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# Steroidal saponins from the roots of *Smilax* sp.: Structure and bioactivity

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ABSTRACT

Phytochemical characterization of a commercial herb sample supplied as *Smilax ornata* Lem. (sarsaparilla) led to the isolation of five steroidal saponins, including two new furostanol saponins sarsaparilloside B (**1**) and sarsaparilloside C (**2**), whose structures were elucidated via a combination of multistage mass spectrometry ($MS^n$), 1D and 2D NMR experiments, and chemical degradation. The previously unreported spectroscopic characterization of sarsaparilloside (**3**), $\Delta^{20(22)}$-sarsaparilloside (**4**), and parillin (**5**) is also provided. The antiproliferative activity of the isolated saponins was compared in six human cell lines derived from different tumor types and one of the structures (**2**) was particularly active against the HT29 colon tumor cell line.



## 1. Introduction

The genus *Smilax* (family Smilacaceae) comprises approximately 260 species of climbing vines that are mostly native to Central and South America. The roots of a number of *Smilax* species (all commonly referred to as "sarsaparilla"), in particular *Smilax glauca* Walter, *Smilax rotundifolia* L., *Smilax aristolochiifolia* Mill., *Smilax regelii* Killip & C.V. Morton,[1] and *Smilax china* L., are used as herbal medicines. Traditional uses of sarsaparilla include the treatment of rheumatism, rheumatoid arthritis, syphilis, leprosy, and skin conditions such as psoriasis, as well as a flavoring in root beer and herbal teas [1,2].

The phytochemistry of the genus *Smilax* is characterized by an abundance of steroidal saponins, a class of plant secondary metabolite thought to be responsible for the biological activity proposed for many medicinal herbs. Steroidal saponins exhibit a range of bioactivities, including cytotoxic, hemolytic, anti-inflammatory, antifungal, and anti-bacterial properties [3]. The most comprehensively characterized *Smilax* species are *S. aspera* L. [4–7], *S. china* L. [8–10], and *S. lebrunii* H.Lév. [11–13], which all contain furostanol and spirostanol steroidal glycosides. The isolation of steroidal saponins and their aglycones has also been reported from *S. excelsa* L. [14], *S. officinalis* Kunth. [15,16], *S. medica* Schltdl. & Cham. (synonymous with *S. aristolochiifolia* Mill.) [17,18], *S. menispermoidea* A. DC. [11,19], *S. riparia* A. DC. [20], and *S. sieboldii* Miq. [21–23]. Despite the long history of medicinal use of sarsaparilla [24] and the extensive phytochemical characterization of the above *Smilax* species, little is known about the chemical constituents of *S. ornata* Lem.,[1] a member of the genus reported to posses antileprotic activity [25,26] and used widely in the herbal medicine industry. Very early literature suggests the presence of saponins and their sapogenins in *S. ornata* [27,28], and we thus chose to investigate any steroidal saponins present in a commercial herb sample that was supplied as *S. ornata* (but unable to be botanically identified). We chose to perform a preliminary screen for antiproliferative activity of the isolated saponins, as this is a common bioactivity of many steroidal saponins. In particular, the cytotoxic activity of a number of saponins from *Smilax* has been investigated with mixed results [4,9,29,30].

In this work we describe the isolation and structure elucidation of two new furostanol steroidal saponins, sarsaparilloside B (**1**) and sarsaparilloside C (**2**). In addition, the previously unreported spectroscopic characterization of sarsaparilloside (**3**), $\Delta^{20(22)}$-sarsaparilloside (**4**), and parillin (**5**) is provided.

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[1] Recently it has been proposed that *S. regelii* Killip &C.V. Morton and *S. ornata* Lem. are the same species [Ferrufino-Acosta L. Taxonomic revision of the genus *Smilax* (Smilacaceae) in Central America and the Caribbean Islands. Willdenowia. 2010;40:227–80]. Both *S. regelii* and *S. ornata* have received only limited phytochemical characterization.

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## 2. Experimental

### 2.1. General methods

Optical rotations were measured on a JASCO P-2000 polarimeter. $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AV750 spectrometer with either the pyridine-$d_5$ signal ($\delta_H$ 8.71 ppm; $\delta_C$ 149.9 ppm) or TMS (in pyridine-$d_5$/$D_2O$, ~9:1 solvent) as internal standard. Low resolution and multistage mass spectra were recorded on a Bruker ESQUIRE HCT instrument (positive and negative ion ESI). High resolution mass spectra were acquired on a Bruker MicrOTOF-Q instrument (positive ion ESI) with internal calibration using Agilent Tune-Mix. Compounds **1**–**5** were purified by semipreparative reverse-phase HPLC performed on a Shimadzu LC-20AT liquid chromatograph (flow rate of 2 mL/min) equipped with an ELSDLT detector (52 °C, $N_2$ pressure: 200 kPa), column oven (40 °C) and a Phenomenex HPLC column (Luna C-18, 5 μm, 250 × 10 mm). Melting points were performed on a melting-point apparatus (Dr. Tottoli) and are uncorrected.

### 2.2. Plant material

Dried plant material was supplied and certified by Heinrich Klenk GmbH & Co KG as *S. ornata*. A specimen was deposited (accession number PHARM-110042) at the Medicinal Plant Herbarium, Southern Cross University, Lismore, Australia.

### 2.3. Extraction and isolation

Powdered roots and rhizome of *Smilax* sp. (12 g) were extracted (80% aq. methanol, 120 mL) with sonication (3 × 10 min). Following filtration and removal of the solvent *in vacuo*, the crude extract was partially purified by solid phase extraction (Phenomenex Strata C-18E cartridge) eluting with $H_2O$ followed by methanol. The methanol fraction was concentrated *in vacuo*, dissolved (90% aqueous methanol, 20 mL), filtered and purified by semipreparative reverse-phase HPLC (gradient of 25–63% aqueous acetonitrile over 35 min). Four different fractions were collected, numbered **I**–**IV** in order of elution. Fractions **I**, **III** and **IV** were pure and corresponded to sarsaparilloside **3** (Rt: 10.8 min, 80.6 mg), $\Delta^{20(22)}$-sarsaparilloside **4** (Rt: 16.4 min, 11.1 mg) and parillin **5** (Rt: 32.7, 16.9 mg). Fraction **II** was subjected to further fractionation by semipreparative reverse-phase HPLC (gradient of 23–28% aqueous acetonitrile over 45 min) to give two pure fractions that corresponded to sarsaparilloside B **1** (Rt: 36.8 min, 3.3 mg) and sarsaparilloside C **2** (Rt: 35.3 min, 8.9 mg).

To obtain the aglycone of **1**–**5**, crude extract (from 10 g plant material) was dissolved in methanol (10 mL) and aqueous HCl (32%, 1 mL) and heated under reflux for 2 h. After cooling, water (10 mL) was added and the mixture extracted with diethyl ether (3 × 40 mL). The combined organic layers were washed with aqueous NaOH (5% w/v, 3 × 40 mL) and dried ($MgSO_4$) before removal of the solvent *in vacuo*. The residue was purified by flash chromatography (28% ethyl acetate in hexane, silica gel 60) to yield sarsasapogenin (7.6 mg), which was identified via comparison of its $^1$H and $^{13}$C NMR data with literature reports [31–33].

### 2.4. 3-O-{[α-L-rhamnopyranosyl(1 → 4)][β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl}-26-O-β-D-glucopyranosyl-(25S)-5β-furostane-3β,22,26-triol (sarsaparilloside B, **1**)

Amorphous solid; $[\alpha]_D^{23}$ − 27.0 (*c* 0.13, $CH_3OH$); $^1$H (pyridine-$d_5$/$D_2O$ ~9:1, 750 MHz) and $^{13}$C NMR (pyridine-$d_5$/$D_2O$ ~9:1, 188 MHz) spectroscopic data, see Tables 1 and 2; positive ion ESIMS: *m*/*z* 1089, 1071, 925, 791; negative ion ESIMS: *m*/*z* 1065, 919, 903, 757, 739, 595; HRESIMS: *m*/*z* 1089.5423 $[M + Na]^+$ (calcd for $C_{51}H_{86}NaO_{23}$, 1089.5452).

### 2.5. 3-O-{[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl}-26-O-β-D-glucopyranosyl-(25S)-5β-furostane-3β,22,26-triol (sarsaparilloside C, **2**)

Amorphous solid; $[\alpha]_D^{22}$ − 33.0 (*c* 0.27, $CH_3OH$); $^1$H (pyridine-$d_5$/$D_2O$ ~9:1, 750 MHz) and $^{13}$C NMR (pyridine-$d_5$/$D_2O$ ~9:1, 188 MHz) spectroscopic data, see Tables 1 and 2; positive ion ESIMS: *m*/*z* 943, 925, 763, 583; negative ion ESIMS: *m*/*z* 919, 757, 595, 433; HRESIMS: *m*/*z* 943.4869 $[M + Na]^+$ (calcd for $C_{45}H_{76}NaO_{19}$, 943.4873).

### 2.6. Sarsaparilloside (**3**)

Off-white solid; m.p. 160–162 °C (decomp.); $[\alpha]_D^{20}$ − 34.9 (*c* 0.17, $CH_3OH$); $[\alpha]_D^{20}$ − 31.7 (*c* 0.14, $H_2O$); Lit. $[\alpha]_D^{20}$ − 44 (*c* 0.86, $H_2O$) [34,35]; $^1$H (pyridine-$d_5$, 750 MHz) and $^{13}$C NMR (pyridine-$d_5$, 188 MHz) spectroscopic data, see Tables 1 and 2; positive ion ESIMS: *m*/*z* 1251, 1233, 1087, 1071, 925, 909; negative ion ESIMS: *m*/*z* 1227, 1081, 1065, 919, 903, 757, 739, 595; HRESIMS: *m*/*z* 1251.6008 $[M + Na]^+$ (calcd for $C_{57}H_{96}NaO_{28}$, 1251.5980).

### 2.7. $\Delta^{20(22)}$-sarsaparilloside (**4**)

Amorphous solid; $[\alpha]_D^{20}$ − 15.7 (*c* 0.06, $CH_3OH$); Lit. $[\alpha]_D^{20}$ − 36 (*c* 0.84, $CH_3OH$) [35]; $^1$H (pyridine-$d_5$/$D_2O$ ~9:1, 750 MHz) and $^{13}$C NMR (pyridine-$d_5$/$D_2O$ ~9:1, 188 MHz) spectroscopic data, see Tables 1 and 2; positive ion ESIMS: *m*/*z* 1233, 1087, 1071, 925, 763; negative ion ESIMS: *m*/*z* 1209, 1063, 1047, 1029, 901, 739; HRESIMS: *m*/*z* 1233.5918 $[M + Na]^+$ (calcd for $C_{57}H_{94}NaO_{27}$, 1233.5875).

### 2.8. Parillin (**5**)

Off-white solid; m.p. 240–244 °C (decomp.); Lit. m.p. 220–223 °C [36]; $[\alpha]_D^{21}$ − 37.1 (*c* 0.10, $CH_3OH$); $[\alpha]_D^{20}$ − 35.7 (*c* 0.06, 80% aq. EtOH); Lit. $[\alpha]_D^{20}$ − 64 (*c* 1.00, 80% aq. EtOH) [36]; $^1$H (pyridine-$d_5$/$D_2O$ ~9:1, 750 MHz) and $^{13}$C NMR (pyridine-$d_5$/$D_2O$ ~9:1, 188 MHz) spectroscopic data, see Tables 1 and 2; positive ion ESIMS: *m*/*z* 1071, 925, 909, 763, 601; negative ion ESIMS: *m*/*z* 1083, 1047, 901, 885, 739, 577; HRESIMS: *m*/*z* 1071.5333 $[M + Na]^+$ (calcd for $C_{51}H_{84}NaO_{22}$, 1071.5346).

### 2.9. Determination of sugar absolute configuration

Each saponin (1 mg) was subjected to acid catalyzed methanolysis, before per-trifluoroacetylation of the resultant methylglycosides and enantioselective GC analysis (Chirasil-L-Val capillary column) according to procedures used previously [37]. The retention times for the TFAA-derivatized standards were for the two anomers of: D-glucose (26.21 and 29.97 min); L-glucose (26.13 and 29.79 min); D-rhamnose (17.63 and 23.60 min); and L-rhamnose (17.33 and 23.30 min). The retention times for the TFAA-derivatized saponin hydrolysates were for: **1** (17.33, 23.31, 26.24 and 29.99 min); **2** (17.31, 23.31, 26.19 and 30.00 min); **3** (17.33, 23.31, 26.20 and 29.99 min); **4** (17.31, 23.30, 26.22 and 29.99 min); and **5** (17.31, 23.31, 26.18 and 29.99 min).

### 2.10. Inhibition of human cell proliferation assay

Cells seeded at 3000–5000 cells/well in a 96-well plate were treated with compounds diluted from 10 mg/mL stock solutions in DMSO, giving a highest final concentration of DMSO (at 50 μg/mL of compound) of 0.5%. After incubation for 6 days, the plates were fixed in ethanol and cell content compared with untreated

**Table 1**
$^1$H (750 MHz) and $^{13}$C (188 MHz) NMR spectroscopic data for the aglycone moiety of **1**–**5**.

| | **1**[a] | | **2**[a] | | **3**[b] | | **4**[a] | | **5**[a] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C |
| 1a | 1.48 m[c] | 30.9 | 1.53 m | 31.0 | 1.46 m[c] | 30.9 | 1.49 m[c] | 30.8 | 1.47 m[c] | 30.8 |
| 1b | 1.70 m[c] | | 1.75 m[c] | | 1.80 m[c] | | 1.85 m[c] | | 1.83 m[c] | |
| 2a | 1.57 m | 26.9 | 1.59 br t (14.2) | 27.0 | 1.46 m[c] | 26.8 | 1.50 m[c] | 26.8 | 1.50 m[c] | 26.7 |
| 2b | 1.95 m[c] | | 2.04 m[c] | | 1.85 m | | 1.89 m[c] | | 1.89 m[c] | |
| 3 | 4.43 m | 74.6 | 4.46 m | 74.6 | 4.26 m[c] | 75.3 | 4.33 m[c] | 75.2 | 4.32 m[c] | 75.2 |
| 4a | 1.82 m[c] | 30.4 | 1.74 m[c] | 30.5 | 1.76 m[c] | 30.7 | 1.81 m[c] | 30.5 | 1.78 m[c] | 30.4 |
| 4b | 1.82 m[c] | | 1.82 br t (13.3) | | 1.76 m[c] | | 1.81 m[c] | | 1.78 m[c] | |
| 5 | 2.03 m[c] | 36.9 | 2.03 m[c] | 37.0 | 2.16 m | 36.7 | 2.21 m[c] | 36.6 | 2.20 m | 36.4 |
| 6a | 1.15 m | 26.9 | 1.10 m[c] | 27.0 | 1.13 m | 27.0 | 1.19 m[c] | 26.9 | 1.16 m[c] | 26.9 |
| 6b | 1.76 m[c] | | 1.75 m[c] | | 1.78 m[c] | | 1.83 m[c] | | 1.78 m[c] | |
| 7a | 0.93 m | 26.7 | 0.93 m | 26.7 | 0.89 m | 26.8 | 0.95 m | 27.0 | 0.92 m | 26.7 |
| 7b | 1.25 m[c] | | 1.26 m[c] | | 1.21 m | | 1.23 m[c] | | 1.22 m[c] | |
| 8 | 1.46 m[c] | 35.4 | 1.49 m | 35.5 | 1.46 m[c] | 35.5 | 1.41 m | 35.2 | 1.46 m[c] | 35.5 |
| 9 | 1.26 m[c] | 40.2 | 1.27 m[c] | 40.2 | 1.23 m | 40.3 | 1.25 m[c] | 40.2 | 1.25 m[c] | 40.2 |
| 10 | | 35.2 | | 35.2 | | 35.3 | | 35.2 | | 35.2 |
| 11a | 1.19 m | 21.1 | 1.20 m | 21.1 | 1.18 m | 21.2 | 1.19 m[c] | 21.3 | 1.18 m[c] | 21.1 |
| 11b | 1.32 m[c] | | 1.33 m[c] | | 1.29 m | | 1.33 m | | 1.30 m | |
| 12a | 1.09 br d (13.8) | 40.3 | 1.08 m[c] | 40.4 | 1.06 m | 40.4 | 1.13 m | 40.1 | 1.06 m | 40.3 |
| 12b | 1.73 m[c] | | 1.72 m[c] | | 1.69 m[c] | | 1.72 m | | 1.68 br d (12.4) | |
| 13 | | 41.2 | | 41.2 | | 41.3 | | 43.8 | | 40.8 |
| 14 | 1.04 m[c] | 56.3 | 1.06 m[c] | 56.4 | 1.04 m[c] | 56.4 | 0.85 m | 54.7 | 1.05 m[c] | 56.4 |
| 15a | 1.41 m | 32.3 | 1.42 dt (6.6, 12.7) | 32.4 | 1.40 dt (6.2, 12.7) | 32.5 | 1.47 m[c] | 34.4 | 1.43 m[c] | 32.1 |
| 15b | 2.02 m[c] | | 2.03 m[c] | | 2.00 m[c] | | 2.12 m | | 2.04 m | |
| 16 | 5.02 m | 81.3 | 5.02 td (7.6, 8.3) | 81.3 | 4.98 td (7.1, 7.9) | 81.3 | 4.86 m[c] | 84.6 | 4.62 m[c] | 81.4 |
| 17 | 2.02 m[c] | 63.6 | 2.01 dd (6.6, 8.3) | 63.8 | 1.94 m[c] | 64.1 | 2.50 d (10.2) | 64.7 | 1.86 dd (6.4, 8.2) | 62.8 |
| 18 | 0.87 s | 16.7 | 0.87 s | 16.7 | 0.86 s | 16.8 | 0.70 s | 14.4 | 0.83 s | 16.6 |
| 19 | 0.85 s | 23.8 | 0.86 s | 23.9 | 0.96 s | 24.1 | 0.99 s | 24.0 | 0.97 s | 24.0 |
| 20 | 2.27 m | 40.5 | 2.26 m | 40.6 | 2.23 m | 40.7 | | 103.7 | 1.94 quin. (6.9) | 42.5 |
| 21 | 1.36 d (6.5) | 16.4 | 1.36 d (6.8) | 16.4 | 1.32 d (6.9) | 16.5 | 1.65 s | 11.8 | 1.18 d (7.0) | 14.9 |
| 22 | | 110.8 | | 110.7 | | 110.7 | | 152.3 | | 109.9 |
| 23a | 2.08 m[c] | 36.9 | 2.02 m[c] | 37.0 | 1.97 m[c] | 37.2 | 2.20 m[c] | 23.6 | 1.48 m[c] | 26.3 |
| 23b | 2.14 m | | 2.13 dt (4.2, 12.2) | | 2.08 m[c] | | 2.27 m | | 1.93 dt (4.9, 13.8) | |
| 24a | 1.71 m[c] | 28.2 | 1.72 m[c] | 28.3 | 1.68 m[c] | 28.4 | 1.48 m[c] | 31.4 | 1.39 m | 26.1 |
| 24b | 2.07 m[c] | | 2.08 m[c] | | 2.05 m[c] | | 1.87 m[c] | | 2.17 m | |
| 25 | 1.95 m[c] | 34.3 | 1.95 m | 34.4 | 1.92 m[c] | 34.5 | 1.98 m | 33.7 | 1.62 m[c] | 27.5 |
| 26a | 3.53 br t (8.0) | 75.4 | 3.51 dd (6.8, 9.4) | 75.4 | 3.48 dd (6.9, 9.4) | 75.4 | 3.52 dd (6.9, 9.4) | 75.2 | 3.42 br d (11.2) | 65.2 |
| 26b | 4.10 m[c] | | 4.10 dd (5.7, 9.4) | | 4.07 dd (5.8, 9.4) | | 4.10 dd (5.7, 9.4) | | 4.11 dd (2.4, 11.2) | |
| 27 | 1.05 d (6.2) | 17.4 | 1.05 d (6.7) | 17.5 | 1.02 d (6.7) | 17.5 | 1.06 d (6.7) | 17.2 | 1.09 d (7.1) | 16.2 |

[a] Recorded in pyridine-$d_5$/$D_2O$ (~9:1).
[b] Recorded in pyridine-$d_5$.
[c] Indicates overlapping signals.

controls by staining with sulforhodamine B and quantitation at 540 nm in an ELISA reader. The $IC_{50}$ values (dose at which cell growth was inhibited by 50%) were determined by interpolation on plots of % control absorbance vs dose. For the non-adherent K562 cell line, MTS was added directly to the cultures after 6 days of treatment and cell numbers compared with the control after 2–4 h at 37 °C, from the absorbance at 490 nm. Compound **4** caused cell lysis as judged by the appearance of greatly enlarged cytoplasm. Cancer cell lines were: cervical carcinoma (HeLa); colon tumor (HT29); breast tumor (MCF7); melanoma (MM96L); and leukemia (K562).

## 3. Results and discussion

### 3.1. Structure elucidation of compounds *1*–*5*

Semipreparative reverse-phase HPLC of the crude methanolic extract of *Smilax* sp. underground parts (roots and rhizome) afforded, as a total of 1% of dry weight plant material, two new furostanol saponins (**1** and **2**) and three known compounds (**3**–**5**), albeit ones that had not been spectroscopically characterized previously (Fig. 1). The extremely limited physical properties reported and the absence of spectroscopic characterization for sarsaparilloside (**3**) [34,35], $\Delta^{20(22)}$-sarsaparilloside (**4**) [35], and parillin (**5**) [34–36] necessitated their complete structural elucidation via a combination of multistage mass spectrometry ($MS^n$), 1D and 2D NMR spectroscopy, and chemical degradation; complete spectroscopic characterization of **3**–**5** is reported here.

Compound **1** was isolated as an amorphous solid and positive ion high resolution (HR) ESIMS provided an ion at $m/z$ 1089.5423, which corresponded to a molecular formula of $C_{51}H_{86}O_{23}$ (calcd for $[M + Na]^+$: 1089.5452). Fragmentation observed in negative ion $ESIMS^n$ showed neutral losses of both 162 and 146 Da from the quasi-molecular ion at $m/z$ 1065 ($[M–H]^-$), indicating the presence of both a terminal hexose and deoxyhexose residue in the saccharide portion of **1**. The $[M–162–H]^-$ precursor ion at $m/z$ 903 was further fragmented to yield neutral losses of either 146 or 164 Da, which was followed by the loss of 162 Da from the $[M–162–146–H]^-$ precursor ion. The observed $ESIMS^n$ fragmentation pattern was consistent with a polyhydroxylated $C_{27}$ steroidal skeleton accompanied by one deoxyhexose and two hexose monosaccharides, while the molecular formula for **1** suggested the presence of one additional $C_6$ sugar unit. The $^1$H NMR spectrum for **1** (in pyridine-$d_5$/$D_2O$, ~9:1) displayed two signals typical of the angular methyl group of a steroid at $\delta_H$ 0.85 (s, $H_3$-19) and 0.87 ppm (s, $H_3$-18), along with two signals at $\delta_H$ 1.05 (d, $J$ = 6.2 Hz, $H_3$-27) and 1.36 ppm (d, $J$ = 6.5 Hz, $H_3$-21) arising from methyl groups attached to methine carbons. An additional methyl signal observed at $\delta_H$ 1.66 (d, $J$ = 5.9 Hz, $H_3$-6, 4′-*O*-α-L-rhamnose) was consistent with the presence of one deoxyhexose

**Table 2**
$^1$H (750 MHz) and $^{13}$C (188 MHz) NMR spectroscopic data for the sugar portion of **1**–**5**.

| | **1**[a] | | **2**[a] | | **3**[b] | | **4**[a] | | **5**[a] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C | $^1$H [$\delta$, mult, $J$ (Hz)] | $^{13}$C |
| | 3–*O*–$\beta$–D-Glucose | | 3–*O*–$\beta$–D-Glucose | | 3–*O*–$\beta$–D-Glucose | | 3–*O*–$\beta$–D-Glucose | | 3–*O*–$\beta$–D-Glucose | |
| 1′ | 4.87 d (7.8) | 102.6 | 4.90 d (7.8) | 103.0 | 4.78 d (7.5) | 101.6 | 4.84 d (7.6) | 101.3 | 4.84 d (7.8) | 101.1 |
| 2′ | 3.95 t (8.2) | 74.9 | 4.03 t (8.4) | 75.0 | 4.16 m[c] | 81.9 | 4.21 t (8.6) | 81.3 | 4.21 t (8.9) | 81.0 |
| 3′ | 4.13 t (8.6) | 76.5 | 4.24 t (8.9) | 78.2 | 4.16 m[c] | 76.4 | 4.17 t (8.6) | 76.4 | 4.16 t (8.9) | 76.4 |
| 4′ | 4.29 t (9.1) | 79.3 | 4.16 t (9.4) | 71.5 | 4.23 t (9.0) | 78.9 | 4.23 t (9.2) | 78.7 | 4.20 t (9.1) | 78.6 |
| 5′ | 3.83 m | 75.2 | 4.12 m | 77.2 | 3.75 m | 75.3 | 3.77 m | 75.2 | 3.76 m | 75.2 |
| 6a′ | 4.12 m[c] | 68.4 | 4.37 dd (6.3, 11.5) | 70.0 | 4.07 dd (5.0, 11.2) | 68.6 | 4.08 dd (3.9, 11.0) | 68.4 | 4.07 dd (4.9, 11.2) | 68.3 |
| 6b′ | 4.60 br d (10.8) | | 4.83 br d (11.2) | | 4.52 br d (11.4) | | 4.56 br d (11.1) | | 4.56 br d (11.2) | |
| | | | | | 2′–*O*–$\beta$–D-Glucose | | 2′–*O*–$\beta$–D-Glucose | | 2′–*O*–$\beta$–D-Glucose | |
| 1 | | | | | 5.39 d (7.7) | 105.5 | 5.42 d (7.4) | 105.0 | 5.43 d (7.7) | 104.8 |
| 2 | | | | | 4.04 t (8.1) | 77.0 | 4.05 t (8.0) | 76.6 | 4.04 t (8.4) | 76.4 |
| 3 | | | | | 4.25 t (8.7) | 78.0 | 4.26 t (8.3) | 77.8 | 4.26 t (9.1) | 77.7 |
| 4 | | | | | 4.28 t (8.9) | 72.0 | 4.24 t (8.7) | 71.8 | 4.21 t (9.1) | 71.8 |
| 5 | | | | | 3.96 m | 78.7 | 3.98 m | 78.6 | 3.98 ddd (2.7, 5.5, 9.5) | 78.5 |
| 6a | | | | | 4.47 dd (4.9, 11.4) | 63.0 | 4.46 dd (4.8, 10.8) | 62.9 | 4.43 dd (5.5, 11.7) | 62.8 |
| 6b | | | | | 4.57 br d (11.4) | | 4.61 br d (11.1) | | 4.60 br d (11.6) | |
| | 4′–*O*–$\alpha$–L-Rhamnose | | | | 4′–*O*–$\alpha$–L-Rhamnose | | 4′–*O*–$\alpha$–L-Rhamnose | | 4′–*O*–$\alpha$–L-Rhamnose | |
| 1 | 5.78 br s | 102.6 | | | 5.79 br s | 102.8 | 5.76 br s | 102.6 | 5.72 br s | 102.5 |
| 2 | 4.71 br s | 72.2 | | | 4.68 br s | 72.5 | 4.69 br s | 72.2 | 4.67 br s | 72.1 |
| 3 | 4.55 br d (9.7) | 72.3 | | | 4.48 dd (2.8, 9.2) | 72.7 | 4.51 dd (2.8, 9.4) | 72.4 | 4.50 dd (3.2, 9.3) | 72.3 |
| 4 | 4.34 t (9.3) | 73.5 | | | 4.29 t (9.3) | 73.8 | 4.33 t (9.4) | 73.6 | 4.32 t (9.4) | 73.5 |
| 5 | 4.82 m | 70.5 | | | 4.78 m | 70.5 | 4.78 m | 70.5 | 4.76 m | 70.4 |
| 6 | 1.66 d (5.9) | 18.4 | | | 1.60 d (6.2) | 18.5 | 1.62 d (6.2) | 18.4 | 1.62 d (6.2) | 18.3 |
| | 6′–*O*–$\beta$–D-Glucose | | 6′–*O*–$\beta$–D-Glucose | | 6′–*O*–$\beta$–D-Glucose | | 6′–*O*–$\beta$–D-Glucose | | 6′–*O*–$\beta$–D-Glucose | |
| 1 | 4.98 d (7.8) | 104.6 | 5.16 d (7.8) | 105.1 | 4.94 d (7.7) | 105.0 | 4.96 d (7.8) | 104.7 | 4.94 d (7.8) | 104.6 |
| 2 | 4.03 t (8.3) | 74.6 | 4.07 t (8.4) | 75.0 | 4.02 t (8.4) | 74.9 | 4.04 t (8.3) | 74.7 | 4.02 t (8.4) | 74.6 |
| 3 | 4.26 t (9.1) | 77.9 | 4.26 t (8.9) | 78.2 | 4.19 t (9.0) | 78.5 | 4.24 t (9.0) | 78.1 | 4.24 t (9.1) | 78.0 |
| 4 | 4.15 t (9.0) | 71.3 | 4.21 t (9.0) | 71.5 | 4.20 t (9.0) | 71.5 | 4.19 t (8.9) | 71.3 | 4.15 t (9.1) | 71.3 |
| 5 | 3.92 m | 78.1 | 3.96 m | 78.3 | 3.85 m | 78.5 | 3.90 m | 78.3 | 3.98 ddd (2.3, 5.5, 9.5) | 78.2 |
| 6a | 4.29 dd (7.1, 12.0) | 62.4 | 4.35 dd (5.9, 11.9) | 62.6 | 4.33 dd (5.6, 11.7) | 62.6 | 4.33 dd (5.6, 11.7) | 62.5 | 4.29 dd (5.7, 11.8) | 62.4 |
| 6b | 4.50 br d (12.0) | | 4.52 br d (11.8) | | 4.48 br d (11.7) | | 4.51 br d (11.7) | | 4.49 br d (11.8) | |
| | 26–*O*–$\beta$–D-Glucose | | 26–*O*–$\beta$–D-Glucose | | 26–*O*–$\beta$–D-Glucose | | 26–*O*–$\beta$–D-Glucose | | | |
| 1 | 4.81 d (7.7) | 104.8 | 4.81 d (7.8) | 105.0 | 4.80 d (7.8) | 105.2 | 4.85 d (7.8) | 105.0 | | |
| 2 | 4.03 t (8.4) | 74.9 | 4.03 t (8.4) | 75.0 | 4.02 t (8.3) | 75.3 | 4.05 t (8.5) | 75.0 | | |
| 3 | 4.28 t (9.0) | 78.1 | 4.27 t (9.0) | 78.3 | 4.23 m[c] | 78.7 | 4.28 t (8.8) | 78.4 | | |
| 4 | 4.18 t (9.2) | 71.5 | 4.21 t (9.1) | 71.5 | 4.23 m[c] | 71.7 | 4.21 t (9.3) | 71.6 | | |
| 5 | 3.96 m | 78.1 | 3.96 m | 78.3 | 3.93 m | 78.5 | 3.98 m | 78.4 | | |
| 6a | 4.33 dd (5.8, 11.4) | 62.5 | 4.36 dd (6.1, 12.0) | 62.6 | 4.38 dd (5.3, 11.8) | 62.9 | 4.37 dd (4.2, 11.6) | 62.7 | | |
| 6b | 4.53 br d (12.2) | | 4.54 br d (12.0) | | 4.54 br d (11.8) | | 4.56 br d (11.4) | | | |

[a] Recorded in pyridine-$d_5$/$D_2O$ (~9:1).
[b] Recorded in pyridine-$d_5$.
[c] Indicates overlapping signals.

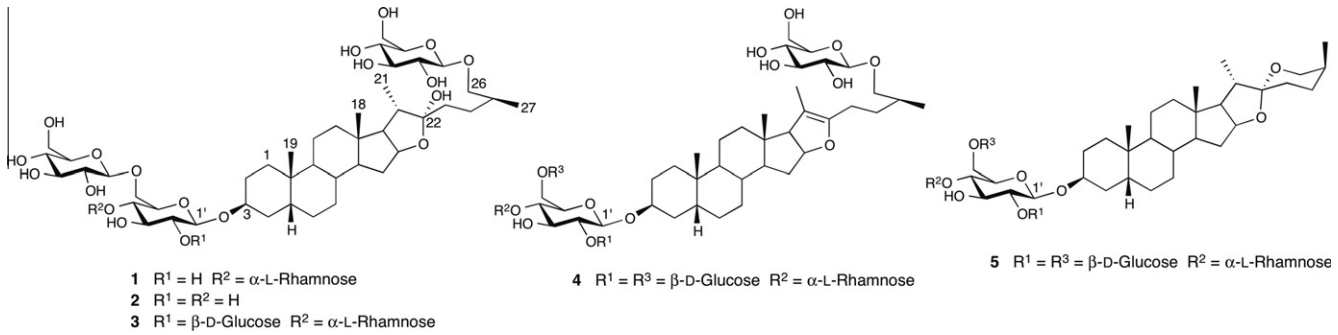


**Fig. 1.** Steroidal saponins **1**–**5** isolated from the roots of *Smilax* sp.

monosaccharide. The $^{13}$C NMR spectrum of **1** contained 51 signals, which included five methyl groups along with 14 methylene, 29 methine, and only three quaternary carbons, at $\delta_C$ 35.2 (C-10), 41.2 (C-13), and 110.8 ppm (C-22). Correlations observed in the HSQC and HMBC spectra of **1** allowed assignment of the steroid methyl groups as $\delta_C$ 16.4 (C-21), 16.7 (C-18), 17.4 (C-27) and 23.8 ppm (C-19). The HMBC correlations of these characteristic methyl groups allowed the rapid assignment of the steroidal skeleton. For example, correlations observed for $H_3$-21 ($\delta_H$ 1.36 ppm) revealed the signals of C-17 ($\delta_C$ 63.6), C-20 ($\delta_C$ 40.5), and C-22 ($\delta_C$ 110.8), with the chemical shift of C-22 clearly indicating the presence of a hemiacetal moiety, characteristic of furostanol saponins [38]. Further HMBC correlations from $H_3$-27 ($\delta_H$ 1.05 ppm) allowed identification of C-24 ($\delta_C$ 28.2), C-25 ($\delta_C$ 34.3), and C-26 ($\delta_C$ 75.4), with the downfield shift of the latter suggesting *O*-glycosylation at C-26. Examination of TOCSY, COSY, HSQC, and HMBC spectra

allowed the complete $^{1}H$ and $^{13}C$ NMR assignment of the steroidal skeleton of **1** and its identification as a saturated furostanol core (Table 1). The downfield shift of C-19 in **1** (to $\delta_C$ 23.8 ppm), when compared with its value in analogous H-5α furostanes ($\delta_C$ 12.3 ± 0.1 ppm) [38], suggested the *cis* fusion of the steroid A and B rings. The β orientation of H-5 was confirmed by an intense cross peak between $H_3$-19 and H-5 in the ROESY spectrum of **1**. The absolute configuration of C-25 in **1** was assigned on the basis of the geminal proton resonances of $H_2$-26 in pyridine-$d_5$/$D_2O$ (~9:1) ($\delta_H$ 3.53 and 4.10 ppm, $\Delta\delta$ = 0.57 ppm), which were in agreement with the 25*S* ($\Delta\delta \geqslant 0.57$ ppm) rather than 25*R* ($\Delta\delta \leqslant 0.48$ ppm) configuration [39]. The α orientation of the C-22 hydroxyl group was indicated by ROESY correlations between H-20 ($\delta_H$ 2.27 ppm) and both H-23a ($\delta_H$ 2.08 ppm) and H-23b ($\delta_H$ 2.14 ppm). The aglycone of **1** was obtained via semipreparative reverse-phase HPLC performed on the acid hydrolysate of a crude *Smilax* sp. extract, and displayed identical spectroscopic ($^{1}H$ and $^{13}C$ NMR) properties to literature reports for sarsasapogenin [31–33], in agreement with the structure and stereochemistry proposed for **1** on the basis of spectroscopic data.

The $^{1}H$ NMR spectrum of **1** also contained four signals typical of the anomeric proton of a glycoside at $\delta_H$ 4.81 (d, *J* = 7.7 Hz, H-1, 26-*O*-β-D-glucose), 4.87 (d, *J* = 7.8 Hz, H-1′, 3-*O*-β-D-glucose), 4.98 (d, *J* = 7.8 Hz, H-1, 6′-*O*-β-D-glucose) and 5.78 ppm (br s, H-1, 4′-*O*-α-L-rhamnose) (Table 2). These were correlated in the HSQC spectrum of **1** with signals at $\delta_C$ 104.8 (C-1, 26-*O*-β-D-glucose), 102.6 (C-1′, 3-*O*-β-D-glucose), 104.6 (C-1, 6′-*O*-β-D-glucose) and 102.6 ppm (C-1, 4′-*O*-α-L-rhamnose), respectively. Examination of the vicinal coupling constants obtained through selective 1D TOCSY experiments identified the sugar units of **1** as one α-rhamnopyranosyl and three β-glucopyranosyl residues. The absolute configuration of the sugar units was determined by enantioselective GC analysis, in which acid catalyzed methanolysis of **1** was followed by per-trifluoroacetylation of the resultant methyl-glycosides [40]. Comparison of the retention times of authentic, TFAA-derivatized standards and co-injection of the saponin **1** hydrolysate confirmed the presence of three units of D-glucose and one unit of L-rhamnose. The sugar linkage pattern was elucidated using correlations observed in the HMBC spectrum of **1**. Correlations between $\delta_H$ 4.87 (H-1′, 3-*O*-β-D-glucose) and $\delta_C$ 74.6 (C-3, aglycone), $\delta_H$ 5.78 (H-1, 4′-*O*-α-L-rhamnose) and $\delta_C$ 79.3 (C-4′, 3-*O*-β-D-glucose), and $\delta_H$ 4.98 (H-1, 6′-*O*-β-D-glucose) and $\delta_C$ 68.4 (C-6′, 3-*O*-β-D-glucose) indicated the presence of a branched trisaccharide moiety linked at position C-3 of the aglycone. An additional correlation between $\delta_H$ 4.81 (H-1, 26-*O*-β-D-glucose) and $\delta_C$ 75.4 (C-26, aglycone) revealed the attachment of a single glucose residue at position C-26 of the aglycone. The structure of **1** was therefore identified as 3-*O*-{[α-L-rhamnopyrnosyl(1 → 4)][β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl}-26-*O*-β-D-glucopyranosyl-(25*S*)-5β-furostane-3β,22,26-triol (sarsaparilloside B).

Compound **2** was isolated as an amorphous solid. Positive ion HRESIMS provided an ion at *m*/*z* 943.4869, consistent with a molecular formula of $C_{45}H_{76}O_{19}$ (calcd for $[M + Na]^+$: 943.4873). The fragmentation pattern observed in negative ion ESIMS$^n$ suggested the presence of three hexose monosaccharides, with the successive neutral loss of three 162 Da units from the quasi-molecular ion at *m*/*z* 919 ($[M–H]^-$). Along with the molecular formula for **2**, this suggested a structure similar to **1**, lacking the deoxyhexose residue. Accordingly, the $^{1}H$ and $^{13}C$ NMR spectroscopic data for the aglycone of **2** (in pyridine-$d_5$/$D_2O$, ~9:1) were very similar to those of **1** (Table 1). The $^{1}H$ NMR spectrum of **2** displayed only three signals typical of the anomeric proton of a glycoside, at $\delta_H$ 4.81 (d, *J* = 7.8 Hz, H-1, 26-*O*-β-D-glucose), 4.90 (d, *J* = 7.8 Hz, H-1′, 3-*O*-β-D-glucose), and 5.16 ppm (d, *J* = 7.8 Hz, H-1, 6′-*O*-β-D-glucose). Examination of selective 1D TOCSY spectra and enantioselective GC analysis confirmed that three β-D-glucopyranosyl units were present in **2**. Long-range HMBC correlations between $\delta_H$ 4.90 (H-1′, 3-*O*-β-D-glucose) and $\delta_C$ 74.6 (C-3, aglycone), $\delta_H$ 5.16 (H-1, 6′-*O*-β-D-glucose) and $\delta_C$ 70.0 (C-6, 3-*O*-β-D-glucose), and $\delta_H$ 4.81 (H-1, 26-*O*-β-D-glucose) and $\delta_C$ 75.4 (C-26, aglycone) revealed a 1 → 6 linked diglucose moiety attached at C-3 of the aglycone, along with a single glucose residue attached at C-26 (Table 2). The upfield shift of C-4′ in **2** ($\delta_C$ 71.5 ppm), when compared with its chemical shift in **1** ($\delta_C$ 79.3 ppm), confirmed that the C-4′ linked rhamnose present in **1** was absent in **2**. The structure of **2** was therefore elucidated as 3-*O*-{[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl}-26-*O*-β-D-glucopyranosyl-(25*S*)-5β-furostane-3β, 22,26-triol (sarsaparilloside C). While **2** has not been previously isolated as a natural product, it has been reported in the patent literature with very limited characterization as a synthetic compound [41]. $^{1}H$ and $^{13}C$ NMR data were available (in pyridine-$d_5$) for the three anomeric positions only, but the reported chemical shifts ($\delta_H$ 5.19, 5.28, and 5.31 ppm; $\delta_C$ 102.1, 103.5, and 105.1 ppm) [41] clearly do not match those for **2**; this discrepancy remains to be resolved.

Compound **3** was isolated as an off-white solid, and positive ion HRESIMS provided an ion *m*/*z* 1251.6008, corresponding to a molecular formula of $C_{57}H_{96}O_{28}$ (calcd for $[M + Na]^+$: 1251.5980). Fragmentation in negative ion ESIMS$^n$ of the quasi-molecular ion at *m*/*z* 1227 ($[M–H]^-$) yielded neutral losses of both 146 and 162 Da (*m*/*z* 1081 and 1065, respectively), suggesting the presence of both a terminal deoxyhexose and a hexose residue in a branched sugar chain. The $[M–162–H]^-$ fragment was selected as a precursor ion and went onto give successive neutral losses of 162, 164, and 142 Da, consistent with the presence of at least one deoxyhexose and three hexose monosaccharides; the molecular formula for **3** indicated the presence of an additional $C_6$ sugar. The $^{1}H$ NMR spectrum of **3** (in pyridine-$d_5$) displayed signals for two methyl groups attached to quaternary carbons, at $\delta_H$ 0.86 (s, $H_3$-18) and 0.96 ppm (s, $H_3$-19), and two methyl groups attached to methine carbons, at $\delta_H$ 1.02 (d, *J* = 6.7 Hz, $H_3$-27) and 1.32 ppm (d, *J* = 6.9 Hz, $H_3$-21). An additional methyl doublet signal was observed at $\delta_H$ 1.60 ppm (d, *J* = 6.2 Hz, $H_3$-6, 4′-*O*-α-L-rhamnose), consistent with the presence of one deoxyhexose monosaccharide. These five methyl group signals were correlated in the HSQC spectrum of **3** with signals at $\delta_C$ 16.8 (C-18), 24.1 (C-19), 17.5 (C-27), 16.5 (C-21), and 18.5 ppm (C-6, 4′-*O*-α-L-rhamnose), respectively, with the downfield chemical shift of C-19 being indicative of a *cis* A/B ring junction. The HMBC correlations of the steroid methyl groups provided important structural information; the chemical shift of C-22 ($\delta_C$ 110.7 ppm) as revealed by a correlation from $H_3$-21 ($\delta_H$ 1.32 ppm) was typical of a 22-hydroxy furostanol, while the shift of C-26 ($\delta_C$ 75.4 ppm) was suggestive of 26-*O*-glycosylation [38]. Complete $^{1}H$ and $^{13}C$ NMR assignment of the aglycone of **3** (Table 1) confirmed that this saponin, like **1** and **2**, is based on sarsasapogenin.

The $^{1}H$ NMR spectrum of **3** also displayed five anomeric signals, at $\delta_H$ 4.78 (d, *J* = 7.5 Hz, H-1′, 3-*O*-β-D-glucose), 4.80 (d, *J* = 7.8 Hz, H-1, 26-*O*-β-D-glucose), 4.94 (d, *J* = 7.7 Hz, H-1, 6′-*O*-β-D-glucose), 5.39 (d, *J* = 7.7 Hz, H-1, 2′-*O*-β-D-glucose), and 5.79 (br s, H-1, 4′-*O*-α-L-rhamnose) (Table 2). These were correlated in the HSQC spectrum of **3** with signals at $\delta_C$ 101.6 (C-1′, 3-*O*-β-D-glucose), 102.8 (C-1, 4′-*O*-α-L-rhamnose), 105.0 (C-1, 6′-*O*-β-D-glucose), 105.2 (C-1, 26-*O*-β-D-glucose), and 105.5 ppm (C-1, 2′-*O*-β-D-glucose). Examination of 1D TOCSY subspectra for the five individual sugar units, along with enantioselective GC analysis of the saponin hydrolysate, led to identification of the sugars of the saccharide portion of **3** as one α-L-rhamnopyranosyl and four β-D-glucopyranosyl residues. The sugar linkage pattern was established through HMBC correlations observed between $\delta_H$ 4.78 (H-1′, 3-*O*-β-D-glucose) and $\delta_C$ 75.3 (C-3, aglycone), $\delta_H$ 5.39 (H-1, 2′-*O*-β-D-glucose) and $\delta_C$ 81.9 (C-2′, 3-*O*-β-D-glucose), $\delta_H$ 5.79 (H-1, 4′-*O*-α-L-rhamnose) and $\delta_C$ 78.9 (C-4′, 3-*O*-β-D-glucose), $\delta_H$ 4.94 (H-1, 6′-*O*-β-D-glucose) and

$\delta_C$ 68.6 (C-6′, 3-*O*-*β*-D-glucose), and $\delta_H$ 4.80 (H-1, 26-*O*-*β*-D-glucose) and $\delta_C$ 75.4 (C-26, aglycone). Compound **3** was thus identified as sarsaparilloside, a bidesmodic furostanol saponin possessing a highly branched tetrasaccharide moiety linked at position C-3 [34,35]. While sarsaparilloside (**3**) has been isolated previously from *S. aristolochiifolia*, as part of a mixture containing "up to 15%" of 22-methoxy sarsaparilloside [34,35], its spectroscopic characterization has not been reported. The optical rotation reported for sarsaparilloside ($[\alpha]_D^{20}$ − 44, *c* 0.86, $H_2O$) [34,35] (the only reported physical property) was in fair agreement with that of **3** ($[\alpha]_D^{20}$ − 31.7, *c* 0.14, $H_2O$). The complete $^1H$ and $^{13}C$ NMR assignment of sarsaparilloside (**3**) (in pyridine-$d_5$) is reported here for the first time (Tables 1 and 2).

Compound **4** was isolated as an amorphous solid, and positive ion HRESIMS gave a molecular formula of $C_{57}H_{94}O_{27}$, from an ion observed at *m*/*z* 1233.5918 (calcd for $[M + Na]^+$: 1233.5875). The fragmentation pattern observed in negative ion ESIMS$^n$ for **4** was similar to that of **3**, with the quasi-molecular ion at *m*/*z* 1209 undergoing sequential neutral losses of one 146 Da and three 162 Da units. The $^1H$ NMR spectrum of **4** (in pyridine-$d_5$/$D_2O$, ~9:1) displayed signals for two methyl groups attached to quaternary carbons at $\delta_H$ 0.70 (s, $H_3$-18) and 0.99 ppm (s, $H_3$-19), along with two signals for methyl groups attached to methine carbons at $\delta_H$ 1.06 (d, *J* = 6.7 Hz, $H_3$-27) and 1.62 ppm (d, *J* = 6.2 Hz, $H_3$-6, 4′-*O*-*α*-L-rhamnose) (Table 1). The methyl doublet signal observed for $H_3$-21 in **3** was absent, but a new methyl singlet was observed at $\delta_H$ 1.65 ppm. The methyl group signals seen in the $^1H$ NMR spectrum of **4** showed HSQC correlations with signals at $\delta_C$ 14.4 (C-18), 24.0 (C-19), 17.2 (C-27), 18.4 (C-6, 4′-*O*-*α*-L-rhamnose), and 11.8 ppm (C-21). The long-range HMBC correlations of $H_3$-21 ($\delta_H$ 1.65 ppm) revealed one methine carbon at $\delta_C$ 64.7 ppm (C-17), which was correlated in the HSQC spectrum with a signal at $\delta_H$ 2.50 ppm (d, *J* = 10.2 Hz, H-17), as well as two quaternary carbons at $\delta_C$ 103.7 (C-20) and 152.3 ppm (C-22) in the olefinic region. The chemical shifts of C-20, C-21, and C-22, and the multiplicity of the H-17 and $H_3$-21 resonances, suggested a $\Delta^{20(22)}$-unsaturated aglycone for **4** and were in agreement with literature values for $\Delta^{20(22)}$-furostanol saponins derived from sarsasapogenin [42,43].

The $^1H$ NMR spectrum of **4** contained five signals corresponding to the anomeric protons of glycosides, at $\delta_H$ 4.84 (d, *J* = 7.6 Hz, H-1′, 3-*O*-*β*-D-glucose), 4.85 (d, *J* = 7.8 Hz, H-1, 26-*O*-*β*-D-glucose), 4.96 (d, *J* = 7.8 Hz, H-1, 6′-*O*-*β*-D-glucose), 5.42 (d, *J* = 7.4 Hz, H-1, 2′-*O*-*β*-D-glucose), and 5.76 ppm (br s, H-1, 4′-*O*-*α*-L-rhamnose), which displayed HSQC correlations with signals at $\delta_C$ 101.3 (C-1′, 3-*O*-*β*-D-glucose), 105.0 (C-1, 26-*O*-*β*-D-glucose), 104.7 (C-1, 6′-*O*-*β*-D-glucose), 105.0 (C-1, 2′-*O*-*β*-D-glucose), and 102.6 ppm (C-1, 4′-*O*-*α*-L-rhamnose), respectively (Table 2). Careful examination of TOCSY, COSY, HSQC, and HMBC spectra for **4** revealed that this saponin shares the same glycosylation pattern as present in sarsaparilloside (**3**). Compound **4** was therefore was identified as $\Delta^{20(22)}$-sarsaparilloside, a $\Delta^{20(22)}$-unsaturated furostanol saponin previously synthesized from sarsaparilloside (**3**) and reported with only limited characterization (optical rotation and melting point) [35]. The optical rotation of **4** ($[\alpha]_D^{20}$ − 15.7, *c* = 0.06, $CH_3OH$) was in only fair agreement with that reported for $\Delta^{20(22)}$-sarsaparilloside ($[\alpha]_D^{20}$ − 36 (*c* = 0.84, $CH_3OH$) [35]; whether this is due to the difference in concentration or other factors is unknown. We provide here the complete $^1H$ and $^{13}C$ NMR assignment of **4** (Tables 1 and 2), a steroidal saponin that has not been previously isolated from a natural source.

Compound **5** was isolated as an off-white solid and HRESIMS provided an ion at *m*/*z* 1071.5333, consistent with a molecular formula of $C_{51}H_{84}O_{22}$ (calcd for $[M + Na]^+$: 1071.5346). Fragmentation in negative ion ESIMS$^n$ of the quasi-molecular ion at *m*/*z* 1047 ($[M–H]^-$) yielded neutral losses of both 146 Da (*m*/*z* 901) and 162 Da (*m*/*z* 885), suggesting the presence of a branched polysaccharide as seen in **3** and **4**. The $[M–146–H]^-$ product ion was further fragmented to yield two successive neutral losses of 162 Da, consistent with the presence of at least one deoxyhexose and two hexose monosaccharides; the molecular formula for **5** indicated the presence of a fourth $C_6$ sugar. The $^1H$ NMR spectrum of **5** (in pyridine-$d_5$/$D_2O$, ~9:1) contained signals for two methyl groups attached to quaternary carbons, at $\delta_H$ 0.83 (s, $H_3$-18) and 0.97 ppm (s, $H_3$-19), and three methyl groups attached to methine carbons, at $\delta_H$ 1.09 (d, *J* = 7.1 Hz, $H_3$-27), 1.18 (d, *J* = 7.0 Hz, $H_3$-21), and 1.62 ppm (d, *J* = 6.2 Hz, $H_3$-6, 4′-*O*-*α*-L-rhamnose). The $^{13}C$ NMR chemical shifts of these methyl groups were assigned via HSQC as $\delta_C$ 16.6 (C-18), 24.0 (C-19), 16.2 (C-27), 14.9 (C-21), and 18.3 ppm (C-6, 4′-*O*-*α*-L-rhamnose), with the chemical shift of C-19 again indicative of the *β* orientation of H-5. Correlations observed in the HMBC spectrum of **5** from $H_3$-21 ($\delta_H$ 1.18 ppm) and $H_3$-27 ($\delta_H$ 1.09 ppm) allowed the assignment of positions 22 ($\delta_C$ 109.9 ppm), 24 ($\delta_H$ 1.39 and 2.17 ppm; $\delta_C$ 26.1 ppm), 25 ($\delta_H$ 1.62 ppm; $\delta_C$ 27.5 ppm), and 26 ($\delta_H$ 3.42 and 4.11 ppm; $\delta_C$ 65.2 ppm), revealing F ring chemical shifts typical of a 25*S*-configured spirostanol [44,45]. Examination of COSY, TOCSY, HSQC, and HMBC spectra, and enantioselective GC analysis, revealed that **5** contains the same branched tetrasaccharide moiety as found in **3** and **4**. Compound **5** was thus identified as parillin, the spirostanolic analogue of sarsaparilloside (**3**), first reported along with compound **3** from *S. aristolochiifolia* [34–36]. The limited physical properties reported for parillin (m.p. 220–223 °C; $[\alpha]_D^{20}$ − 64, *c* 1.00, 80% aq. EtOH) [36] were in fair agreement with those of **5** (m.p. 240–244 °C, decomp.; ($[\alpha]_D^{21}$ − 35.7, *c* 0.06, 80% aq. EtOH). The previously unreported $^1H$ and $^{13}C$ NMR assignment of **5** is provided in Tables 1 and 2.

**Table 3**
$IC_{50}$ of the isolated saponins **1**–**5** for inhibition of human cell proliferation against normal fibroblasts (NFF) and different cancer cell lines (μg/mL).

| Compound | Cell line | | | | | |
|---|---|---|---|---|---|---|
| | NFF | HeLa | HT29 | MCF7 | MM96L | K562 |
| **1** | >50 | >50 | >50 | >50 | >50 | >50 |
| **2** | 27 | 42 | 4.8 | 24 | 23 | 28 |
| **3** | 13 | 12 | 5 | 9.5 | 14 | 22 |
| **4** | 4.5 | 40 | 14 | 3.4 | 3.8 | 4.3 |
| **5** | >50 | >50 | >50 | >50 | >50 | >50 |

### 3.2. Biological evaluation

The isolation of a structurally related series of saponins, differing in aglycone structure and glycosylation pattern, provides a means to systematically probe structure–activity relationships of the components of this widely used medicinal herb. The prolonged treatment time of sparsely-seeded cultures allowed for comparison of clonogenic survival of treated cells. Three of the five isolated saponins showed cytotoxic activity in cultured cells at <10 μg/mL, a range with potential for treatment *in vivo*. Compounds **2** and **3** selectively inhibited the proliferation of the HT29 colon tumor cell line, whereas **4** was more generally active across the six cell lines studied (Table 3). Interestingly, HT29 is not particularly sensitive to other classes of saponin [46,47]. The variation in selectivity for different tumor types suggests a mechanism more specific than lysis of the cell membrane. Many studies have demonstrated that saponins induce apoptosis in tumor cells by a variety of pathways [48] but few specific ligands have been identified.

## 4. Conclusions

Phytochemical investigation of a commercial herb sample supplied as *S. ornata* (but unable to be botanically identified) led to the

isolation of steroidal saponins **1**–**5**. The structures of these saponins represent an interesting and apparently almost complete biosynthetic pathway, allowing us to propose a route for their biosynthesis. The presence of a glucose residue at position C-3 in all of the isolated saponins indicates that this glycosylation step is an early elaboration of the furostanol steroidal skeleton. Similarly, C-26 glucosylation is chemically logical to permit formation of the hemiacetal at position C-22 that can later close to give the C-22 spiroacetal. Glucosylation at position C-6′ gives the $1 \rightarrow 6$ linked disaccharide moiety common to **1**–**5** and completes the biosynthesis of sarsaparilloside C (**2**), while further glycosylation at C-4′ (by a rhamnose unit) yields sarsaparilloside B (**1**). Additional glucosylation of C-2′ then yields sarsaparilloside (**3**) and completes the highly branched tetrasaccharide moiety present in **3**–**5**. Compound **3** may then undergo dehydration to give $\Delta^{20(22)}$-sarsaparilloside (**4**) or specific cleavage of the C-26 glucose residue to yield its spirostanolic analogue, parillin (**5**).

The complex taxonomy of the genus *Smilax* makes it difficult to comment on the chemotaxonomy of this group. The isolated furostanol (**1**–**4**) and spirostanol (**5**) saponins are all derived from the sarsasapogenin aglycone, which is characterized by 25*S* stereochemistry and the presence of a *cis* A/B ring junction. Compounds **3** and **5** have been previously reported from *S. artistolochiifolia* [34–36], while the remaining saponins **1**, **2**, and **4** all posses closely related structures. Interestingly, the *cis* fusion of the steroid A and B rings seen in **1**–**5** appears to be typical of saponins isolated from *S. aristolochiifolia* (also known as *S. medica*) [17,18,34–36] and *S. aspera* [4,5], and has also been found in a saponin reported from *S. officinalis* along with the corresponding 5$\alpha$ glycoside [15]. In contrast, saponins possessing $\Delta^{5(6)}$-unsaturation are found mainly in *S. china* [8–10] as well as *S. menispermoidea* [11,19], *S. excelsa* [14], and *S. lebrunii* [12], while *trans* A/B ring fusion predominates in the saponins of *S. sieboldii* [21–23]. The isolation of exclusively 5$\beta$ saponins from a commercial sample supplied as *S. ornata* suggests that this species may belong to a sub-group within *Smilax* that contains *S. artisotlochiifolia* and *S. aspera* in which the biosynthesis of 5$\beta$ saponins is favored.

The antiproliferative activity of the isolated saponins **1**–**5** was compared against six human cancer cell lines derived from different tumor types, with compound **2** showing particular activity against the HT29 colon cancer cell line. The specificity of **2**, a more hydrophobic structure than **1** or **3,** for inhibition of HT29 might provide an opportunity to explore the determinants of both saponin potency and selectivity.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2012.01.009.

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