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Steroidal saponins from *Asparagus acutifolius*

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Abstract

Six new steroidal saponins (**1**–**6**) were isolated from the roots of *A. acutifolius* L., together with a known spirostanol glycoside (**7**). Their structures were elucidated mainly by extensive spectroscopic analysis (1D and 2D NMR, FABMS and HRESIMS). Compounds **4**–**7** demonstrated antifungal activity against the human pathogenic yeasts *C. albicans*, *C. glabrata* and *C. tropicalis* with MICs values between 12.5 and 100 μg/ml.

Keywords: *Asparagus acutifolius*; Liliaceae; Steroidal saponins; Antifungal activity

1. Introduction

Asparagus acutifolius L. belonging to the family Liliaceae is a native plant species widely distributed throughout the Mediteranean areas (Sica et al., 2005). A survey of the literature showed that several *Asparagus* species have already been chemically studied and found to contain steroidal saponins (Sharma et al., 1982; Sati and Pant, 1985; Shimoyamada et al., 1996; Oketch-Rabah and Dossaji, 1997; Ahmad et al., 1999; Debella et al., 1999; Zhang et al., 2004; Yang et al., 2004a; Kim et al., 2005). From a biological point of view, some species were documented to exhibit antifungal (Shimoyamada et al., 1996), antiprotozoal (Oketch-Rabah and Dossaji, 1997) and cytotoxic (Zhang et al., 2004; Yang et al., 2004a) activity. Previous phytochemical investigations carried out on aerial part of *A. acutifolius* have resulted in the obtention of flavonoids (Panova et al., 1984) but there has been no detailed report on the phytochemistry and biological activity of constituents contained in the underground part of this plant. As part of our ongoing search for new antifungal steroid saponins (Sautour et al., 2004a,b, 2005) we have investigated the roots of *A. acutifolius* L. The present paper reports the isolation and characterization of six new steroidal saponins (**1**–**6**) along with a known spirostanol saponin (**7**). Their structures were determined by spectroscopic methods including 1D and 2D NMR experiments and FABMS and HRESIMS. Furthermore, their antifungal activity was tested against three human pathogenic yeasts (*Candida albicans*, *C. glabrata*, and *C. tropicalis*).

2. Results and discussion

The *n*-BuOH-soluble fraction of the MeOH–H_2O (7:3) extract of the roots of *A. acutifolius* 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 C_{18} silica gel and medium-pressure liquid chromatography (MPLC) on normal silica gel to yield compounds **1**–**6** and the known (25*S*)-5β-spirostan-3β-ol 3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranoside (**7**) isolated from *Asparagus officinalis* (Shimoyamada et al., 1990).

After acid hydrolysis of compounds **1**–**6**, an artifactual aglycon was obtained. Glucose and xylose were identified by comparison on TLC with an authentic sample and the

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absolute configurations of the sugars were determined to be D by GC analysis of chiral derivatives of the sugars in the acid hydrolysate (see Section 3).

Compound **1**, a white amorphous powder, exhibited in FABMS (negative-ion mode) a quasimolecular ion peak at *m/z* 1035 $[M-H]^-$, indicating a molecular weight of 1036. The molecular formula was established as $C_{50}H_{84}O_{22}$ by the positive ion-mode HRESIMS showing a pseudo-molecular ion peak at *m/z* 1059.5392 $[M+Na]^+$ (calcd for 1059.5352 $C_{50}H_{84}O_{22}Na$). Other fragment ion peaks were observed in the FABMS spectrum at *m/z* 903 $[(M-H)-132]^-$, corresponding to the loss of one pentosyl moiety. The comparison of NMR data of **1** with literature data allowed the identification of the aglycon as the previously reported (25*S*)-3β,5 β,22α-22-methoxyfurostane-3,26-diol (the aglycon of timosaponin BI) (Bian et al., 1996; Sautour et al., 2005). The A/B *cis*-ring fusion was deduced by the signals at δ_C 35.5 (C-5), 39.9 (C-9), and 23.3 (C-19), indicating that **1** is a 5β-steroidal sapogenin (Yang et al., 2004b). The 25*S* stereochemistry of the Me-27 group was deduced from the resonances of protons and carbons at C-25 (δ_C 33.5), C-26 (δ_C 74.6), and C-27 (δ_C 16.8) in comparison with literature data (Agrawal, 2004, 2005). The differences observed in ^{1}H NMR chemical shifts of the geminal protons H-26a and H-26b ($\delta_a - \delta_b = 0.72$) supported a 25*S* furostane-type steroid since this difference is usually >0.57 ppm in 25*S* compounds and <0.48 ppm in 25*R* compounds (Agrawal, 2004, 2005). The configuration of 22-methoxy group was deduced to be α by the observed NOESY correlation between the methoxy protons at δ_H 3.28 (*s*) and the H-16 proton signal at δ_H 4.44 (Braca et al., 2004). The ^{1}H NMR spectrum of **1** displayed signals for four anomeric protons at δ_H 4.78 (*d*, $J = 7.2$ Hz), 5.06 (*d*, $J = 6.9$ Hz), 4.82 (*d*, $J = 7.9$ Hz) and 4.70 (*d*, $J = 7.6$ Hz), which gave correlations, in the HSQC spectrum, with ^{13}C NMR signals at δ_C 99.8, 105.6, 104.2 and 103.7, respectively. Evaluation of chemical shifts and spin-spin couplings allowed the identification of two β-glucopyranosyl units (Glc I, Glc II) and two β-xylopyranosyl units (Xyl I, Xyl II). The sequence of the oligosaccharide chain was determined from the HMBC and NOESY spectra. Correlations observed in the HMBC spectrum between the ^{1}H NMR signals at δ_H 4.78 (*d*, $J = 7.2$ Hz, Glc I H-1) and the ^{13}C NMR signal at δ_C 74.5 (Agly C-3), and in the NOESY spectrum between δ_H 4.78 (Glc I H-1) and δ_H 4.30 (*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 ^{1}H NMR signal at δ_H 5.06 (*d*, $J = 6.9$ Hz, Xyl I H-1) and δ_C 81.0 (Glc I C-2) and a NOESY cross-peak between δ_H 5.06 (Xyl I H-1) and δ_H 3.95 (Glc I H-2), revealed a (1 → 2) linkage between these two sugars. The linkage of Xyl II to the 4-position of Glc I was deduced by the HMBC correlation observed between δ_H 4.82 (Xyl II H-1) and δ_C 79.5 (Glc I C-4) and the NOESY cross-peak between δ_H 4.82 (Xyl II H-1) and δ_H 4.00 (Glc I H-4). The linkage of the sugar at the C-26 position was indicated by long-range coupling (^3J) in the HMBC spectrum between the anomeric proton of Glc II at δ_H 4.70 (*d*, $J = 7.9$ Hz) and δ_C 74.6 (Agly C-26). On the basis of the above results, the structure of **1** was established as (25*S*)-3β,5β,22α-22-methoxyfurostane-3,26-diol 3-*O*-β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranosyl 26-*O*-β-D-glucopyranoside. Since the 22-hydroxyfurostanols are readily converted to the corresponding 22-methoxyfurostanols in the solution containing MeOH (Sharma et al., 1982), the presence of **1** as a natural product was confirmed by TLC after an extraction of 10 g of the roots of *A. acutifolius* in refluxed water (without MeOH) (Yin et al., 2003).

Compound **2** was isolated as a white amorphous powder. Its HRESIMS (positive-ion mode) exhibited a pseudo-molecular ion peak at *m/z* 1045.5145 $[M+Na]^+$ (calcd for 1045.5195 $C_{49}H_{82}O_{22}Na$), ascribable to a molecular formula $C_{49}H_{82}O_{22}$. Its FABMS spectrum (negative-ion mode) showed a quasi-molecular ion peak at *m/z* 1021 $[M-H]^-$ indicating a molecular weight of 1022. Other fragment ion peaks were observed at *m/z* 859 $[(M-H)-162]^-$ and at *m/z* 727 $[(M-H)-162-132]^-$ corresponding to the loss of one hexosyl and one pentosyl moiety. The comparison of NMR data of **1** with literature data allowed the identification of the aglycon as the previously reported (25*S*)-3β,5β,22α-furostane-3,22,26-triol (the aglycon of aspafilioside D) (Li et al., 2005). Scrutiny of the 2D NMR (COSY, NOESY, HMQC) data of compound **2** (Tables 1 and 2) and detailed comparison of the ^{1}H and ^{13}C NMR chemical shifts with those of **1** showed that **2** differed from **1** only by the absence of the methoxy group at C-22 of the aglycon. The absence of signal at δ_C 47.1 /δ_H 3.28 (22-OMe in **1**) and the presence of the signal at δ_C 110.5 (instead δ_C 112.8 in **1**, C-22) confirmed the presence of a C-22 hydroxy derivative. Accordingly, the structure of **2** was determined as (25*S*)-3β,5β,22α-furostane-3,22,26-triol 3-*O*-β-D-xylopyranosyl-(1 → 2)-[β-D- xylopyranosyl-(1 → 4)]-β-D-glucopyranosyl 26-*O*-β-D-glucopyranoside.

Compound **3** was obtained as a white amorphous powder. Its positive-ion mode HRESIMS showed a pseudo-molecular ion peak at *m/z* 927.4968 $[M+Na]^+$ (calcd for 927.4929 $C_{45}H_{76}O_{18}Na$) consistent with a molecular formula of $C_{45}H_{76}O_{18}$. The negative-ion mode FABMS spectrum exhibited a quasi-molecular ion peak at *m/z* 903 $[M-H]^-$, indicating a molecular mass of 904. Other fragment ion peak were observed at *m/z* 741 $[(M-H)-162]^-$ corresponding to the loss of one hexosyl moiety. Scrutiny of the 2D NMR (COSY, NOESY, HMQC) data of compound **3** (Tables 1 and 2) and detailed comparison of the ^{1}H and ^{13}C NMR chemical shifts with those of **1** showed that **3** differed from **1** only in the absence of Xyl II linked at C-4 of Glc I. This was confirmed by the upfield chemical shift at δ_C 71.0 (Glc I C-4) instead at δ_C 79.5 in **1**. Therefore, the structure of **3** was assigned as (25*S*)-3β,5β,22α-22-methoxyfurostane-3,26-diol 3-*O*-β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranosyl 26-*O*-β-D-glucopyranoside. Its natural presence in the plant was also verified after an extraction of 10 g of the roots in refluxed water (without MeOH) (Yin et al., 2003).

Compound **4**, exhibited in FABMS (negative-ion mode) a quasimolecular ion peak at *m/z* 857 $[M-H]^-$, indicating a

Table 1
^{1}H-and ^{13}C NMR data[a] of the aglycone part of **1-6** (in pyridine-d_5), δ in ppm, J in Hz

δ	**1**		**2**		**3**		**4**		**5**		**6**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	30.1	1.40[b], 1.82[b]	30.3	1.42[b], 1.74 [b]	30.5	1.43[b], 1.76[b]	30.3	1.42[b], 1.76 [b]	30.3	1.42[b], 1.76[b]	30.4	1.42[b], 1.75 [b]
2	26.3	1.10[b], 1.66[b]	26.6	1.44[b], 1.78 [b]	26.6	1.46[b], 1.74[b]	26.4	1.44[b], 1.70 [b]	26.4	1.44[b], 1.70[b]	26.4	1.44[b], 1.70 [b]
3	*74.5*	*4.30*[b]	*74.3*	*4.24 m*	*74.5*	*4.20 m*	*74.4*	*4.16 m*	*74.4*	*4.16 m*	*74.3*	*3.84 m*
4	30.1	1.62[b], 1.76[b]	30.3	1.65[b], nd	30.7	1.76[b], 1.84[b]	30.3	1.78[b], nd	30.3	1.78 [b], nd	30.4	1.42[b], 1.78[b]
5	35.5	2.08 *m*	35.5	2.14 *m*	36.0	2.14 *m*	35.8	2.14 [b]	35.8	2.14[b]	35.8	2.14 *m*
6	26.3	1.65[b], 1.80 *brd* (13.5)	26.6	1.70[b], 1.78 *brd* (12.4)	26.6	1.72[b], 1.82[b]	26.5	1.70[b], 1.86[b]	26.5	1.70[b], 1.86[b]	26.5	1.68[b], 1.88[b]
7	26.1	1.10[b], nd	26.4	1.06[b], 1.44[b]	26.4	1.10[b], 1.46[b]	26.4	1.08[b], 1.40[b]	26.4	1.08[b], 1.40[b]	26.4	1.10[b], 1.42[b]
8	34.9	1.29 *m*	35.2	1.36 *m*	34.8	1.36 *m*	35.8	1.44 *m*	35.8	1.44 *m*	35.7	1.42 *m*
9	39.9	1.10[b]	40.0	1.15[b]	39.8	1.16[b]	39.9	1.22[b]	39.9	1.22[b]	39.9	1.20[b]
10	34.5		34.8		34.5		34.9		34.9		34.9	
11	20.5	1.18[b], nd	20.8	1.10[b], 1.22[b]	20.7	1.10[b], 1.22[b]	20.7	1.18[b], 1.38[b]	20.8	1.18[b], 1.38[b]	20.8	1.18[b], 1.32[b]
12	39.5	1.14[b], 1.60[b]	41.1	1.00[b], 1.64 [b]	40.0	1.00[b], 1.62[b]	32.2	1.48[b], nd	32.2	1.48[b], nd	32.2	1.44[b], nd
13	40.7		40.9		40.9		45.2		40.6		45.1	
14	55.8	0.90[b]	56.0	0.95[b]	56.0	0.96[b]	52.5	2.01[b]	56.2	1.00[b]	52.4	1.98[b]
15	31.4	1.26[b], 1.90 *m*	31.9	1.32[b], 1.90 *m*	32.0	1.30[b], 1.92 *m*	31.7	1.35[b], 1.97[b]	31.7	1.40[b], 1.97[b]	31.7	1.35[b], 1.94
16	81.0	4.44 *m*	81.4	4.90 *m*	81.2	4.46 *m*	89.8	4.40 *m*	81.1	4.54 *m*	89.8	4.40 *m*
17	63.4	1.72[b]	63.3	1.91 *m*	62.1	1.74 *m*	89.8		62.4		89.8	
18	15.9	0.70 *s*	16.3	0.78 *s*	16.0	0.74 *s*	17.1	0.88 *s*	16.3	0.76 *s*	17.1	0.86 *s*
19	23.3	0.84 *s*	23.5	0.87 *s*	23.5	0.88 *s*	23.5	0.90 *s*	23.5	0.90 *s*	23.7	0.88 *s*
20	39.9	2.16 *m*	40.0	2.16 *m*	39.8	2.18 *m*	45.2	2.14 *m*	42.2	1.87 *m*	45.0	2.12 *m*
21	15.6	1.10 *d* (6.4)	16.0	1.24 *d* (6.7)	16.1	1.10 *d* (6.7)	9.2	1.16 *d* (7.1)	14.5	1.10 *d* (6.9)	9.1	1.16 *d* (7.1)
22	112.8		110.5		112.6		110.2		109.7		110.2	
23	39.5	0.90[b], 1.60[b]	36.5	1.94[b], 2.06 [b]	39.8	1.00[b], 1.60[b]	26.0	1.30[b], nd	26.0	1.30[b], nd	26.0	1.30[b], nd
24	27.5	1.34[b], 1.60[b]	27.8	1.61[b], 1.95 [b]	27.8	1.36[b], 1.62[b]	26.4	1.07[b], nd	26.4	1.07[b], nd	26.4	1.06[b], nd
25	33.5	1.83 *m*	34.0	1.84 *m*	33.9	1.82[b]	27.1	1.54 [b]	27.1	1.54[b]	27.0	1.52[b]
26	*74.6*	*3.47 m, 4.22*[b]	*75.0*	*3.43 m, 3.98*[b]	*74.5*	*3.50 m, 4.00*[b]	64.8	3.26 *m*, 4.02[b]	64.9	3.34 *m*, 4.02[b]	64.8	3.28[b], 4.02[b]
27	16.8	0.98 *d* (6.4)	17.0	0.95 *d* (6.4)	17.1	0.98 *d* (6.4)	15.9	0.98 *d* (5.7)	15.9	0.98 *d* (5.7)	15.9	0.96 *d* (5.9)
22-OMe	47.1	3.28 *s*			47.2	3.24 *s*						

nd: not determined.
[a] ^{1}H- and ^{13}C NMR chemical shifts of substituted residues are italicized.
[b] Overlapped with other signals.

Table 2
^{1}H- and ^{13}C NMR data [a] of the sugar moieties of **1**–**6** (in pyridine-d_5), δ in ppm, J in Hz

	1		**2**		**3**		**4**		**5**		**6**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Glc I-1	*99.8*	*4.78 d (7.6)*	*100.2*	*4.80 d (7.6)*	*100.3*	*4.80 d (7.8)*	*100.2*	*4.77 d (8.2)*	*100.2*	*4.79 d (8.1)*	*100.6*	*4.78 d (7.9)*
2	*81.0*	*3.95*[b]	*81.4*	*4.02 t (7.8)*	*82.8*	*3.98 t (7.3)*	*81.4*	*4.04*[b]	*81.4*	*4.04*[b]	*79.9*	*4.20*[b]
3	75.2	4.12[b]	75.6	4.17[b]	77.7	4.22[b]	75.7	4.13[b]	75.7	4.13[b]	75.9	4.18[b]
4	*79.5*	*4.00*[b]	*79.9*	*4.10*[b]	71.0	4.04[b]	*80.0*	*4.00*[b]	*80.0*	*4.00*[b]	*80.0*	*4.02*[b]
5	75.2	3.75 *m*	75.7	3.74 *m*	77.6	3.82[b]	75.7	3.72[b]	75.7	3.72[b]	75.8	3.74[b]
6	60.5	4.28[b], nd	60.9	4.30[b], 4.36[b]	61.0	4.30[b], 4.34[b]	60.9	4.30[b], 4.37[b]	60.9	4.30[b], 4.37[b]	60.9	4.30[b], 4.35[b]
Xyl I-1	*105.6*	*5.06 d (6.9)*	*105.6*	*5.17 d (7.4)*	*106.0*	*5.10 d (7.6)*	*105.6*	*5.16 d (7.4)*	*105.6*	*5.17 d (7.5)*	*104.8*	*4.86 d (7.0)*
2	74.0	3.85 *t* (8.5)	74.5	3.92 *t* (8.6)	74.0	3.92 *t* (9.0)	75.7	3.94[b]	75.7	3.94[b]	74.3	3.84[b]
3	76.6	4.04[b]	76.9	4.10[b]	77.0	4.08[b]	76.9	4.12[b]	76.9	4.12[b]	77.4	4.04[b]
4	70.1	4.10 *m*	70.5	4.18 *m*	70.5	4.15[b]	70.5	4.20[b]	70.5	4.20[b]	70.2	4.05[b]
5	66.3	3.58 *t* (10.9), 4.28[b]	66.8	3.64 *t* (10.5), 4.35[b]	67.0	3.62 *t* (10.9), 4.33[b]	66.8	3.66[b], 4.38[b]	66.8	3.66[b], 4.38[b]	66.6	3.58[b], 4.12[b]
Xyl II-1	*104.2*	*4.82 d (7.9)*	*104.8*	*4.92 d (7.6)*			*104.8*	*4.91 d (7.3)*	*104.8*	*4.93 d (7.4)*		
2	73.8	3.78 *t* (8.3)	74.3	3.85 *t* (8.3)			74.3	3.86[b]	74.3	3.86[b]		
3	76.4	4.08[b]	76.9	4.10[b]			77.4	4.06[b]	77.4	4.06[b]		
4	69.7	3.98 *m*	70.2	4.06[b]			70.2	4.08[b]	70.2	4.08[b]		
5	66.0	3.54 *t* (10.2), 4.08[b]	66.6	3.56[b], 4.10[b]			66.6	3.58[b], 4.10[b]	66.6	3.58[b], 4.10[b]		
Glc II-1	*103.7*	*4.70 d (7.6)*	*104.4*	*4.70 d (7.8)*	*104.3*	*4.72 d (7.9)*					*104.1*	*5.34 d (7.2)*
2	75.2	3.82 *t* (8.3)	75.7	3.92 *t* (8.6)	75.7	3.92 *t* (9.0)					75.8	3.90[b]
3	77.0	4.11[b]	77.7	4.15[b]	77.6	4.18[b]					77.4	4.16[b]
4	70.7	3.92[b]	71.1	4.04[b]	71.0	4.04[b]					71.4	4.06[b]
5	77.0	3.83 *m*	77.7	3.84 *m*	77.7	3.88[b]					78.1	3.90[b]
6	61.7	4.10[b], 4.32[b]	62.1	4.20[b], 4.38[b]	62.1	4.19[b], 4.40[b]					62.5	4.28[b], 4.50[b]

nd: not determined.
[a] ^{1}H- and ^{13}C NMR chemical shifts of substituted residues are italicized.
[b] Overlapped with other signals.

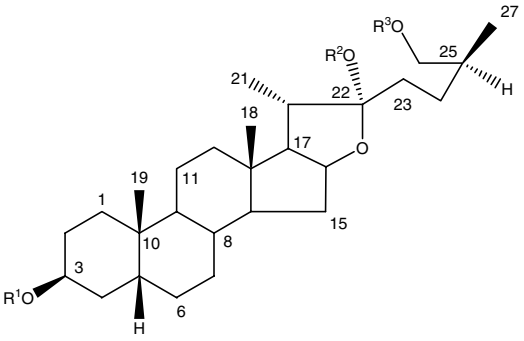
molecular weight of 858. The molecular formula was established as $C_{43}H_{70}O_{17}$by the positive ion-mode HRESIMS showing a pseudo-molecular ion peak at *m/z* 881.4561 $[M+Na]^+$ (calcd for 881.4511 $C_{43}H_{70}O_{17}Na$). Other fragment ion peaks were observed at *m/z* 725 $[(M-H)-132]^-$ and 593 $[(M-H)-132\ -132]^-$, corresponding to the successive loss of two pentosyl moieties. The aglycon was identified as 17α-hydroxysarsapogenin [(25*S*)-5β-spirostane-3β,17α-diol] (Kim et al., 2005; Huang and Kong, 2006) from the 1D and 2D NMR spectra of **4** (see Tables 1 and 2). The A/B *cis*-ring fusion was confirmed by observation of the signals at δ_C 35.8 (C-5), 39.9 (C-9), and 23.5 (C-19), indicating that the aglycon of **4** is a 5β-steroidal sapogenin (Yang et al., 2004a,b). The 25*S* stereochemistry of the 27-methyl group was deduced based on the presence of the two proton signals [δ_H 4.02 (1H, *m*) and 3.26 (1H, *d*, $J = 10.9$ Hz)], which corresponded to the H_2-26 in the ^{1}H NMR (Debella et al., 1999) and the higher field resonance of C-27 (δ_C 15.9) when compared to the ^{13}C NMR shift of 25*R*-spirostanes (δ_C 17.1–17.2) (Agrawal et al., 1995). In the ^{13}C NMR, a quaternary carbon signal was observed at δ_C 89.8. In the HMBC analysis, this carbon showed a $^3J_{C-H}$ correlation with the signal at δ_H 1.16 (3H, *d*, $J = 7.1$ Hz), which was assigned as H_3-21 (CH_3). These protons (H_3-21) in turn have $^3J_{C-H}$ correlation with C-22 (δ_C 110.2). The correlation between δ_C 89.8 and δ_H 0.88 (3H, *s*, H-18) was also observed. These observations allowed the assignment of the quaternary carbon C-17 at δ_C 89.8 suggesting this position to be hydroxylated. The NOESY correlation between H_3-18 (δ_H 0.88) and H-20 (δ_H 2.14) indicated the α-orientation of the C-17 hydroxyl group (Mimaki et al., 1999). The ^{1}H NMR spectrum of **4** displayed signals for three anomeric protons at δ_H 4.77 (*d*, $J = 8.6$ Hz), 5.16 (*d*, $J = 4.8$ Hz) and 4.91 (*d*, $J = 7.4$ Hz), which showed correlations, in the HSQC spectrum, with three anomeric carbon signals at δ_C 100.2, 105.6 and 104.8, respectively. The ring protons of the monosaccharide residues were assigned starting from the readily identifiable anomeric protons by means of COSY, TOCSY, NOESY, HSQC, and HMBC experiments (Table 2). Evaluation of spin-spin couplings and chemical shifts allowed the identification of one β-glucopyranosyl unit (Glc I) and two β-xylopyranosyl units (Xyl I and Xyl II). The sugar sequence investigated by extensive 2D NMR experiments revealed that the ^{1}H and ^{13}C NMR signals of the oligosaccharide chain at C-3 were almost superimposable with those of β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranosyl characterized in **1** (Table 2). Furthermore, the correlation observed in the HMBC spectrum between the ^{1}H NMR signal at δ_H 4.77 (Glc I H-1) and the ^{13}C NMR signal at δ_C 74.4 (Agly C-3), and in the NOESY spectrum between δ_H 4.77 (Glc I H-1) and δ_H 4.16 (Agly H-3) confirmed Glc I to be linked at C-3 of the aglycon. On the basis of the above results, the structure of **4** was established as (25*S*)-5β-spirostane-3β,17α-diol 3-*O*-β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranoside.

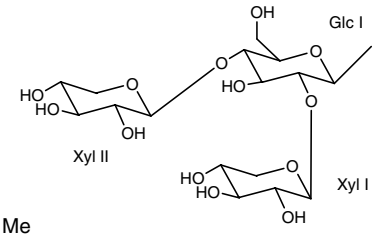
Compound **5**, a white amorphous powder, exhibited in FABMS (negative-ion mode) a quasi-molecular ion peak at *m/z* 841 $[M-H]^-$, indicating a molecular weight of 842. The molecular formula was established as $C_{43}H_{70}O_{16}$ by the positive ion-mode HRESIMS showing a pseudo-molecular ion peak at *m/z* 865.4601 $[M+Na]^+$ (calcd for 865.4562 $C_{43}H_{70}O_{16}Na$). Other fragment ion peaks were observed at *m/z* 709 $[(M-H)-132]^-$ corresponding to the loss of one pentosyl moiety. The aglycon was identified as sarsapogenin [(25*S*)-5β-spirostane-3β-ol] (Agrawal et al., 1998; Agrawal, 2003) from the 1D and 2D NMR spectral data of **5** (see Tables 1 and 2). The sugar sequence investigated by extensive 2D NMR experiments revealed that the ^{1}H and ^{13}C NMR signals of the oligosaccharide chain at C-3 were almost superimposable with those of β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl- (1 → 4)]-β-D-glucopyranosyl characterized in **1** and **4** (Table 2). Furthermore, the correlation observed in the HMBC spectrum between the ^{1}H NMR signal at δ_H 4.79 (Glc I H-1) and the ^{13}C NMR signal at δ_C 74.4 (Agly C-3), and in the NOESY spectrum between δ_H 4.79 (Glc I H-1) and δ_H 4.16 (Agly H-3) confirmed Glc I to be linked at C-3 of the aglycon. On the basis of the above results, the structure of **5** was established as (25*S*)-5β-spirostane-3β-ol 3-*O*-β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranoside.

Compound **6** was isolated as a white amorphous powder. Its HRESIMS (positive-ion mode) exhibited a pseudo-molecular ion peak at *m/z* 911.4656 $[M+Na]^+$ (calcd for $C_{44}H_{72}O_{18}Na$, 911.4616), ascribable to a molecular formula $C_{44}H_{72}O_{18}$. Its FABMS spectrum (negative-ion mode) showed a quasi-molecular ion peak at *m/z* 887 $[M-H]^-$ indicating a molecular weight of 888. Other fragment ion peaks were observed at *m/z* 755 $[(M-H)-132]^-$ and 593 $[(M-H)-132-162]^-$, corresponding to the successive loss of one pentosyl and one hexosyl moieties. The aglycone was identified as 17α-hydroxysarsapogenin [(25*S*)-5β-spirostane-3β,17α-diol] (Kim et al., 2005; Huang et al., 2006) from the 2D NMR spectra of **6**. Scrutiny of the 2D NMR (COSY, NOESY, HMQC) data of compound **6** (Tables 1 and 2) showed that **6** differed from **4** only in the presence of glucose at the C-2 position of Glc I. The HMBC correlation between the Glc II H-1 ($\delta_H = 5.34$) and Glc I C-II ($\delta_C = 79.9$) and the NOESY correlation between Glc II H-1 ($\delta_H = 5.34$) and Glc I H-2 ($\delta_H = 4.20$) indicated the attachment of Glc II at the C-2 position of Glc I. On the basis of these results, **6** was deduced as (25*S*)-5β-spirostane-3β,17α-diol 3-*O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranoside.

The antifungal activity of saponins **1**–**7** (Table 3) was evaluated at concentrations less than 200 μg/ml against strains of *C. albicans*, *C. glabrata* and *C. tropicalis*. Compounds **4**–**7** (spirostanol saponins) showed antifungal activity against *Candida* species with MIC values between 12.5 and 100 μg/ml. Compounds **1**–**3**, having a furostane skeleton, exhibited MIC values above 200 μg/ml and were considered inactive against the tested yeasts. Regarding the aglycon structure, we confirmed the presence of an anti-

fungal activity only for the spirostanol derivatives whereas none was observed for the furostanol derivatives (Sautour et al., 2004a,b, 2005). Concerning the oligosaccharidic chain, the results showed the importance of the nature of sugar at the C-2 position of glucose. We found here a better antifungal activity when glucose at C-3 of the aglycone is

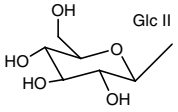


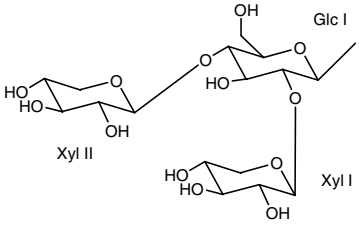


1 R^1 =

R^2 = Me

R^3 =

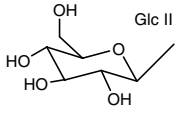


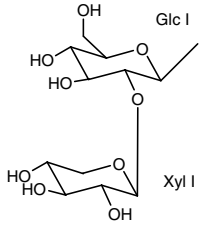


2 R^1 =

R^2 = H

R^3 =





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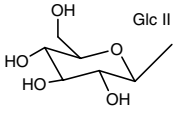
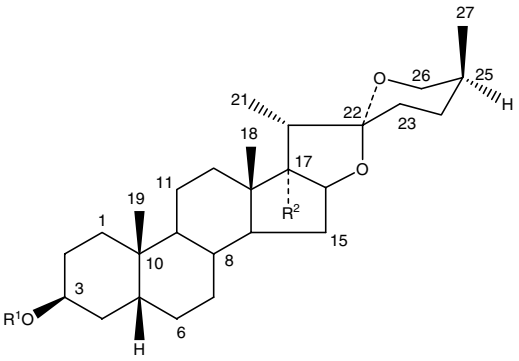
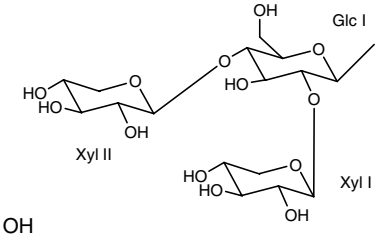
R^2 = Me

R^3 =

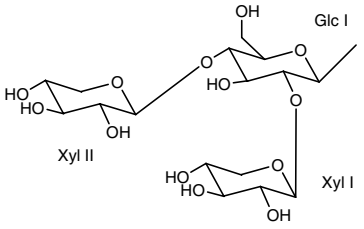






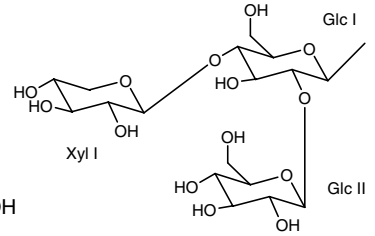
4 R^1 =

R^2 = OH



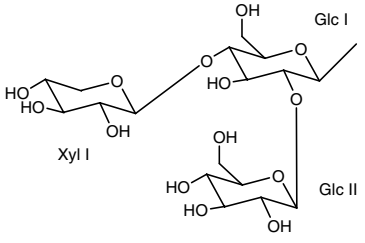
5 R^1 =

R^2 = H



6 R^1 =

R^2 = OH



7 R^1 =

R^2 = H

Table 3
Antifungal activity of **1**–**7** against *Candida* species given as MIC (μg/ml)[a]

Compounds	*Candida albicans*	*Candida glabrata*	*Candida tropicalis*
1	>200	>200	>200
2	>200	>200	>200
3	>200	>200	>200
4	12.5	12.5	50
5	12.5	12.5	50
6	50	50	100
7	50	50	100
ketoconazole[b]	0.78	0.78	1.56

[a] Compounds with MIC values >200 μg/ml are considered not active.
[b] Positive control.

substitued at the C-2 position by xylose rather than by glucose.

3. Experimental

3.1. General procedures

Optical rotations were taken with a AA-10R automatic polarimeter. IR spectra ($CHCl_3$) were recorded on a Perkin–Elmer 881 spectrophotometer. 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). Conventional pulse sequences were used for COSY, HSQC, and HMBC spectra. TOCSY spectra were acquired using the standard MLEV17 spin-locking sequence and a 90 ms mixing time. The mixing time in the NOESY experiment was set to 500 ms. The carbon type (CH_3, CH_2, CH) was determined by DEPT experiments. 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). FABMS (negative-ion mode, glycerol matrix) was conducted on a JEOL SX 102 spectrometer. HRESIMS was carried out on a Q-TOF 1 micromass spectrometer. GC analysis was carried out on a Thermoquest gas chromatograph using a DB-1701 capillary column (30 m × 0.25 mm, i.d.) (J&W Scientific), with detection by FID, and the initial temperature maintained at 80 °C for 5 min and then raised to 270 °C at the rate of 15 °C/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 × 25 mm and 460 × 15 mm), Büchi precolumn (110 × 15 mm)] and silica gel 60 (Merck, 15–40 μm). Vacuum-liquid chromatography (VLC) was performed on a C_{18} reversed phase (Merck, 25–40 μm) (12 × 3 cm). TLC and HPTLC employed precoated silica gel 60 F_{254} 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.

3.2. Plant material

The roots of *A. acutifolius* were collected in may 2005 near the city of Venasque (Vaucluse, France) and identified by Mr. G. Ducerf, 7110, Briant, France.

A voucher specimen (No. 6623) is deposited in the Herbarium of the Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Burgundy, France.

3.3. Extraction and isolation

Dried powdered roots (330 g) of *A. acutifolius* were refluxed three times with MeOH-H_2O (7:3, 2 l) for 1 h and evaporated to dryness yielding 79.3 g of a MeOH-H_2O extract. This extract was suspended in water (500 ml) and partitioned successively with hexane and *n*-BuOH (each 3 × 200 ml), yielding after evaporation of the solvents the corresponding hexane (160 mg) and *n*-BuOH (21.4 g) fractions. A 6 g aliquot of the *n*-BuOH residue was dissolved in MeOH and purified by precipitation with diethyl ether (3 × 300 ml), yielding a crude saponin mixture (4.3 g). The latter was submitted to VLC on C_{18} reversed phase using H_2O (100 ml), MeOH–H_2O mixtures (5:5; 4:1, each 100 ml), and finally MeOH (100 ml) as eluents. After evaporation of the solvents, four fractions were obtained: VLC-F1 (H_2O), VLC-F2 (MeOH-H_2O, 5:5), VLC-F3 (MeOH–H_2O, 4:1) and VLC-F4 (MeOH). VLC-F3 (1.94 g) was submitted to MPLC [system a: silica gel (15–40 μm), $CHCl_3$-MeOH-H_2O (13:7:2, lower phase)] to give 11 fractions (1–11). Fraction 5 was rechromatographed by MPLC (system a) yielding 3 fractions (1–3). Fractions 1 and 2 were concentrated to dryness, affording the pure compounds **1** (9 mg) and **3** (6 mg). Fraction 3 was rechromatographed in the same conditions (system a) to give the pure compound **2** (6 mg). VLC-F4 (410 mg) was submitted to MPLC [system a: silica gel (15–40 μm), $CHCl_3$–MeOH–H_2O (13:7:2, lower phase)] to give 11 fractions (1–11). Fraction 3 was rechromatographed in the same conditions (system a) to give the pure compounds **4** (8 mg) and **5** (8 mg). Fraction 5 rechromatographed by MPLC (system a) yielded the pure compounds **6** (7 mg) and **7** (7 mg).

Ten grams of powdered roots of *A. acutifolius* were refluxed with H_2O (2 × 100 ml) for 1 h and evaporated to dryness yielding an aqueous extract which was analyzed by TLC (solvent system a) in comparison with the MeOH-H_2O extract confirming the presence of **1** and **3** as natural and not artefactual compounds in the plant.

3.3.1. (25S)-3β,5β,22α-22-Methoxyfurostane-3,26-diol 3-O-β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranosyl 26-O-β-D-glucopyranoside (**1**)

White amorphous powder; $[\alpha]_D^{20}$ −71.4° (MeOH, *c* 0.042); IR ($CHCl_3$) ν_{max} 3376 (OH), 2956 (CH), 1023 (C–O–C)

cm^{-1}; Negative FABMS (glycerol matrix) *m/z* 1035 [M−H]$^-$, *m/z* 903 [(M−H)−132]$^-$, HRESIMS (positive-ion mode) *m/z* 1059.5392 [M+Na]$^+$ (calcd for $C_{50}H_{84}O_{22}Na$, 1059.5352); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz): see Tables 1 and 2.

*3.3.2. (25S)-3β,5β,22α-Furostane-3,22,26-triol 3-O-β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranosyl 26-O-β-D-glucopyranoside (**2**)*

White amorphous powder; $[\alpha]_D^{20}$ −40.0° (MeOH, *c* 0.025); IR ($CHCl_3$) ν_{max} 3355 (OH), 2927 (CH), 1041 (C–O–C) cm^{-1}; Negative FABMS (glycerol matrix) *m/z* 1021 [M−H]$^-$, *m/z* 859 [(M−H)−162]$^-$, *m/z* 727 [(M−H)−162−132]$^-$; HRESIMS (positive-ion mode) *m/z* 1045.5145 [M+Na]$^+$ (calcd for $C_{49}H_{82}O_{22}Na$, 1045.5195); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz): see Tables 1 and 2.

*3.3.3. (25S)-3β,5β,22α-22-Methoxyfurostane-3,26-diol 3-O-β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranosyl 26-O-β-D-glucopyranoside (**3**)*

White amorphous powder; $[\alpha]_D^{20}$: −78.4° (MeOH, *c* 0.051); IR ($CHCl_3$) ν_{max} 3356 (OH), 2933 (CH), 1032 (C–O–C) cm^{-1}; Negative FABMS (glycerol matrix) *m/z* 903 [M−H]$^-$, *m/z* 741 [(M−H)−162]$^-$, HRESIMS (positive-ion mode) *m/z* 927.4968 [M+Na]$^+$ (calcd for $C_{45}H_{76}O_{18}Na$, 927.4929); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz): see Tables 1 and 2.

*3.3.4. (25S)-5β-Spirostane-3β,17α-diol 3-O-β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranoside (**4**)*

White amorphous powder; $[\alpha]_D^{20}$ −108.1° (MeOH, *c* 0.037); IR ($CHCl_3$) ν_{max} 3355 (OH), 2927 (CH), 1041 (C–O–C) cm^{-1}; Negative FABMS (glycerol matrix) *m/z* 857 [M−H]$^-$, *m/z* 725 [(M−H)−132]$^-$, *m/z* 593 [(M−H)−132−132]$^-$; HRESIMS (positive-ion mode) *m/z* 881.4561 [M+Na]$^+$ (calcd for $C_{43}H_{70}O_{17}Na$, 881.4511); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz): see Tables 1 and 2.

*3.3.5. (25S)-5β-Spirostane-3β-ol 3-O-β-D-xylopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranoside (**5**)*

White amorphous powder; $[\alpha]_D^{20}$ −101.1° (MeOH, *c* 0.037); IR ($CHCl_3$) ν_{max} 3355 (OH), 2927 (CH), 1041 (C–O–C) cm^{-1}; Negative FABMS (glycerol matrix) *m/z* 841 [M−H]$^-$, *m/z* 709 [(M−H)−132]$^-$; HRESIMS (positive-ion mode) *m/z* 865.4601 [M+Na]$^+$ (calcd for $C_{43}H_{70}O_{16}Na$, 865.4562); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz): see Tables 1 and 2.

*3.3.6. (25S)-5β-Spirostane-3β,17α-diol 3-O-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 4)]-β-D-glucopyranoside (**6**)*

White amorphous powder; $[\alpha]_D^{20}$ −80.0° (MeOH, *c* 0.025); IR ($CHCl_3$) ν_{max} 3364 (OH), 2926 (CH), 1460, 1037 (C–O–C) cm^{-1}; Negative FABMS (glycerol matrix) *m/z* 887 [M−H]$^-$, *m/z* 755 [(M−H)−132]$^-$, *m/z* 593 [(M−H)−132−162]$^-$; HRESIMS (positive-ion mode) *m/z* 911.4656 [M+Na]$^+$ (calcd for $C_{44}H_{72}O_{18}Na$, 911.4616); ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz): see Tables 1 and 2.

3.4. Acid hydrolysis

A solution of each saponin (3 mg) in 2 N aqueous CF_3COOH (5 ml) was refluxed on a water bath for 3 h. After extraction with CH_2Cl_2 (3 × 5 ml), the aqueous layer was evaporated to dryness with MeOH until neutral, and glucose was identified by TLC with a standard sugar (solvent system c). Furthermore, a silylated derivative of the sugar was prepared according to the procedure previously described (Haddad et al., 2003). L-Cysteine methyl ester hydrochloride (0.06 mol/l) and HMDS-TMCS (hexamethyldisilazane-trimethylchlorosilane, 3:1) (150 μl) were added to the aqueous residue. After centrifugation of the precipitate, the supernatant was concentrated and partitioned between *n*-hexane and H_2O (0.1 ml each), and the hexane layer (1 μl) was analyzed by GC. D-Glucose and D-xylose were detected, giving single peaks at 18.50 and 13.49, respectively.

3.5. Antifungal activity

Evaluation of pure saponins was performed using a dilution test (Quiroga et al., 2001). For these bioassays, three human pathogenic yeasts were used: *C. albicans* (IP 1180-79), *C. glabrata*, and *C. tropicalis*. These clinical isolates were provided by Dr. A. Bonnin, Laboratory of Parasitology and Mycology, Hopital du Bocage, Dijon, France. Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtiter plates. Pure saponins were dissolved in 180 μl of malt extract broth (15 g malt extract and 5 g peptone in 1 l distilled water) to achieve final concentrations of 3.12–200 μg/ml and were dispensed in triplicate into the wells. Each well was inoculated with 10 μl of 10^5 CFU/ml fungal suspension. The microplates were incubated at 30 °C for 48 h and growth was evaluated spectrophotometrically at 630 nm with a ELX 808 automatic microplate reader. The MIC was defined as the lowest concentration of saponin at which no yeast growth was observed after incubation at 30 °C for 48 h. The reference compound ketoconazole (Sigma) (Favel et al., 1994) was used as positive control.

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