-5‴	68.2	4.92	68.4	4.78
C-6‴	18.7	1.59	18.8	1.52
rhamnose C				
C-1⁗	103.1	6.28	103.2	6.18
C-2⁗	72.4	4.90	72.5	4.85
C-3⁗	72.7	4.52	72.8	4.44
C-4⁗	73.8	4.31	73.8	4.27
C-5⁗	70.2	4.36	70.4	4.25
C-6⁗	18.3	1.60	18.4	1.54
C-26 glucose				
C-1′′′′′			104.8	4.77
C-2′′′′′			75.1	3.99
C-3′′′′′			78.4	4.24
C-4′′′′′			71.6	4.16
C-5′′′′′			78.3	3.92
C-6′′′′′			62.7	4.31, 4.50

While there was, in general, a correspondence to within ±0.3 ppm between the ^{13}C NMR chemical shifts observed by us and those given by Miyamura et al. (1982) for the glucose and rhamnose carbons of 2, some differences were apparent in the respective tabulations of ^{13}C NMR data. For example, Miyamura et al. (1982) reported four sugar resonances in the region 68–70 ppm, whereas we observed only three carbon signals (68.2, 69.4, and 70.2 ppm) in this region.

The recognition of this and some other assignment discrepancies (see below) prompted us to perform an extensive series of high-resolution one- and two-dimensional NMR experiments in order that an unequivocal assignment of the ^{13}C and ^{1}H NMR resonances of dichotomin (1a), and its enzymatic hydrolysis product (2), might be obtained and the location and stereochemistry of the glycosidic linkages might be confirmed.

NMR Signal Assignments. The proton signals of 2 were correlated in an absolute value COSY experiment in which 20 of the 22 cross peaks anticipated from the protons of the three rhamnose units and the glucose unit were observed. This led to the conclusion that two pairs of sugar methine protons possessed identical chemical shifts. Analysis of the cross peaks identified one of the pairs of superimposed signals as being H-2′ and H-3′ of the glucose unit, since H-4′ exhibited a cross peak at 4.22 ppm (H-3′), while H-1′ (4.95 ppm) exhibited a cross peak at 4.21 ppm (H-2′), thereby accounting for the absence of a resolvable off-diagonal cross peak between H-2′ and H-3′. The ^{13}C–^{1}H correlated spectrum of 2 subsequently confirmed the occurrence of two methine proton resonances in the region 4.21–4.22 ppm (see below). In a like manner it emerged that H-2‴ and H-3‴ of the rhamnose B unit possessed similar chemical shifts.

A COSY experiment performed with an additional delay of 0.1 s inserted in the t_n period resulted in the detection of additional cross peaks attributable to weak long-range ^{4}J and ^{5}J couplings. The recently developed family of TOCSY and HOHAHA two-dimensional proton correlated experiments (Rahman, 1989) are also well suited to the detection of long-range proton couplings. A phase-sensitive HOHAHA experiment performed with a mixing time of 300 ms (i.e., optimized for the detection of couplings of the order 0.33 Hz) substantiated the proton chemical shift assignments presented in Table II. For example, in each of the glucose and rhamnose units the anomeric protons (H-1) exhibited correlations at the resonance frequencies of the H-2, H-3, H-4, H-5, and H-6 protons within the same sugar ring system. The quality of the cross-section profiles available from HOHAHA experiments performed with varying mixing times is remarkable; provided the anomeric proton resonance can be distinguished, a complete assignment of the sugar resonances within that ring system can readily be achieved by performing a series of experiments using various mixing times.

Comparison of the chemical shifts and coupling constants thus determined for the protons of rhamnose A of 2 (see Table III) with those observed for the corresponding α-L-rhamnose unit of (25*R*)-spirost-5-ene-3β,27-diol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (3) (Mimaki and Sashida, 1990) and for a specimen of α-L-rhamnose supports the assignments given in Table II.

The resolution of the ^{13}C–^{1}H correlated spectrum of 2, initially determined in magnitude mode, was increased by using phase-sensitive acquisition and by restricting the ^{1}H axis to the region 3.2–6.5 ppm and the ^{13}C axis to the region 40–160 ppm. Under these conditions, folding of most of the steroidal signals occurred in either, or both, of the F1 and F2 dimensions; however, none of the sugar methine or methylene signals were folded. We have previously shown (Miles et al., 1992b, and references cited therein) that provided sufficient F1 data points are employed, the resolution of phase-sensitive ^{13}C–^{1}H correlated cross-section profiles is sufficient to distinguish the axially and equatorially inclined protons of steroids and triterpenes. The axially and equatorially inclined protons of sugar molecules can be similarly distinguished. Thus, the C-2′, C-3′, and C-4′ cross sections of the β-D-glucosyl unit of 2 exhibited triplet-like profiles, while the C-2, C-3, and C-4 cross-section profiles of the α-L-rhamnosyl units exhibited broadened singlet-, doublet-, and triplet-like appearances, respectively, consistent with the multiplicity patterns also observed for the appropriate protons in a *J*-resolved experiment.

Table III. ^{1}H NMR Chemical Shifts (δ) and Coupling Constants (Hertz) for α-L-Rhamnose Units in C_5D_5N

	in 3[a]	rhamnose A in 2	α-L-rhamnose
H-1	6.39 (br s)	6.39 (br s)	5.90 (br s)
H-2	4.81 (br d, *J* 3.2)	4.85 (br s)	4.70 (br s)
H-3	4.64 (d, d, *J* 3.2, 9.3)	4.63 (d, d, *J* 3.1, 9.4)	4.74 (d, d, *J* 3.2, 9.2)
H-4	*b* (~t, *J* 9.4)	4.37 (t, *J* 9.4)	4.33 (t, *J* 9.3)
H-5	5.01 (d, q, *J* 9.5, 6.2)	4.94 (d, q, *J* 9.4, 6.3)	4.63 (d, q, *J* 9.3, 6.2)
H-6	1.78 (d, *J* 6.2)	1.78 (d, *J* 6.3)	1.66 (d, *J* 6.2)

[a] (25*R*)-Spirost-5-ene-3β,27-diol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (Mimaki and Sashida, 1990). [b] Not recorded.

Miyamura et al. (1982) assigned the C-2′ resonance of the glucose unit of 2 to the signal at 80.0 ppm; however, the following results indicate that C-2′ resonates at 77.8 ppm and that the signal at 80.2 ppm arises from C-4‴: The ^{13}C–^{1}H correlated spectrum of 2 demonstrated that the C-1′ glucose signal (100.1 ppm) correlated with the proton resonance (H-1′) at 4.95 ppm (d, *J* 7.8 Hz). In the COSY spectrum this signal (H-1′) correlated with a signal (H-2′) at 4.21 ppm, which in the ^{13}C–^{1}H correlated spectrum correlated with the carbon resonance 77.8 ppm. On the other hand, the methine carbon signal at 80.2 ppm correlated with the rhamnose B proton which resonated at 4.44 ppm. In an NOE difference experiment this proton experienced a negative enhancement upon irradiation of the H-1⁗ (anomeric proton) of rhamnose C.

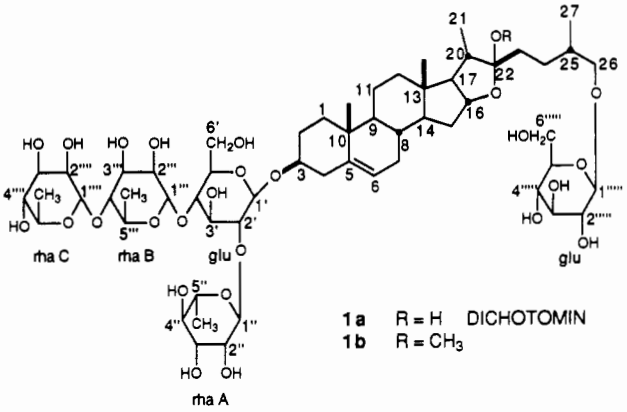
The proton resonances of 1a were correlated in a similar series of COSY, long-range COSY, and HOHAHA spectra. In the latter spectrum the anomeric proton (H-1⁗′) of the C-26 glucose unit exhibited cross peaks at the resonance frequencies of H-2⁗′ to H-6⁗′. The ^{13}C NMR spectrum of 1a included six signals not present in the spectrum of 2, attributable to the C-26 glucose unit; transformation of the ^{13}C FID with a line-broadening factor of 1 Hz or less readily located the six additional signals, since they exhibited line widths 30–40% smaller than those of the other sugar carbons and, in consequence, their peak heights were appreciably greater. This difference in line widths can be ascribed to the differing correlation times of the carbon atoms of the comparatively unrestrained C-26 glucose unit (part of a flexible side chain having a relatively high degree of rotational freedom), compared to the bulky, and more constrained, rhamnoglucosyloxy system at C-3.

This observation prompted us to determine the two-dimensional NOESY spectrum of 1a in phase-sensitive mode. We observed that suitably oriented protons of the 3β-[tris(rhamanosyl)glucosyloxy] unit exhibited negative enhancements (in-phase cross peaks), whereas those of the 26-glucosyloxy unit exhibited positive enhancements (anti-phase cross peaks). A similar series of negative and positive enhancement peaks were observed in one-dimen-

Table IV. Selected NOE Correlations Observed for 1a in NOE Difference and Two-Dimensional NOESY Experiments[a]

irradiated signal	enhanced (negative unless indicated) signals
4.91 (3-glu H-1′)	4.15 (glu H-3′), 3.86 (H-3α), 3.60 (glu H-5′), 2.6–2.8 (H-4α,H-4β)
4.77 (26-glu H-1‴″)	3.60, 3.93 (H-26)[b]
6.27 (rha-A H-1″)	4.80 (rha-A H-2″), 4.15 (glu H-2′)
5.72 (rha-B H-1‴)	4.52 (rha-B H-2‴), 4.32 (glu H-4′), 4.04, 4.16 (glu H-6′)
6.18 (rha-C H-1⁗)	4.85 (rha-C H-2⁗), 4.36 (rha-B H-4‴)
3.86 (H-3α)	4.91 (3-glu, H-1′)
3.60 (H-26)	3.93 (H-26),[b] 4.77 (26-glu H-1‴″)[b]
3.93 (H-26)	3.60 (H-26),[b] 4.77 (26-glu H-1‴″)[b]

[a] δ in C_5D_5N, glu = glucose, rha = rhamnose. [b] Positive enhancement.



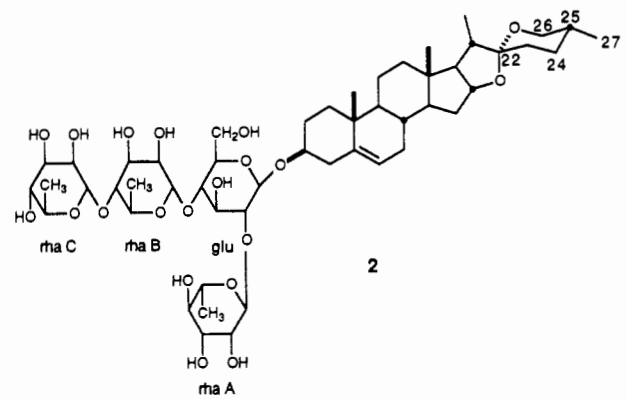


Figure 1. Structures of dichotomin (**1a**), its 22-methoxy analogue (**1b**), and its β-glucosidase hydrolysis product (**2**).

sional NOE difference experiments upon irradiation of the anomeric (H-1) protons of the five sugar units and the steroidal H-3α and H-26 protons. The one- and two-dimensional NOE results defined the site and stereochemistry of the glycosidic linkages in **1a** (Table IV). It is also apparent from these data that H-1′ of the 3β-glucosyl is inclined toward the steroidal H-3α, H-4α, and H-4β protons, while the H-1‴ of rhamnose B is inclined toward the glucosyl H-4′ and H-6′ protons.

Equivalent in-phase cross peaks (negative NOEs) were also observed in the phase-sensitive two-dimensional NOESY spectrum of **2** and in the corresponding one-dimensional NOE difference spectra of **2** upon irradiation of the four anomeric sugar protons and the steroidal H-3α proton.

Thus, the structures of dichotomin (**1a**), the major saponin isolated from *P. dichotomiflorum*, and of its enzymatic hydrolysis product (**2**) and the stereochemistry of their glycosidic linkages are as shown in Figure 1.

The saponin content of other plants involved in ovine hepatogenous photosensitization diseases associated with bile crystal formation have been investigated. *N. ossifragum* contains furostanol saponins similar to **1a**, but these are derived from sarsasapogenin (Ceh and Hauge, 1981) rather than from diosgenin. *T. terrestris* (de Kock and Enslin, 1958) and *P. schinzii* (Miles et al., 1992b) contain saponins of diosgenin, while *P. coloratum* (Patamalai et al., 1990) and *P. miliaceum* (unpublished work) contain saponins derived from both diosgenin and yamogenin. *A. lecheguilla* contains saponins derived from a sapogenin that has been tentatively identified as smilagenin (Camp et al., 1988). Recently, a mixture of two spirostan-3-ols has been isolated from the ruminal contents of photosensitive sheep grazing *B. decumbens* (Abdullah et al., 1992), indicating the presence of plant steroidal saponins in the diet.

There is now evidence, therefore, that all of the plants clearly implicated in bile crystal-associated photosensitization of sheep contain steroidal saponins. That the bile crystals of sheep grazing many of these plants are derived from ingested plant steroidal saponins (Miles et al., 1992b) suggests that the presence of steroidal saponins in the plant is necessary for bile crystal formation; the question still remains, however, whether the plant saponins are solely responsible for the liver damage or whether other factors are involved in the etiology of the disease process.

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