

## New Epimeric Spirostanol and Furostanol-Type Steroidal Saponins from *Cestrum laevigatum* L.

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Four new epimeric spirostanol and furostanol-type steroidal saponins, (25*R,S*)-5 $\alpha$ -spirostan-2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside, (25*R,S*)-5 $\alpha$ -spirostan-2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside, 26-*O*- $\beta$ -D-glucopyranosyl-(25*R,S*)-5 $\alpha$ -furost-20-ene,2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-galactopyranoside and 26-*O*- $\beta$ -D-glucopyranosyl-(25*R,S*)-5 $\alpha$ -furost-20-ene,2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside, in addition to the known (25*R,S*)-5 $\alpha$ -spirostan-2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-galactopyranoside, were isolated from *Cestrum laevigatum*. Compounds were submitted to cytotoxic activity assays using colorectal adenocarcinoma (HCT-116), human promyelocytic leukemia (HL-60), ovarian carcinoma (OVCAR-8), glioma (SF-295) human cancer cell lines, and the antimicrobial activity was evaluated against *Candida parapsilosis*, *C. albicans*, *C. krusei*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*.

**Keywords:** *Cestrum laevigatum*, saponins, spirostanol, furostanol, cytotoxic, antimicrobial

### Introduction

Steroidal saponins are constituted mainly by spirostanol and furostanol-type glycosides. Spirostanol glycosides are the largest group and comprises aglycones with the spirostan skeleton containing a sugar chain generally at C-3 position and a spiro-bicyclic acetal at C-22, while furostanol derivatives usually present a hemiacetal with a hydroxy or methoxy moiety at C-22 or yet a  $\Delta^{(20,22)}$ -unsaturation, besides a glycosidic linkage

at C-26. In both skeletons, C-25 is naturally found with either *R* or *S* configuration, or as inseparable epimeric mixtures.<sup>1,2</sup> Furostanol are recognized as biogenetic precursors of spirostanol, since the 26-*O* glucoside unit can be enzymatically cleaved and a ring closure to 26-OH takes place with dehydration of 22-OH.<sup>3</sup> So, the co-occurrence of spirostanol saponins with the correspondent furostanols, in innumerable plants, is the consequence of this ready conversion.

The isolation of bioactive steroidal saponins has been reported to several species of reputed poisonous plants belonging to the Solanaceae.<sup>1,4,5</sup> Based on these findings, we have investigated *Cestrum laevigatum*, one of the most toxic plants lethal to mammals in the Brazilian livestock,

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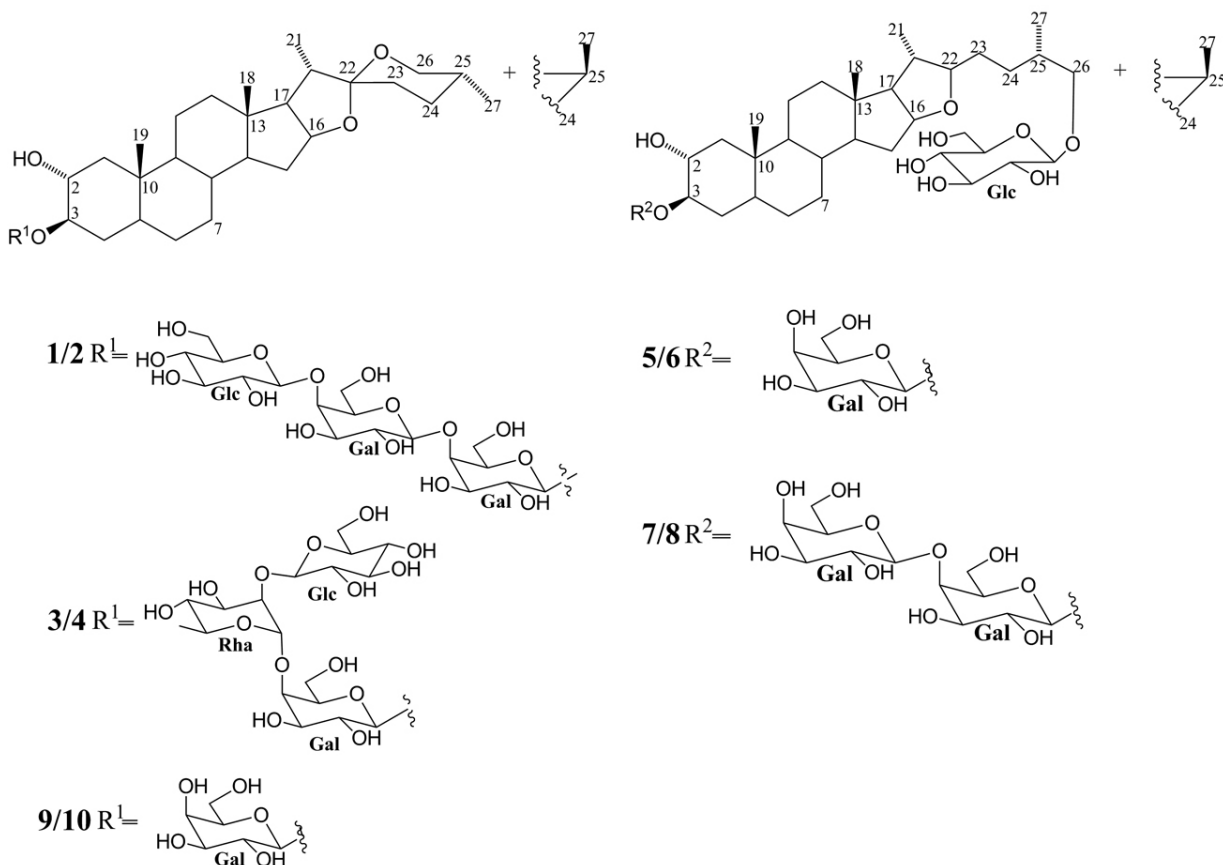
among the group of common invasive species that cause liver damage.<sup>6,7</sup>

In this study, we have isolated epimeric mixtures of the new (25*R,S*)-5 $\alpha$ -spirostan-2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside (**1/2**), (25*R,S*)-5 $\alpha$ -spirostan-2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside (**3/4**), 26-*O*- $\beta$ -D-glucopyranosyl-(25*R,S*)-5 $\alpha$ -furost-20-ene,2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-galactopyranoside (**5/6**) 26-*O*- $\beta$ -D-glucopyranosyl-(25*R,S*)-5 $\alpha$ -furost-20-ene,2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside (**7/8**), in addition to the known (25*R,S*)-5 $\alpha$ -spirostan-2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-galactopyranoside (**9/10**) (Figure 1). The cytotoxicity of all compounds was evaluated against four human tumor cell lines: colorectal adenocarcinoma (HCT-116), human promyelocytic leukemia (HL-60), ovarian carcinoma (OVCAR-8) and glioma (SF-295). The screening for antifungal activities was performed against *Candida parapsilosis* ATCC 22019, *Candida albicans* ATCC 10231 and *Candida krusei* ATCC 14243, while the antimicrobial activity was assayed against *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633.

## Experimental

### General experimental procedures

Melting points were obtained on a Mettler Toledo FP82HT apparatus (Columbus, OH, USA) and were uncorrected. Infrared (IR) spectra were recorded as KBr pellets on a PerkinElmer FT-IR Spectrum 1000 (Waltham, MA, USA). The nuclear magnetic resonance (NMR) spectra were performed on an Agilent VNMR-600 (Santa Clara, CA, USA) or Bruker Avance DRX 500 (Billerica, MA, USA) spectrometers. The proton (<sup>1</sup>H) and carbon 13 (<sup>13</sup>C) chemical shifts are expressed in the  $\delta$  scale and were referenced to tetramethylsilane (TMS) through the residual solvent. High resolution mass spectra were recorded on a Waters Acquity UPLC system (Milford, MA, USA) coupled with a quadrupole/time-of-flight (TOF) system (UPLC/Qtof MSE spectrometer) in the positive mode. TOF conditions were as follow: source temperature 120 °C; desolvation temperature 350 °C; desolvation gas flow of 500 L h<sup>-1</sup>; capillary voltage 3.2 kV; collision energy ramp 20 eV. Data were recorded from *m/z* 110 to 1200 Da. High performance liquid chromatography (HPLC) analyses were performed on a Shimadzu chromatograph equipped with a ternary



**Figure 1.** Steroidal saponins isolated from *Cestrum laevigatum*.

pump (Shimadzu LC-20AT) and UV detector (Shimadzu SPD-M20A; Tokyo, Japan), using Phenomenex RP-18 column (analytical: 250 × 4.6 mm, 5 μm; semi-preparative: 250 × 10 mm, 10 μm; Torrance, CA, USA). HPLC grade solvents were purchased from Tedia Co (Brasil, São Paulo) and the HPLC grade water was obtained by a Milli-Q purification system (Millipore, Bedford, MA, USA). Gas chromatography (GC) analyses were performed on an Agilent 7890B/5977A GC/MSD (Santa Clara, CA, USA) using a CP-ChiraSil-L-Val (30 m × 0.25 mm × 0.25 mm) column. Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and solid phase extraction (SPE) C18 cartridge (Strata, 5 g × 20 mL; Phenomex, Torrance, CA, USA). Thin layer chromatography (TLC) was performed on precoated silica gel aluminum sheets (Kieselgel 60 F<sub>254</sub>, 0.20 mm; Merck, Darmstadt, Germany). Compounds were visualized by ultraviolet (UV) detection and by spraying with vanillin/perchloric acid/ethanol (EtOH) solution, followed by heating.

#### Plant material

*Cestrum laevigatum* L. was collected at Guaramiranga Mountain, Pacoti, Ceará State, Northeast of Brazil (August 2012). Voucher specimens (#38643) were deposited at the Herbário Prisco Bezerra (EAC) and identified by MSc Edson de Paula Nunes, Departamento de Biologia, Universidade Federal do Ceará, Ceará State, Brazil.

#### Extraction and isolation

Stems of *C. laevigatum* (3.03 kg) were pulverized and extracted with hexane (3 × 5 L) at room temperature. The solvent was removed under reduced pressure to yield the hexane extract (0.3 g). The plant residue was then extracted with EtOH (3 × 6 L) to yield the corresponding EtOH extract (30.1 g), after evaporation of the solvent.

An aliquot of the EtOH extract (2.8 g) was chromatographed over Sephadex LH-20 (2.0 × 10 cm) by elution with methanol (MeOH, 250 mL) to afford forty fractions that were pooled together into three main fractions after TLC analysis: F-1 (50 mL, 400 mg), F-2 (120 mL, 830 mg), and F-3 (80 mL, 600 mg). Fraction F-2 was chromatographed on flash silica gel column (2.5 × 28 cm) using CH<sub>2</sub>Cl<sub>2</sub> 100% (100 mL), CH<sub>2</sub>Cl<sub>2</sub>/MeOH 2:1 (50 mL), CH<sub>2</sub>Cl<sub>2</sub>/MeOH 1:1 (120 mL), CH<sub>2</sub>Cl<sub>2</sub>/MeOH 1:3 (130 mL) and MeOH 100% (50 mL) to give ninety sub-fractions (5 mL), which were combined into eight main sub-fractions according to TLC analysis: F-1 (180.0 mg), F-2 (80.0 mg), F-3 (65.0 mg), F-4 (72.7 mg), F-5 (78.3 mg), F-6 (73.4 mg), F-7 (50.0 mg), and F-8 (140.0 mg).

Fractions F-2 (80.0 mg) and F-3 (65.0 mg) yielded the pure compounds **9/10** (7.2 mg) and **1/2** (6.0 mg), respectively, after recrystallization with MeOH.

Roots (4.43 kg) were pulverized and extracted with hexane (3 × 7 L) at room temperature. The solvent was removed under reduced pressure to yield the hexane extract (0.45 g). The residue was extracted with EtOH (3 × 7 L) to yield the corresponding extract (22.5 g).

An aliquot of the EtOH extract (600 mg) was chromatographed on a SPE C18 cartridge by elution with MeOH/H<sub>2</sub>O 1:1 (40 mL), MeOH/H<sub>2</sub>O 7:3 (50 mL), MeOH/H<sub>2</sub>O 8:2 (50 mL), MeOH/H<sub>2</sub>O 9:1 (30 mL) and MeOH (40 mL), to yield five fractions: F-1 (92.0 mg), F-2 (140.0 mg), F-3 (130.0 mg), F-4 (102.2 mg), and F-5 (90.6 mg). Recrystallization of the fraction F-4 (102.2 mg) with MeOH yielded the compounds **3/4** (9 mg). Fraction F-3 (130 mg) was submitted to semi-preparative RP-18 HPLC chromatography, using a UV detector (210-400 nm), flow 4.5 mL min<sup>-1</sup> and isocratic elution with MeOH/H<sub>2</sub>O (8:2) to afford **5/6** (7 mg) and **7/8** (9 mg).

#### Acid hydrolysis, silylation and sugar analysis

The epimeric mixtures (ca. 5.0 mg) were dissolved in 2 mol L<sup>-1</sup> HCl (dioxane/H<sub>2</sub>O 1:1, 2 mL) and stirred at 90 °C for 2 h. After cooling, the reaction mixture was neutralized with solution of 1 mol L<sup>-1</sup> NaOH, extracted with CH<sub>2</sub>Cl<sub>2</sub>, and the aqueous layer was evaporated to give a mixture of monosaccharides. The residue was dissolved in hexadimethyldisilazane/trimethylchlorosilan/pyridine (3:1:9), excess, and stirred at 70 °C for 60 min. The supernatants (3 μL) were analyzed by GC Agilent model 7890B/5977A GC/MSD (quadrupole), under the following conditions: CP-ChiraSil-L-Val column, 0.25 mm × 25 m; temperatures for detector and injector 150 and 200 °C, respectively; temperature of gradient system for the oven, 100 °C for 1 min and then raised to 180 °C, rate 5 °C min<sup>-1</sup> kept for 5 min. The configurations of sugars were determined by comparison of the retentions times of the corresponding derivatives with those of standards treated simultaneously with same silylating reagents (L-rhamnose Rt:11.15, D-galactose Rt:16.02, and D-glucose Rt:16.57). Peaks of the hydrolysates were detected at Rt:11.20 (L-rhamnose), Rt:16.01 (D-galactose) and Rt:16.57 (D-glucose). Co-injection of each hydrolysate with the respective standard gave single peaks.

#### Cytotoxic activity

The tested tumor cell lines (colorectal adenocarcinoma HCT-116, ovarian carcinoma OVCAR-8, human

promyelocytic leukemia HL-60 and glioma SF-295) were kindly donated by the National Cancer Institute (Bethesda, MD, USA). Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 2 mmol L<sup>-1</sup> glutamine, 100 U mL<sup>-1</sup> penicillin, and 100 µg mL<sup>-1</sup> streptomycin at 37 °C with 5% CO<sub>2</sub>. The cytotoxicity of the isolated compounds was tested against tumor cell lines using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) reduction assay. Cells were plated in 96-well plates (10<sup>5</sup> cells *per* well for adherent cells or 3 × 10<sup>5</sup> cells *per* well for suspended cells in 100 µL of medium) and compounds (0.05-25 µg mL<sup>-1</sup>) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), added to each well using the high-throughput screening (HTS) Biomek 3000 (Beckman Coulter, Brea, CA, USA), and incubated for 72 h. Doxorubicin was used as the positive control. Control groups received the same amount of DMSO. After 69 h of incubation, the supernatant was replaced by fresh medium containing tetrazolium dye (MTT, 0.5 mg mL<sup>-1</sup>). Three hours later, MTT formazan product was dissolved in 150 µL of DMSO, and the absorbance was measured at 595 nm (DTX 880 Multimode Detector, Beckman Coulter, Brea, CA, USA).

#### Antimicrobial activity

##### *In vitro* antibacterial activity

Tests were performed according to the M02-A11 (CLSI, 2012) protocol with modifications.<sup>8</sup> Wells with 6 mm diameter were made in the agar overlay of the Petri dish. To those wells, a volume of 20 µL (1000 µg mL<sup>-1</sup>) of the obtained compounds was applied. The plates were incubated for 20 h at 35 °C. The solvents and diluents used in the compounds dissolution were used as negative control. The assayed microorganisms used in this study were *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, and *Bacillus subtilis* ATCC 6633. The antibiotic disk used in antimicrobial sensitivity test was Cefepime (CPM) 30 µg (Specialized Diagnostics Microbiology, DME, São Paulo, SP, Brazil).

##### *In vitro* antifungal activity

The broth microdilution (BMD) antifungal susceptibility test was performed according to M27-A3 protocol using RPMI broth (pH 7.0) buffered with 0.165 mol L<sup>-1</sup> 3-(*N*-morpholino)propanesulfonic acid (MOPS; Sigma-Aldrich, St Louis, MO, USA).<sup>9</sup> Compounds were dissolved in DMSO and tested at concentrations ranging from 1.95 to 1000 µg mL<sup>-1</sup>. Fluconazole was used as positive

control. The yeasts and compounds were incubated in 96-well culture plates at 35 °C for 24 h and the results were examined visually. The minimum inhibitory concentration (MIC) of each compound was determined as the concentration that inhibited 50% of fungal growth. The assayed microorganisms used in this study were *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 14243 and *Candida albicans* ATCC 10231.

##### (25-*R,S*)-5 $\alpha$ -Spirostan-2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside (**1/2**)

White solid; m.p. 190-192 °C; R.f. 0.24 (CH<sub>2</sub>Cl<sub>2</sub>/H<sub>2</sub>O 15%); IR (KBr)  $\nu$  / cm<sup>-1</sup> 3350, 2927, 1638, 1450, 1377, 1241, 1156, 1085, 982, 920, 897; <sup>1</sup>H NMR (500 MHz, C<sub>5</sub>D<sub>5</sub>N) and <sup>13</sup>C NMR (125 MHz, C<sub>5</sub>D<sub>5</sub>N), see Tables 1 and 2; HRESIMS *m/z* calc. for C<sub>45</sub>H<sub>75</sub>O<sub>19</sub> [M + H]<sup>+</sup> 919.4931; found: 919.4902.

##### (25-*R,S*)-5 $\alpha$ -Spirostan-2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside (**3/4**)

White solid; m.p. 194-196 °C; R.f. 0.25 (CH<sub>2</sub>Cl<sub>2</sub>/H<sub>2</sub>O 15%); IR (KBr)  $\nu$  / cm<sup>-1</sup> 3432, 2915, 1650, 1446, 1365, 1041, 906; <sup>1</sup>H NMR (500 MHz, C<sub>5</sub>D<sub>5</sub>N) and <sup>13</sup>C NMR (125 MHz, C<sub>5</sub>D<sub>5</sub>N), see Tables 1 and 2; HRESIMS *m/z* calc. for C<sub>45</sub>H<sub>75</sub>O<sub>18</sub> [M + H]<sup>+</sup> 903.4979; found: 903.4953.

##### 26-*O*- $\beta$ -D-Glucopyranosyl-(25-*R,S*)-5 $\alpha$ -furost-20-ene,2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-galactopyranoside (**5/6**)

White solid; m.p. 126-128 °C; R.f. 0.40 (CH<sub>2</sub>Cl<sub>2</sub>/H<sub>2</sub>O 15%); IR (KBr)  $\nu$  / cm<sup>-1</sup> 3367, 2934, 1643, 1397, 1036, 906; <sup>1</sup>H NMR (600 MHz, CD<sub>3</sub>OD) and <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OH), see Tables 1 and 2; HRESIMS *m/z* calc. for C<sub>39</sub>H<sub>65</sub>O<sub>14</sub> [M + H]<sup>+</sup> 757.4372; found: 757.4374.

##### 26-*O*- $\beta$ -D-Glucopyranosyl-(25-*R,S*)-5 $\alpha$ -furost-20-ene,2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside (**7/8**)

White solid; m.p. 184-186 °C; R.f. 0.25 (CH<sub>2</sub>Cl<sub>2</sub>/H<sub>2</sub>O 10%); IR (KBr)  $\nu$  / cm<sup>-1</sup> 3369, 2926, 1635, 1439, 1374, 1033, 902; <sup>1</sup>H NMR (600 MHz, CD<sub>3</sub>OD) and <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD), see Tables 1 and 2; HRESIMS *m/z* calc. for C<sub>45</sub>H<sub>75</sub>O<sub>19</sub> [M + H]<sup>+</sup> 919.4902; found: 919.4902.

## Results and Discussion

Compounds **1/2** were isolated as a pair of inseparable epimers, which showed only a single peak in reverse-

phase (RP) HPLC and could not be separated by a variety of column chromatographies. This was evident from the observation of several signals of closely similar chemical shifts in the  $^{13}\text{C}$  NMR spectra. The HRESI/MS spectrum showed the protonated molecular ion peak at  $m/z$  919.4931  $[\text{M} + \text{H}]^+$ , suggesting the molecular formula  $\text{C}_{45}\text{H}_{74}\text{O}_{19}$ . The sequential loss of the three hexose moieties was observed by the fragment ion peaks at  $m/z$  757.4390, 595.3856 and 433.3330, respectively.

The most shielded region of the  $^1\text{H}$  NMR spectrum showed three methyl singlets at  $\delta_{\text{H}}$  0.72 (s, H-19), 0.79 (s, H-18), 0.80 (s, H-18), and four doublets at  $\delta_{\text{H}}$  0.70 ( $J$  5.3 Hz, H-27), 1.06 ( $J$  5.3 Hz, H-27), 1.12 ( $J$  3.6 Hz, H-21) and 1.13 (d,  $J$  3.6 Hz, H-21). Furthermore, several signals were observed in the range of  $\delta_{\text{H}}$  3.30–4.70, in addition to three doublets at  $\delta_{\text{H}}$  4.89 (d,  $J$  7.7 Hz, H-1'), 5.19 (d,  $J$  7.7 Hz, H-1''') and 5.60 (d,  $J$  7.9 Hz, H-1''). These data suggested for **1** a steroidal core structure containing three sugar units.

The  $^{13}\text{C}$  NMR and distortionless enhancement by polarization transfer (DEPT) spectra displayed sixty-three signals, several of which related to oxygenated carbons with a high degree of overlapping. The presence of typical signals of spiroacetal carbons at  $\delta_{\text{C}}$  109.5 and 110.1 (C-22), and secondary methyl groups at  $\delta_{\text{C}}$  30.9 and 27.8 (C-25), suggested for **1/2** the structure of one mixture of steroidal saponins containing a spirostane skeleton as aglycone. The methyl groups at  $\delta_{\text{C}}$  16.9 and 17.6 (C-27) in axial and equatorial positions, respectively, besides the steric compression of the methyl group in axial position protecting the carbon at  $\delta_{\text{C}}$  26.7 (C-23) indicated the *R/S* epimeric pair at C-25. The aglycones were characterized as gitogenin and neogitogenin after comparison with literature data.<sup>10–12</sup> The relative configuration of the hydroxyl and sugar portion at C-2 and C-3 was established by comparison with the  $^{13}\text{C}$  NMR spectral data described to the gitogenin and its 2 $\beta$ ,3 $\alpha$ -dihydroxy isomer, through the characteristic chemical shifts relative to C-1 to C-5. The chemical shifts at  $\delta_{\text{C}}$  46.0 (C-1), 70.7 (C-2), 85.0 (C-3), 34.3 (C-4) and 44.9 (C-5) observed in **1/2** were in accordance with the 2 $\alpha$ , 3 $\beta$ -orientation.<sup>13</sup>

All proton and carbon signals were fully assigned through heteronuclear single-quantum correlation spectroscopy (HSQC) and heteronuclear multiple-bond correlation spectroscopy (HMBC) spectra analyses (Tables 1 and 2). The three sugar units were characterized by the HSQC correlations of the acetal carbon at  $\delta_{\text{C}}$  103.7 (C-1') with the anomeric proton at  $\delta_{\text{H}}$  4.89 (d,  $J$  7.7 Hz, H-1'); the carbon at  $\delta_{\text{C}}$  105.3 (C-1'') with the proton at  $\delta_{\text{H}}$  5.60 (d,  $J$  7.9 Hz, H-1''), and the carbon at  $\delta_{\text{C}}$  107.4 (C-1''') with the proton at  $\delta_{\text{H}}$  5.19 (d,  $J$  7.7 Hz, H-1''').

The monosaccharides were identified as one glucose and two galactose units through NMR analyses followed by comparison with the literature data.<sup>12,14</sup> The absolute configurations were determined as D, on the basis of the acid hydrolysis followed by silylation of the sugars and GC analysis. The  $\beta$ -anomeric configurations of the glucopyranosyl and galactopyranosyl moieties were supported by the relatively large vicinal coupling constant values (ca. 8.0 Hz) observed for the anomeric protons.

The HMBC spectrum analysis allowed to establish the sequence of all sugar units by long-range correlations between the proton signal at  $\delta_{\text{H}}$  5.19 (H-1''') of the glucopyranose unit with the carbon at  $\delta_{\text{C}}$  80.4 (C-4'') of the galactopyranose, which anomeric proton at  $\delta_{\text{H}}$  5.60 (H-1'') showed correlation with the carbon at  $\delta_{\text{C}}$  80.4 (C-4') of the second galactopyranose unit. The anomeric proton of the second galactopyranose at  $\delta_{\text{H}}$  4.89 (H-1') in turn showed correlation with the C-3 carbon of the aglycone portion at  $\delta_{\text{C}}$  85.3 (C-3). These findings confirmed the attachment sequence of the sugar chain at C-3 to be 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside.

Thus, the structure of **1/2** was elucidated as the epimeric mixture of the new (25-*R,S*)-5 $\alpha$ -spirostan-2 $\alpha$ ,3 $\beta$ -diol 3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside.

Compounds **3/4** was isolated as a yellow resin and showed only a single peak in RP HPLC. The support for an epimeric mixture with a spirostanol steroid skeleton came from the comparative analysis of its NMR data with those observed for compounds **1/2**.

The  $^1\text{H}$  NMR spectrum of **3/4** showed resonances quite similar to those of **1/2**, except the presence of an additional methyl doublet at  $\delta_{\text{H}}$  1.77 (d,  $J$  6.0 Hz, H-6''). As seen for compound **1/2**, the presence of several signals in the range of  $\delta_{\text{H}}$  3.30–5.00 besides the deshielded signals corresponding to the anomeric protons at  $\delta_{\text{H}}$  4.92 (d,  $J$  7.8 Hz, H-1'), 5.53 (d,  $J$  7.3 Hz, H-1''') and 6.28/6.38 (s, H-1''), revealed the presence of the monosaccharide moieties.

Comparison of the  $^{13}\text{C}$  NMR data of **3/4** with those of **1/2** supported the assignment of the aglycones as gitogenin/neogitogenin. As to the glycosidic portions, a difference was the presence of an additional methyl carbon at  $\delta_{\text{C}}$  19.1 (C-6''). In addition, the sugar anomeric carbons at  $\delta_{\text{C}}$  105.4 (C-1'), 102.3/102.4 (C-1'') and 104.1 (C-1''') showed HSQC correlations with corresponding protons signals at  $\delta_{\text{H}}$  4.92 (H-1'), 6.28/6.38 (H-1'') and 5.53 (H-1'''), respectively (Tables 1 and 2).

The assignment of the sugar units as glucose, galactose and rhamnose was made by comparison of their carbon chemical shifts with those reported in literature.<sup>10–15</sup> The

$\beta$ -orientations of glucose and galactose and the  $\alpha$ -orientation of rhamnose were ascertained by the coupling constant values of the anomeric protons. A further coupling constant corresponding to a proton signal at  $\delta_{\text{H}}$  4.27 (t,  $J$  9.5 Hz, H-4'') confirms the galactose unit. The absolute configuration of the glucose and galactose units was determined as D and that of rhamnose as L, through acid hydrolysis, followed by silylation and GC analysis. In addition, the quasimolecular ion peak at  $m/z$  903.4979 (calcd. for 903.4953) in the HRESIMS, and the subsequent fragmentations signals at  $m/z$  741.4476, 595.3871 and 433.3298 relative to the loss of the three sugar moieties, confirmed the proposed structure.

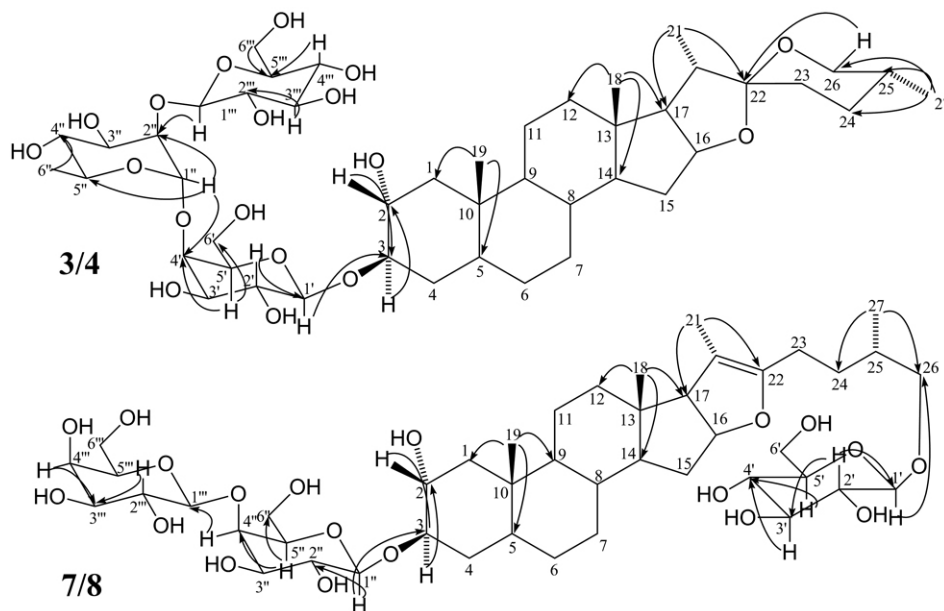
The attachment of the sugar moieties to C-3 of the aglycone was deduced by long-range correlations between the anomeric proton of the galactopyranosyl unit at  $\delta_{\text{H}}$  4.92 (H-1'') and the carbons at  $\delta_{\text{C}}$  85.6/85.2 (C-3) in the HMBC spectrum. Moreover, the correlations between the anomeric proton of the glucopyranosyl unit at  $\delta_{\text{H}}$  5.53 (H-1''') with the carbon at  $\delta_{\text{C}}$  76.5 (C-2'') of the rhamnopyranosyl, whose anomeric proton at  $\delta_{\text{H}}$  6.38/6.28 (H-1''') correlated with the carbons at  $\delta_{\text{C}}$  78.4/78.0 (C-4') of the galactopyranosyl, established the sugar sequence (Figure 2).

The nuclear overhauser effect spectroscopy (NOESY) spectrum provided certain information for the stereostructure assignments. This spectrum showed NOE correlations between the signals at  $\delta_{\text{H}}$  0.70 (H-19) and 3.91 (H-2), and between  $\delta_{\text{H}}$  1.05 (H-5) and 3.82 (H-3), and the junction of rings A/B was confirmed to be *trans*. Figure 3 shows all diagnostic dipolar-dipolar interactions observed for compounds **3/4**.

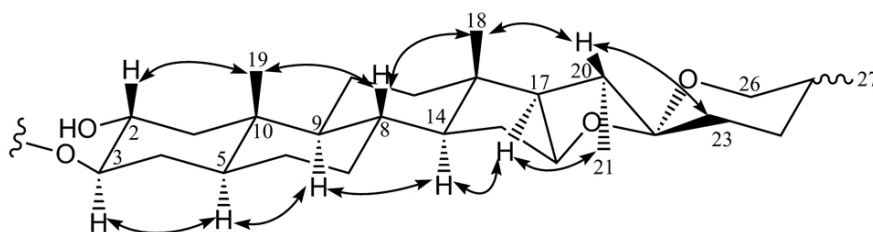
Thus, the structure of **3/4** was elucidated as the new (25-*R,S*)-5 $\alpha$ -spirostan-2 $\alpha$ ,3 $\beta$ -diol-3-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside.

Compounds **5/6**, white solid, were isolated as one single peak by RP HPLC. The existence of several signals with closely similar chemical shifts in the NMR spectra led to the conclusion that this compound, as the other previously described, was also an epimeric mixture.

Inspection of the  $^1\text{H}$  NMR spectrum also suggested a steroidal saponin for compounds **5/6**, by the signals relative to methyl groups at  $\delta_{\text{H}}$  0.70 (s, H-18), 0.90 (s, H-19), 1.60 (s, H-21) and 0.95 (d,  $J$  6.0 Hz, H-27), and the protons of the sugar units in the range of  $\delta_{\text{H}}$  3.2-3.9, besides the



**Figure 2.**  $^1\text{H}$ ,  $^{13}\text{C}$  heteronuclear multiple-bond correlation spectroscopy (HMBC) of compounds **3/4** and **7/8**.



**Figure 3.** Nuclear overhauser effect (NOE) correlations for the aglycone moiety of compounds **3/4**.

two anomeric at  $\delta_{\text{H}}$  4.22 (d,  $J$  7.8 Hz, H-1') and 4.32 (d,  $J$  7.4 Hz, H-1'').

A typical  $\Delta^{20,22}$ -furostanol skeleton was deduced after comparative analysis of the  $^{13}\text{C}$  NMR data with those of compounds **1/2** and **3/4**, that revealed the absence of the carbons relative to a spiroketal, and the presence of typical signals of unsaturated carbons at  $\delta_{\text{C}}$  105.2/105.3 (C-20) and 153.0/153.1 (C-22), besides one oxymethylene at  $\delta_{\text{C}}$  75.9 (C-26).<sup>16,17</sup> The presence of the  $2\alpha,3\beta$ -dioxy-decalin moiety was supported by the oxymethine carbons at  $\delta_{\text{C}}$  71.7 (C-2) and 85.1 (C-3).

The sugar units were confirmed as glucose and galactose by comparison of their chemical shifts with those reported for sugar moieties.<sup>10-15</sup> Their  $\beta$ -orientations were as ascertained by the relatively large coupling constants of the anomeric protons. This was further confirmed by the precursor ion peak at  $m/z$  757.4372 (calcd. for 757.4374) in the HRESIMS, and the subsequent loss of the two sugar units at  $m/z$  595.3828 and 433.3308.

The attachment of the sugar moieties was deduced through the HMBC analysis by the correlation peaks observed for the anomeric proton of the glucose unit at  $\delta_{\text{H}}$  4.22 (H-1') with the oxymethylene carbon at  $\delta_{\text{C}}$  75.9/75.8 (C-26), and for the anomeric proton of the galactopyranosyl unit at  $\delta_{\text{H}}$  4.22 (H-1'') with the carbon at  $\delta_{\text{C}}$  85.1 (C-3) of the aglycone. In addition, long range correlations were also detected for the proton at  $\delta_{\text{H}}$  0.95 (H-27) with the carbons at  $\delta_{\text{C}}$  75.9 (C-26) and 32.2 (C-24), and for the proton at  $\delta_{\text{H}}$  4.70 (H-16), 2.48 (H-17) and 1.71 (H-24) with the unsaturated carbons at  $\delta_{\text{C}}$  153.1/153.0 (C-22). The C-25 configuration was assigned as *25R* and *25S* based on the observed difference of the  $^1\text{H}$  NMR chemical shifts related to the H-26 geminal protons ( $\Delta_{\text{ab}} = \delta_{\text{a}} - \delta_{\text{b}}$ ). The values of  $\Delta_{\text{ab}} = 0.34$  and  $0.46$  were in agreement with those reported for *25R* and *25S* furostane-type steroidal saponins ( $\Delta_{\text{ab}} < 0.35$  for *25R*;  $\Delta_{\text{ab}} > 0.45$  for *25S*).<sup>17</sup> From the above mentioned data compound **5/6** was assigned as the new 26-*O*- $\beta$ -D-glucopyranosyl-(*25R,S*)-5 $\alpha$ -furost-20-ene,2 $\alpha,3\beta$ -diol-3-*O*- $\beta$ -D-galactopyranoside.

Compounds **7/8** were isolated as white solid. As observed previously, the several signals with closely similar chemical shifts in the NMR spectra revealed it to be an epimeric mixture.

Comparison of the NMR data of **7/8** with those correspondents of **5/6** revealed that these compounds were quite similar, except for the presence of an additional hexose monosaccharide. Three anomeric protons at  $\delta_{\text{H}}$  4.22 (d,  $J$  7.2 Hz, H-1'), 4.33 (d,  $J$  7.2 Hz, H-1'') and 4.82 (m, H-1''') were observed in the  $^1\text{H}$  NMR spectrum. In addition, the resonances of the aglycone moiety containing one glucose unit linked to C-26 appeared almost identical

in the  $^{13}\text{C}$  NMR, while some slight differences were detected among the resonances of the galactose that was linked to C-3 (Tables 1 and 2). In particular, the signals for C-4'' ( $\delta_{\text{C}}$  70.5) and for H-4'' ( $\delta_{\text{H}}$  3.82) of **5/6** were a lot more shielded than the corresponding C-4'' ( $\delta_{\text{C}}$  79.4) and H-4'' ( $\delta_{\text{H}}$  4.02) on **7/8**. These data suggested the additional sugar moiety to be linked at C-4''. It was identified as  $\beta$ -D-galactopyranosyl by comparison of its  $^{13}\text{C}$  NMR chemical shifts with those reported for sugar moieties and by GC analysis.<sup>14</sup> The quasimolecular ion peak at  $m/z$  919.4902 (calcd. for 919.4902) in the HRESIMS, and the subsequent fragmentations at  $m/z$  757.4390, 595.3831 and 433.3283, corroborated the above data.

The HMBC long range correlations of the anomeric proton at  $\delta_{\text{H}}$  4.82 (H-1''') with the carbon at  $\delta_{\text{C}}$  79.4 (C-4'') confirmed the attachment of the additional galactose unit at C-4''. Similarly, the anomeric proton at  $\delta_{\text{H}}$  4.33 (H-1'') showed correlation with the carbon at  $\delta_{\text{C}}$  85.3 (C-3) of the aglycone (Figure 2). Thus, compounds **7/8** was elucidated as the new 26-*O*- $\beta$ -D-glucopyranosyl-(*25R,S*)-5 $\alpha$ -furost-20-ene,2 $\alpha,3\beta$ -diol-3-*O*- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-galactopyranoside.

The genus *Cestrum* is a known as an abundant source of saponins. The natural role of these compounds in plants is to protect them against potential pathogens, that would be related with their antimicrobial activity.<sup>18-20</sup> Compounds **1/2** exhibited antimicrobial activity against *Candida albicans* and *Candida parapsilosis* with MIC<sub>50</sub> 3.9 and 7.0  $\mu\text{g mL}^{-1}$ , while compounds **9/10** showed moderated activity with IC<sub>50</sub> 7.0  $\mu\text{g mL}^{-1}$  (Table 3). Compounds **3/4** showed moderated activity against *C. krusei* and *C. albicans* with IC<sub>50</sub> 7.8  $\mu\text{g mL}^{-1}$ , and compounds **5/6** and **7/8** were inactive to all microorganisms. In addition, the cytotoxic activity of all compounds was evaluated against HCT-116, OVCAR-8, HL-60, and SF-295 cell lines (Table 4). Compounds **1/2** exhibited high cytotoxic activity to HL-60, with IC<sub>50</sub> value of 2.22  $\mu\text{g mL}^{-1}$ , and moderate activity against the others with IC<sub>50</sub> ranging from 6.88 to 10.8  $\mu\text{g mL}^{-1}$ . Compounds **3/4** showed only moderate activity to all cell lines with IC<sub>50</sub> ranging from 7.28 to 15.30  $\mu\text{g mL}^{-1}$ . Compounds **9/10** showed moderate activity against HL-60, OVCAR-8 and HCT-116 with IC<sub>50</sub> ranging from 11.3 to 16.7  $\mu\text{g mL}^{-1}$ , and were inactive against SF-295 cell lines. In contrast, compounds **5/6** and **7/8** were inactive to all cell lines.

## Conclusions

Chemical investigation of *C. laevigatum* yielded four new epimeric mixtures of spirostane and furostan-type saponins. Compounds containing the spirostane

**Table 1.** <sup>1</sup>H nuclear magnetic resonance (NMR) chemical shifts of compounds **1/2**, **3/4**, **5/6** and **7/8** (*J* in Hz)

H	<b>1/2</b> <sup>a,c</sup>		<b>3/4</b> <sup>a,c</sup>		<b>5/6</b> <sup>b,d</sup>		<b>7/8</b> <sup>b,d</sup>	
	$\delta_{\text{H}} / \text{ppm}$		$\delta_{\text{H}} / \text{ppm}$		$\delta_{\text{H}} / \text{ppm}$		$\delta_{\text{H}} / \text{ppm}$	
	25R	25S	25R	25S	25R	25S	25R	25S
1	2.21 (d, 12.0)	e	2.16 (m)	e	1.98 (m)	e	1.98 (m)	e
	1.07 (m)		1.15 (m)		0.90 (m)		0.93 (m)	
2	3.92 (m)	e	3.91 (m)	e	3.68 (m)	e	3.61 (m)	e
3	3.82 (m)	e	3.82 (m)	e	3.48 (m)	e	3.46 (m)	e
4	1.81 (m)	e	1.80 (m)	e	1.73 (m)	e	1.60 (m)	e
	1.42 (m)		1.42 (m)		1.42 (m)		1.25 (m)	
5	1.00 (m)	e	1.05 (m)	e	1.05 (m)	e	1.20 (t, 12.6)	e
6	1.15 (m)	e	1.15 (m)	e	1.41 (m)	e	1.35 (m)	e
	1.04 (m)		1.03 (m)		1.23 (m)		1.24 (m)	
7	1.53 (m)	e	1.51 (m)	e	1.71 (m)	e	1.70 (m)	e
	0.78 (m)		0.78 (m)		0.96 (sl)			
8	1.40 (m)	e	1.39 (m)	e	1.52 (m)	e	1.53 (m)	e
9	0.60 (t, 9.6)	e	0.57 (m)	e	0.78 (m)	e	0.70 (m)	e
10	–	e	–	e	–	e	–	e
11	1.48 (m)	e	1.44 (m)	e	1.57 (m)	e	1.58 (m)	e
	1.22 (m)		1.19 (m)		1.40 (m)		1.39 (m)	
12	1.62 (m)	e	1.62 (m)	e	1.80 (m)	e	1.79 (m)	e
	1.04 (m)		1.03 (m)		1.22 (m)		1.25 (m)	
13	–	e	–	e	–	e	–	e
14	1.03 (m)	e	1.05 (m)	e	1.05 (m)	e	1.04 (m)	e
15	2.00 (m)	e	2.00 (m)	e	2.10 (m)	e	2.15 (m)	e
	1.40 (m)		1.38 (m)		1.39 (m)		1.38 (m)	
16	4.51 (m)	4.51 (m)	4.54 (m)	e	4.70 (dd, 15.6, 7.8)	e	4.70 (dd, 15.7, 7.8)	e
17	1.78 (m)	e	1.78 (m)	e	2.48 (d, 10.0)	e	2.47 (d, 9.6)	e
18	0.80 (s)	0.79 (s)	0.78 (s)	0.79 (s)	0.70 (s)	e	0.70 (s)	e
19	0.72 (s)	e	0.70 (s)	e	0.90 (s)	e	0.90 (s)	e
20	1.93 (m)	1.89	1.88 (m)	1.92 (m)	–	–	–	–
21	1.13 (d, 3.6)	1.12 (d, 3.6)	1.13 (m)	e	1.60 (s)	e	1.60 (s)	e
22	–	–	–	–	–	–	–	–
23	1.66 (m)	1.38 (m)	1.67 (m)	1.34 (m)	2.14	e	2.12 (m)	e
24	1.55 (m)	e	1.70 (m)	e	1.71 (m)	e	1.61 (m)	e
	1.35 (m)		1.62 (m)		1.40 (m)		1.25 (m)	
25	1.59 (m)	e	1.56 (m)	1.59 (m)	1.53 (m)	e	1.76 (m)	e
26	3.49 (d, 9.6)	4.04 (m)	3.58 (dd, 7.9, 2.5)	4.09 (m)	3.71 (m)	3.78 (m)	3.72 (m)	3.78 (m)
	3.58 (m)	3.36 (d, 10.8)	3.52 (t, 10.4)	3.38 (d, 11.0)	3.37 (m)	3.32 (m)	3.37 (m)	3.33 (m)
27	0.70 (d, 5.3)	1.06 (d, 5.3)	0.70 (d, 8.7)	1.06 (d, 7.0)	0.95 (d, 6.0)	e	0.95 (d, 6.0)	e
1'	4.89 (d, 7.7)	e	4.92 (m)	e	4.22 (d, 7.8)	e	4.22 (d, 7.2)	e
2'	4.42 (m)	e	4.62 (m)	e	3.19 (t, 7.6)	e	3.17 (t, 9.0)	e
3'	4.22 (m)	e	4.22 (m)	e	3.32 (m)	e	3.35 (m)	e
4'	4.65 (m)	e	3.95 (d, 4.5)	e	3.51 (m)	e	3.25 (m)	e
5'	4.09 (m)	e	4.10 (m)	e	3.53 (m)	e	3.29 (m)	e
6'	4.52 (m)	e	4.56 (m) 4.21 (m)	e	3.85 (m)	e	3.85 (m)	e
	4.17 (m)						3.67 (m)	
1''	5.60 (d, 7.9)	e	6.38 (s)	6.28 (s)	4.32 (d, 7.4)	e	4.33 (d, 7.2)	e
2''	4.04 (d, 4.5)	e	4.76 (sl)	e	3.52 (m)	e	3.56 (m)	e
3''	4.22 (m)	e	4.76 (sl)	e	3.51 (m)	e	3.58 (m)	e
4''	4.62 (m)	e	4.27 (t, 9.5)	4.22 (m)	3.82 (m)	e	4.02 (d, 7.8)	e
5''	4.10 (m)	e	4.92 (m)	4.89 (m)	3.27 (m)	e	3.25 (m)	e
6''	4.54 (m)	e	1.77 (d, 6.0)	e	3.68 (m)	e	3.85 (m)	e
	4.19 (m)						3.66 (m)	
1'''	5.19 (d, 7.7)	e	5.53 (d, 7.3)	–	–	–	4.82 (m)	e
2'''	4.10 (m)	e	4.25 (m)	–	–	–	3.38 (m)	e
3'''	4.19 (m)	e	4.20 (m)	–	–	–	3.58 (m)	e
4'''	4.04 (m)	e	4.15 (m)	–	–	–	3.43 (m)	e
5'''	3.98 (m)	e	4.07 (m)	–	–	–	3.72 (m)	e
6'''	4.60 (m)	e	4.55 (m)	–	–	–	3.85 (m)	e
	4.23 (m)		4.14 (m)				3.58 (m)	

<sup>a</sup><sup>1</sup>H NMR data recorded at 500 MHz; <sup>b</sup>and at 600 MHz; <sup>c</sup>solvent C<sub>3</sub>D<sub>5</sub>N; <sup>d</sup>solvent CD<sub>3</sub>OD; <sup>e</sup>show the same chemical shifts.



**Table 2.**  $^{13}\text{C}$  nuclear magnetic resonance (NMR) chemical shifts of compounds **1/2**, **3/4**, **5/6**, **7/8** and **9/10**

C	<b>1/2<sup>a,c</sup></b>		<b>3/4<sup>a,c</sup></b>		<b>5/6<sup>a,d</sup></b>		<b>7/8<sup>b,d</sup></b>		<b>9/10<sup>a,c</sup></b>	
	$\delta_{\text{C}} / \text{ppm}$		$\delta_{\text{C}} / \text{ppm}$		$\delta_{\text{C}} / \text{ppm}$		$\delta_{\text{C}} / \text{ppm}$		$\delta_{\text{C}} / \text{ppm}$	
	25R	25S	25R	25S	25R	25S	25R	25S	25R	25S
1	46.0	e	45.8	e	46.0	46.1	45.9	46.0	46.1	e
2	70.7	e	70.9	70.5	71.7	e	71.6	e	71.0	e
3	85.0	e	85.6	85.2	85.1	e	85.3	e	85.5	e
4	34.3	e	34.2	34.0	34.3	e	34.3	e	34.6	e
5	44.9	e	45.0	e	46.0	e	45.9	e	45.0	e
6	28.4	e	28.4	e	29.2	e	29.2	e	28.5	e
7	32.4	e	32.5	e	33.6	e	33.6	e	32.6	e
8	34.9	e	34.9	e	35.8	e	35.8	e	35.0	e
9	54.7	e	54.7	e	55.9	e	55.8	e	54.8	e
10	37.2	e	37.2	e	38.1	e	38.0	e	37.2	e
11	21.7	e	21.7	e	22.6	e	22.6	e	21.8	e
12	40.4	e	40.3	e	41.0	e	41.0	e	40.4	e
13	41.1	e	41.1	41.0	44.8	e	44.8	e	41.1	e
14	56.6	e	56.6	e	56.1	e	56.1	e	56.7	e
15	32.5	e	32.4	e	35.2	e	35.2	e	32.5	e
16	81.4	81.5	81.4	81.5	85.8	e	85.8	e	81.5	81.6
17	63.3	63.4	63.1	63.2	65.4	e	65.7	e	63.2	63.4
18	16.9	e	16.9	e	14.9	15.0	15.0	15.9	16.9	e
19	13.7	e	13.7	e	13.9	e	13.9	e	13.8	e
20	42.3	42.8	42.2	42.3	105.2	105.3	105.3	104.8	42.4	42.8
21	15.2	15.3	15.1	15.3	12.0	e	12.0	e	15.2	15.4
22	109.5	110.1	109.5	110.0	153.0	153.1	153.0	e	109.6	110.0
23	32.1	26.7	32.1	26.6	24.2	e	24.3	e	32.2	26.8
24	29.6	26.5	29.5	e	32.2	e	32.2	e	29.6	26.6
25	30.9	27.8	30.9	27.9	34.3	e	35.1	e	31.0	27.9
26	67.2	65.4	67.2	65.4	76.0	75.9	75.9	75.8	67.2	65.5
27	17.6	16.9	17.6	16.6	17.5	e	17.5	17.4	17.7	16.7
1'	103.7	e	105.4	e	104.6	e	104.6	e	104.5	e
2'	73.3	e	72.9	e	75.3	e	75.3	e	72.7	e
3'	75.2	e	72.3	e	78.3	e	78.3	e	75.7	e
4'	80.4	e	78.0	78.4	71.8	e	71.8	e	70.6	e
5'	76.2	e	75.7	e	77.0	e	78.0	e	77.6	e
6'	63.6	e	61.3	e	62.9	e	62.9	e	62.7	e
1''	105.3	e	102.3	102.4	103.5	e	103.3	e	—	—
2''	73.6	e	76.5	e	72.6	e	75.8	e	—	—
3''	75.4	e	72.7	72.9	75.0	e	75.2	e	—	—
4''	80.4	e	74.7	74.3	70.5	e	79.4	e	—	—
5''	76.0	e	70.7	71.0	78.0	e	78.0	e	—	—
6''	63.6	e	19.1	e	62.7	e	61.6	e	—	—
1'''	107.4	e	104.1	e	—	—	104.0	e	—	—
2'''	76.2	e	73.1	e	—	—	73.3	e	—	—
3'''	79.0	e	77.7	e	—	—	75.3	e	—	—
4'''	69.8	e	71.0	e	—	—	69.4	e	—	—
5'''	78.8	e	78.4	e	—	—	76.0	e	—	—
6'''	61.2	e	63.5	e	—	—	63.6	e	—	—

<sup>a</sup> $^{13}\text{C}$  NMR data were recorded at 125 MHz; <sup>b</sup>and at 150 MHz; <sup>c</sup>solvent  $\text{C}_5\text{D}_5\text{N}$ ; <sup>d</sup>solvent  $\text{CD}_3\text{OD}$ ; <sup>e</sup>show the same chemical shifts.

**Table 3.** Antimicrobial activity

Compound	<i>C. krusei</i> MIC <sub>50</sub> / (µg mL <sup>-1</sup> )	<i>C. parapsilosis</i> MIC <sub>50</sub> / (µg mL <sup>-1</sup> )	<i>C. albicans</i> MIC <sub>50</sub> / (µg mL <sup>-1</sup> )	<i>P. aeruginosa</i> halo <sup>a</sup> / mm	<i>B. subtilis</i> halo <sup>a</sup> / mm	<i>S. aureus</i> halo <sup>a</sup> / mm
<b>1/2</b>	125.0	7.0	3.9	NA	NA	NA
<b>3/4</b>	7.8	125.0	7.8	NA	NA	NA
<b>5/6</b>	> 500	> 500	> 250	NA	NA	NA
<b>7/8</b>	> 500	> 500	> 500	NA	NA	NA
<b>9/10</b>	31.2	> 500	7.0	NA	NA	NA
Fluconazole	16.0	0.125	4.0	–	–	–
Cefepime	–	–	–	33	26	30
Control <sup>b</sup>	NA	NA	NA	NA	NA	NA

<sup>a</sup>Geometric means of at least three halo determined on different days; <sup>b</sup>DMSO. NA: No activity.

**Table 4.** Cytotoxicity against four human cancer cell lines

Compound	HL-60 IC <sub>50</sub> <sup>a</sup> / (µg mL <sup>-1</sup> )	OVCAR-8 IC <sub>50</sub> <sup>a</sup> / (µg mL <sup>-1</sup> )	HCT-116 IC <sub>50</sub> <sup>a</sup> / (µg mL <sup>-1</sup> )	SF-295 IC <sub>50</sub> <sup>a</sup> / (µg mL <sup>-1</sup> )
<b>1/2</b>	2.22 (1.55-3.17)	10.80 (9.51-12.27)	7.27 (5.93-8.90)	6.88 (4.49-10.56)
<b>3/4</b>	7.28 (6.68-7.95)	15.30 (11.91-19.64)	11.41 (9.63-13.51)	12.90 (10.78-15.43)
<b>5/6</b>	ND	ND	ND	ND
<b>7/8</b>	ND	ND	ND	ND
<b>9/10</b>	16.68 (11.85-23.49)	11.30 (9.21-13.87)	16.50 (14.3-19.1)	> 25
Doxorubicin <sup>b</sup>	0.02 (0.01-0.02)	0.26 (0.17- 0.3)	0.12 (0.09-0.17)	0.24 (0.02-0.27)

<sup>a</sup>95% of confidence interval; <sup>b</sup>positive control. Experiments were performed in triplicate. ND: Not determined.

skeleton **1/2**, **3/4** and **5/6** exhibited antimicrobial activity against *Candida albicans*, *Candida krusei* and *Candida parapsilosis* and cytotoxic activity against HCT-116, OVCAR-8, HL-60 and SF-295 cancer cell lines, while the furostanols **5/6** and **7/8** were inactive. These results indicate that the ring closure in C-22/C-26 typical of spirostanol skeleton appears to play an important role in the antimicrobial and cytotoxicity activities.

## Supplementary Information

Supplementary data are available free of charge at <http://jbcbs.s bq.org.br> as PDF file

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