A New Bioactive Steroidal Saponin from *Agave shrevei*

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A new steroidal saponin was isolated from the leaves of *Agave shrevei* Gentry. Its structure was established as 26-(*β*-D-glucopyranosyloxy)-22-methoxy-3-{*O*-*β*-D-glucopyranosyl-(1→2)-*O*-[*O*-*β*-D-glucopyranosyl-(1→4)-*O*-[*O*-*β*-D-glucopyranosyl-(1→6)]-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranosyl]oxy}-(3*β*,5*α*,25*R*)-furostane. The structural identification was performed using detailed analyses of ^{1}H and ^{13}C NMR spectra including 2D NMR spectroscopic techniques (COSY, HETCOR, and COLOC) and chemical conversions. The steroidal saponin showed absence of haemolytic effects in the *in vitro* assay, but demonstrated a significant inhibition of the capillary permeability activity.

Key words: Agave shrevei, Steroidal Saponin, Anti-inflammatory Activity

Introduction

The *Agave* genus belongs to the Agavaceae family and most of the species are distributed in tropical and subtropical regions (Lorenzi and Souza, 1995). Although some *Agave* species have for several years been an important source of steroidal saponins (Blunden *et al.*, 1986; Ding *et al.*, 1993), a few have a pharmacological background, in particular the steroidal sapogenins (hecogenin and tigogenin) of *A. americana* that showed *in vivo* anti-inflammatory activity and a slight ulcerogenic action (Peana *et al.*, 1997), hecogenin tetraglycoside isolated from this species showed cytotoxic activity against HL-60 human promyelocytic leukemia cells (Yokosuka *et al.*, 2000), a saponin fraction of *A. lophanta* showed analgesic and anti-inflammatory activities and ulceroprotective action (Abdel-Khalik *et al.*, 2002), a steroidal saponin isolated from *A. attenuata* showed *in vivo* anti-inflammatory activity with absence of haemolytic effects (da Silva *et al.*, 2002) and chlorogenin hexasaccharide isolated from *A. fourcroydes* showed cytotoxic and cell cycle inhibitory activities (Ohtsuki *et al.*, 2004). *A. shrevei* Gentry is cultivated in Brazil with ornamental purposes but no medicinal use and chemical study are reported. As part of our ongoing investigation of bioactive steroidal saponins, we have now examined the leaves of this plant. We isolated a new steroidal saponin from *A. shrevei* and evaluated its haemolytic effect and anti-inflammatory property.

Materials and Methods

Plant material

Fresh leaves of *Agave shrevei* were obtained from the Ornamental Garden of UFRJ, Rio de Janeiro, in February 2003 and a voucher specimen is maintained in the Laboratory of Chemistry of Medicinal Plants at Federal University of Rio de Janeiro.

General procedures

Melting points were determined by an IA 9200 digital melting point apparatus (Electrothermal Engineering Ltd., Southend-on-Sea, UK) and are uncorrected. Optical rotations were measured on a Perkin Elmer 243B polarimeter. IR spectra were measured on a Perkin Elmer 599B. Negative LSI-MS were carried out using thioglycerol as the matrix and Cs ions accelerated at 35 kV; acceleration voltage: 8 kV. Mass spectra and GC-MS were taken on a VG Auto SpecQ spectrometer. NMR spectra were measured in C_5D_5N (100 mg of steroidal saponin **1** in 0.5 ml) at 25 °C with a Varian Gemini 200 NMR spectrometer, with tetramethylsilane (δ = 0.00) used as internal standard. ^{1}H NMR spectra were recorded at 200 MHz and ^{13}C NMR spectra at 50 MHz. Silica gel columns (230–400 mesh ASTM, Merck) and Sephadex LH-20 (Pharmacia) were used for CC. TLC was performed on silica gel plates (Kieselgel 60F_{254}, Merck) using the following solvent systems: (A)

$CHCl_3$/MeOH/H_2O (65:35:10 v/v/v, lower phase) for steroidal saponin **1**, (B) $CHCl_3$/MeOH (12:1 v/v) for pseudosapogenin and (C) *n*-BuOH/Me_2CO/MeOH (4:5:1 v/v/v) for monosaccharides. Spray reagents were orcinol/H_2SO_4 for steroidal saponin **1** and monosaccharides, and $CeSO_4$ for pseudosapogenin.

Extraction and isolation

Fresh leaves (3 kg) were extracted with 80% aqueous EtOH (6 l) for 72 h at room temperature. The extract was concentrated under reduced pressure to remove most of the EtOH and the resulting aqueous phase (600 ml) was shaken with *n*-BuOH [water/*n*-BuOH (1:1 v/v)]. This procedure was repeated and the resulting organic phase was evaporated *in vacuo* to give a crude material (17.3 g) The residue was roughly chromatographed on Sephadex LH-20 with MeOH. The fractions were combined based on the TLC profiles to give the saponin mixture (2.7 g). Further purification by chromatography on a silica gel column eluted with $CHCl_3$/MeOH/H_2O (70:30:10 v/v/v) afforded one TLC-homogeneous compound **1** (974 mg), R_f 0.38, which gave a dark green colour with orcinol/H_2SO_4.

Compound **1**

Colorless needles. – $[a]_D^{25}$ – 80° (*c* 0.1, MeOH). – M.p. 278–280 °C. – IR (KBr): ν_{max} = 3407 (O-H), 2929 (C-H), 1651, 1455, 1424, 1379, 1259, 1163, 1074 (C-O), 1042 (C-O), 914, 894 and 632 cm^{-1} [(25*R*)-furostanol, intensity 914 < 894]. – Negative LSI-MS: *m/z* = 1419 $[M\text{-}H]^-$. – ^1H and ^{13}C NMR data: see Tables I and II.

Acid hydrolysis of **1**

A solution of compound **1** (200 mg) in 2 N H_2SO_4/50% EtOH (20 ml) was refluxed on a water bath for 4 h. The reaction mixture was diluted with water (40 ml) and filtered. The precipitate was cristallized with MeOH to give (53 mg). The identity of tigogenin was established by comparison with an authentic sample through m.p., IR, ^1H and ^{13}C NMR and EI-MS. The sugar mixture was dissolved in pyridine and analyzed by silica gel-TLC in the above described solvent system. After spraying, glucose gave a blue spot at R_f 0.70 and galactose gave a purple spot at R_f 0.66.

Molar carbohydrate composition and D,L configurations

The molar carbohydrate composition of compound **1** was determined by GC-MS analysis of its monosaccharides as their trimethylsilylated methylglycosides obtained after methanolysis (0.5 M HCl in MeOH, 24 h, 80 °C) and trimethylsilylation (Kamerling *et al.*, 1975). The configurations of the glycosides were established by capillary GC of their trimethylsilylated (–)-2-butylglycosides (Gerwig *et al.*, 1978).

Methylation analysis

Compound **1** was methylated with dimethyl sulfoxide/lithium methylsulfinyl carbanion/methyl iodide (Parente *et al.*, 1985). The methyl ethers were obtained after hydrolysis (4 N trifluoroacetic acid, 2 h, 100 °C) and analyzed as partially alditol acetates by GC-MS (Sawardeker *et al.*, 1965).

Animals

Male BALB/c mice, weighing 15–20 g, were used. The animals were housed under standard environmental conditions and fed with standard rodent diet and water *ad libitum.*

Statistical analysis

The experimental data were tested for statistical differences using the Student's *t* test. IC_{50} values were obtained from the regression lines.

Haemolytic activity

Normal human red blood cell suspension (0.6 ml of 0.5%) was mixed with 0.6 ml diluent containing 5, 10, 20, 30, 40, 50, 100, 250 and 500 mg/ml of compound **1**, aluminum hydroxide, and 5–500 ml/ml of Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) in saline solution. Mixtures were incubated for 30 min at 37 °C and centrifuged at 70 × *g* for 10 min. Saline and distilled water were included as minimal and maximal haemolytic controls. The haemolytic percents developed by the saline control were subtracted from all groups. The adjuvant concentration inducing 50% of the maximum haemolysis was considered as HD_{50} (halmolytic dose, graphical interpolation). Experiments were performed in triplicate at each concentration (Santos *et al.*, 1997).

Table I. Selected ^{1}H NMR assignments (d [ppm], *J* [Hz]) of compound **1** in C_5D_5N.

Aglycone characteristic proton signals			Sugar anomeric proton signals		
Position	Compound **1**	^{2}H-^{1}H-COSY	Position	Compound **1**	^{1}H-^{1}H-COSY
H-3	3.89 m	H-2, H-4	Gal-H-1	4.80 d (7.7)	Gal-H-2
H-5	0.88 m	H-4, H-6	Glc-H-1	4.90 d (7.3)	Glc-H-2
Me-18	0.77 s		Glc-H-1′	5.16 d (7.3)	Glc-H-2′
Me-19	0.83 s		Glc-H-1″	5.49 d (7.3)	Glc-H-2″
Me-21	1.22 d (6.8)	H-20	Glc-H-1‴	5.31 d (7.3)	Glc-H-2‴
Me-27	0.98 d (6.6)	H-25	Glc-H-1⁗	4.85 d (7.8)	Glc-H-2⁗
OMe-22	3.25 s				

Anti-inflammatory activity

Anti-inflammatory activity was evaluated by measuring acetic acid-induced vascular permeability (Whittle, 1964). Male mice (BALB/c, 15–20 g) in groups of five were dosed orally with different concentrations of compound **1** (10, 25, 50 and 100 mg/kg body weight) and a positive control, indomethacin (10 mg/kg body weight). After injection of the dye, 0.1 N acetic acid (10 ml/kg body weight) was injected intraperitoneally. 20 min later, the mice were killed with an overdose of diethyl ether and the viscera were exposed after a 1 min period to allow blood to drain away from the abdominal wall. The animal was held by a flap of the abdominal wall and the viscera were irrigated with 10 ml of saline over a petri dish. The washing was filtered through glass wool and transferred to a test tube. To each tube 100 ml of 1 N NaOH were added in order to clear any turbidity due to protein, and the absorbance was read at 590 nm.

Results and Discussion

Compound **1** was obtained as colorless needles and gave a positive Liebermann-Burchard test for a steroidal saponin. It revealed a quasi-molecular ion peak at *m/z* 1419 [M-H] in the negative mode LSI-MS. In the ^{13}C NMR spectrum (Table II), 64 carbon signals observed belong to five methyl groups (one of which was oxygenated), seventeen methylene groups (seven of which were oxygenated), thirty-nine methine groups (thirty-two of which were oxygenated) and three quaternary carbon atoms (one of which was oxygenated). On the basis of above mentioned MS and NMR spectral data, compound **1** was supposed to be a saponin with the molecular formula $C_{64}H_{108}O_{34}$, bearing a chain of six sugar moieties.

In addition to this, the furostanol glycosidic nature of compound **1** was indicated by the strong absorption bands at 3407, 1074 and 1042 cm^{-1} and a (25*R*)-furostan steroidal structure (894 and

Table II. ^{13}C NMR data of the aglycone and carbohydrate moieties of compound **1** in C_5D_5N[a].

C	**1**	C	**1**
1	37.83	Glc 1	105.21
2	30.50	2	81.94
3	77.31	3	74.92
4	34.69	4	81.81
5	44.35	5	75.71
6	28.91	6	68.47
7	32.04	Glc 1′	105.03
8	35.47	2′	75.91
9	55.07	3′	77.95
10	35.92	4′	71.52
11	21.86	5′	78.43
12	40.66	6′	62.13
13	41.10	Glc 1″	105.31
14	56.98	2″	76.51
15	32.68	3″	78.12
16	81.82	4″	71.68
17	64.49	5″	78.38
18	17.10	6″	62.23
19	12.91	Glc 1‴	105.41
20	40.66	2‴	75.33
21	16.79	3‴	78.18
22	113.25	4‴	71.74
23	32.68	5‴	78.37
24	29.55	6‴	63.08
25	34.79	Glc 1⁗	105.20
26	75.26	2⁗	75.51
27	17.74	3⁗	78.93
Ome-22	47.88	4⁗	72.13
Gal 1	103.03	5⁗	78.71
2	73.33	6⁗	63.21
6	75.42		
6	80.31		
6	76.72		
6	60.63		

[a] The assignments were made on the basis of DEPT, HETCOR and COLOC experiments.

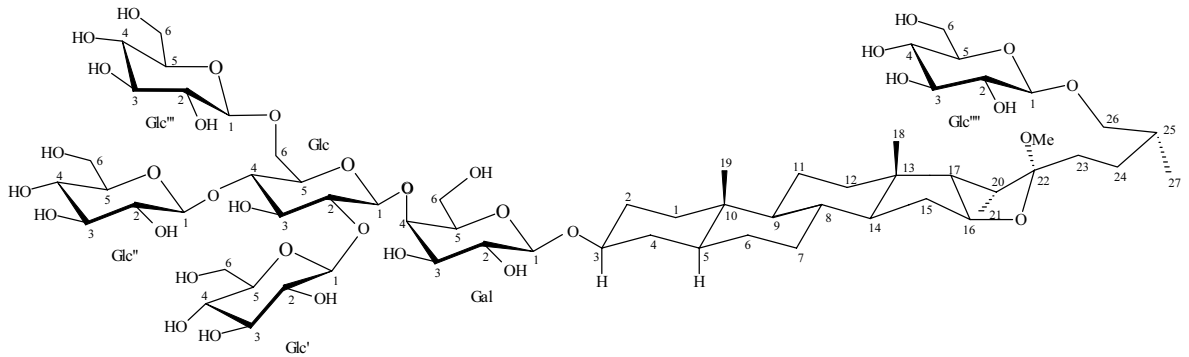

Fig. 1. Chemical structure of compound **1**.

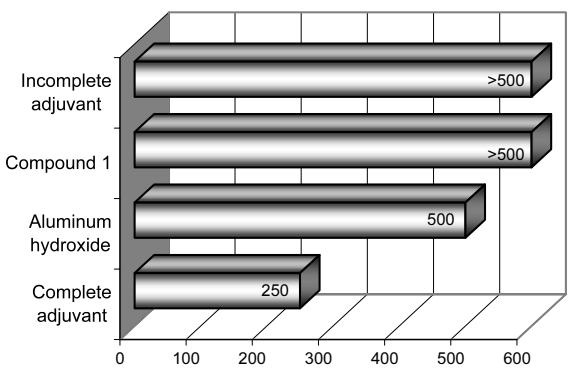

Fig. 2. 50% Haemolytic dose (mg/ml) of compound **1** and adjuvants.

914 cm^{-1}, intensity 914 < 894) in the IR spectrum (Wall *et al.*, 1952), confirmed by ^{1}H and ^{13}C NMR spectra (Tables I and II) (Agrawal *et al.*, 1985; Ding *et al.*, 1993; da Silva *et al.*, 2002). The ^{1}H NMR spectral data (Table I) contained signals for two protons at δ 3.89 (m) and 0.88 (m), methoxyl protons at δ 3.25 (s), two secondary methyl protons at δ 1.22 (d, J = 6.8 Hz) and 0.98 (d, J = 6.6 Hz) and two angular methyl protons at δ 0.77 and 0.83 (each s). The above ^{1}H NMR spectral data and a comparison of the ^{13}C NMR signals of the aglycone moiety of compound **1** (Table II) with those described in the literature (Agrawal *et al.*, 1985; Ding *et al.*, 1993) showed the structure of the aglycone to be (3β,5α,25*R*)-furostan-3-ol. In addition to this, the ^{1}H NMR spectrum of **1**

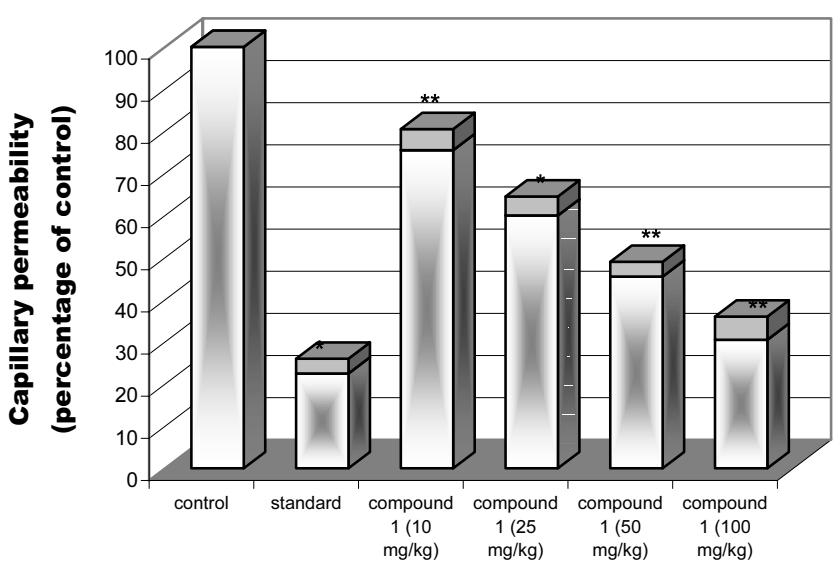

Fig. 3. Anti-inflammatory property of compound **1** tested by capillary permeability. Significantly different from the control group; *p < 0.01,**p < 0.05.

showed six anomeric protons at δ 4.80 (d, J = 7.7 Hz), 4.85 (d, J = 7.8 Hz), 4.90 (d, J = 7.3 Hz), 5.16 (d, J = 7.3 Hz), 5.31 (d, J = 7.3 Hz) and 5.49 (d, J = 7.3 Hz) corresponding to Gal-H-1, Glc-H-1‴, Glc-H-1, Glc-H-1′, Glc-H-1‴ and Glc-H-1″, respectively.

In the ^{13}C NMR spectrum of **1**, a 1,4-linked inner β-D-galactopyranosyl unit, a 1,2,4,6-linked inner β-D-glucopyranosyl unit and four terminal β-D-glucopyranosyl units were observed. As shown in Tables I and II, ^{1}H and ^{13}C NMR chemical shift assignments were made by standard 1D and 2D NMR techniques. The COLOC spectrum displayed long range couplings between galactose-H-1 at δ 4.80 and aglycone-C-3 at δ 77.31, between glucose-H-1‴ at δ 4.85 and aglycone-C-26 at δ 75.26, between glucose-H-1 at δ 4.90 and galactose-C-4 at δ 80.31, between glucose-H-1′ at δ 5.16 and glucose-C-2 at δ 81.94, between glucose-H-1″ at δ 5.49 and glucose-C-4 at δ 81.81 and between glucose-H-1‴ at δ 5.31 and glucose-C-6 at δ 68.47. In addition to this, the methylation analysis of **1** (Parente *et al.*, 1985) furnished 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol and 1,2,4,5,6-penta-*O*-acetyl-3-mono-*O*-methyl glucitol. These results indicated that compound **1** is undoubtedly as shown in Fig. 1.

On acid hydrolysis, compound **1** gave a pseudosapogenin, galactose and glucose. The pseudosapogenin was identified as tigogenin by direct comparison of TLC, m.p., IR, ^{1}H and ^{13}C NMR and EI-MS results with an authentic sample. The molar carbohydrate composition of **1** indicated the presence of six neutral monosaccharides: galactose:glucose (1:5) (Kamerling *et al.*, 1975). Their absolute configurations were determined by GC of their trimethylsilylated (−)-2-butylglycosides (Gerwig *et al.*, 1978). D-Glucose and D-galactose were identified. Consequently, on the basis of IR, ^{1}H and ^{13}C NMR spectroscopy, LSI-MS and chemical reactions, the structure of compound **1** was established as 26-(β-D-glucopyranosyloxy)-22-methoxy-3-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-β-D-glucopyranosyl-(1→4)-*O*-[*O*-β-D-glucopyranosyl-(1→6)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl]oxy}-(3β,5α,25*R*)-furostane.

The chemical structure of compound **1** is shown in Fig. 1. On the basis of recent reports of haemolytic (Oda *et al.*, 2000) and anti-inflammatory (Abdel-Khalik *et al.*, 2002) activities of steroidal saponins, these pharmacological properties of compound **1** were investigated. The absence of haemolytic effects (HD_{50} > 500 mg/ml) demonstrates that the membranolytic activity is related to the amphipathic nature of the molecule (Fig. 2). In addition to this, the capillary permeability assay (Whittle, 1964) was used to evaluate the anti-inflammatory activity of compound **1** in different concentrations (Fig. 3), which showed inhibition of the increase in vascular permeability caused by acetic acid (IC_{50} = 55 mg/kg or IC_{50} = 0.75 mM for each animal), which is a typical model of first stage inflammatory reaction. The results obtained may help to explain some biological properties attributed to steroidal saponins reported in the literature.

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