New Steroidal Saponins from Rhizomes of *Costus spiralis*

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Two new steroidal saponins were isolated from the rhizomes of *Costus spiralis* Rosc. Their structures were established as (3β,25*R*)-26-(β-D-glucopyranosyloxy)-22-hydroxyfurost-5-en-3-yl *O*-D-apio-β-D-furanosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (**1**) and (3β,25*R*)-26-(β-D-glucopyranosyloxy)-22-hydroxyfurost-5-en-3-yl *O*-D-apio-β-D-furanosyl-(1→4)-*O*-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**2**). Their structural identifications were performed using detailed analyses of ^{1}H and ^{13}C NMR spectra including 2D NMR spectroscopic techniques (DEPT, COSY, HETCOR and COLOC) and chemical conversions. The steroidal saponins were evaluated for anti-inflammatory activity.

Key words: Costus spiralis, Steroidal Saponins, Anti-inflammatory Activity

Introduction

Costus spiralis Rosc. (Costaceae) is used in the Brazilian folk medicine as a diuretic to relieve complaints of the bladder and urethra and to expel kidney stones (Corrêa, 1984; Cruz, 1965). Pharmacological evaluation of the antiurolithiac activity of the water extract of this plant in rats confirmed the folk information (Viel *et al.*, 1999). Previous phytochemical studies on *C. spiralis* have revealed the occurrence of sterols and furostanol glycosides (Willuhn and Pretzsch, 1985) and flavonol glycosides (Antunes *et al.*, 2000). As part of our program of the chemical investigation of bioactive steroidal saponins, we have now examined the rhizomes of this plant. We isolated two new steroidal saponins from *C. spiralis*, along with evaluations of their anti-inflammatory properties.

Materials and Methods

Plant material

Fresh rhizomes of *Costus spiralis* were obtained from the Ornamental Plant Garden of Federal University of Rio de Janeiro, in September 2000 and a voucher specimen is maintained in the Laboratory of Chemistry of Medicinal Plants.

General procedures

Melting points were determined by an Electrothermal 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 LSIMS 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 and a Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer, respectively. GC was carried out with FID, using a glass capillary column (0.25 m× 25 m, 0.25 micron, J. W. Scientific Inc.) DB-1. NMR spectra were measured in C_5D_5N (100 mg of steroidal saponin 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 saponins **1** and **2**, (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 saponins **1** and **2** and monosaccharides and $CeSO_4$ for pseudosapogenin.

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Extraction and isolation

Fresh rhizomes (1 kg) were extracted with 80% aqueous MeOH (3 l) for 72 h at room temperature. The extract was concentrated under reduced pressure to remove most of the MeOH and the resulting aqueous phase 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*, the residue dissolved in MeOH, precipitated by Et_2O addition, and washed with Et_2O to give a crude material (3.8 g). The residue was dissolved in MeOH and roughly chromatographed on Sephadex LH-20 with MeOH. The fractions were combined based on the TLC profiles to give the saponin mixture (1.3 g). Further purification by chromatography on a silica gel column provided several fractions which two TLC homogeneous compounds **1** (270 mg, R_f 0.52) and **2** (153 mg, R_f 0.55) which gave dark green colors with orcinol/H_2SO_4.

Compound **1**

Colorless needles. – $[\alpha]_D^{25}$ – 106° (*c* 0.1, MeOH). – M.p. 216–218 °C. – IR (KBr): ν_{max} = 3430 (OH), 1050 (C–O), 913, 838, 813, 638 cm^{-1} [(25*R*)-furostanol, intensity 913 < 838]. – Negative LSIMS: *m/z* = 1033 $[M-H]^-$. – ^1H and ^{13}C NMR data: Tables I and II.

Compound **2**

Colorless needles. – $[\alpha]_D^{25}$ – 110° (*c* 0.1, MeOH). – M.p. 218–220 °C. – IR (KBr): ν_{max} = 3430 (OH), 1050 (C–O), 913, 838, 813, 638 cm^{-1} [(25*R*)-furostanol, intensity 913 < 838]. – Negative LSIMS: *m/z* = 1033 $[M-H]^-$. – ^1H and ^{13}C NMR data: Tables I and II.

Acid hydrolysis of **1** *and* **2**

A solution of each compound (100 mg) in 1 M HCl/1,4-dioxan (1:1 v/v; 10 ml) was heated in a sealed tube for 1 h at 100 °C. After cooling, the reaction mixture was neutralized with 3% KOH in 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 (93 mg) which was chromatographed on silica gel CC with $CHCl_3$/MeOH/H_2O (7:3:0.2) to yield the diosgenin (30 mg) and a sugar mixture. Identity of diosgenin was established by comparison with an authentic sample through m.p., IR, ^1H and ^{13}C NMR and EIMS. The sugar mixture was dissolved in pyridine and analyzed by silica gel-TLC in the above described solvent system. After spraying, apiose gave a weak yellow spot at R_f 0.78, rhamnose gave a green spot at R_f 0.75 and glucose gave a blue spot at R_f 0.70.

Molar carbohydrate composition and D,L *configurations*

The molar carbohydrate composition of **1** and **2** were determined by GC-MS analyses of their 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

Compounds **1** and **2** were 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).

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.*

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 compounds **1** and **2** (100 μg/g body weight) and a positive control, indomethacin (10 μg/g body weight). After injection 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

Table I. Selected ^{1}H NMR assignments (δ(ppm), J [Hz]) of compounds **1** and **2** in C_5D_5N[a].

Position	**1**	**2**	^{1}H-^{1}H-COSY
Aglycone characteristic proton signals			
H-6	5.30 br s	5.30 br s	H-7
Me-18	0.85 s	0.85 s	
Me-19	1.10 s	1.10 s	
Me-21	1.22 d (6.8)	1.22 d (6.8)	H-20
Me-27	0.98 d (6.6)	0.98 d (6.6)	H-25
Sugar methyl group and anomeric protons			
Rha-Me	1.78 d (6.3)	1.76 d (6.3)	Rha-H-5
iGlc-H-1	4.98 d (7.7)	4.96 d (7.7)	iGlc-H-2
tGlc-H-1	4.82 d (7.8)	4.84 d (7.8)	tGlc-H-2
Rha-H-1	6.30 br s	5.80 br s	Rha-H-2
Api-H-1	5.95 d (3.5)	5.90 d (3.5)	Api-H-2

[a] The following conventions were used: iGlc = inner glucose, tGlc = terminal glucose.

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 rhizomes of *C. spiralis* 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 compounds **1** and **2** which were detected with orcinol/H_2SO_4 reagent. Compounds **1** and **2** were obtained as colorless needles and gave positive Liebermann-Burchard test for steroidal saponins. The LSIMS showed an ion peak $[M\text{-}H]^-$ at *m/z* 1033 which, together with ^{13}C NMR spectral data (Table II), suggested the molecular formula as $C_{50}H_{82}O_{22}$ for compounds **1** and **2**.

In addition to this, the furostanol glycosidic nature of **1** and **2** was indicated by the strong absorption bands at 3430 cm^{-1} for the hydroxyl group and a 25*R*-furostan steroidal structure (813, 838 and 913 cm^{-1}, intensity 913 < 838 cm^{-1}) 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; Shao *et al.*, 1997). The ^{1}H NMR spectral data (Table I) of compounds **1** and **2** contained a signal for the olefinic proton at δ 5.30 (br s, H-6), two secondary methyl protons at δ 1.22 (d, J = 6.8 Hz, 3H-21) and 0.98 (d, J = 6.6 Hz, 3H-27) and two angular methyl protons at δ 1.10 (s, 3H-19) and 0.85 (s, 3H-18). In addition to this, the COLOC

Table II. ^{13}C NMR data of the aglycone and carbohydrate moieties of compounds **1** and **2** in C_5D_5N[a].

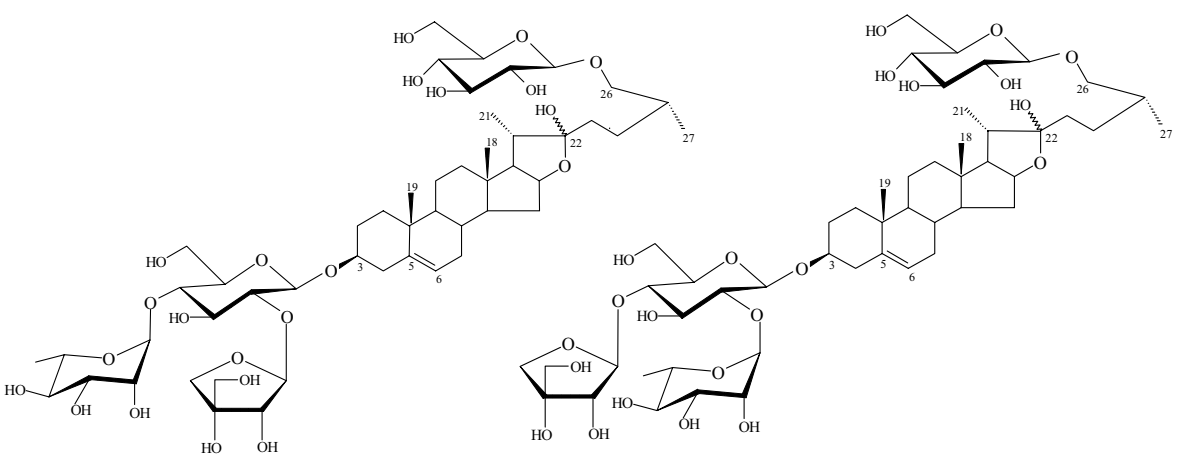
C	**1**	C	**1**	C	**2**	C	**2**
1	37.2	iGlc 1	100.1	1	37.1	iGlc 1	100.0
2	29.7	2	77.8	2	29.5	2	77.8
3	77.9	3	78.1	3	77.9	3	78.0
4	38.5	4	78.8	4	38.5	4	78.8
5	140.3	5	76.9	5	140.3	5	76.7
6	121.5	6	61.2	6	121.4	6	61.2
7	32.2	tGlc 1	104.5	7	32.2	tGlc 1	104.7
8	31.2	2	74.8	8	31.3	2	74.7
9	50.2	3	78.1	9	49.9	3	78.1
10	36.9	4	71.8	10	36.7	4	71.9
11	20.9	5	78.8	11	20.7	5	78.7
12	39.6	6	62.7	12	39.5	6	62.3
13	40.7	Rha 1	102.7	13	40.5	Rha 1	101.8
14	56.5	2	72.3	14	56.3	2	72.2
15	31.9	3	72.2	15	31.7	3	72.3
16	80.9	4	73.7	16	80.7	4	73.7
17	63.7	5	69.3	17	63.5	5	69.3
18	16.3	6	18.2	18	16.2	6	18.2
19	18.5	Api 1	110.5	19	18.3	Api 1	110.7
20	40.5	2	77.3	20	40.3	2	77.2
21	16.5	3	79.9	21	16.3	3	79.6
22	110.7	4	74.8	22	110.5	4	74.8
23	30.3	5	64.3	23	30.1	5	64.2
24	27.9			24	27.7		
25	33.9			25	33.7		
26	74.9			26	74.7		
27	16.9			27	16.7		

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

spectrum displayed long range couplings between H-1 of the terminal glucose and C-26 of the aglycone. The above ^{1}H NMR spectral data and a comparison of the ^{13}C NMR signals of the aglycone moiety of **1** and **2** (Table II) with those described in the literature (Agrawal *et al.*, 1985; Shao *et al.*, 1997) showed the structure of the aglycone to be (3β,25*R*)-22-hydroxyfurost-5-ene-3,22,26-triol.

In the ^{13}C NMR spectrum of **1** and **2**, a 2,4-linked inner β-D-glucopyranosyl unit, a terminal β-D-glucopyranosyl unit, a terminal α-L-rhamnopyranosyl unit and a terminal β-D-apiofuranosyl unit were clearly observed. The COLOC spectrum of compound **1** displayed long range couplings between inner glucose-H-1 at δ 4.98 and aglycone-C-3 at δ 77.9, between terminal glucose-H-1 at δ 4.82 and aglycone-C-26 at δ 74.9, between apiose-H-1 at δ 5.95 and inner glucose-C-2 at δ 77.8, and between rhamnose-H-1 at δ 6.30 and inner glucose-C-4 at δ 78.8, indicating that the apiose and rhamnose were linked to the C-2 and C-4 of the inner glucose, respectively. The COLOC spectrum of compound **2** displayed long range couplings between inner glucose-H-1 at δ 4.96 and aglycone-C-3 at δ 77.9, between terminal glucose-H-1 at δ 4.84 and aglycone-C-26 at δ 74.7, between apiose-H-1 at δ 5.90 and inner glucose-C-4 at δ 78.8, and between rhamnose-H-1 at δ 5.80 and inner glucose-C-2 at δ 77.8, indicating that the apiose and rhamnose were linked to the C-4 and C-2 of the inner glucose, respectively. The signal at δ 1.78 and 1.76 were due to the methyl group of rhamnose of **1** and **2**, respectively. The methylation analyses (Parente *et al.*, 1985) showed a terminal glucopyranose, a terminal apiofuranose, a terminal rhamnopyranose and a 2,4-linked glucopyranose for **1** and **2**.

On acid hydrolysis, compounds **1** and **2** gave a pseudosapogenin, glucose, rhamnose and apiose. The pseudosapogenin was identified as diosgenin by direct comparison of TLC, m.p., IR, ^{1}H and ^{13}C NMR and EIMS with an authentic sample. The molar carbohydrate composition of **1** and **2** indicated the presence of four neutral monosaccharides: glucose/rhamnose/apiose (2.0:0.9:0.9; the molar responses of rhamnose and apiose are taken as 1.0) (Kamerling *et al.*, 1975). Their absolute configurations were determined by GC of their trimethylsilylated (−)-2-butylglycosides (Gerwig *et al.*, 1978). D-glucose, L-rhamnose and D-apiose were detected. Consequently, on the basis of IR, ^{1}H and ^{13}C NMR spectroscopy, LSIMS and chemical reactions, the structures of the steroidal saponins **1** and **2** were established as (3β,25*R*)-26-(β-D-glucopyranosyloxy)-22-hydroxyfurost-5-en-3-yl *O*-D-apio-β-D-furanosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside and (3β,25*R*)-26-(β-D-glucopyranosyloxy)-22-hydroxyfurost-5-en-3-yl *O*-D-apio-β-D-furanosyl-(1→4)-*O*-[α-L-rham-



1 **2**

Fig. 1. (3β,25*R*)-26(β-D-glucopyranosyloxy)-22-hydroxyfurost-5-en-3-yl *O*-D-apio-β-D-furanosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (**1**) and (3β,25*R*)-26(β-D-glucopyranosyloxy)-22-hydroxyfurost-5-en-3-yl *O*-D-apio-β-D-furanosyl-(1→4)-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**2**).

nopyranosyl-(1→2)]-β-D-glucopyranoside, respectively (Fig. 1).

In order to confirm popular informations about the use of this plant against inflammatory conditions and based on literature reports of anti-inflammatory activities of steroidal saponins (Lacaille-Dubois and Wagner, 1996), this pharmacological property was evaluated for compounds **1** and **2** using the capillary permeability assay (Whittle, 1964). In the regular dose of 100 mg/kg, compounds **1** and **2** showed inhibition of the increase in vascular permeability (Fig. 2) caused by acetic acid, which is a typical model of first stage inflammatory reaction. This result suggests that the steroidal saponins **1** and **2** may be the potential therapeutic agents involved in inflammatory disorders justifying the use of *C. spiralis* in Brazilian traditional medicine.

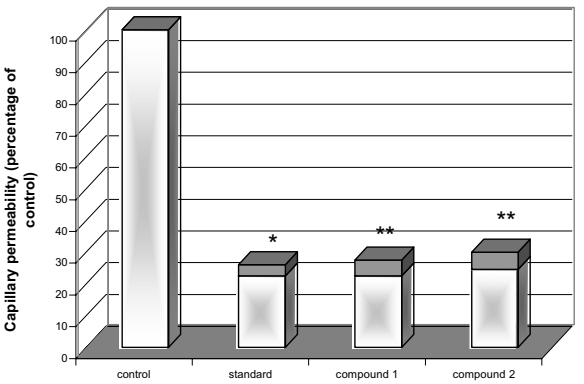


Fig. 2. Effect of compounds **1** and **2** (100 mg/kg, p.o.) on acetic acid-induced vaular permeability in mice. Standard: indomethacin (10 mg/kg, p.o.). Results are mean +/– S.E.M. (n : 5); * $p < 0.05$, ** $p < 0.01$ *vs.* control; Student's t-test.

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