New Polyhydroxylated Steroidal Sapogenin and Saponin from the Leaves of *Cestrum sendtenerianum*

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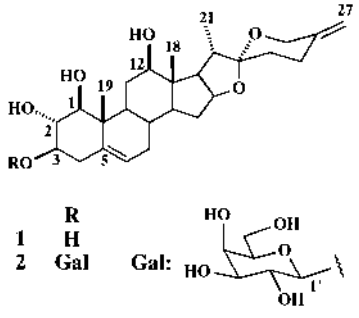
New polyhydroxylated spirostanol sapogenin (1) and saponin (2) were isolated from the leaves of *Cestrum sendtenerianum* (Solanaceae). Their structures were determined to be spirosta-5,25(27)-diene-1β,2α,3β,12β-tetrol (1) and spirosta-5,25(27)-diene-1β,2α,3β,12β-tetrol 3-*O*-β-D-galactopyranoside (2), respectively, on the basis of spectroscopic analysis, including two-dimensional NMR techniques, and the result of hydrolytic cleavage.

Key words *Cestrum sendtenerianum*; Solanaceae; spirostanol sapogenin; spirostanol saponin

The genus *Cestrum* (Solanaceae), with more than 300 species, is mainly distributed in South America. Although there are several applications of the *Cestrum* plants in folk medicine,[1)] the toxicity of these species to humans and livestock has been frequently reported.[2)] The search for toxic principles has been conducted, and a glycoside of 1,25-dihydroxycholecalciferol, which causes mortal carcinosis in cattle, from *C. diurnum*,[2a,d)] and a kaurene type diterpene glycoside, carboxyparquin, from *C. parqui*,[2b)] have been revealed to be responsible for the toxicity of the plants. *C. sendtenerianum* is indigenous to Brazil and has also been reputed to be a poisonous plant. A survey of literature, however, showed that no chemical analysis has been done on the plant. We have now carried out a phytochemical analysis of the leaves of *C. sendtenerianum* and isolated new polyhydroxylated spirostanol sapogenin (**1**) and saponin (**2**). This paper provides detailed evidence which is consistent with the structural assignment of **1** and **2** as spirosta-5,25(27)-diene-1β,2α,3β,12β-tetrol (**1**) and spirosta-5,25(27)-diene-1β,2α,3β,12β-tetrol 3-*O*-β-D-galactopyranoside (**2**), respectively.

The ethanolic extract of the leaves of *C. sendtenerianum* (dry weight 500 g) was partitioned between 1-butanol and H_2O. The 1-butanol-soluble portion was fractionated by subjecting it to chromatography on a silica gel column followed by preparative medium-pressure liquid chromatography (MPLC) and preparative HPLC to furnish compounds **1** (12.0 mg) and **2** (12.4 mg).

Compound **1**, isolated as an amorphous solid, $[\alpha]_D$ −61.4° (methanol), showed in the high-resolution (HR) FAB-MS (positive mode) an $[M+H]^+$ peak at *m/z* 461.2900 in accordance with an empirical molecular formula $C_{27}H_{40}O_6$, also deduced on the basis of ^{13}C-NMR data. The IR spectrum showed a broad absorption band attributable to hydroxyl groups near 3380 cm^{-1}. The ^{1}H-NMR spectrum in pyridine-d_5 showed signals for two tertiary methyl groups at δ 1.37 and 1.17 (each 3H, s), a secondary methyl group at δ 1.41 (3H, d, J=6.7 Hz), an exomethylene group at δ 4.83 and 4.79 (each 1H, br s), and an olefinic proton at δ 5.62 (1H, br d, J=5.4 Hz). These ^{1}H-NMR data in addition to two pairs of olefinic carbon signals at δ 139.3 (C)/124.5 (CH) and 144.6 (C)/108.6 (CH_2) and an acetalic quaternary carbon at δ 109.7 in the ^{13}C-NMR spectrum suggested **1** to be a steroidal sapogenin based upon spirosta-5,25(27)-diene.[3)] From a preliminary inspection of the ^{13}C-NMR spectrum of **1**, seven signals at δ 82.5 (CH), 81.5 (CH), 79.4 (CH), 78.1 (CH), 73.2 (CH), 65.0 (CH_2) and 63.1 (CH) could be identified between 60—90 ppm, and three of them, at δ 81.5 (CH), 65.0 (CH_2) and 63.1 (CH), were assignable to the C-16, C-26 and C-17 positions, respectively, in a spirostanol skeleton.[3)] Consequently, the remaining four signals, at δ 82.5 (CH), 79.4 (CH), 78.1 (CH) and 73.2 (CH), were due to hydroxymethine groups. The presence of four hydroxyl groups in the molecule was further ascertained by the fact that the treatment of **1** with acetic anhydride in pyridine gave the corresponding tetraacetate (**1a**). The locations of the hydroxyl groups were fixed by analysis of the ^{1}H–^{1}H shift correlation spectroscopy (^{1}H–^{1}H COSY), homonuclear Hartmann–Hahn (HOHAHA), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) and ^{1}H-detected heteronuclear multiple-bond connectivities (HMBC) spectra. The hydroxymethine proton signal at δ 4.04 was revealed to be coupled with the two hydroxymethine proton resonances at δ 3.65 and 3.91. The signal at δ 3.91 had spin-coupling links with a geminal pair of the protons appearing at δ 2.67 and 2.80, while the resonance at δ 3.65 showed no additional coupling correlation. In the HMBC spectrum, the methine proton signal at δ 3.65 showed correlation peaks with the carbon signals at δ 50.9 (C-9), 43.7 (C-10) and 15.0 (C-19). The signal of one of the geminal pair protons at δ 2.67 exhibited HMBC correlations with the olefinic carbon signals at δ 139.3 (C-5) and 124.5 (C-6), as well as with the C-10 carbon. The geminal protons were shown to be attached to a carbon with a resonance at δ 41.1, from which a long-range correlation was detected to



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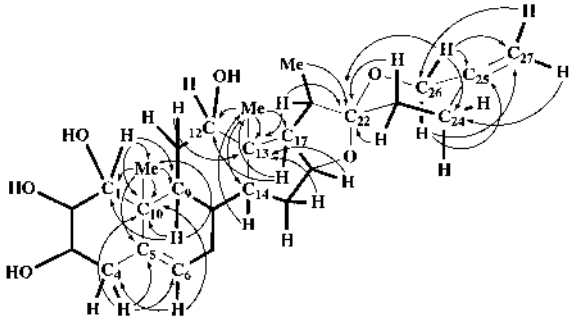

Fig. 1. HMBC Correlations of **1**

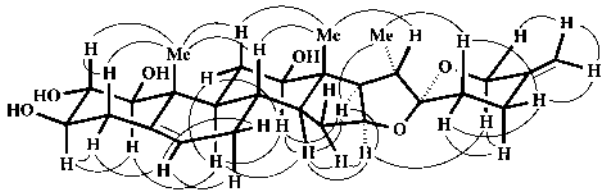

Fig. 2. NOE Correlations of **1**

the H-6 proton. The above findings allowed us to deposit the three hydroxyl groups at the C-1, C-2 and C-3 positions. Tracing out the successive proton spin-couplings through the ^{1}H–^{1}H COSY and HOHAHA spectra, the three-proton doublet at δ 1.41 (J=6.7 Hz) assignable to Me-21, being used as the starting point of analysis, led us to confirm the locus of the remaining hydroxyl group at C-12. This was further supported by long-range correlations from the C-12 carbon resonance at δ 79.4 to the H-17 signal at δ 2.22 and Me-18 at δ 1.17. Thus, the plane structure of **1** was established as a spirosta-5,25(27)-diene derivative with four hydroxyl groups at C-1, C-2, C-3 and C-12. The nuclear Overhauser effect (NOE) correlations from Me-19 to H-4ax, H-8 and H-11ax, H-14 to H-9, H-16 and H-17, Me-18 to H-8, H-11ax and H-20, H-16 to H-17 and H-26ax, and Me-21 to H-23ax in the phase-sensitive NOE correlation spectroscopy (NOESY) spectrum indicated that **1** had the usual spirostanol ring junctions and configurations of B/C *trans*, C/D *trans*, D/E *cis*, C-20α and C-22α. All the hydroxyl functions were revealed to be present in the equatorial orientations by the coupling constants of the hydrogens bearing hydroxyl groups, $^3J_{\text{H-1,H-2}}$= 9.2 Hz, $^3J_{\text{H-2,H-3}}$=9.2 Hz, $^3J_{\text{H-3,H-4ax}}$=11.6 Hz and $^3J_{\text{H-3,H-4eq}}$= 5.3 Hz, and $^3J_{\text{H-12,H-11ax}}$=11.2 Hz and $^3J_{\text{H-12,H-11eq}}$=4.3 Hz in the ^{1}H-NMR spectrum of **1**. This was supported by the strong NOEs from H-1 to H-3 and H-9, H-2 to H-4ax and Me-19, and H-12 to H-9, H-14 and H-17 in the phase-sensitive NOESY spectrum. All of these data were consistent with the structure spirosta-5,25(27)-diene-1β,2α,3β,12β-tetrol for **1**.

Compound **2** was also obtained as an amorphous solid, $[\alpha]_D$ −47.6° (methanol). The HR FAB-MS showed an $[M+Na]^+$ ion at *m/z* 645.3245, consistent with the molecular formula $C_{33}H_{50}O_{11}$. The prominent ^{1}H-NMR signals at δ 4.82 and 4.77 (each 1H, br s), 1.38 (3H, d, J=6.5 Hz), 1.23 and 1.14 (each 3H, s), and 5.51 (1H, br s, J=5.2 Hz) were essentially analogous to those of **1**. In addition, an anomeric proton signal due to a hexopyranose could be identified at δ 5.01 (1H, d, J=7.7 Hz). The ^{1}H–^{1}H COSY and HOHAHA experiments allowed the sequential assignment of the resonances for the hexopyranosyl residue, starting from the anomeric proton signal. Multiplet patterns and measurements of coupling constants were strongly suggestive of the presence of a β-galactopyranosyl moiety in the form of the $^4C_1$ conformation in the molecule. The six carbon signals at δ 103.8 (CH), 72.3 (CH), 75.2 (CH), 70.2 (CH), 77.2 (CH) and 62.4 (CH_2) in the ^{13}C-NMR spectrum of **2** agreed with those of authentic methyl β-galactopyranoside. Acid hydrolysis of **2** with 1 M hydrochloric acid in dioxane–H_2O (1 : 1) gave **1** as a sapogenin and D-galactose. Thus, it was found that **2** was a β-D-galactopyranoside of **1**. The galactosyl group was revealed to be attached at the C-3 hydroxyl position of the aglycon by a long-range correlation between the anomeric proton at δ 5.01 and the C-3 carbon of the aglycon at δ 81.3. Accordingly, the structure of **2** was formulated as spirosta-5,25(27)-diene-1β,2α,3β,12β-tetrol 3-*O*-β-D-galactopyranoside.

Compounds **1** and **2** are therefore a new polyhydroxylated spirostanol sapogenin and saponin, respectively.

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for Compounds **1** and **2**[a]

	1			**2**
	^{1}H-NMR	J (Hz)	^{13}C-NMR	^{13}C-NMR
1	3.65 d	9.2	82.5	82.1
2	4.04 dd	9.2, 9.2	78.1	75.5
3	3.91 ddd	11.6, 9.2, 5.3	73.2	81.3
4eq	2.67 dd	13.3, 5.3	41.1	37.9
ax	2.80 dd	13.3, 11.6		
5	—		139.3	138.0
6	5.62 br d	5.4	124.5	125.1
7α	1.56		32.1	32.0
β	1.97			
8	1.68		31.6	31.5
9	1.55		50.9	50.7
10	—		43.7	43.2
11eq	3.32 ddd	13.6, 4.3, 4.3	34.3	34.2
ax	2.04 dd-like	13.6, 11.2		
12	3.69 dd	11.2, 4.3	79.4	79.3
13	—		45.9	45.9
14	1.18		55.3	55.2
15α	2.10		32.1	32.0
β	1.65			
16	4.62 q-like	7.3	81.5	81.4
17	2.21 dd	7.3, 6.6	63.1	63.0
18	1.17 s		11.3	11.2
19	1.37 s		15.0	14.8
20	2.22		42.9	42.9
21	1.41 d	6.7	14.3	14.3
22	—		109.7	109.7
23eq	1.83		33.3	33.3
ax	1.87 ddd	12.9, 12.9, 4.8		
24eq	2.26		29.0	29.0
ax	2.76 ddd	12.9, 12.9, 4.8		
25	—		144.6	144.6
26eq	4.06 d	12.1	65.0	65.0
ax	4.52 d	12.1		
27	4.83 br s		108.6	108.5
	4.79 br s			
1′				103.8
2′				72.3
3′				75.2
4′				70.2
5′				77.2
6′				62.4

[a] Spectra were measured in pyridine-d_5.

Experimental

Optical rotations were measured using a JASCO DIP-360 automatic digital polarimeter. IR spectra were recorded on a JASCO A-100 spectrophotometer. NMR spectra were recorded on a Bruker DPX-400 spectrometer (400 MHz for ^{1}H-NMR) or on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H-NMR) using XWIN-NMR pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as an internal standard. MS were recorded on a VG AutoSpec E mass spectrometer. Silica gel 60 (Merck, Germany) was used for open column chromatography. MPLC was performed with a CIG column system (Kusano Scientific, Japan) equipped with a glass column [22 mm i.d.×300 mm, octadecylsilanized (ODS) silica gel, 20 μm]. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck, Germany) and RP-18 F_{254} S (0.25 mm thick, Merck) plates, and spots were visualized by spraying the plates with 10% H_2SO_4 solution, followed by heating. Preparative HPLC was performed using an Inertsil PREP-ODS column (20 mm i.d.×250 mm, ODS, 10 μm, GL Science, Japan,).

Plant Material *Cestrum sendtenerianum* was collected in the fields of Itapetininga City, São Paulo State. It was identified by Dr. Sylvio Panizza, Universidade de São Paulo, and a voucher of the plant is on file in our laboratory.

Extraction and Isolation The exsiccated leaves of the plant material (500 g) were extracted with hot EtOH three times (each 960 ml). The EtOH extract was concentrated under reduced pressure to give a crude residue (71 g). This was partitioned between *n*-BuOH saturated with H_2O and H_2O. Column chromatography of the *n*-BuOH-soluble phase (39 g) silica gel and elution with stepwise gradients of $CHCl_3$–MeOH (9 : 1; 4 : 1; 7 : 3), followed by $CHCl_3$–MeOH–H_2O (20 : 10 : 1; 7 : 4 : 1), and finally with MeOH alone, gave six fractions, I (0.112 g), II (0.123 g), III (0.177 g), IV (0.737 g), V (4.79 g) and VI (18.4 g). Fraction V was further fractionated by submitting it to MPLC using MeOH–H_2O (4 : 1) to collect 22 fractions (V-1—V-22). Fractions V-15—V-17 were combined (0.224 g) and repeatedly subjected to preparative HPLC using MeCN–H_2O (2 : 3) to afford **2** (12.4 mg). Fraction V-20 (0.173 g) was separated by preparative HPLC using MeOH–H_2O (7 : 3) to furnish **1** (12.0 mg).

Compound **1**: An amorphous solid, $[\alpha]_D^{25}$ −61.4° (MeOH, c=0.14). HR FAB-MS (positive mode) *m/z*: 461.2900 $[M+H]^+$ ($C_{27}H_{41}O_6$, Calcd for 461.2903). IR ν_{max} (KBr) cm^{-1}: 3380 (OH), 2950, 2920 and 2850 (CH), 1450, 1365, 1340, 1255, 1225, 1180, 1155, 1065, 1040, 1015, 975, 955, 915, 890, 875, 830.

Acetylation of **1**: Compound **1** (3 mg) was acetylated with Ac_2O (0.5 ml) in pyridine (0.5 ml) containing 3 mg of 4-(dimethylamino)pyridine at room temperature for 24 h. The crude acetate was chromatographed on silica gel and eluted with hexane–Me_2CO (3 : 1) to afford the corresponding tetraacetate (**1a**) (2.5 mg). Compound **1a**: An amorphous solid. IR ν_{max} (KBr) cm^{-1}: 2920 and 2855 (CH), 1755 (C=O), 1440, 1365, 1245, 1090, 1070, 1040, 1020, 975, 950, 915, 870, 835, 795. ^{1}H-NMR (chloroform-*d*) δ: 5.73 (1H, br d, J=6.1 Hz, H-6), 5.25 (1H, dd, J=10.2, 10.2 Hz, H-2), 4.86 (1H, d, J=10.2 Hz, H-1), 4.78 and 4.74 (each 1H, br s, H_2-27), 4.74 (1H, ddd, J=12.0, 10.2, 6.2 Hz, H-3), 4.45 (1H, q-like, J=7.2 Hz, H-16), 4.39 (1H, dd, J=10.6, 4.3 Hz, H-12), 4.27 and 3.84 (each 1H, br d, J=12.2 Hz, H_2-26), 2.03, 2.02, 2.00 and 1.98 (each 3H, s, Ac×4), 1.20 (3H, s, Me-19), 0.90 (3H, d, J=6.5 Hz, Me-21), 0.88 (3H, s, Me-18).

Compound **2**: Amorphous solid, $[\alpha]_D^{25}$ −47.6° (MeOH, c=0.25). HR FAB-MS (positive mode) *m/z*: 645.3245 $[M+Na]^+$ ($C_{33}H_{50}O_{11}$·Na, Calcd for 645.3251). IR ν_{max} (KBr) cm^{-1}: 3390 (OH), 2950 and 2905 (CH), 1445, 1365, 1225, 1065, 1035, 975, 955, 915, 890, 870, 830. ^{1}H-NMR (pyridine-d_5) δ: 5.51 (1H, br d, J=5.2 Hz, H-6), 5.01 (1H, d, J=7.7 Hz, H-1′), 4.82 and 4.77 (each 1H, br s, H_2-27), 4.62 (1H, q-like, J=7.3 Hz, H-16), 4.55 (1H, d, J=3.0 Hz, H-4′), 4.51 and 4.06 (each 1H, br d, J=12.3 Hz, H_2-26), 4.50 (1H, dd, J=9.5, 7.7 Hz, H-2′), 4.48 (1H, dd, J=11.1, 6.7 Hz, H-6′a), 4.42 (1H, dd, J=11.1, 5.2 Hz, H-6′b), 4.21 (1H, dd, J=9.5, 3.0 Hz, H-3′), 4.14 (1H, dd, J=6.7, 5.2 Hz, H-5′), 3.98 (1H, dd, J=8.8, 8.8 Hz, H-2), 3.96 (1H, m, H-3), 3.66 (1H, dd, J=11.0, 4.2 Hz, H-12), 3.56 (1H, d, J=8.8 Hz, H-1), 1.38 (3H, d, J=6.5 Hz, Me-21), 1.23 (3H, s, Me-19), 1.14 (3H, s, Me-18).

Acid Hydrolysis of **2**: A solution of **2** (5 mg) in 1 M HCl (dioxane–H_2O, 1 : 1, 2 ml) was heated at 100 °C for 2 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo) column and chromatographed on silica gel using a gradient mixture of $CHCl_3$–MeOH (9 : 1; 4 : 1; 1 : 1) to give an aglycon (**1**) (2.6 mg) and a sugar fraction (0.9 mg). HPLC analysis of the sugar fraction under the following conditions showed the presence of D-galactose. Column: Kaseisorb NH_2-60-5 (4.6 mm i.d.×250 mm, Tokyo-Kasei, Japan); detector: Shodex OR-2 (Showa-Denko, Japan) and Tosoh RI-8010 (Tosoh, Japan); solvent: MeCN–H_2O (3 : 1); flow rate: 0.6 ml/min. t_R 18.86 min (positive polarity).

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