Isolation of the Steroidal Sapogenin Epismilagenin from the Bile of Sheep Affected by *Panicum dichotomiflorum* Toxicosis

Patrick T. Holland,*,† Christopher O. Miles,† Peter H. Mortimer,† Alistair L. Wilkins,‡ Allan D. Hawkes,† and Barry L. Smith†

MAF Technology, Ruakura Agricultural Centre, Hamilton, New Zealand, and Chemistry Department, University of Waikato, Hamilton, New Zealand

Crystals formed in the bile of sheep affected by a hepatogenous photosensitization after grazing *Panicum dichotomiflorum* (smooth witch grass) have been investigated. The birefringent crystals were essentially insoluble in common solvents but partially dissolved in warm glacial acetic acid. Direct probe MS and FT-IR indicated the presence of spirostanol sapogenins. Acidic hydrolysis of the crystals released a sapogenin which was identified by TLC, MS, and NMR as epismilagenin [(25*R*)-5β-spirostan-3α-ol]. The crystals contained only traces of free epismilagenin. They were principally composed of an acidic and a neutral derivative of epismilagenin. There was evidence that saponins from plants involved in this disease, and other similar diseases resulting in bile crystal formation, undergo transformation in the animal.

INTRODUCTION

Panicum dichotomiflorum (smooth witch grass) is a warm zone annual grass weed that is spreading in the northern districts of New Zealand. Sheep, particularly lambs, dairy cattle, and goats grazing stands of this highly palatable grass frequently become photosensitized. Affected animals exhibit the classical clinical signs of photosensitization similar to those found with facial eczema, the mycotoxic disease caused by the saprophytic pasture fungus *Pithomyces chartarum* (Mortimer et al., 1978). In herbivores these cholestases result in the accumulation of phylloerythrin, a photodynamic compound derived from chlorophyll, inducing severe photodermatitis in the presence of sunlight. The *P. dichotomiflorum* induced liver lesions are characterized by the presence in and about the bile ducts of birefringent crystals which are often arrayed in aciculated sheaves. Similar photosensitization diseases involving the deposition of crystalline material in the bile ducts have been reported for livestock grazing *Panicum miliaceum* (Clare, 1955), *Panicum coloratum* (kleingrass) (Bridges et al., 1987; Patamalai et al., 1990), *Narthecium ossifragum* (bog asphodel) (Ceh and Hauge, 1981), *Tribulis terrestris* (Coetzer et al., 1983), and *Agave lecheguilla* (Camp et al., 1988). These crystals appear to be associated with the saponin content of the plants (Bridges et al., 1987). Camp et al. (1988) reported that crystals from the bile of sheep fed *A. lecheguilla* contained a steroidal sapogenin which was tentatively identified as either smilagenin or sarsasapogenin.

In this study we report the isolation of the steroidal sapogenin epismilagenin from crystals obtained from the bile ducts of sheep showing clinical signs of *P. dichotomiflorum* toxicosis.

MATERIALS AND METHODS

Thin-Layer Chromatography. Silica gel coated plates (0.2 mm, E. Merck 5554) were used with the following solvent systems: sapogenins, chloroform–methanol 97:3 v/v; saponins, chloroform–methanol–water 65:35:10 v/v (bottom layer). The plates were visualized with anisaldehyde spray reagent: 0.5 mL of *p*-anisaldehyde, 50 mL of glacial acetic acid, 1 mL of concentrated sulfuric acid (Stahl, 1965).

Mass Spectrometry. Electron impact spectra from direct probe sample introduction were obtained under low- and high-resolution conditions using a Kratos MS80 RFA instrument. Gas chromatography–mass spectrometry (GC–MS) was carried out on a Hewlett-Packard 5970 MSD using split–splitless injection on a 25 m × 0.25 mm FSOT column, 0.25 μm HP1 (Hewlett-Packard), with temperature programming (70 °C 1 min, 30 °C/min to 150 °C, 10 °C/min to 280 °C).

Infrared Spectrometry. A Bruker IFS 85 FT-IR instrument was used with samples of crystals or reference standards (1 mg) finely ground with KBr (200 mg) and pressed into disks. Spectra were obtained in the region 4000–500 cm^{-1}.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H and ^{13}C NMR spectra were obtained from deuterochloroform solutions using a Bruker AC 300 instrument at 300 and 75 MHz, respectively.

Isolation and Characterization of Crystals. Sheep were grazed on pastures containing mainly young *P. dichotomiflorum*. Several clinically affected animals were slaughtered and the livers removed. Crystalline material was obtained from the bile and by washing the larger bile ducts and gall bladder with distilled water. Further material was obtained by scraping the epithelium of the ducts. The crude crystals were washed with water and collected by centrifugation. Percol density gradient centrifugation removed epithelial debris. Following further washes with water the crystals were air-dried to provide 16 mg of material.

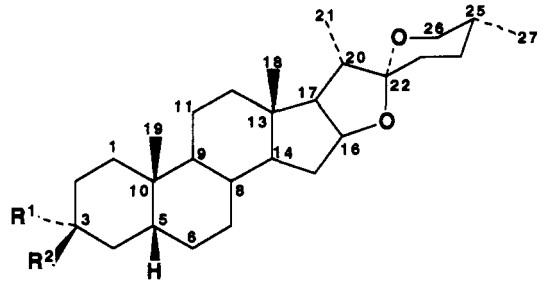
The crystals were essentially insoluble in common solvents (boiling water, acetone, methanol, chloroform). By microscopic examination the crystals were birefringent and principally consisted of clusters of small colorless rhomboidal platelets. There was no melting or decomposition on the melting point stage up to 245 °C.

Powder X-ray diffraction on a sample of crushed crystals gave a distinct spectrum (lines at 12.9°, 15.5°, and 21.5°) which did not correspond to known insoluble calcium salts such as oxalate. Direct probe MS of crushed crystals gave weak spectra at probe temperatures above 250 °C with base peak m/z 139 and peaks at masses 255, 273, 329, and 344. These fragment masses are characteristic of sapogenins from the spirostanol class (Budzikiewicz et al., 1964). However, the high probe temperature and lack of molecular ions are not characteristic of free sapogenins.

The IR spectrum (see Figure 2) of the crystals displayed prominent absorption bands at 864, 901, 922, and 982 cm^{-1}, characteristic of spirostanes. The 901-cm^{-1} band was more intense than the 922-cm^{-1} band, suggesting the 25*R* configuration (Eddy et al., 1953). The spectrum more closely matched that of smi-

† Ruakura Agricultural Centre.
‡ University of Waikato.

0021-8561/91/1439-1963$02.50/0



R 1=OH, R 2=H Epismilagenin
R1=H, R 2=OH Smilagenin

Figure 1. Structures of smilagenin and epismilagenin.

lagenin [(25*R*)-5β-spirostan-3β-ol] (Upjohn Laboratories) than that of tigogenin [(25*R*)-5α-spirostan-3β-ol] (Sigma Chemical Co.) reference standards.

Isolation of Sapogenins. Bile crystals (2.5 mg) were heated with 1 mL of 5 M HCl in a sealed vial for 16 h at 100 °C. The cooled hydrolysate was extracted with chloroform and the solvent evaporated under nitrogen. The oily residue (2.1 mg) gave a single spot on TLC (R_f 0.20, tigogenin R_f 0.23, smilagenin R_f 0.28). The sapogenin was identified by MS and NMR.

The direct probe mass spectrum (EI) gave base peak m/z 139, M^+ 416.3305, 7% ($C_{27}H_{44}O_3$ requires 416.3291) and prominent high-mass peaks at masses 344 (14%), 329 (7%), 302 (8%), 287 (11%), 284 (13%), 273 (20%), and 255 (17%). The spectrum more closely matched that for smilagenin than that for tigogenin reference materials. However, the smilagenin spectrum had some significant differences in relative intensities, particularly at m/z 284 (4%) and 255 (8%). GC–MS gave a single peak at the same retention time (15.8 min) as that for a smilagenin standard, but the spectra had the characteristic differences outlined above.

The ^{13}C NMR spectrum of the sapogenin fraction was virtually identical to that previously reported for epismilagenin (Agrawal et al., 1985), with the following line assignments: 14.54 (C-21), 16.49 (C-18), 17.18 (C-27), 20.66 (C-11), 23.42 (C-19), 26.73 (C-7), 27.14 (C-6), 28.86 (C-24), 30.35 (C-25), 30.56 (C-2), 31.44 (C-23), 31.85 (C-15), 34.75 (C-10), 35.41 (C-1), 35.53 (C-8), 36.50 (C-4), 40.29 (C-12), 40.60 (C-9), 40.62 (C-13), 41.66 (C-20), 42.07 (C-5), 56.41 (C-14), 62.29 (C-17), 66.90 (C-26), 71.85 (C-3), 80.95 (C-16), 109.28 (C-22).

The aqueous phase from the acid hydrolysis was diluted with water to 5.0 mL. The calcium content, determined by atomic absorption spectroscopy, corresponded to 2–3% by weight of the crystals. The amino acid content determined by ion-exchange chromatography was negligible.

Isolation of Saponin Fractions. Further crystals (12 mg) were washed with small volumes of dichloromethane followed by methanol–water (1:1) and then several volumes of warm ethanol–water (85:15). The dichloromethane fraction contained trace amounts of epismilagenin (Figure 1) (TLC, MS). The alcoholic fractions contained significant material (1.1 mg) which was purified by flash chromatography on silica gel (solvent as for TLC). Upon TLC it gave a single yellow spot (R_f 0.15) characteristic of saponins. Although insufficient material was present for definitive NMR, the fraction yielded epismilagenin (TLC, MS) upon acid hydrolysis (0.1 M HCl–methanol 1:1, 70 °C, 5 h).

The crystal residue remaining after the above washings was gently warmed with glacial acid. After filtration, solvent was removed under reduced pressure (toluene azeotrope) to give a fraction with a single yellow spot by TLC (R_f 0.08 shifted to R_f 0.15 with 1% acetic acid added to the eluant). This acid-soluble saponin fraction was insufficient for NMR but yielded epismilagenin (TLC, MS) upon acid hydrolysis.

RESULTS AND DISCUSSION

The IR spectrum of the crystals (Figure 2) strongly indicated the presence of a spirostane derivative. Acid hydrolysis of the crystals produced a sapogenin fraction

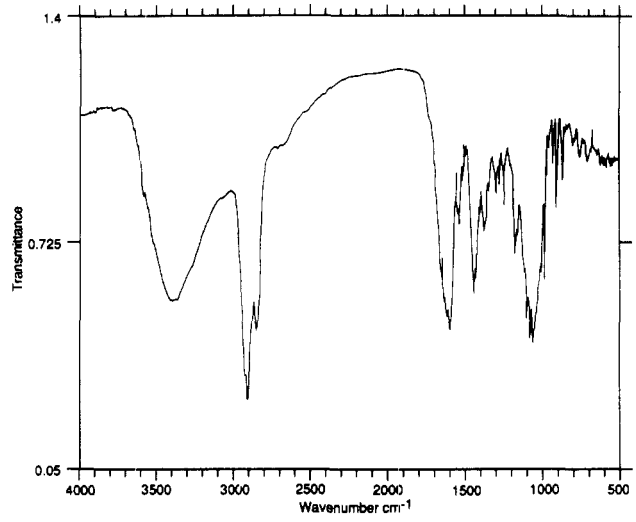


Figure 2. Infrared spectrum (KBr disk) of crystals isolated from the bile of sheep affected by *P. dichotomiflorum* toxicity.

in high yield (80–90%) which was essentially a single compound by TLC. The NMR data showed the compound to be epismilagenin [(25*R*)-5β-spirostan-3α-ol]. The chemical shifts for C-23–C-27 are characteristic of a (25*R*)-spirostane (Agrawal, 1985). The 3-H signal at δ 3.63 showed an approximate t ($J \approx 10$ Hz) of t ($J \approx 5$ Hz) pattern indicating the presence of a 3α-OH,5β-H- or a 3β-OH,5α-H-spirostanol, i.e., epismilagenin or tigogenin. The signal for C-19 at δ 23.42 is diagnostic for the configuration at the A–B ring junction: typically δ C-19 is 24 ppm for 5β-spirostane skeletons, while that for 5α-spirostanes is 12 ppm (Agrawal et al., 1985). The ^{13}C NMR data fit those reported for epismilagenin to within 0.1 ppm, while those for tigogenin differ by as much as 14 ppm, particularly for the CH_3 resonances (Agrawal et al., 1985).

The MS data for the sapogenin fraction support the epismilagenin structure. Although the mass spectrum closely resembled that of smilagenin (Figure 1), the relative intensities of the m/z 284 and 255 fragment ions were significantly higher for the product from the crystals than for smilagenin. Rules have been formulated by Blunden et al. (1980) for the relative abundances of water elimination ions in the mass spectra of isomeric spirostan-3-ols. The observed intensity ratios for masses 284/302 and 255/273 are characteristic of the 3α-OH isomer. The low solubility, high melting point, and direct probe MS characteristics of the crystals showed that a sapogenin such as smilagenin could only be present in a combined rather than in a free form.

The aqueous alcohol extractable compound from the crystals, approximately 8% by weight, displayed TLC and chemical characteristics consistent with a neutral saponin of epismilagenin.

The acetic acid extractable compound also displayed TLC and chemical characteristics consistent with an epismilagenin saponin. The large increase in R_f upon acidification of the TLC eluent suggests the saponin is acidic. The free acidic saponin was soluble in aqueous alcohol, whereas it could not be extracted from the crystals using this solvent. This suggests that it was originally present in the crystals as an insoluble salt. The IR spectrum of the crystals is consistent with a salt of an acidic saponin as a major component. In particular, the O–H stretching band at 3700–2500 cm^{-1} is consistent with a carbohydrate moiety, while the intense absorptions at 1600 and 1430 cm^{-1} are characteristic of a carboxylate salt C–O stretch rather than a free acid (Nakanishi, 1962).

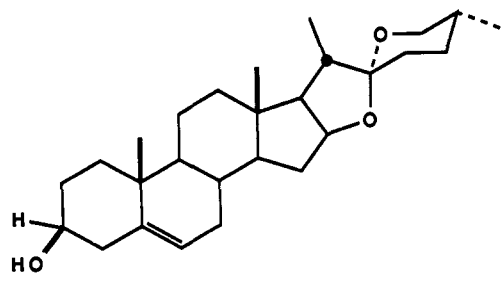


Figure 3. Structure of diosgenin.

The presence of significant levels of calcium in the crystal hydrolysate suggests that at least some of the acidic conjugate may have been present as the calcium salt. The formation of acidic sugar conjugates of spirostanols would be fully consistent with normal mammalian metabolism (Keglevic, 1979).

Thus, the crystals principally consisted of epismilagenin in combined forms with only traces of the free sapogenin present. One acidic and one neutral conjugate were isolated but not identified. Further studies on the composition of the combined forms await isolation of larger amounts of crystals.

The crystalline inclusions in bile of sheep affected by *A. lecheguilla* have been tentatively identified as either smilagenin or its 25*S* isomer, sarsasapogenin (Camp et al., 1988). Their published mass spectra of smilagenin and the sapogenin from the *A. lecheguilla* induced bile crystals showed similar differences to those described here for smilagenin and the *P. dichotomiflorum* induced crystals. This indicates that the sapogenin in bile crystals derived from *A. lecheguilla* may also have the 3α (epi) configuration, although solubility, TLC, and other evidence presented by Camp et al. (1989) showed the sapogenin was in a free rather than a combined form such as described here for *P. dichotomiflorum*.

The presence of a high proportion of epismilagenin in the crystals points to the saponin content of *P. dichotomiflorum* as the origin of the crystalline hepatic inclusions. Preliminary studies on the hydrolyzed saponin fraction from *P. dichotomiflorum* foliage have indicated that diosgenin [(25*R*)-spirost-5-en-3β-ol] (Figure 3) is the major component. No spirostan-3-ols were detected (unpublished work). Certainly the most common 3-OH spiroketal sapogenins in plants have the 3β configuration, although the epi (3α) configurations are known, particularly from *Cordyline* species (Blunden et al., 1981). *P. coloratum* also contains diosgenin plus its 25*S* isomer, yamogenin (Patamalai et al., 1990). The nature of the bile crystals produced in animals consuming *P. coloratum* has not been reported. The principal sapogenin in *A. lecheguilla* has been reported as smilagenin (Wall et al., 1954), whereas the data of Camp et al. (1988) may be interpreted as showing that this plant gave rise to bile crystals containing epismilagenin. Thus, the present study suggests that spirostan-3β-ols in toxic feeds may undergo metabolic transformation in the animal prior to crystallizing in the bile. These transformations appear to include epimerization of 3β-ol to 3α-ol and reduction of the 5–6 double bond. An analogous epimerization, of glycyrretic acid to epiglycyrretic acid, has been reported for human intestinal flora (Hattori et al., 1985), and reduction of the 5–6 double bond of cholesterol to the 5β isomer by microbial action in the mammalian intestine is also well-known (Gould and Cook, 1958). Further studies are underway on the nature of the saponins in *Panicum* species, their fate in animals, and their role in the hepatopathy.

ACKNOWLEDGMENT

G. C. Upreti assisted with Percol density gradient purification. B. K. Nicholson, University of Waikato, provided X-ray powder diffraction analyses. C. J. Clark provided amino acid analyses. We thank the University of Waikato Research Committee and the New Zealand Lottery Grants Board for grants toward the purchase of the NMR spectrometer.

LITERATURE CITED

Agrawal, P. K.; Jain, D. C.; Gupta, R. K.; Thakur, R. S. Carbon-13 NMR spectrometry of steroidal sapogenins and steroidal saponins. *Phytochemistry* **1985**, *24*, 2479–2496.

Blunden, G.; Jaffer, J. A.; Jewers, K.; Robinson, J. M. Configurational assignments to spirostan-3-ols by mass spectrometry. *Steroids* **1980**, *36*, 299–303.

Blunden, G.; Jaffer, J. A.; Jewers, K.; Griffin, W. J. Steroidal sapogenins from leaves of *Cordyline* species. *J. Nat. Prod.* **1981**, *44*, 441–447.

Bridges, C. H.; Camp, B. J.; Livingston, C. W.; Bailey, E. M. Kleingrass (*Panicum coloratum* L) poisoning in sheep. *Vet. Pathol.* **1987**, *24*, 525–531.

Budzikiewicz, H.; Djerassi, C.; Williams, D. H. *Structure Elucidation of Natural Products Vol. II: Steroids, terpenoids, sugars and miscellaneous classes*; Holden-Day: San Francisco, CA, 1964; pp 110–120.

Camp, B. J.; Bridges, C. H.; Hill, D. W.; Patamalai, B.; Wilson, S. Isolation of a steroidal sapogenin from the bile of a sheep fed *Agave lecheguilla*. *Vet. Hum. Toxicol.* **1988**, *30*, 533–535.

Ceh, L.; Hauge, J. G. Alveld producing saponins. I. Chemical studies. *Vet. Scand.* **1981**, *22*, 391–402.

Clare, N. T. Photosensitization in Animals. *Adv. Vet. Sci.* **1955**, *2*, 182–211.

Coetzer, J. A. W.; Kellerman, T. S.; Sadler, W.; Bath, G. F. Photosensitivity in South Africa. V. A comparative study of the pathology of the ovine hepatogenous photosensitivity diseases facial eczema and Geeldikkop (Tribulosis ovis). *Onderstepoort J. Vet. Res.* **1983**, *50*, 59–71.

Eddy, C. R.; Wall, M. E.; Scott, M. K. Catalog of Infra-red Absorption Spectra and Steroidal Sapogenin Acetates. *Anal. Chem.* **1953**, *25*, 266–271.

Gould, R. G.; Cook, R. P. The Metabolism of Cholesterol and Other Sterols in the Animal Organism. In *Cholesterol*; Cook, R. P., Ed.; Academic Press: New York, 1958.

Hattori, M.; Sakamoto, T.; Yamagishi, T.; Sakamoto, K.; Konishi, K.; Kobashi, K.; Namba, T. Metabolism of Glycyrrhizin by Human Intestinal Flora. II. Isolation and Characterization of Human Intestinal Bacteria Capable of Metabolizing Glycyrrhizin and Related Compounds. *Chem. Pharm. Bull.* **1985**, *33*, 210–217.

Keglevic, D. Glycosiduronic Acids and Related Compounds. In *Advances in Carbohydrate Chemistry and Biochemistry*; Tipson, R. S., Horton, D., Eds.; Academic Press: New York, 1979.

Mortimer, P. H.; White, E. P.; di Menna, M. E. Pithomycotoxicosis "facial eczema" in sheep. In *Mycotoxic fungi, mycotoxins, mycotoxicoses. An encyclopaedic handbook*; Wyle, T. D., Morehouse, G. L., Eds.; Dekker: New York, 1978; Vol. II, pp 195–203.

Nakanishi, K. *Infrared Absorption Spectroscopy—Practical*; Holden-Day: San Francisco, CA, 1962; pp 43–44.

Patamalai, B.; Hejtmancik, E.; Bridges, C. H.; Hill, D. W.; Camp, B. J. The isolation and identification of steroidal sapogenins in Kleingrass. *Vet. Hum. Toxicol.* **1990**, *32*, 314–318.

Stahl, E. Ed. *Thin layer chromatography—A laboratory handbook*; Academic Press: New York, 1965.

Wall, M. E.; Krider, M. M.; Krewson, C. J.; Eddy, C. R.; Willaman, J. J.; Correl, D. S.; Gentry, H. S. *Steroidal Sapogenins XIII*, Suppl. table of data for steroidal sapogenins, VII; USDA: Washington, DC, 1954; AIC-363.

Received for review April 25, 1991. Accepted August 9, 1991.

Registry No. I, 16653-88-6.