

Steroidal Saponins from the Roots of *Smilax aspera* subsp. *mauritanica*

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Two new steroidal saponins (**1**, **2**) were isolated from the roots of *Smilax aspera* subsp. *mauritanica* (POIR.) ARCANG. (Liliaceae), together with the known curillin G (**3**), asparagoside E (**4**), asparoside A (**5**), asparoside B (**6**) and the phenolic compound resveratrol (**7**). Their structures were established mainly on the basis of 600 MHz 2D-NMR spectral data. **3** exhibited antifungal activity against the human pathogenic yeasts *Candida albicans*, *C. glabrata* and *C. tropicalis* (minimum inhibitory concentrations of 25, 25 and 50 µg/ml, respectively) whereas the other compounds were inactive.

Key words *Smilax aspera* subsp. *mauritanica*; Liliaceae; steroidal saponin; resveratrol; 2D-NMR; antifungal activity

The genus *Smilax* contains 350 species, which are distributed widely mainly in tropical regions of east Asia, and South and North America. Several *Smilax* species have already been studied chemically and found to contain steroidal saponins.^{1–6} From a biological point of view, some species were documented to exhibit antiinflammatory,⁷ NO-modulating⁸ and antileptotic⁹ activity and recently, antifungal steroidal saponins were isolated from the rhizome of *S. medica*.^{5,6} As part of our ongoing search for new antifungal steroid saponins,^{5,6,10–12} we have investigated the roots of *Smilax aspera* subsp. *mauritanica*. The present paper reports the isolation and characterization of two new steroidal saponins (**1**, **2**) along with four known saponins (**3**–**6**) and the phenolic compound resveratrol (**7**). Their structures were determined by spectroscopic methods including 1D and 2D NMR experiments and FAB-MS and HR-ESI-MS. Furthermore, their antifungal activity was tested against three human pathogenic yeasts (*Candida albicans*, *C. glabrata*, and *C. tropicalis*).

The *n*-BuOH-soluble fraction of the MeOH–H₂O (7:3) extract of the roots of *S. aspera* subsp. *mauritanica* was purified by precipitation with diethyl ether to give a crude saponin mixture. This mixture was submitted to multiple chromatographic steps involving vacuum-liquid chromatography (VLC) on reversed-phase C₁₈ silica gel and medium-pressure liquid chromatography (MPLC) on normal silica gel to yield compounds **1**–**6**. The diethyl ether fraction was submitted to multiple MPLC to give the pure phenolic compound **7**.

Compound **1**, a white amorphous powder, exhibited in FAB-MS (negative-ion mode) a quasimolecular ion peak at *m/z* 885 [M–H][–], indicating a molecular weight of 886. Its molecular formula was established as C₄₅H₇₄O₁₇ by the positive ion-mode HR-ESI-MS showing a pseudo-molecular ion peak at *m/z* 909.5392 [M+Na]⁺ (Calcd for C₄₅H₇₄O₁₇Na: 909.5352). Other fragment ion peaks were observed at *m/z* 739 [(M–H)–146][–] and 577 [(M–H)–146–162][–], corresponding to the successive loss of one deoxy-hexosyl and one hexosyl moieties. Acid hydrolysis of **1** yielded glucose, rhamnose (TLC) and their absolute configuration was determined to be D and L by GC analysis of chiral derivatives of the sugars in the acid hydrolysate (see Experimental). The

aglycon was identified as sarsapogenin [(25S)-5β-spirostane-3β-ol]^{13,14} from the 1D and 2D NMR spectral data of **1** (see Table 1). The A/B *cis*-ring fusion was confirmed by observation of the signals at δ_C 35.3 (C-5), 39.8 (C-9), and 23.6 (C-19), indicating that the aglycon is a 5β-steroidal saponin.¹⁵ The 25S stereochemistry of the 27-methyl group was deduced based on the presence of the two proton signals [δ_H 3.34 (1H, d, *J*=10.9 Hz) and 4.00 (1H, m)], which corresponded to the H₂-26 in the ¹H-NMR¹⁶ and the higher field resonance of C-27 (δ_C 15.8) when compared to the ¹³C-NMR shift of 25R-spirostanes (δ_C 17.1–17.2).¹⁷ The ¹H-NMR spectrum of **1** displayed signals for three anomeric protons at δ_H 4.84, 5.54 (d, *J*=7.5 Hz) and 6.0 (s), which gave correlations, in the HSQC spectrum, with ¹³C-NMR signals at δ_C 100.0, 101.2 and 101.9, respectively. Evaluation of chemical shifts and spin–spin couplings allowed the identification of two β-glucopyranosyl units (Glc I, Glc II) and one α-rhamnopyranosyl units (Rha I). The sequence of the oligosaccharide chain was determined from the HMBC and NOESY spectra. Correlations observed in the HMBC spectrum between the ¹H-NMR signal at δ_H 4.84 (Glc I H-1) and the ¹³C-NMR signal at δ_C 74.7 (Agly C-3), and in the NOESY spectrum between δ_H 4.84 (Glc I H-1) and δ_H 4.26 (m) (Agly H-3) proved the Glc I to be linked at C-3 of the aglycon. The correlation in the HMBC spectrum between the ¹H-NMR signal at δ_H 5.54 (d, *J*=7.5 Hz) (Glc II I H-1) and δ_C 78.2 (Glc I C-2) and a NOESY cross-peak between δ_H 5.54 (d, *J*=7.5 Hz) (Glc II H-1) and δ_H 4.34 (Glc I H-2), revealed a (1→2) linkage between these two sugars. The linkage of Rha I to the 2-position of Glc II was deduced by the HMBC correlation observed between δ_H 6.0 (s) (Rha I H-1) and δ_C 79.4 (Glc II C-2) and the NOESY cross-peak between δ_H 6.0 (s) (Rha I H-1) and δ_H 3.94 (Glc II H-2). On the basis of the above results, the structure of **1** was established as (25S)-5β-spirostane-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

Compound **2** was isolated as a white amorphous powder. Its HR-ESI-MS (positive-ion mode) exhibited a pseudo-molecular ion peak at *m/z* 1089.5195 [M+Na]⁺ (Calcd for C₅₁H₈₆O₂₃Na: 1045.5245), ascribable to a molecular formula C₅₁H₈₆O₂₃. Its FAB-MS spectrum (negative-ion mode) showed a quasi-molecular ion peak at *m/z* 1065 [M–H][–] indicating a

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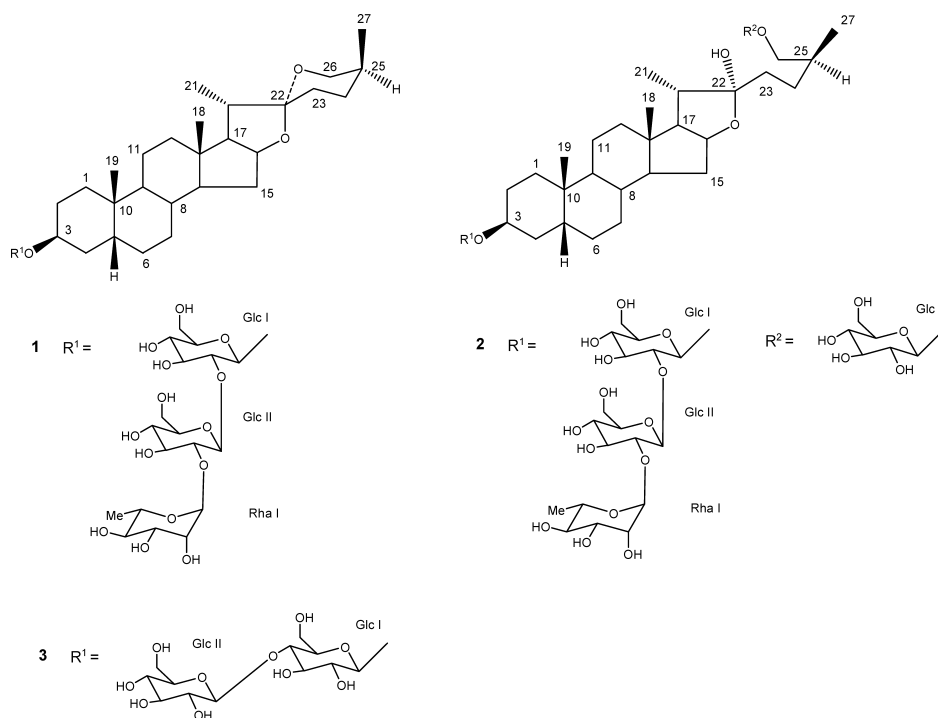


Fig. 1. Chemical Structures of 1–3

molecular weight of 1066. A fragment ion peak was observed at m/z 919 $[(M-H)-146]^-$ corresponding to the loss of one deoxy-hexosyl moiety. The comparison of NMR data of **2** (Table 1) with literature data allowed the identification of the aglycon as the previously reported (25*S*)-3 β ,5 β ,22 α -furostane-3,22,26-triol (the aglycon of aspaflioside D).¹⁸⁾ The 25*S* stereochemistry of the Me-27 group was deduced from the resonances of protons and carbons at C-25 (δ_C 33.1), C-26 (δ_C 74.5), and C-27 (δ_C 17.0) in comparison with literature data.^{19,20)} The differences observed in 1H -NMR chemical shifts of the geminal protons H-26a and H-26b ($\delta_a - \delta_b = 0.58$) supported a 25*S* furostane-type steroid since this difference is usually more than 0.57 ppm in 25*S* compounds and less than 0.48 ppm in 25*R* compounds.^{19,20)} The 1H -NMR spectrum of **2** displayed signals for four anomeric protons at δ_H 4.90 (d, $J=7.5$ Hz), 5.60 (d, $J=7.5$ Hz), 6.06 (s) and 4.70 (d, $J=7.6$ Hz), which gave correlations, in the HSQC spectrum, with ^{13}C -NMR signals at δ_C 99.4, 101.6, 101.9 and 104.3, respectively. Evaluation of chemical shifts and spin–spin couplings allowed the identification of three β -glucopyranosyl units (Glc I, Glc II, Glc III) and one α -rhamnopyranosyl unit (Rha I). The sequence of the oligosaccharide chain was determined from the HMBC and the NOESY spectra. 1H - and ^{13}C -NMR signals of the oligoside at C-3 were superimposable with those of **1** revealing the same sequence of sugars as described above. The linkage of the sugar at the C-26 position was indicated by the correlation in the NOESY spectrum between the anomeric proton of Glc III at δ_H 4.70 (d, $J=7.9$ Hz) and δ_H 3.42 (Agly C-26). On the basis of the above results, the structure of **2** was established as (25*S*)-3 β ,5 β ,22 α -furostane-3,22,26-triol 3-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosyl 26-*O*- β -D-glucopyranoside.

Compounds **3**–**7** were identified by interpretation of their spectral data, mainly FAB-MS and 2D-NMR (COSY, TOCSY,

Table 1. 1H - and ^{13}C -NMR Data^{a)} of the Aglycone Part of 1–2, δ in ppm (J in Hz)

	1		2	
	δ_C	$\delta_H^{b),c)}$	δ_C	$\delta_H^{b),c)}$
1	30.2	1.28, ^{b)} nd	29.4	1.20, ^{b)} nd
2	26.4	1.52, ^{b)} 1.80 ^{b)}	26.5	1.10, ^{b)} 1.84 ^{b)}
3	74.7	4.26 m	74.5	4.29 m
4	29.6	1.70, ^{b)} nd	29.4	1.20, ^{b)} nd
5	35.3	2.35 ^{b)}	35.2	2.36 ^{b)}
6	25.6	1.30, ^{b)} 1.40 ^{b)}	30.3	1.80, ^{b)} nd
7	25.8	nd, nd	26.4	1.10, ^{b)} nd
8	35.1	1.30 m	34.9	1.33 m
9	39.8	1.28 ^{b)}	39.9	1.20 ^{b)}
10	34.8		34.8	
11	20.7	1.10, ^{b)} nd	21.6	nd
12	39.9	1.00, ^{b)} 1.60 ^{b)}	39.9	1.20, ^{b)} 1.65 ^{b)}
13	40.5		40.9	
14	56.0	1.00 ^{b)}	55.9	0.98 ^{b)}
15	31.6	1.30, ^{b)} 1.62 ^{b)}	31.8	nd
16	81.1	4.55 m	81.0	4.94 m
17	62.2	1.80 dd (6.7, 8.8)	63.2	1.92 ^{b)}
18	16.1	0.74 s	16.3	0.79 s
19	23.6	0.88 s	23.6	0.90 s
20	42.1	1.84 m	40.2	2.16 m
21	14.4	1.10 d (6.7)	16.0	1.26 ^{b)}
22	109.7		110.5	
23	31.6	1.25, ^{b)} 1.95 ^{b)}	36.4	1.96, ^{b)} 2.07 ^{b)}
24	26.3	1.30, ^{b)} 2.08 ^{b)}	27.8	1.63, ^{b)} 1.97 ^{b)}
25	26.9	1.80	33.1	1.86 m
26	64.9	3.34 d (10.9), 4.00 ^{b)}	74.5	3.42, ^{b)} 4.00 ^{b)}
27	15.8	0.99 d (6.9)	17.0	0.98 d (6.5)

a) Measured at 600 MHz for 1H and 150 MHz for ^{13}C with reference to pyridine- d_5 .

b) Overlapping 1H -NMR signals are reported without designated multiplicities. c) nd: not determined.

NOESY, HSQC and HMBC) as well by comparison with literature data as curillin G (**3**),²¹⁾ asparagosome E (**4**),²²⁾ asparosome A (**5**),²³⁾ asparosome B (**6**)²³⁾ and resveratrol (**7**),²⁴⁾ re-

Table 2. ^1H - and ^{13}C -NMR Data of the Sugar Moieties of **1**–**2** (in Pyridine- d_5)^{a),b),c)} δ in ppm (J in Hz)

	1		2	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}
Glc I				
1	100.0	4.84 ^{b)}	99.4	4.90 d (7.5)
2	78.2	4.34 ^{b)}	78.4	4.39 ^{b)}
3	79.4	4.02	79.2	4.52
4	71.0	3.94 ^{b)}	71.1	4.04 ^{b)}
5	77.6	3.82 br d (9.3)	77.6	3.85 ^{b)}
6	62.8	4.12, ^{b)} 4.50 ^{b)}	62.1	4.20, ^{b)} 4.40 ^{b)}
Glc II				
1	101.2	5.54 d (7.5)	101.6	5.60 d (7.5)
2	79.4	3.94 ^{b)}	79.4	4.00 dd (9.3, 9.8)
3	78.3	4.02 dd (8.6, 8.8)	78.4	4.08 ^{b)}
4	72.1	3.78 (8.6, 9.8)	72.2	3.84 ^{b)}
5	77.6	3.84 m	77.6	3.84 ^{b)}
6	61.7	4.12, ^{b)} 4.32 ^{b)}	62.9	4.19, ^{b)} 4.52 ^{b)}
Rha I				
1	101.9	6.0 s	101.9	6.06 s
2	71.6	4.63 br s	71.7	4.57 br s
3	71.7	4.52 ^{b)}	71.7	4.57 dd (7.6, 11.2)
4	73.5	4.22 ^{b)}	73.1	4.26 dd (9.9, 9.1)
5	68.7	4.86 dq (5.9, 9.3)	68.8	4.89 ^{b)}
6	18.4	1.70 d (6.2)	18.5	1.72 d (4.5)
Glc III				
1			104.3	4.70 d (7.6)
2			73.7	3.92 ^{b)}
3			77.6	4.18 dd (8.1, 8.3)
4			71.1	3.97 dd (8.6, 8.3)
5			77.6	3.85 ^{b)}
6			62.9	4.19, ^{b)} 4.38 ^{b)}

a) The assignments were based on the DEPT, HSQC and HMBC experiment (150 MHz for ^{13}C -NMR, 600 MHz for ^1H -NMR). b) Overlapping ^1H -NMR signals are reported without designated multiplicities. c) ^1H - and ^{13}C -NMR chemical shifts of substituted residues are italicized.

spectively. The presence of resveratrol in some plants species was of no interest until 1992 when it was reported the occurrence of this compound as a potent antioxidant.²⁵⁾ This compound was previously isolated in some Liliaceae such as *Veratrum* sp.²⁶⁾ or *Yucca* sp.²⁷⁾ but to our knowledge, this is the first account of the isolation of resveratrol from the *Smilax* genus.

The antifungal activity of compounds **1**–**6** was evaluated at concentrations less than 200 $\mu\text{g/ml}$ against strains of *Candida albicans*, *C. glabrata* and *C. tropicalis* (Table 3). According to our previous observations, saponins having a furostan skeleton (**2**, **4**, **5**, **6**) were devoid of activity against the tested fungi.^{5,6,10–12)} Concerning the spirostanol saponins, only compound **3**, having two sugars for the osidic chain showed an antifungal activity against the yeast tested with minimum inhibitory concentration (MIC) values between 25 and 50 $\mu\text{g/ml}$ (Table 4). Antifungal activities were also observed previously with SS₁ which differ of **3** by the (1 $\rightarrow$ 6) linkage of the second glucose. Sautour *et al.* showed that spirostanol saponins (SS₂ and SS₃) from *Smilax medica* having three sugars for the osidic chain exhibited antifungal activities with MIC values between 12.5 to 50 $\mu\text{g/ml}$.⁵⁾ In the present study, compound **1** was devoid of activity. The difference between the osidic part of **1** and other saponins having three sugars (SS₂ and SS₃) is the presence of a rhamnose unit, but especially the number of saccharides connected at Glc I. Indeed, SS₂ and SS₃ possess a branched-chain

Table 3. Antifungal Activity of **1**–**6** and Ketoconazole against *Candida* Species Given as MIC ($\mu\text{g/ml}$)^{a)}

Compounds	<i>Candida albicans</i>	<i>Candida glabrata</i>	<i>Candida tropicalis</i>
1	>200	>200	>200
2	>200	>200	>200
3	25	25	50
4 – 6	>200	>200	>200
Ketoconazole ^{b)}	1.56	1.56	3.12

a) Compounds with MIC values >200 $\mu\text{g/ml}$ are considered not active. b) Positive control.

Table 4. Antifungal Activity of **1**, **3** and Other Spirostanol Saponins (SS₁–SS₃) According to the Osidic Part at the Position C-3

Osidic part at the position C-3	MIC values against	
	<i>C. albicans</i> , <i>C. glabrata</i>	Ref. and <i>C. tropicalis</i>
3 : β -D-Glc-(1 $\rightarrow$ 4)- β -D-Glc	25 to 50 $\mu\text{g/ml}$	
SS ₁ : β -D-Glc-(1 $\rightarrow$ 6)- β -D-Glc	6.25 to 25 $\mu\text{g/ml}$	6
SS ₂ : β -D-Glc-(1 $\rightarrow$ 6)-[β -D-Glc-(1 $\rightarrow$ 4)]- β -D-Glc	12.5 to 50 $\mu\text{g/ml}$	5
SS ₃ : β -D-Glc-(1 $\rightarrow$ 6)-[β -D-Glc-(1 $\rightarrow$ 2)]- β -D-Glc	25 to 50 $\mu\text{g/ml}$	5
1 : α -L-Rha-(1 $\rightarrow$ 2)- β -D-Glc-(1 $\rightarrow$ 2)- β -D-Glc	>200 $\mu\text{g/ml}$	

trisaccharide moiety (Table 4), while the osidic chain of **1** is linear. The primary mode of action of saponins is believed to be through interaction with sterols of the plasma membrane,²⁸⁾ and the presence of a linear-chain trisaccharide moiety at the position C-3 seems to reduce the antifungal activity.

Experimental

General Experimental Methods IR spectra (CHCl_3) were recorded on a Perkin-Elmer 881 spectrophotometer. FAB-MS (negative-ion mode, glycerol matrix) was conducted on a JEOL SX 102 spectrometer. HR-ESI-MS was carried out on a Q-TOF 1 micromass spectrometer. Optical rotations were taken with a AA-10R automatic polarimeter. The 1D and 2D NMR spectra (^1H - ^1H COSY, TOCSY, NOESY, HSQC and HMBC) were performed using a UNITY-600 spectrometer at the operating frequency of 600 MHz on a Varian INOVA 600 instrument equipped with a SUN 4 L-X computer system (600 MHz for ^1H and 150 MHz for ^{13}C spectra). All chemical shifts (δ) are given in ppm and the samples were solubilized in pyridine- d_5 (δ_{C} 150.3, 135.9, 123.9). GC analysis was carried out on a Thermoquest gas chromatograph using a DB-1701 capillary column (30 m $\times$ 0.25 mm, i.d.) (J & W Scientific), with detection by FID, and the initial temperature maintained at 80 $^\circ\text{C}$ for 5 min and then raised to 270 $^\circ\text{C}$ at the rate of 15 $^\circ\text{C}/\text{min}$; carrier gas: He. Compound isolations were carried out using a medium-pressure liquid chromatography (MPLC) system [Gilson M 305 pump, 25 SC head pump, M 805 manometric module, Büchi column (460 $\times$ 25 mm and 460 $\times$ 15 mm), Büchi precolumn (110 $\times$ 15 mm)] and silica gel 60 (Merck, 15–40 μm). Vacuum-liquid chromatography (VLC) was performed on a C₁₈ reversed phase (Merck, 25–40 μm) (12 $\times$ 3 cm). TLC and HPTLC employed precoated silica gel 60 F₂₅₄ plates (Merck). The following TLC solvent systems were used: for saponins (a) CHCl_3 -MeOH- H_2O (13:7:2, lower phase); for sapogenins (b) CHCl_3 -MeOH (9:1); for monosaccharides (c) CHCl_3 -MeOH- H_2O (8:5:1). Spray reagents for the saponins were: Komarowsky reagent, a mixture (5:1) of *p*-hydroxybenzaldehyde (2% in MeOH) and H_2SO_4 50%; for the sugars: diphenylaminephosphoric acid reagent.

Plant Material The roots of *Smilax aspera* subsp. *mauritanica* (POIR.) ARCAN. were collected in Mas de Jau from Case de Pènes (Roussillon, France) and identified by Gerard Ducerf. A voucher specimen (No. 6624) is deposited in the Herbarium of the Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Burgundy, France.

Extraction and Separation Dried powdered roots (410 g) of *S. aspera* subsp. *mauritanica* were refluxed three times with MeOH- H_2O (7:3, 21),

evaporated to dryness yielding a MeOH–H₂O extract. This was partitioned successively with hexane, CH₂Cl₂ and *n*-BuOH (each 3×200 ml) yielding after evaporation of the solvents the corresponding hexane (1 g), CH₂Cl₂ (1.5 g) and *n*-BuOH (17 g) fractions. Nine grams of the *n*-BuOH residue was dissolved in MeOH and purified by precipitation with diethyl ether (2×250 ml), yielding a crude saponin mixture (5.2 g). This latter was submitted to the VLC on C₁₈ reversed-phase (12×3 cm) using H₂O (100 ml), MeOH–H₂O mixtures (5:5; 4:1, each 100 ml) and finally MeOH (100 ml). The fraction eluted with MeOH–H₂O (4:1) (435 mg) was submitted to MPLC column chromatography (Si gel (15–40 μm), system a), yielding 11 fractions, 1–11. Fraction 1 was rechromatographed in the same conditions to give the pure compound **6** (5 mg). Fraction 3 was rechromatographed in the same conditions to give the pure compound **2** (6 mg). Fractions 4, 6 and 9 were rechromatographed in the same conditions to give the pure compounds **3** (9 mg), **4** (5 mg) and **5** (11 mg), respectively. The fraction eluted with 100% MeOH (200 mg) was submitted to MPLC (system a) to give 10 fractions, 1–10. Fraction 6 was concentrated to dryness, yielding the pure compound **1** (21 mg). The diethyl ether fraction (1.13 g) was submitted to MPLC column chromatography (system a) yielding the pure compound **7** (9 mg).

(25S)-5-β-Spirostane-3β-ol 3-*O*-α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (**1**): White amorphous powder, HR-ESI-MS (positive ion-mode) *m/z*: 909.5392 [M+Na]⁺, (Calcd for C₄₅H₇₄O₁₇Na: 909.5352). FAB-MS (negative ion mode) *m/z*: 885 [M–H][–]. [α]_D²⁰ –33.3° (*c*=0.07, MeOH). IR *v*_{max} (CHCl₃) cm^{–1}: 3255 (OH), 2980 (CH), 1040 (C–O–C). ¹H- and ¹³C-NMR: see Tables 1 and 2.

(25S)-3β,5β,22α-Furostane-3,22,26-triol 3-*O*-α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl 26-*O*-β-D-Glucopyranoside (**2**): White amorphous powder, HR-ESI-MS (positive ion-mode) *m/z*: 1089.5195 [M+Na]⁺, (Calcd for C₅₁H₈₆O₂₃Na: 1045.5245). FAB-MS (negative ion mode) *m/z*: 1065 [M–H][–]. [α]_D²⁰ –69.2° (*c*=0.053, MeOH). IR *v*_{max} (CHCl₃) cm^{–1}: 3355 (OH), 2927 (CH), 1067 (C–O–C). ¹H- and ¹³C-NMR: see Tables 1 and 2.

Acid Hydrolysis A solution of each saponin (3 mg) in 2N aqueous CF₃COOH (5 ml) was refluxed on a water bath for 3 h. After extraction with CH₂Cl₂ (3×5 ml), the aqueous layer was repeatedly evaporated to dryness with MeOH until neutral and glucose was identified by TLC with a standard using CHCl₃–MeOH–H₂O (8:5:1). Furthermore, a silylated derivated of the sugar was prepared according to the procedure previously described.²⁹⁾ L-Cysteine methyl ester hydrochloride (0.06 mol/l) and HMDS–TMCS (hexamethyldisilazane–trimethylchlorosilane, 3:1) were added to the aqueous residue. After centrifugation of the precipitate, the supernatant was concentrated and partitioned between *n*-hexane and H₂O, and the hexane layer was analyzed by GC. D-Glucose and L-rhamnose were detected.

Antifungal Activity Minimum inhibitory concentrations (MICs) were performed using the broth dilution test.³⁰⁾ For these bioassays three human pathogenic yeasts were used: *Candida albicans* (IP 1180-79), *C. glabrata* and *C. tropicalis* (clinical isolates). The reference compound ketoconazole (Sigma) was used as positive control.

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