A New Bioactive Steroidal Saponin from *Agave attenuata*

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Agave attenuata, Steroidal Saponin, Anti-inflammatory Activity

A new steroidal saponin was isolated from the leaves of *Agave attenuata* Salm-Dyck. Its structure was established as (3β,5β,22α,25*S*)-26-(β-D-glucopyranosyloxy)-22-methoxyfurostan-3-yl *O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside. 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 haemolytic potential of the steroidal saponin was evaluated and the anti-inflammatory activity was performed using the capillary permeability assay.

Introduction

The occurrence of steroidal saponins in *Agave* genus is well documented (Blunden *et al*., 1980; Blunden *et al*., 1986; Ding *et al*., 1989; Ding *et al*., 1993). Some species have an ethnopharmacological background, in particular *A. sisalana* which in the Bahama Islands, the central bud is boiled with salt and the decoction given as a remedy for jaundice; said to be effective within 24 hours (Eldridge, 1975). *Agave attenuata* Salm-Dyck (Agavaceae) is a native species from Tropical America. In Brazil, this plant is an evergreen, perennial succulent species that lacks spines. It is non-invasive, but a widespread ornamental plant often cultivated in gardens and parks (Lorenzi and Souza, 1995). Recently, the aqueous extract of *A. attenuata* was evaluated for activity against *Bulinus africanus*, *Daphnia pulex*, *Anopheles arabiensis* and *Oreochromis mossambicus* demonstrating molluscicidal, piscicidal and larvicidal properties (Brackenbury and Appleton, 1997). Previous phytochemical study on *A. attenuata* has revealed the occurrence of sarsapogenin (Wall *et al*., 1954). As part of our program of the chemical investigation of bioactive steroidal saponins, we have now examined the leaves of this plant. We isolated a new steroidal saponin from *A. attenuata*, along with an evaluation of its haemolytic effect and anti-inflammatory properties.

Materials and Methods

Plant material

Fresh leaves of *Agave attenuata* were obtained from the Ornamental Plant Garden of FIOCRUZ, Rio de Janeiro, in January 2000 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 Ellectrotermal 9200 micro-melting point 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 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 **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

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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 (95:5 v/v) for pseudosapogenin and (C) *n*-BuOH–C_5H_5N–H_2O (60:40:30 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

The fresh leaves of the plant (3 kg) were extracted with 80% aqueous EtOH (6 l) followed by concentration to 600 ml and extraction with an equal volume of *n*-BuOH gave a crude material (12.5 g). It was roughly chromatographed on Sephadex LH-20 with MeOH to give crude steroidal glycoside (2.5 g). Further purification by chromatography on a silica gel column eluted with $CHCl_3$–MeOH–H_2O (70:30:10 v/v/v) to afford one TLC homogeneous compound **1** (635 mg), R_f 0.43 which gave a dark green color with orcinol–H_2SO_4.

Compound **1**

Colorless needles; $[\alpha]_D^{25}$ −280° (*c* 1.0, MeOH); m.p. 225–235 °C (dec.); IR ν_{max} (KBr): 3435, 2936, 1641, 1592, 1513, 1461, 1403, 1381, 1313, 1241, 1184, 1055, 985, 915, 845, 815 cm^{-1} [(25*S*)-furostanol, intensity 915 > 845]. LSI-MS (neg.), *m/z* 1419 $[M-H]^-$; ^1H and ^{13}C NMR data (Tables I and II).

Acid hydrolysis of **1**

Compound **1** (200 mg) was hydrolyzed with 2 M HCl–1,4-dioxan (1:1 v/v; 10 ml) in a sealed tube for 3 h at 100 °C. After cooling, the reaction mixture was neutralized with 3% KOH MeOH and evaporated to dryness. The salts that deposited on addition of MeOH were filtered off and the filtrate was passed through a Sephadex LH-20 with MeOH to give the hydrolysate (192 mg) which was chromatographed on silica gel CC with $CHCl_3$–MeOH–H_2O (7:3:0.2 v/v/v) to yield the sarsapogenin (62 mg) and a sugar mixture. Identity of sarsapogenin 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 DMSO-lithium methylsulfinyl carbanion–CH_3I (Parente *et al.*, 1985). The methyl ethers were obtained after hydrolysis (4 N TFA, 2 h, 100 °C) and analyzed as partially alditol acetates by GC-MS (Sawardeker *et al.*, 1965).

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 $\mu g \cdot ml^{-1}$ concentrations of compound **1**, aluminum hydroxide, and 5–500 $\mu l \cdot ml^{-1}$ 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 was subtracted from all groups. The adjuvant concentration inducing 50% of the maximum haemolysis was considered the HD_{50} (graphical interpolation). Experiments included triplicate at each concentration (Santos *et al.*, 1997).

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 compound **1** (100 μg/g body weight) and a positive control, indomethacin (10 μg/g body weight). After injec-

tion of the dye, 0.1 N acetic acid (10 µl/g body weight) was injected intraperitoneally. Twenty minutes later, the mice were killed with an overdose of 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 was added 100 µl of 1 N NaOH in order to clear any turbidity due to protein, and the absorbance was read at 590 nm.

Results and Discussion

The fresh leaves of *Agave attenuata* were extracted with methanol. After concentration under reduced pressure, the methanol extract was partitioned between water and *n*-butanol. Chromatographic separations of the organic phase on Sephadex LH-20 and silica gel gave compound **1** which was detected with orcinol–H_2SO_4 reagent. Compound **1** was obtained as colorless needles and gave a positive Liebermann-Burchard test for a steroidal saponin. The LSI-MS showed an ion peak $[M-H]^-$ at *m/z* 1419 which, together with ^{13}C NMR spectral data (Table II), suggested the molecular formula as $C_{64}H_{108}O_{34}$.

In addition to this, the furostanol glycosidic nature of **1** was indicated by the strong absorption bands at 3450 and 1055 cm^{-1} and a 25*S*-furostan steroidal structure (815, 845 and 915 cm^{-1}, intensity 915 > 845 cm^{-1}) in the IR spectrum (Wall *et al.*, 1952), confirmed by ^1H and ^{13}C NMR spectra (Tables I and II) (Tori *et al.*, 1981; Agrawal *et al.*, 1985). The ^1H NMR spectral data (Table I) contained signals for two protons at δ 3.63 (m) and 1.40 (m), methoxyl protons at δ 3.28 (s), two secondary methyl protons at δ 1.07 (d, J = 5.3 Hz) and 1.04 (d, J = 5.9 Hz) and two angular methyl protons at δ 0.82 and 0.88 (each s). The above ^1H NMR spectral data and a comparison of the ^{13}C NMR signals of the aglycone moiety of **1** (Table II) with those described in the literature (Agrawal *et al.*, 1985; Tori *et al.*, 1981) showed the structure of the aglycone to be (3β,5β,22α,25*S*)-22-methoxyfurostan-3,26-diol. In addition to this, the

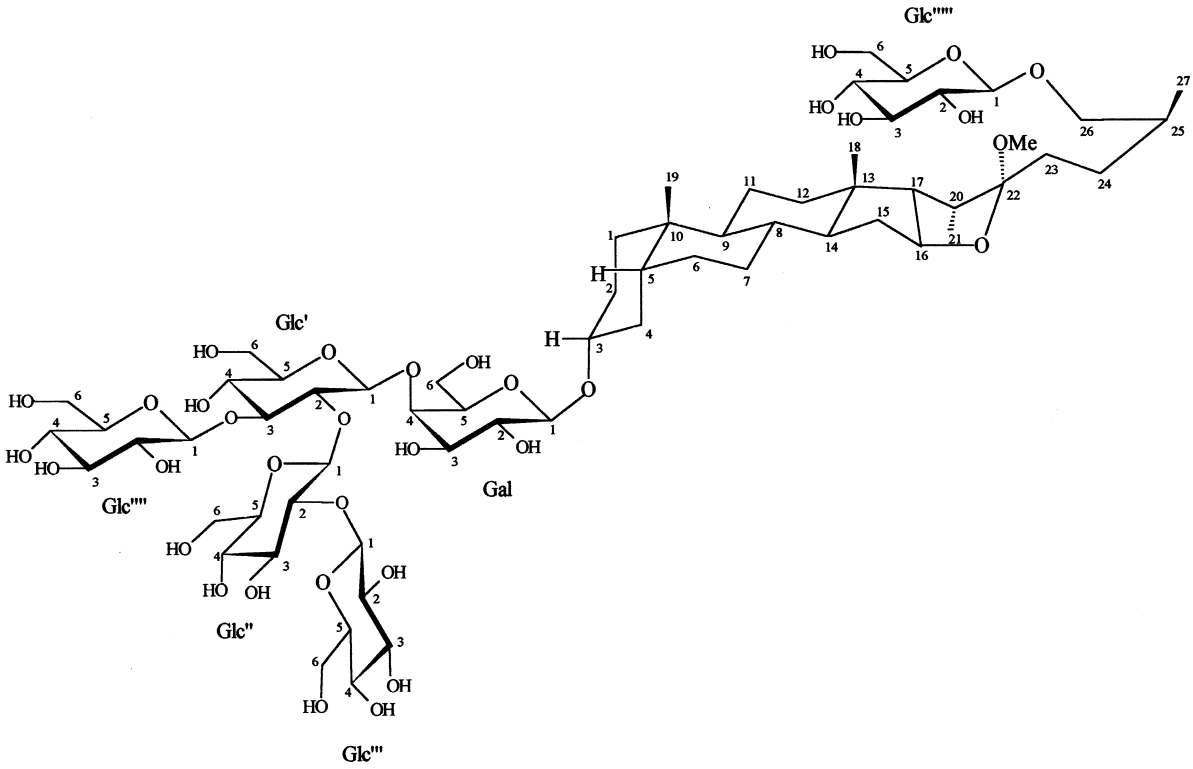

Table I. Selected ^{1}H NMR assignments (δ[ppm]), *J* [Hz] of compound **1** in C_5D_5N.

Aglycone characteristic proton signals			Sugar anomeric proton signals		
Position	Compound **1**	^{1}H–^{1}H-COSY	Position	Compound **1**	^{1}H–^{1}H-COSY
H-3	3.63 m	H-2, H-4	Gal–H-1	4.90 d (6.7)	Gal–H-2
H-5	1.40 m	H-4, H-6	Glc–H-1′	4.75 d (7.5)	Glc–H-2′
Me-18	0.82 s		Glc–H-1″	4.70 d (7.8)	Glc–H-2″
Me-19	0.88 s		Glc–H-1‴	4.82 d (7.3)	Glc–H-2‴
Me-21	1.07 d (5.3)	H-20	Glc–H-1⁗′	5.28 d (7.3)	Glc–H-2⁗′
Me-27	1.04 d (5.9)	H-25	Glc–H-1⁗″	4.80 d (7.7)	Glc–H-2⁗″
OMe-22	3.28 s				

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

Carbon number	Compound **1**	Carbon number	Compound **1**
1	36.40	Glc 1′	104.84
2	30.45	2′	80.81
3	77.88	3′	85.55
4	34.77	4′	71.20
5	35.05	5′	77.82
06	26.45	6′	62.23
7	26.32	Glc 1″	104.53
8	34.75	2″	74.67
9	40.18	3″	85.55
10	35.05	4″	69.31
11	20.73	5″	77.46
12	39.76	6″	62.27
13	39.76	Glc 1‴	107.31
14	55.92	2‴	75.90
15	40.78	3‴	77.84
16	80.74	4‴	71.31
17	62.34	5‴	78.20
18	15.96	6‴	62.42
19	23.51	Glc 1⁗′	105.49
20	40.78	2⁗′	74.93
21	15.86	3⁗′	78.43
22	112.19	4⁗′	71.30
23	31.68	5⁗′	78.43
24	27.64	6⁗′	62.51
25	27.82	Glc 1⁗″	104.85
26	74.67	2⁗″	74.87
27	15.96	3⁗″	78.04
OMe-22	46.88	4⁗″	71.82
Gal 1	101.95	5⁗″	78.42
2	73.28	6⁗″	62.33
3	74.93		
4	80.11		
5	74.93		
6	61.62		

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

^{1}H NMR spectrum of **1** showed five anomeric protons at δ 4.70 (d, *J* = 7.8 Hz), 4.75 (d, *J* = 7.5 Hz), 4.80 (d, *J* = 7.7 Hz), 4.82 (d, *J* = 7.3 Hz), 4.90 (d, *J* = 6.7 Hz) and 5.28 (d, *J* = 7.3 Hz) corresponding to Glc–H-1″, Glc–H-1′, Glc–H-1⁗″, Glc–H-1‴, Gal–H-1 and Glc–H-1⁗′, respectively.

In the ^{13}CNMR spectrum of **1**, 1,4-linked inner β-D-galactopyranosyl unit, 1,2,3-linked inner β-D-glucopyranosyl unit, 1,2-linked inner β-D-glucopyranosyl unit and three terminal β-D-glucopyranosyl units were observed. Its COLOC spectrum displayed long range couplings between galactose–H-1 at δ 4.90 and aglycone–C-3 at δ 77.81, between glucose–H-1⁗″ at δ 4.80 and aglycone–C-26 at δ 74.67, between glucose–H-1′ at δ 4.75 and galactose–C-4 at δ 80.11, between glucose–H-1″ at δ 4.70 and glucose–C-2′ at δ 80.81, between glucose–H-1‴ at δ 4.82 and glucose–C-2″ at δ 74.67 and between glucose–H-1⁗′ at δ 5.28 and glucose–C-3′ at δ 85.55. These results were confirmed by comparison with those reported in the literature (Matsuura *et al.*, 1989; Ding *et al.*, 1993) and by methylation analysis (Parente *et al.*, 1985).

On acid hydrolysis, compound **1** gave a pseudosapogenin, galactose and glucose. The pseudosapogenin was identified as sarsapogenin by direct conparison of TLC, m.p., IR, ^{1}H and ^{13}C NMR and EI-MS with an authentic sample. The molar carbohydrate composition of **1** indicated the presence of six neutral monosaccharides: galactose: glucose (1.0:5.0) (Kamerling *et al.*, 1975). Their absolute configurations were determined by GC of their trimethylsilylated (–)-2-butylglycosides (Gerwig *et al.*, 1978). D-galactose and D-glucose were identified. Consequently, on the basis of IR, ^{1}H and ^{13}C NMR spectroscopy, LSI-MS and chemical reactions, the structure of **1** was established as (3β,5β,22α,25*S*)-26-(β-D-glucopyranosyloxy)-22-methoxyfurostan-3-yl *O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-*O*-[β-D-glucopyrano-

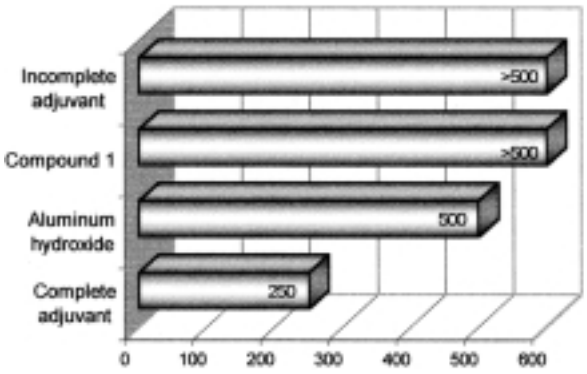

Fig. 1. 50% haemolytic dose (μg/ml) of compound **1** and adjuvants.

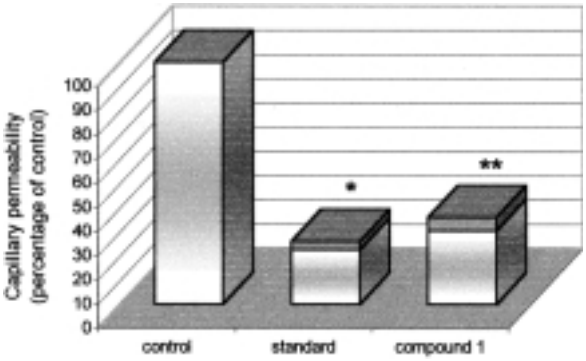

Fig. 2. Anti-inflammatory property of compound **1**. Significantly different from the control group; $*p < 0.01$, $**p < 0.05$.

syl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside.

According to the literature, steroidal saponins are shown to possess anti-inflammatory properties (Lacaille-Dubois and Wagner, 1996). However, this activity is sometimes accomplished by an undesirable haemolytic effect (Oda *et al.*, 2000). In order to evaluate the pharmacological properties of the steroidal saponin **1**, it was screened for haemolytic activity *in vitro* (Santos *et al.*, 1997) and compared to adjuvants commonly used in animal and human experimental models. Generally, steroidal saponins possess elevated haemolytic activity because steroids have higher affinities for cholesterol on erythrocyte membranes (Oda *et al.*, 2000). Nonetheless, this is not the case for compound **1** (Fig. 1), which demonstrated absence of haemolytic effects. This particular behavior can be easily explained by the assumption that the saponin **1** possesses sugar units distributed in opposite sides of the aglycone moiety, which considerably reduces its hydrophobicity, resulting in the loss of the amphipathic features. In addition to this, compound **1** inhibited the increase in vascular permeability caused by acetic acid, which is a typical model of first stage inflammatory reaction (Whittle, 1964). The standard drug indomethacin also reduced the leakage (Fig. 2). The biological results obtained may help explain some biological properties attributed to several steroidal saponins reported in the literature (Lacaille-Dubois and Wagner, 1996).

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