Photosensitivity in South Africa. IX. Structure elucidation of a β-glucosidase-treated saponin from *Tribulus terrestris*, and the identification of saponin chemotypes of South African *T. terrestris*

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ABSTRACT

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Saponin C, a β-glucosidase-treated saponin isolated from ethanol-water extracts of a South African collection of *Tribulus terrestris*, was shown by one- and two-dimensional NMR spectroscopy to be ruscogenin 1-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-6-*O*-acetylglucopyranoside. GC-MS analysis of the hydrolysed ethanol-water (4:1) extracts of *T. terrestris* specimens from two of four sites, revealed high levels of ruscogenin and potentially lithogenic diosgenin saponins. Specimens from two other sites contained non-lithogenic saponins derived predominantly from tigogenin, neotigogenin, gitogenin and neogitogenin.

Keywords: β-glucosidase-treated saponin, geeldikkop, photosensitivity, saponin chemotypes, structure elucidation, *Tribulus terrestris*

INTRODUCTION

The photosensitization disease of ruminants, known in South Africa as geeldikkop, is associated with the ingestion of *Tribulus terrestris* and the deposition in the biliary system of optically active crystalloid material (Theiler 1918; Coetzer, Kellerman, Sadler & Bath 1983; Kellerman, Miles, Erasmus, Wilkins & Coetzer 1994). In the late 1950s, the possible involvement of steroidal saponins in the aetiology of geeldikkop was investigated (De Kock & Enslin 1958; Brown & De Kock 1959), but interest diminished when attempts to induce the disease by dosing with saponins, failed. Subsequently (Kellerman, Van der Westhuizen, Coetzer, Roux, Marasas, Minne, Bath & Basson 1980), geeldikkop was experimentally induced in sheep by the simultaneous ingestion of *Tribulus terrestris* plants and cultures of *Pithomyces chartarum* containing the mycotoxin sporidesmin. More recently, geeldikkop was induced by dosing sheep only with a crude *T. terrestris* saponin extract, in the absence of sporides-

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min (Kellerman, Erasmus, Coetzer, Brown & Maartens 1991). The resulting biliary crystals were found to be composed of the calcium salt of a 6:1 mixture of the β-D-glucuronides of epismilagenin and episarsasapogenin (Miles, Wilkins, Erasmus, Kellerman & Coetzer 1994a). The ovine metabolism of *T. terrestris* saponins in experimentally induced geeldikkop has also been investigated (Miles, Wilkins, Erasmus & Kellerman 1994b).

Hitherto, a variety of saponins, including tribulosin and dioscin, have been isolated from geographically diverse collections of *T. terrestris* (Mahato, Ganguly & Sahu 1982). However, little is known about the occurrence and distribution of potentially lithogenic saponins in South African populations of *T. terrestris*. In an early investigation, De Kock (1960) isolated four saponins from *T. terrestris* extracts by means of the procedures of Wall, Krider, Rothman & Eddy (1952) and of Rothman, Wall & Eddy (1952) (summarized in Fig. 1), in which the crude saponin extract was acetylated and separated by column chromatography on alumina (method B). Treatment with $NaOCH_3$ regenerated the parent saponins. An alternative procedure (Hérissey 1932) in which the crude extract was cleaned up by means of $Pb(OAc)_2$ precipitation, followed by β-glucosidase treatment and recrystallization from methanol-water (method A, Fig. 1) afforded two saponins, designated saponins C and Dn. Acid hydrolysis of these saponins afforded ruscogenin [1b] and diosgenin [1a] (Fig. 2), respectively. More recently, Miles *et al.* (1994a) reported that acid hydrolysis of the plant material utilized by Kellerman *et al.* (1991) and Miles *et al.* (1994b) in their metabolic investigations afforded diosgenin, yamogenin, epismilagenin, tigogenin, neotigogenin, gitogenin and neogitogenin in the ratio 10:7:1:11:7:35:25.

Most of the saponins and β-glucosidase-treated saponins prepared by De Kock (1960) were consumed in dosing experiments that failed to induce geeldikkop (Brown & De Kock 1959). However, a small quantity of saponin C was retained and recently became available for structure elucidation. Given the central involvement of saponins in the aetiology of geeldikkop, we considered it desirable that the structure of saponin C be defined by means of modern NMR instrumentation. Additionally, samples of *T. terrestris* from different localities both inside and outside the endemic geeldikkop areas of South Africa, have been collected for sapogenin analysis.

MATERIALS AND METHODS

General

The ^1H and ^{13}C NMR spectra of saponin C (δ, C_5D_5N) were determined at 300,13 and 75,47 MHz with the use of a Bruker AC-300 spectrometer. NOE-difference and two-dimensional NMR spectra were acquired and processed as reported elsewhere (Munday, Wilkins, Miles & Holland 1993). A specimen of saponin C was kindly supplied by Prof. J.M.M. Brown. Physical constants and microanalytical data for saponin C prepared by De Kock (1960), are given below. Chemical structures [2a], [2b], and [2c] (Fig. 2) were assigned in the present investigation.

Saponin data

Saponin C [2a] had m.p. 187 °C (from methanol-water), $[\alpha]_D^{22}$ -89° (c 0,73% in chloroform), IR: λ_{max} 865, 898, 920, 980, 1740 cm^{-1}. (Found C, 58,9; H, 8,4. $C_{41}H_{64}O_{14}\cdot3H_2O$ requires C, 59,0; H, 8,5%). Hydrolysis of [2a] with 0,05 M KOH afforded desacetyl saponin C [2b], m.p. 204 °C (from methanol-water), $[\alpha]_D^{29}$-42,7° (c 0,65% in ethanol), IR: λ_{max} 840, 900, 920, 985 cm^{-1}. (Found C, 59,1; H, 8,7. $C_{39}H_{62}O_{13}\cdot3H_2O$ requires C, 59,1; H, 8,7%). Acetylation of [2a] with acetic anhydride–pyridine afforded saponin C peracetate [2c], m.p. 238–240 °C (from methanol), $[\alpha]_D^{20}$ -44,2° (c 0,85% in ethanol). (Found C, 61,4; H, 7,4. $C_{53}H_{76}O_{20}$ requires C, 61,6; H, 7,4 %). Acidic hydrolysis of saponin C yielded equimolar quantities of ruscogenin, glucose, rhamnose and acetate.

Saponin X peracetate, isolated from the acetylated *T. terrestris* extracts as outlined in Fig. 1, had m.p. 197–203 °C (from methanol), $[\alpha]_D^{21}$ -10,4° (c 1,0% in chloroform) (Found C, 58,3; H, 6,9. $C_{65}H_{92}O_{28}\cdot CH_3OH$ requires C, 58,6; H, 7,1%). Hydrolysis of saponin X peracetate with 0,3 M $NaOCH_3$ afforded an amorphous specimen of saponin X, which, when treated with β-glucosidase afforded desacetylsaponin C [2b], identical to that prepared by hydrolysis of [2a] (saponin C) with 0,05 M KOH.

Extraction and GC-MS analysis of *T. terrestris* specimens

Plant samples were collected from farms in the vicinity of Grassdale and Vredelust in the district of Graaff-Reinet, Theronsville in the district of Fraserburg (regions in the Karoo where geeldikkop is endemic) and Rooibokkraal in the district of Ellisras, Northern Province (remote from the endemic area). *T. terrestris* specimens (3 g) were first extracted with hexane in a Soxhlet apparatus for 7 h and then with ethanol-water (4:1) for 16 h. Evaporation of the latter solvent under reduced pressure afforded a residue which was hydrolysed with 2 M HCl and extracted into $CHCl_3$ by the procedure of De Kock & Enslin (1958). Sarsasapogenin propionate (300 μℓ of a 0,5 mg/mℓ solution in chloroform as internal standard for the quantification of sapogenins), was added to the extract, which was acetylated and analysed by gas chromatography–mass spectroscopy (GC-MS), as described previously (Wilkins, Miles, Smith, Meagher & Ede 1994), on a 20 m x 0,22 mm HP-1 methylsilicone capillary column installed in an HP5890 GC interfaced to an HP5970B mass-selective detector. Quantification

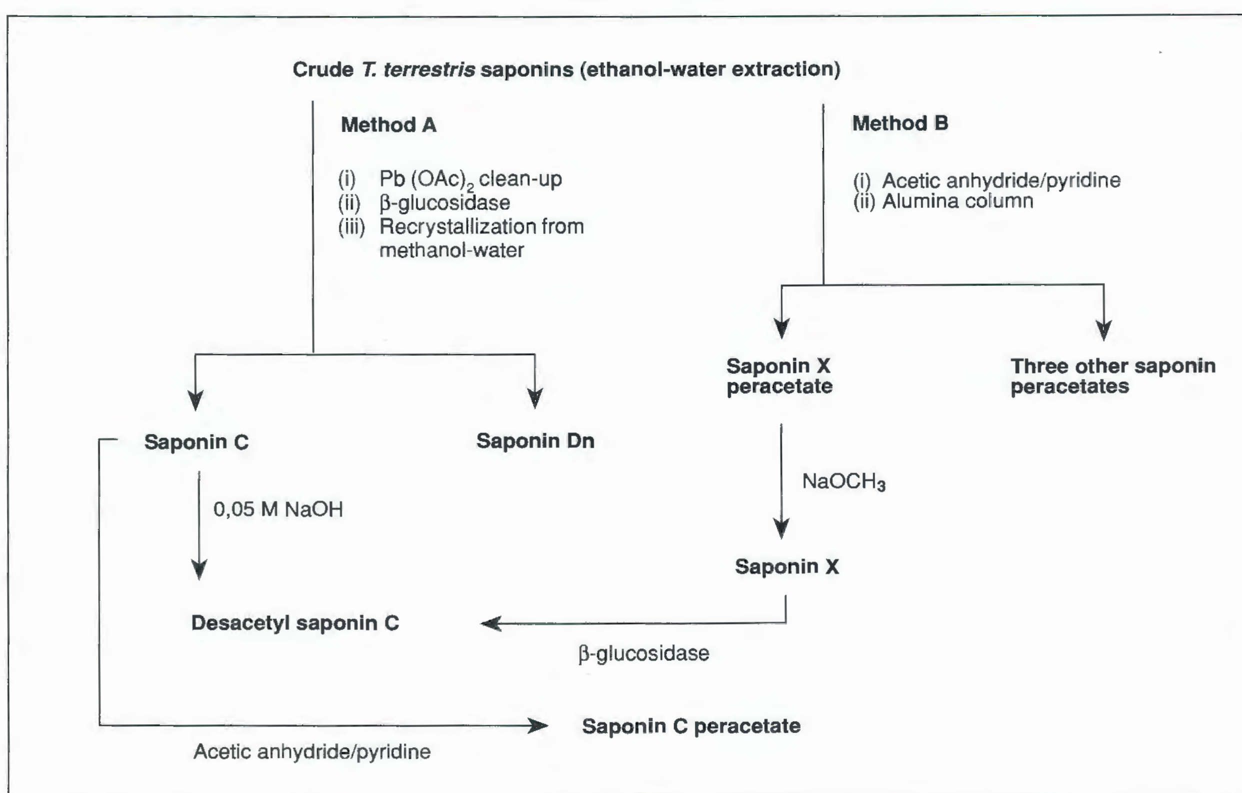

FIG. 1 Isolation of *T. terrestris* saponins, after De Kock (1960)

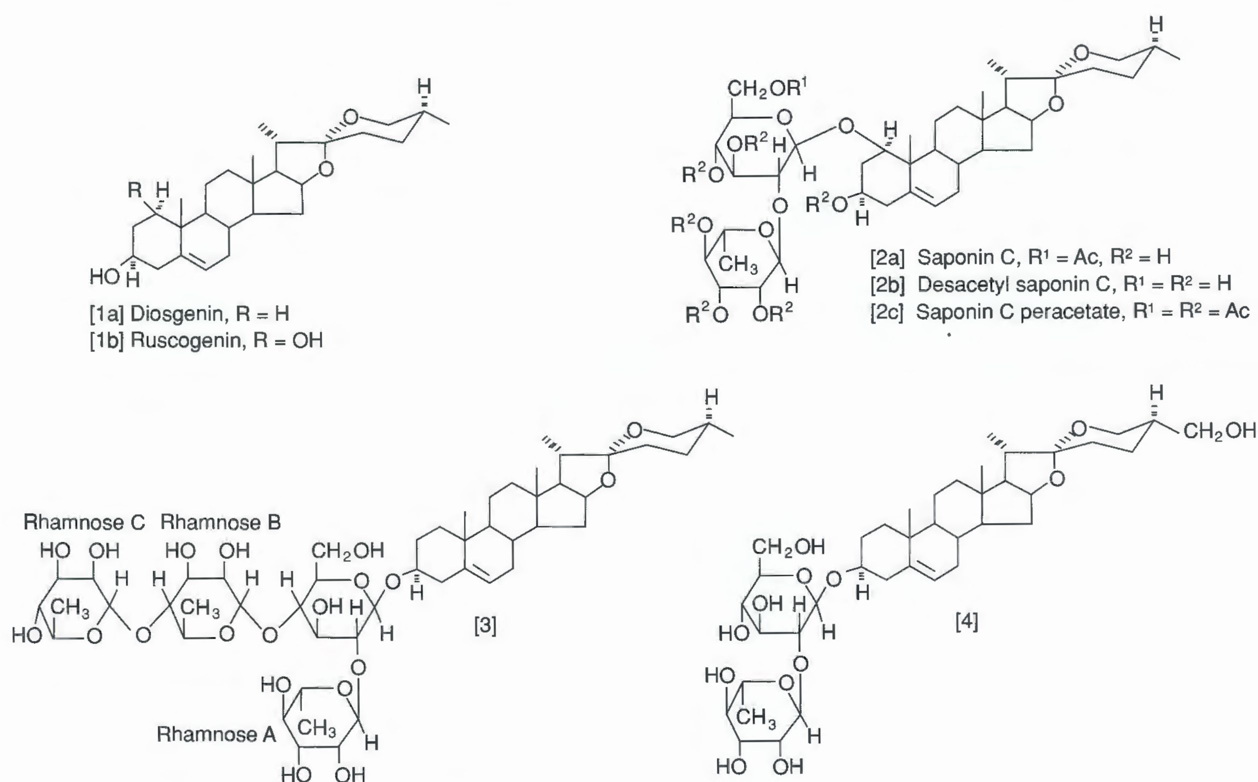

FIG. 2 Structural formulae of some sapogenins and saponins

was performed on integrated *m/z* 139 ion profiles, acquired in selected-ion mode, with a unit response factor applied to all of the sapogenin acetate, diacetate and propionate (internal standard) peaks.

RESULTS

The structure of saponin C was elucidated by a combination of one- and two-dimensional NMR techniques. NMR spectral data are given in Tables 1–4. GC-MS analysis of the hydrolysed ethanol-water (4:1) extracts of *T. terrestris* specimens from two of four sites revealed high levels of ruscogenin and diosgenin saponins, whereas specimens from two other sites contained saponins derived predominantly from tigogenin, neotigogenin, gitogenin and neogitogenin. Sapogenin levels are given in Table 5.

Structure elucidation of saponin C

The ^1H NMR spectrum of saponin C determined in C_5D_5N (see Table 1) included signals attributable to an acetoxyl group, two tertiary methyl groups, three

TABLE 1 ^{13}C and ^1H NMR chemical shifts (δ in C_5D_5N) of diosgenin [1a], ruscogenin [1b] and saponin C [2a]

	Diosgenin			Ruscogenin[a]	Saponin C		
	^{13}C	$^1H\alpha$	$^1H\beta$	^{13}C	^{13}C	$^1H\alpha$	$^1H\beta$
Aglycone signals							
C-1	38,0	1,11	1,81	78,2	85,2	3,63	
C-2	32,4	1,77	2,07	44,0	37,8	2,56	2,40
C-3	71,4	3,82		68,3	68,2	3,77	
C-4	34,6	2,57–2,60[b]		43,7	43,6	2,55–2,66[b]	
C-5	142,2			140,5	139,3		
C-6	121,0	5,38		124,3	124,9		
C-7	32,7	1,53	1,93	33,2	32,0	1,51	1,89
C-8	32,0		1,57	32,5	33,1		1,54
C-9	50,8	0,95		51,6	50,5	1,48	
C-10	37,3			43,7	42,6		
C-11	21,4	1,45–1,50[b]		24,4	24,2	2,85	1,59
C-12	40,2	1,14	1,73	40,8	40,2	1,36	1,66
C-13	40,7			40,4	40,4		
C-14	57,0	1,08		57,2	57,2	1,24	
C-15	32,6	2,02	1,43	32,6	32,4	2,03	1,45
C-16	81,3	4,53		81,2	81,2	4,53	
C-17	63,2	1,81		63,4	63,1	1,91	
C-18	16,5	0,85		16,7	16,9	0,90	
C-19	19,8	1,04		14,0	15,0	1,38	
C-20	42,2	1,95		42,2	42,0	1,95	
C-21	15,1	1,13		15,1	15,1	1,12	
C-22	109,4			109,3	109,4		
C-23	32,0	1,55–1,69[b]		32,1	31,8	1,64[b]	
C-24	29,5	1,56[b]		29,5	29,2	1,53[b]	
C-25	30,8	1,57		30,7	30,6	1,54	
C-26	67,1	3,48	3,55	66,1	66,8	3,47	3,55
C-27	17,4	0,69		17,4	17,3	0,65	
Glucose signals							
C-1'					100,6	4,67	
C-2'					79,4	4,13[c]	
C-3'					76,4	4,13[c]	
C-4'					71,5	3,90	
C-5'					74,4	3,79	
C-6'					64,6	4,71[b]	
$OCOCH_3$ (**CH₃**)					21,1	2,02	
$OCOCH_3$ (**C**O)					170,8		
Rhamnose signals							
C-1"					101,5	6,37	
C-2"					72,4	4,67	
C-3"					72,5	4,56	
C-4"					74,1	4,31	
C-5"					69,4	4,83	
C-6"					19,0	1,72	

[a] Assignments taken from Agrawal *et al.* (1985)
[b] Two-proton multiplet
[c] Overlapping multiplets

secondary methyl groups, an olefinic proton and an anomeric sugar proton (6,37 ppm, d, *J* 1,5 Hz), together with a series of signals (3,4–4,9 ppm, total 15 protons) attributable to oxygenated methylene and methine protons. The ^{13}C NMR spectrum consisted of five quaternary, 20 methine, ten methylene and six methyl carbons, the chemical shifts of which (see Table 1) indicated (Agrawal, Jain, Gupta & Thakur 1985) saponin C to be a ring-A disubstituted 25**R**-spirost-5-ene possessing acetoxyl, glucopyranosyl and rhamnopyranosyl groups.

The ^{13}C and ^{1}H NMR resonances of saponin C were correlated in a two-dimensional NMR experiment and compared with those reported (Agrawal *et al.* 1985) for ruscogenin [1b]. A feature of the ^{13}C NMR spectrum of saponin C was the occurrence of one of the ring-A aglycone carbon resonances at 85,2 ppm. A ^{13}C-^{1}H correlation experiment, optimized for the detection of long-range couplings, established (Table 2) that this resonance was attributable to C-1 (rather than to C-3). The downfield shift of 7 ppm exhibited by C-1 (relative to the equivalent resonance of ruscogenin) (Table 1) indicated (Agrawal *et al.* 1985) saponin C to be a 1β-glycosylated analogue of ruscogenin [1b].

Analysis of the ^{1}H and ^{13}C NMR signal correlations, established for the glycosyl atoms of saponin C in two-dimensional ^{1}H-^{1}H (COSY) and ^{13}C-^{1}H correlated experiments, together with NOE-difference and coupling constant data, established saponin C to be ruscogenin 1-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-6-*O*-acetylglucopyranoside [2a]. The resolution of the 300 MHz ^{1}H NMR spectrum of [2a] was such that the chemical shifts and coupling constants of all of the rhamnosyl protons could be recognized (Table 3). Not unexpectedly, there was a close correspondence between the chemical shifts and coupling constants determined for [2a] and those reported elsewhere (Munday *et al.* 1993; Mimaki & Sashida 1990) for the equivalent α-L-rhamnosyl unit of saponins such as [3] and [4] (Fig. 2).

The complete assignment of the carbon and proton resonances of the glucosyl units was complicated by the coincidence of the glucosyl H-2' and H-3' signals at 4,13–4,14 ppm. In the COSY spectrum, this pair of signals exhibited cross-peaks with H-1' (4,67 ppm) and H-4' (3,90 ppm), while H-5' (3,79 ppm) exhibited cross-peaks with H-4' (3,90 ppm), and H-6' (4,71 ppm). The ^{1}J ^{13}C-^{1}H coupling constant of the glucosyl methylene carbon (147,8 Hz, compared with 144 Hz in β-D-glucose) located the acetoxyl group at C-6' (Agrawal *et al.* 1985). The occurrence of the H-6' resonances of saponin C at 4,79 ppm (Table 1) compared with 4,05 and 4,20 ppm in [3] (Munday *et al.* 1993), was also indicative (Agrawal 1992) of a 6-*O*-acetyl (acetoxyl) group.

The coupling constant of the H-1' proton (7,5 Hz) established that the glucosyl unit was β- (rather than α-) linked (Agrawal 1992). The ^{13}C resonances of the C-2' and C-3' (79,4 and 76,4 ppm) were consistent with a rhamnopyranosyl-(1→2)-β-D-glucopyranosyl linkage, rather than a rhamnopyranosyl-(1→3)-β-D-glucopyranosyl linkage, because in the latter case the C-3' resonance would be expected to occur around 88–89 ppm (Agrawal *et al.* 1985). The chemical shift of the glucosyl C-2' resonance of saponin C [2a]

TABLE 2 Selected long-range two-dimensional ^{13}C–^{1}H NMR correlations (δ, C_5D_5N) observed for saponin C [2a]

^{1}H signal	Correlated ^{13}C signals
0,65 (H-27)	17,3 (C-27), 66,8 (C-26)
0,90 (H-18)	16,9 (C-18), 40,4 (C-13), 57,2 (C-14)
1,12 (H-21)	15,1 (C-21), 42,0 (C-20), 63,1 (C-17), 109,4 (C-22)
1,38 (H-19)	15,0 (C-19), 42,6 (C-10), 85,3 (C-1), 139,3 (C-5)
1,72 (rh H-6")	19,0 (rh C-6"), 69,4 (rh C-5"), 74,1 (rh C-4")
6,37 (rh H-1")	69,4 (rh C-5"), 72,5 (rh C-3"), 101,5 (rh C-1")

TABLE 3 ^{1}H NMR chemical shifts (δ in C_5D_5N) and coupling constants (Hz) for α-L-rhamnopyranose units

	In [4][a]	Rhamnose A in [3]	Saponin C [2a]	α-L-rhamnose
H-1	6,39 (br s)	6,39 (br s)	6,37 (d, *J* 1,5)	5,90 (br s)
H-2	4,81 (br d, *J* 3,2)	4,85 (br s)	4,67 (dd, *J* 1,5, 3,4)	4,70 (br s)
H-3	4,64 (dd, *J* 3,2, 9,3)	4,65 (dd, *J* 3,1, 9,4)	4,56 (dd, *J* 3,4, 9,4)	4,74 (dd, *J* 3,2, 9,2)
H-4	* (~t, *J* 9,4)	4,37 (t, *J* 9,4)	4,31 (t, *J* 9,4)	4,33 (t, *J* 9,3)
H-5	5,01 (dq, *J* 9,5, 6,2)	4,94 (dq, *J* 9,4, 6,3)	4,83 (dq, *J* 9,3, 6,2)	4,63 (dq, *J* 9,3, 6,2)
H-6	1,78 (d, *J* 6,2)	1,78 (d, *J* 6,3)	1,72 (d, *J* 6,2)	1,66 (d, *J* 6,2)

* Not recorded
[a] Mimaki & Sashida (1990)

TABLE 4 Selected NOE enhancements for saponin C [2a] (δ, C_5D_5N)

Irradiated signal	Enhanced signal(s)
6,37 (rh H-1")	4,67 (rh H-2"), 4,13 (gl H-2')
4,83 (rh H-5")	1,72 (rh H-6")
4,71 (gl H-6')	3,90 (gl H-4'), 3,79 (gl H-5')
4,67 (gl H-1')[a]	4,13 (gl H-3'), 3,79 (gl H-5'), 3,63 (H-1α), 2,56 (H-2α)
4,67 (rh H-2")[a]	6,37 (rh H-1")
4,13 (gl H-2' & H-3')	6,37 (rh H-1"), 4,67 (gl H-1')
3,79 (gl H-5')	4,71 (gl H-6'), 4,67 (gl H-1')
3,63 (H-1α)	4,67 (gl H-1')

[a] Co-incident signals

TABLE 5 Concentrations (mg/kg) of conjugated sapogenins detected in some South African *Tribulus terrestris* specimens (collected and extracted February 1994)

Genin	Karoo			Rooibokkraal, Northern Province		
	Grassdale[a]	Vredelust[a]	Theronsville[a]	Whole plant	Leaf	Stalks
Diosgenin	1,0	62	54	0,9	0,7	0,5
Yamogenin	trace	4,0	13	trace	trace	–
Tigogenin	44	15	3,4	11	8,1	7,5
Neotigogenin	2,2	2,0	2,1	3,0	3,4	0,5
Ruscogenin	–	203	69	–	–	–
Neoruscogenin	–	5,7	13	–	–	–
Gitogenin	233	–	–	307	268	180
Neogitogenin	12	–	–	96	121	21

[a] Whole plant, "trace" indicates < 0,2 mg/kg
"–" Indicates not detected

(79,4 ppm) is similar to that of the equivalent resonance of [4] (79,7 ppm) (Mimaki & Sashida 1990).

NOE-difference results (Table 4) readily located the glycosidic linkages. Irradiation of the glucose H-1' signal, which together with the rhamnose H-2" signal resonated at 4,67 ppm, enhanced the glucose H-3' and H-5' signals, the aglycone H-1α and H-2α signals, and the rhamnose H-1" signal. Irradiation of the rhamnose H-1" signal enhanced the coincident glucose H-2' and H-3' resonances (4,13–4,14 ppm). However, ^{13}C NMR data (see above) clearly defined the rhamnosyl unit to be 1→2, rather than 1→3, linked.

The structure of saponin C is therefore ruscogenin 1-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-6-*O*-acetylglucopyranoside [2a] (Fig. 2).

DISCUSSION

Structure of Saponin X

Elucidation of the structure of saponin C partially defines the structure of the naturally occurring saponin (saponin X) from which saponin C is derived (Fig. 1 and 2). Brown & De Kock (1959) reported that hydrolysis of saponin C [2a] with 0,05 M KOH afforded desacetyl saponin C. This compound, to which structure [2b] can now be assigned, was shown to be identical to that prepared by β-glucosidase treatment of saponin X, isolated by De Kock (1960) from crude *T. terrestris* extracts as outlined in Fig. 1. Saponin C is unusual in that an acetoxyl group is attached to C-6' of the glucose unit. However, it does not necessarily follow that the parent saponin possesses an acetoxyl group; the crude *T. terrestris* extract was treated with basic $Pb(OAc)_2$ during purification (Fig. 1), and it is possible that this resulted in acetylation of the comparatively unhindered 6'-OH group. Similarly, it is not possible to determine whether saponin X also possessed an acetoxyl group, because the purification procedure employed by De Kock (1960) involved its complete acetylation (Fig. 1). Microanalytical data are consistent with the formulation of saponin X peracetate as ruscogenin 3-*O*-β-D-glucopyranosyl-1-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside peracetate. In accordance with this, base hydrolysis of saponin X peracetate, followed by β-glucosidase treatment (Fig. 1) afforded [2b] (desacetylsaponin C).

Aetiological implications

The biliary crystalloid deposits that characterize geeldikkop, *Narthecium* and *Panicum* photosensitizations, have been identified as the insoluble calcium salts of the β-D-glucuronides of epismilagenin and

episarsasapogenin (Miles *et al.* 1994a; Miles, Wilkins, Munday, Flaoyen, Holland & Smith 1993; Miles, Wilkins, Munday, Holland, Smith, Lancaster & Embling 1992b; Miles, Munday, Holland, Lancaster & Wilkins 1992a). The biliary deposits are thought to be involved in the pathogenesis of geeldikkop (Kellerman *et al.* 1991; Kellerman *et al.* 1994). Ruminal metabolism of ingested *T. terrestris* saponins has been found to afford epismilagenin and episarsasapogenin (Miles *et al.* 1994b). Epismilagenin and episarsasapogenin have been identified (Lajis, Abdullah, Khan, Jalaludin, Salim & Bremner 1993) as ruminal metabolites of sheep intoxicated by *Brachiaria decumbens*. Epismilagenin has also been isolated from the bile of sheep affected by *Panicum dichotomiflorum* toxicoses (Holland, Miles, Mortimer, Wilkins, Hawkes & Smith 1991).

Evidence has been presented that only saponins capable of being metabolised to epismilagenin and episarsasapogenin are lithogenic (Miles *et al.* 1994a; Miles *et al.* 1994b). If it is the biliary deposits that are responsible for the intoxication, then only plants containing such saponins would cause geeldikkop. In contrast, saponins based on tigogenin, neotigogenin, gitogenin and neogitogenin, do not seem to be lithogenic (Miles *et al.* 1994a; Miles *et al.* 1994b), so plants containing only these saponins would not be expected to cause geeldikkop. Since other dihydroxysapogenins (gitogenin, neotigogenin) are not lithogenic, ruscogenin saponins are also unlikely to be lithogenic. This may account, at least in part, for the failure of early attempts (Brown & De Kock 1959) to induce geeldikkop experimentally. Alternatively, these experiments might have failed simply because too little of the saponins were dosed to either form microliths in the bile ducts, or to otherwise induce photosensitization. The absence of ruscogenin saponins in the *T. terrestris* extracts which were used to induce geeldikkop experimentally, prompted us to investigate the possible existence of potentially lithogenic and non-lithogenic populations (chemotypes) of South African *T. terrestris*.

T. terrestris chemotypes

GC-MS analysis of the hydrolysed and acetylated ethanol-water extracts of *T. terrestris* collections from three sites in the Karoo (a region in which geeldikkop is endemic) and from a site in North West Province (a non-endemic area), identified two chemical populations (chemotypes) (Table 5). One of the chemotypes (represented by the Vredelust and Theronsville collections) exhibited the ruscogenin and diosgenin saponin chemistry of Brown & De Kock (1959), while the other chemotype (represented by the Grassdale and Rooibokkraal collections) contained mainly saturated tigogenin, gitogenin and neogitogenin saponins. In the case of the Rooibokkraal sample, generally similar saponin levels were found in leaf, stalk and whole-plant material.

We have demonstrated (Miles *et al.* 1994b) that ovine metabolism of diosgenin and yamogenin predominantly affords the lithogenic 5β-spirostanols epismilagenin and episarsasapogenin, respectively. It is, however, apparent (see Table 5) that in plant tissue, biosynthetic saturation of diosgenin and yamogenin can proceed to afford the non-lithogenic 5α-spirostanols tigogenin and neotigogenin, respectively, which may subsequently be hydroxylated to give gitogenin and neogitogenin, respectively.

It is notable that mainly unsaturated spirost-5-ene saponins (diosgenin and ruscogenin) were present in the Vredelust and Theronsville collections, whereas elevated levels of saturated 5α-spirostanol (tigogenin) and 5α-spirostanediol saponins (gitogenin and neogitogenin) were identified in the Grassdale and Rooibokkraal collections. Because diosgenin is the precursor substance from which gitogenin is biosynthetically derived, there may, however, be circumstances under which significant levels of potentially lithogenic diosgenin saponins occur in the chemotype represented by the Grassdale and Rooibokkraal collections. Similarly, ruscogenin is biosynthesized by hydroxylation of diosgenin; if this hydroxylation were curtailed in response to environmental or other factors, the affected plant would instead contain high levels of lithogenic diosgenin saponins. Grassdale and Vredelust are in the same district and the samples were collected almost simultaneously, but the samples are of different chemotypes. It is uncertain whether the chemotype differences observed are genetically or environmentally determined.

The quantities of lithogenic saponins that need to be ingested to cause deposition of crystalline material in the biliary system of ruminants, have not yet been determined. However, it is known that factors such as plant maturity can have marked effects on the concentration of lithogenic saponins (e.g. those derived from diosgenin) in some species (Wilkins *et al.* 1994). Wang and Lu (1991) have reported seasonal variations in the concentrations of saponins in the leaf and stem of Chinese *T. terrestris*. The concentrations of tigogenin, hecogenin and gitogenin saponins were greatest in material gathered in June (mid-summer in the Northern hemisphere) (9,1, 66,0 and 60,7 mg/100 g, respectively), while lesser concentrations were present in specimens gathered in August (6,0, 3,3 and 5,2 mg/100 g, respectively) and in October (3,2, 16,5 and 41,3 mg/100 g, respectively). It is possible that physiological stress on the plant, for example drought, could also have similarly profound effects on saponin levels. Anecdotal evidence that young, wilted *T. terrestris* plants have increased toxicity (Kellerman *et al.* 1980) suggests that an investigation of the effect of water stress and plant maturity on the saponin levels in this plant might be worthwhile.

The geographic variation in saponin chemotype observed in the present study suggests that a more extensive investigation of the distribution of *T. terrestris*

chemotypes in South Africa, might lead to an improved understanding of some of the factors contributing to outbreaks of geeldikkop. This should include the investigation of genetical variance, since our results indicate the likely existence of potentially lithogenic (diosgenin-containing) and non-lithogenic (ruscogenin-containing) populations of South African *T. terrestris*.

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