Two New Biologically Active Triterpene Saponins from *Acanthophyllum squarrosum*

Ghezala Gaidi,[†] Tomofumi Miyamoto,[‡] Abdolhossein Rustaiyan,[§] Véronique Laurens,[⊥] and Marie-Aleth Lacaille-Dubois*[,†]

Laboratoire de Pharmacognosie, Unité MIB, Faculté de Pharmacie, Université de Bourgogne, 7 Bd Jeanne d'Arc, BP 87900, 21079 Dijon Cedex, France, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan, Department of Chemistry, Shahid Beheshty University, Eveen, Tehran, Iran, and Groupe Immunologie Comparée, UMR CNRS 5548, Faculté des Sciences, Université de Bourgogne, 6 Bd Gabriel, 21079 Dijon Cedex, France

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Two novel triterpenoid saponins (**1** and **2**) have been isolated from the roots of *Acanthophyllum squarrosum*. The structures were established mainly by a combination of 2D NMR techniques as 3-*O*-*β*-D-galactopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucuronopyranosylgypsogenin-28-*O*-*β*-D-xylopyranosyl-(1→3)-*β*-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→3)]-*β*-D-fucopyranoside (**1**) and 3-*O*-*β*-D-glucopyranosylgypsogenin-28-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**2**). Compound **1** showed a moderate concentration-dependent immunomodulatory effect in an in vitro lymphocyte proliferation assay.

Acanthophyllum squarrosum Boiss. [syn.: *A. pungens* (Bunge) Boiss. var. *squarrosum* Golenk.] (Caryophyllaceae) is one of 23 species of the section Oligosperma endemic to Iran.[1] Their roots have been used as a detergent. Our previous phytochemical studies on the methanolic extract of *A. squarrosum* roots led to the isolation of a new triterpene saponin, squarroside A, a gypsogenin glycoside.[2] Further investigation of the same extract has led to the isolation of two additional major triterpene saponins, **1** and **2**. Their structures were elucidated mainly by 1D and 2D NMR experiments (COSY, TOCSY, HSQC, and HMBC). This paper deals with the isolation and structure determination of **1** and **2**. The immunological properties of these new compounds have been investigated.

Results and Discussion

The methanolic extract of the roots of *A. squarrosum* was extracted successively with chloroform and *n*-butanol. The concentrated *n*-butanol-soluble fraction was purified by precipitation with diethyl ether and subjected to Si gel column chromatography to afford a saponin fraction that was further separated by repeated medium-pressure liquid chromatography (MPLC) over reversed-phase and normal Si gel,[3] yielding the pure compounds **1** and **2** (Chart 1).

Compound **1** was obtained as an amorphous powder. The IR spectrum showed absorptions at 3400 (OH), 2930 (CH), 1740 (C═O of ester group), and 1710 cm $^{-1}$ (C═O of carboxylic acid group). The FABMS (thioglycerol matrix, negative ion mode) of compound **1** showed a quasimolecular ion peak at *m*/*z* 1641 $[M - H]^-$, indicating a molecular weight of 1642, compatible with the molecular formula $C_{75}H_{118}O_{39}$. Other significant ion peaks visible at *m*/*z* 1495 $[(M - H) - 146]^-$ and 1333 $[(M - H) - 146 - 132 - 132]^-$ corresponded in turn to the loss of one terminal desoxyhexose unit and two pentosyl moieties. Other fragment ion peaks at *m*/*z* 939 and 469 corresponded to a hexosyl-pentosyl-hexosyluronic acid-aglycon fragment and to the aglycon, respectively.

Table 1. Cross-Peaks (*δ* Values) in the ^{1}H-Detected Long-Range ^{1}H−^{13}C (HMBC) NMR Spectrum Used for Defining the Aglycon of **1**

proton resonance		carbon resonances
1.04 (Me-24)	→	C-3 (81.3), C-4 (53.9), C-5 (47.1), C-23 (209.9)
0.88 (Me-25)	→	C-1 (37.5), C-5 (47.1), C-9 (46.9), C-10 (35.4)
0.65 (Me-26)	→	C-7 (31.7), C-8 (37.5), C-9 (46.9), C-14 (41.1)
1.08 (Me-27)	→	C-8 (37.5), C-13 (143.0), C-14 (41.1), C-15 (27.3)
0.86 (Me-29)	→	C-19 (45.5), C-20 (30.2), C-21 (33.2)
0.85 (Me-30)	→	C-19 (45.5), C-20 (30.2), C-21 (33.2), C-29 (32.6)

The mineral acid hydrolysis of **1** with 2 N TFA at 100 °C afforded an aglycon identified as gypsogenin from the ^{1}H NMR, ^{13}C NMR, DEPT, HSQC, and HMBC spectra of **1**[4−6] and by TLC comparison with an authentic sample. Most of the signals were assigned through ^2J and ^3J connectivities in the HMBC spectrum from the six methyl proton resonances (Table 1). The sugars obtained from the saponin hydrolysates were identified as galactose, xylose, rhamnose, and fucose in a molar ratio of 1:3:2:1 (estimated by GLC analysis) and glucuronic acid (co-TLC with an authentic sample).

The alkaline hydrolysis of **1** performed with 5% KOH yielded the prosapogenin **1a**, which furnished by further acid hydrolysis glucuronic acid, xylose, galactose, and gypsogenin (co-TLC with authentic samples). The FABMS (thioglycerol matrix, negative ion mode) of compound **1a** exhibited a quasimolecular ion peak at *m*/*z* 939 $[M - H]^-$, compatible with the molecular formula $C_{47}H_{72}O_{19}$, and other significant ion peaks at *m*/*z* 777 $[(M - H) - 162]^-$, corresponding to the loss of one hexosyl moiety, and at *m*/*z* 469 $[(M - H) - 162 - 132 - 176]^-$, corresponding to the loss of one hexosyl, one pentosyl, and one hexosyluronic acid unit. These data indicated that **1** must be a bidesmosidic saponin in which glucuronic acid, xylose, and galactose are bound to the aglycon by a glycosidic linkage at C-3 while the five remaining sugars are bound to the genin by a glycosidic ester linkage at C-28. This was confirmed by the signals observed in the ^{13}C NMR spectrum of **1** at δ_C 81.3 (downfield shift of C-3 of the aglycon) and δ_C 175.3 (upfield shift of C-28 of the aglycon).

* To whom correspondence should be addressed. Tel.: 0033-3-80 39 32 29. Fax: 0033-3-80 39 33 00. E-mail: malacd@u-bourgogne.fr.
[†] Laboratoire de Pharmacognosie, Université de Bourgogne.
[‡] Kyushu University.
[§] Shahid Beheshty University.
[⊥] Groupe Immunologie Comparée, UMR CNRS 554, Université de Bourgogne.

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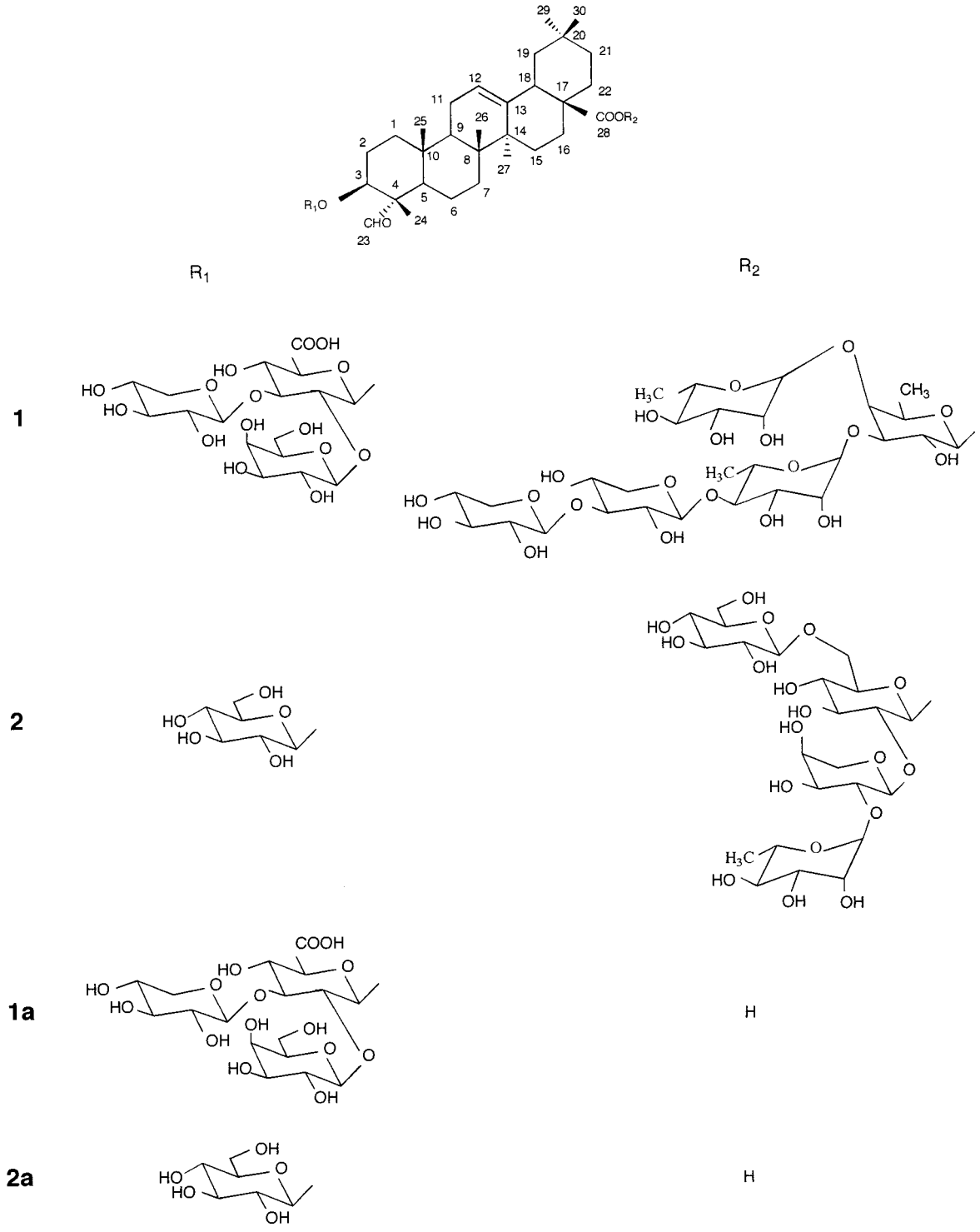
Chart 1



The ^{1}H NMR spectrum of **1** revealed the presence of six tertiary methyl groups at δ 0.65, 0.85, 0.86, 0.88, 1.04, and 1.08, three secondary methyl groups [δ 1.04 (d, J = 6.0 Hz), 1.09 (d, J = 6.4 Hz), 1.12 (d, J = 5.8 Hz)], one trisubstituted olefinic proton [δ 5.16 (br s)], one aldehydic proton [δ 9.40 (s)], and eight anomeric protons in the δ 5.28−4.13 range. In the ^{13}C NMR spectrum of **1** (Tables 2 and 3), there were signals corresponding to two olefinic carbons (δ 143.0, 120.8), one aldehydic carbon (δ 209.9), one carboxylic carbon (δ 172.1), one ester carbon (δ 175.3), and eight anomeric carbons between δ 104.9 and 93.0. The last value was attributed to an anomeric carbon of a sugar linked by an ester linkage to the aglycon.

Compound **1** was shown to contain eight sugar residues from the HSQC NMR spectrum. The anomeric ^{1}H NMR signals at δ 5.28 (d, J = 8.2 Hz), 5.16 (s), 4.93 (s), 4.57 (d, J = 7.9 Hz), 4.52 (d, J = 7.1 Hz), 4.40 (d, J = 7.0 Hz), 4.39 (d, J = 6.9 Hz), and 4.13 (d, J = 7.5 Hz) gave correlations with ^{13}C NMR signals at δ 93.0, 99.6, 100.0, 102.6, 102.3, 104.1, 104.9, and 101.0, respectively. Evaluation of spin−spin couplings and chemical shifts allowed the identification of one β-fucopyranosyl (Fuc), two α-rhamnopyranosyl (Rha), one β-xylopyranosyl (Xyl), one β-galactopyranosyl (Gal), two β-xylopyranosyl (Xyl), and one β-glucuronopyranosyl (GlcA) unit, respectively. The common D-configuration for Fuc, Gal, Xyl, and GlcA and the L-configuration

Table 2. ^{13}C NMR and ^{1}H NMR Data of the Aglycons of **1**, **2**, and **2a** (DMSO-d_6)[a,b]

		1		2		2a
position	mult.[a]	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1	CH_2	37.5	0.92,1.52	37.5	1.56, nd	37.0
2	CH_2	24.2	1.59,1.92	24.4	1.62, 1.74	24.0
3	CH	81.3	3.68	87.9	3.40	83.6
4	C	53.9		54.4		56.0
5	CH	47.1	1.22	46.9	1.29	47.2
6	CH_2	22.4	1.48, nd	19.6	nd	19.9
7	CH_2	31.7	1.38,1.60	31.5	1.39, 1.50	30.5
8	C	37.5		40.6		40.6
9	CH	46.9	1.52	46.8	1.56	46.5
10	C	35.4		35.3		35.9
11	CH_2	23.6	1.78, nd	22.8	1.80, nd	22.9
12	CH	120.8	5.16 (br s)	121.5	5.17	120.5
13	C	143.0		143.3		144.9
14	C	41.1		40.0		nd
15	CH_2	27.3	1.05, nd	27.1	0.92, 1.94	28.6
16	CH_2	22.4	1.94, 1.48	22.5	nd	20.0
17	C	45.9		45.9		45.6
18	CH	41.2	2.70	40.6	2.70	41.4
19	CH_2	45.5	1.05, 1.64	45.5	1.02, 1.62	46.5
20	C	30.2		30.2		29.1
21	CH_2	33.2	1.11, 1.31	33.1	1.14, 1.30	33.1
22	CH_2	28.9	1.20, nd	28.5	nd	29.0
23	CHO	209.9	9.4 (s)	207.3	9.39 (s)	208.5
24	CH_3	10.0	1.04 (s)	9.7	0.99 (s)	12.7
25	CH_3	15.3	0.88 (s)	15.1	0.88 (s)	15.5
26	CH_3	16.2	0.65 (s)	16.6	0.68 (s)	16.6
27	CH_3	25.4	1.08 (s)	25.4	1.09 (s)	25.6
28	C	175.3		174.9		177.2
29	CH_3	32.6	0.86 (s)	32.6	0.86 (s)	32.4
30	CH_3	23.4	0.85 (s)	23.3	0.85 (s)	23.6

[a] Multiplicities were assigned from DEPT spectra. [b] The assignments were based on the HMBC, HSQC, and DEPT experiments (150 MHz for ^{13}C and 600 MHz for ^{1}H NMR).

for Rha were assumed, according to those most often encountered among the plant glycosides in each case.[2]

The ring protons of the monosaccharide residues of **1** were assigned starting from the anomeric protons by means of its COSY, TOCSY, HSQC, and HMBC spectra (Table 3), and the sequence of the oligosaccharide chains was obtained from the HMBC spectrum. The cross-peaks in the HMBC spectrum between δ_H (GlcA-1) 4.13 and δ_C (Agly C-3) 81.3, at δ_H (Xyl-1) 4.57 and δ_C (GlcA-3) 82.6, and at δ_H (GlcA-2) 3.37 and δ_C (Gal-1) 102.3 showed that a trisaccharide moiety, 3-*O*-β-D-galactopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucuronopyranoside, was linked to the gypsogenin aglycon at C-3. After subtraction of the anomeric signals of the sugars linked at the C-3 position from the total HSQC spectrum, the signals of five sugars linked to the aglycon through an ester linkage (Fuc, Rha, Rha, Xyl, and Xyl) remained.

From the TOCSY spectrum, it was possible to assign the proton chemical shifts in fucose from the anomeric ^{1}H NMR signal at δ 5.28 (d, J = 8.2 Hz). It showed two cross-peaks at δ_H 3.58 and δ_H 3.68 assigned to H-2 and H-5, respectively, which were confirmed by correlations in the COSY spectrum between δ_{H-6} 1.04 and δ_{H-5} 3.68 and δ_{H-1} 5.28 and δ_{H-2} 3.58. The cross-peaks from these protons observed in the HSQC spectrum allowed the ^{13}C NMR assignments of the fucose residue to be determined. The HMBC experiment showed long-range coupling between δ_H (Fuc-6) 1.04 and δ_C (Fuc-5) 69.5 (^2J) and δ_C (Fuc-4) 82.4 (^3J), confirming that Fuc C-4 was substituted. Furthermore, the correlation in the COSY spectrum at δ_{H-2} 3.58 and δ_{H-3} 3.89 allowed the assignment of Fuc C-3 at δ_C 85.2 in the HSQC spectrum. The correlation in the HSQC spectrum at δ_C /δ_H 93.0/5.28 showed that the fucose residue was attached to the carboxylic acid function of the aglycon by an ester linkage. This conclusion was confirmed by the HMBC experiment which showed a correlation between δ_H (Fuc-1) 5.28 (d, J = 8.2 Hz) and δ_C (Agly C-28) 175.3. These assignments showed that the sugar linked at C-28 of the genin of **1** was a 1,3,4-trisubstituted Fuc unit.

From the cross-peaks in the $^{1}H-^{1}H$ COSY spectrum of **1**, the ^{1}H NMR chemical shifts in rhamnose were assigned from the anomeric signal at δ 5.16 (s). Furthermore in the TOCSY spectrum, the ^{1}H NMR signal of Rha H-6 at δ 1.12 (d, J = 5.8 Hz) showed two correlations with δ_{H-5} (3.58) and δ_{H-4} (3.34). The sugar was assigned as a 1,4-disubstituted rhamnose (Rha-1,4) from the HMBC experiment, which showed long-range couplings between δ_H (Rha-6) 1.12 and δ_C (Rha-5) 66.8 (^2J) and δ_C (Rha-4) 82.6 (^3J), confirming that Rha C-4 was substituted. Additionally, cross-peaks between δ_H (Rha-1) 5.16 and δ_C (Rha-5) 66.8 and δ_C (Rha-3) 69.5 supported the above observation.

Assignments of the ^{1}H NMR and ^{13}C NMR signals from the TOCSY, HSQC, and HMBC spectra of **1** showed that the three remaining sugars were a terminal Rha (T-Rha), a 1,3-disubstituted Xyl, and a terminal Xyl (T-Xyl). All the carbon signals due to these sugar moieties were in good agreement with literature data.[4] The following cross-peaks in the HMBC spectrum between anomeric ^{1}H NMR signals and ^{13}C NMR signals in adjacent systems and the reverse correlations between proton-ring signals and anomeric carbon signals allowed the complete sequencing of the glycosidic ester chain linked at C-28: δ_H (T Xyl-1) 4.40 → δ_C (Xyl-3) 85.4, δ_H (Xyl-1) 4.39 → δ_C (Rha-4) 82.6, δ_H (Rha-1) 5.16 → δ_C (Fuc-4) 82.4, δ_H (T Rha-1) 4.93 → δ_C (Fuc-3) 85.2, δ_H (Xyl-3) 3.34 → δ_C (T Xyl-1) 104.1, δ_H (Rha-4) 3.34 → δ_C (Xyl-1) 104.9.

On the basis of the above results the structure of compound **1** can be represented as 3-*O*-β-D-galactopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucuronopyranosylgypsogenin-28-*O*-β-D-xylopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→3)]-β-D-fucopyranoside. According to several reports on the distribution of gypsogenin glycosides in Caryophyllaceae and an updated literature search, **1** is a new natural compound.[2−12]

Compound **2** was obtained as an amorphous powder. The IR spectrum showed absorptions at 3400 (OH), 2930 (CH), and 1735 (C=O of ester group) cm^{-1}. Its FABMS (thioglycerol matrix, negative-ion mode) showed a quasimolecular ion peak at m/z 1233 $[M - H]^-$, indicating a molecular weight of 1234, compatible with the molecular formula $C_{59}H_{94}O_{27}$. Other fragment ion peaks visible at m/z 1071 $[(M - H) - 162]^-$, 909 $[(M - H) - 162 - 162]^-$, 763 $[(M - H) - 162 - 162 - 146]^-$, and 469 $[(M - H) - 162 - 162 - 146 - 132 - 162]^-$ indicated the successive loss of one hexosyl, one hexosyl, one deoxyhexosyl, and one pentosylhexosyl moiety. The fragment ions at m/z 469 corresponded to the pseudomolecular ion of the aglycon.

Mineral acid hydrolysis of **2** with 2 N TFA at 100 °C afforded an aglycon identified as gypsogenin from the ^{1}H NMR, ^{13}C NMR, DEPT, HSQC, and HMBC spectra of **2** and by TLC comparison with an authentic sample. The sugars were identified as glucose, arabinose, and rhamnose (co-TLC) in a molar ratio of 3:1:1, respectively, as estimated by GLC after conversion into their alditol acetates.

The alkaline hydrolysis of **2** performed with 5% KOH yielded the prosapogenin **2a**, which furnished glucose and gypsogenin (co-TLC with authentic samples) by further acid hydrolysis. This conclusion was supported by the presence of the anomeric ^{13}C NMR signal at δ_C102.6 and

Table 3. ^{13}C NMR and ^{1}H NMR Data of the Sugar Moieties of Compounds **1**, **2**, and **2a** (DMSO-d_6)[a–c]

	1			**2**		**2a**
	δ ^{13}C	δ ^{1}H		δ ^{13}C	δ ^{1}H	δ ^{13}C
3-*O*-sugars			3-*O*-sugars			
GlcA1	*101.0*	4.13, d, J = 7.5 Hz	Glc1	*104.1*	4.30, d, J = 7.5 Hz	*102.6*
2	*77.8*	3.37	2	73.7	3.06	74.4
3	*82.6*	3.53	3	76.8	3.20	76.4
4	70.3	3.19	4	69.9	3.04	70.1
5	76.1	3.15	5	76.7	3.02	76.7
6	172.1	-	6	60.9	3.36, 3.68	61.1
Xyl 1	*102.6*	4.57, d, J = 7.9 Hz				
2	73.6	3.06				
3	76.1	3.15				
4	69.3	3.25				
5	65.7	2.98, 3.64				
Gal 1	*102.3*	4.52, d, J = 7.1 Hz				
2	73.6	3.22				
3	73.4	3.20				
4	69.5	3.45				
5	74.6	3.27				
6	59.7	3.39, 3.56				
28-*O*-sugars			28-*O*-sugars			
Fuc 1	*93.0*	5.28, d, J = 8.2 Hz	Glc1	*93.2*	5. 35, d, J = 7.7 Hz	
2	70.3	3.58	2	*78.6*	3.80	
3	*85.2*	3.89	3	75.9	3.48	
4	*82.4*	3.62	4	70.8	3.31	
5	69.5	3.68	5	75.9	3.18	
6	16.1	1.04, d, J = 6.0 Hz	6	*67.4*	3.38, 3.90	
T-Rha 1	*100.0*	4.93(s)	T-Glc 1	*103.1*	4.16, d, J = 7.9 Hz	
2	69.3	3.72	2	73.4	2.94	
3	70.3	3.56	3	76.6	3.09	
4	73.6	3.08	4	70.0	3.40	
5	71.6	3.15	5	76.7	3.02	
6	17.7	1.09, d, J = 6.4 Hz	6	61.0	3.43, 3.72	
Rha 1	*99.6*	5.16 (s)	Ara 1	*100.3*	4.07, d, J = 5.2 Hz	
2	71.4	3.68	2	*73.7*	3.40	
3	69.5	3.60	3	71.9	3.43	
4	*82.6*	3.34	4	67.1	3.54	
5	66.8	3.58	5	63.6	3.26, 3.64	
6	17.8	1.12, d, J = 5.8 Hz				
Xyl 1	*104.9*	4.39, d, J = 6.9 Hz	T-Rha 1	*99.6*	4.93 (s)	
2	73.6	3.19	2	70.3	3.59	
3	*85.4*	3.34	3	70.3	3.40	
4	67.5	3.34	4	71.9	3.16	
5	65.6	3.06, 3.64	5	68.0	3.62	
T-Xyl 1	*104.1*	4.40, d, J = 7.0 Hz	6	17.6	1.08, d, J = 6.4 Hz	
2	73.6	3.15				
3	76.3	3.09				
4	69.3	3.27				
5	65.7	3.06, 3.72				

[a] Multiplicities were assigned from DEPT spectra. [b] ^{13}C chemical shifts of substituted residues are italicized. [c] The assignments were based on the COSY, TOCSY, HSQC, HMBC, and DEPT experiments (150 MHz for ^{13}C and 600 MHz for ^{1}H NMR).

the signal at δ_C 83.6 (glycosylated Agly C-3) in the ^{13}C NMR spectrum of **2a** (Table 3).

Compound **2** was investigated by TOCSY, COSY, HSQC, and HMBC experiments, and full assignments of all ^{1}H and ^{13}C NMR resonances were obtained. The presence of signals at δ_C 87.9 (Agly C-3) and δ_C 174.9 (Agly C-28) in the ^{13}C NMR spectrum of **2** enabled the conclusion that **2** is a 3,-28-bidesmosidic saponin. It was shown to contain five sugar residues from the HSQC spectrum. The anomeric ^{1}H NMR signals at δ_H 5.35 (d, J = 7.7 Hz), 4.93 (s), 4.30 (d, J = 7.5 Hz), 4.16 (d, J = 7.9 Hz), and 4.07 (d, J = 5.2 Hz) gave correlations with anomeric ^{13}C NMR signals at δ_C 93.2, 99.6, 104.1, 103.1, and 100.3, respectively. ^{1}H NMR subspectra of the sugar moieties were obtained from the signals corresponding to their anomeric proton resonances and to their other well-resolved resonances (Rha H-6, Ara H-5) in the TOCSY experiment. The ^{13}C NMR signals of each sugar moiety were assigned by means of direct H−C correlation in the HSQC spectrum. Evaluation of spin−spin coupling and chemical shifts allowed the identification of three β-glucopyranosyl (Glc), one α-arabinopyranosyl (Ara), and one α-rhamnopyranosyl (Rha) residue. The common D-configuration for Glc and the L-configuration for Rha and Ara were assumed, according to those most encountered among the plant glycosides in each case. A cross-peak due to a long-range correlation in the HMBC spectrum between δ_H (T-Glc-1) 4.30 and δ_C (Agly-3) 87.9 and a reverse correlation between δ_H (Agly-3) 3.40 and δ_C (T-Glc-1) 104.1 confirmed that glucose was linked to C-3 of the aglycon. After subtraction of the anomeric signal of Glc linked at the C-3 position (δ_C104.1) from the total HSQC spectrum, the signals of four sugars linked to the aglycon by an ester linkage (Glc, Glc, Rha, and Ara) remained. Assignments of the ^{1}H NMR and ^{13}C NMR signals from the TOCSY, HSQC, and HMBC spectra showed that the four remaining sugars were T-Glc, a 1,2,6-trisubstituted glucose (Glc-1,2,6), a 1,2-disubstituted arabinose (Ara-1,2), and a T-Rha (Table 3). The sequence of the tetrasaccharide chain at C-28 was defined by HMBC experiments. The appearance of cross-peaks between δ_H (Ara-1) 4.07 and δ_C (Glc-2) 78.6, δ_H (T Glc-1) 4.16 and δ_C (Glc-6) 67.4, at δ_H (T Rha-1) 4.93 and δ_C (Ara-2) 73.7, and

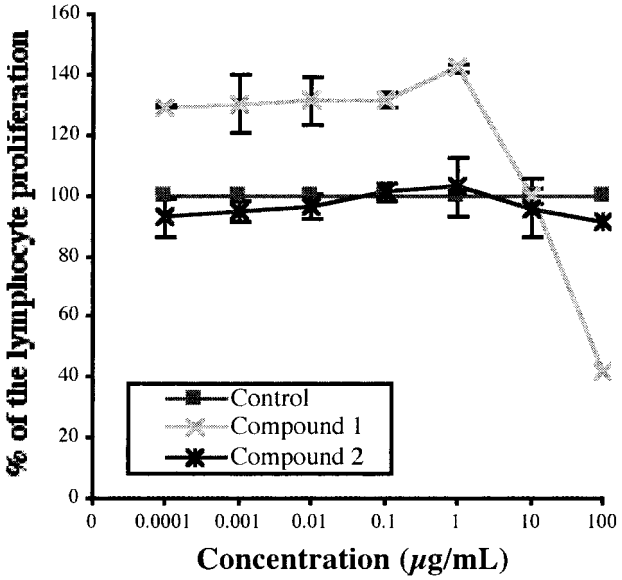


Figure 1. Actions of compounds **1** and **2** in the lymphocyte proliferation test. Each point represents the mean ± SD of four replicates.

δ_H (Glc-1) 5.35 and δ_C (Agly C-28) 174.9 indicated that an α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→6)]-β-D-glucopyranose unit was linked to the aglycon at the C-28 position in **2**. The chemical shifts of the α-L-rhamnopyranosyl-(1→2)- α-L-arabinopyranosyl unit were in good agreement with literature data.[13]

On the basis of the above results, the structure of compound **2** could be represented as 3-*O*-β-D-glucopyranosylgypsogenin-28-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→6)]-β-D-glucopyranoside, a new natural compound.[3−12] It is of interest to note that among the gypsogenin glycosides of Caryophyllaceae, the prosapogenin (3-*O*-β-D-glucopyranosylgypsogenin) was obtained as compound **2a** for the second time. This compound was reported previously from *Dianthus superbus*.[14]

Since some saponins have been reported to exert immunostimulant activities,[15,16] saponins **1** and **2** were tested in an in vitro lymphocyte proliferation test system according to a literature procedure.[17,18] In this test, the percentage of lymphocyte proliferation was quantified by determination of the formation of formazan from exogenous MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2*H*-tetrazolium] in lymphocytes. In the absence of the two mitogens, concanavalin A (con A) and a bacterial lipopolysaccharide (LPS), only saponin **1** showed an immunomodulatory effect dependent on concentration (Figure 1). Compound **1** was not cytotoxic to lymphocytes in culture up to the concentration of 10 μg/mL. However at higher concentrations, a marked cytotoxicity was noted (58% at 100 μg/mL), whereas **1** displayed immunostimulant activity at low concentration (1 μg/mL → 100 pg/mL). In the concentration range 1−0.1 μg/mL, the immunostimulant activity of **2** was low compared with that of saponin **1**, and it was not cytotoxic at 100 μg/mL. The B-lymphocyte proliferation induced by LPS was moderatly increased by saponins **1** and **2** at 1 μg/mL. The T-lymphocyte proliferation stimulated by con A was moderately increased by **2** at 1 μg/mL, whereas **1** suppressed it by 40% at 1 μg/mL.

Experimental Section

General Experimental Procedures. Optical rotations were taken with a Perkin-Elmer 241 polarimeter. IR spectra (KBr disk) were recorded on a Perkin-Elmer 281 spectrophotometer. The 1D and 2D NMR spectra (^{1}H−^{1}H COSY, TOCSY, HSQC, and HMBC) were performed using a UNITY-600 spectrometer at an operating frequency of 600 MHz on a Varian INOVA 600 instrument equipped with a SUN 4 L-X computer system (600 MHz for ^{1}H and 150 MHz for ^{13}C). Conventional pulse sequences were used for COSY, HSQC, and HMBC. TOCSY spectra were acquired using the standard MLEV17 spin-locking sequence and 90 ms mixing time. 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 DMSO-d_6 (δ 39.5). Fast-atom bombardment (FAB) mass spectra (negative-ion mode, thioglycerol matrix) were obtained on a JEOL SX 102 mass spectrometer. TLC and HPTLC employed precoated Si gel plates 60 F_{254} (Merck). The following TLC solvent systems were used: for saponins (a) $CHCl_3$−MeOH−AcOH−H_2O (15:8:3:2); for sapogenins (b) toluene−Me_2CO (4:1); for monosaccharides (c) $CHCl_3$−MeOH−H_2O (8:5:1). Spray reagents were as follows: for the saponins, Komarowsky reagent, a mixture (5:1) of *p*-hydroxybenzaldehyde (2% in MeOH) and 50% H_2SO_4; for the sugars, diphenylaminephosphoric acid reagent. Isolations were carried out using a medium-pressure liquid chromatography (MPLC) system [Gilson pump M 303, head pump 25 SC, manometric module M 802, Rheodyne 7125 injector, Büchi column (460 × 25 mm and 460 × 15 mm), Büchi precolumn (110 × 15 mm)]. GLC analysis: Perkin-Elmer 900 B, glass column (200 × 0.3 cm) packed with 0V 225, carrier gas Ar, 30 mL/min.

Plant Material. *A. squarrosum* was collected in July 1989, 40 km north of Sabzewar, Khorassan Province, Iran. A voucher specimen (No. 48-10) is deposited in the Herbarium of the Department of Botany, Shahid Beheshty University, Tehran, Iran.

Extraction and Isolation. Dried powdered roots (500 g) were defatted in a Soxhlet with 3 L of petroleum ether for 24 h. The air-dried plant was extracted with 5 L of MeOH for 48 h, yielding after evaporation a syrupy brown residue (30 g). The MeOH extract was dissolved in 400 mL of H_2O and partitioned with *n*-BuOH and H_2O to give the *n*-BuOH fraction (10 g). It was solubilized in MeOH (10 mL) and precipitated in Et_2O (3 × 250 mL), yielding 7 g of a crude saponin mixture, of which 2 g was submitted to column chromatography over Sephadex LH-20 eluted by MeOH, yielding 1.5 g of a white powder. This was first fractionated by column chromatography on Si gel 60 (70−230 mesh) using as eluent $CHCl_3$−MeOH−H_2O (8:5:1). Further separations were performed by successive MPLC on the reversed-phase material, Lichroprep RP-18 (Merck, 40−63 μm), eluted with MeOH−H_2O (linear gradient 50−70%) to give compounds **1** (28 mg) and **2** (25 mg). Final purification of both compounds was carried out on a Sephadex LH-20 column eluted with MeOH.

Compound 1: white amorphous powder; $[\alpha]^{20}_D$ −12° (*c* 0.1, MeOH); IR ν_{max} 3500−3300 (OH), 2930 (CH), 1740 (C=O ester), 1710 (CO carboxylic acid), 1610, 1450, 1390, 1300 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz), see Tables 2 and 3; long-range correlations in the HMBC spectrum used for defining the aglycon of **1**, see Table 1; negative FABMS (thioglycerol matrix) *m/z* 1641 [M − H]$^-$, 1495 [(M − H) − 146]$^-$, 1333 [(M − H) − 146 − 132 − 132]$^-$; TLC R_f 0.2 (system a); blue spots by spraying with Komarowsky reagent.

Compound 2: white amorphous powder; $[\alpha]^{20}_D$ +3° (*c* 0.1, MeOH); IR ν_{max} 3400 (OH), 2930 (CH), 1735 (C=O ester) cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz), see Tables 2 and 3; negative FABMS (thioglycerol matrix) *m/z* 1233 [M − H]$^-$, 1071 [(M − H) − 162]$^-$, 909 [(M − H) − 162 − 162]$^-$, 763 [(M − H) − 162 − 162 − 146]$^-$, 469 [(M − H) − 162 − 162 − 146 − 132 − 162]$^-$; TLC R_f 0.45 (system a); blue violet spots by spraying with Komarowsky reagent.

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 this period, the reaction mixture was diluted with H_2O

(15 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The combined CH_2Cl_2 extracts were washed with H_2O and dried with Na_2SO_4. Evaporation of the solvent gave gypsogenin (co-TLC with an authentic sample). After repeated evaporations of the solvent of the aqueous layer by adding MeOH to remove the acid, the sugars were analyzed by silica gel TLC in comparison with standard sugars (solvent system c). A 2 mg quantity of saponin was refluxed in 2 N aqueous CF_3COOH (2 mL) in a sealed serum vial at 100 °C for 3 h. After this period, sugars in the hydrolysate were converted into the alditol acetates and then subjected to GLC analysis according to a method previously described.[19]

Alkaline Hydrolysis. Each saponin (7 mg) was refluxed with 5% aqueous KOH (10 mL) for 1 h. The reaction mixture was adjusted to pH 6 with dilute HCl and then extracted with H_2O-saturated *n*-BuOH (3 × 10 mL). The combined *n*-BuOH extracts were washed (H_2O). Evaporation of the *n*-BuOH gave the prosapogenins **1a** (2 mg) and **2a** (4 mg), respectively, from **1** and **2**.

Compound 1a: white amorphous powder; negative FABMS (thioglycerol matrix) *m/z* 939 $[M - H]^-$, 777 $[(M - H) - 162]^-$, 469 $[(M - H) - 162 - 132 - 176]^-$.

Compound 2a: white amorphous powder; ^{13}C NMR (DMSO-d_6, 150 MHz), see Tables 2 and 3.

Bioassay. The in vitro lymphocyte proliferation assay was performed according to refs 17 and 18. Spleens obtained from mice were removed aseptially and teased with forceps in cold Dulbecco's modified Eagle's medium (DMEM, Gibco BRL Life Technologies, Grand Island, NY). The splenocyte suspension was filtered and washed twice in this medium. Splenocytes were resuspended at 3×10^6 cells/mL in DMEM supplemented with1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 10% FCS (fetal calf serum). A 1 mL aliquot of cell suspension was seeded in a 24-well culture plate (NUNC Roskilde, Denmark), and 100 μL of the saponin sample solution, mitogen LPS (lipopolysaccharide from *Escherichia coli* serotype 026:B6, Sigma) (40 μg/mL), and con A (concanavalin A, Polylabo, Strasbourg, France) (5 μg/mL) were added. The splenocytes were cultured at 37 °C under a 5% CO_2 atm for 3 days. At day 3, 100 μL aliquots of each well cell suspension were transferred into 96-well half-area culture plates (NUNC, Roskilde, Denmark), and four replicates were tested for each dilution of saponin and mitogen. Lymphocyte proliferation was determined using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt] and an electron coupling reagent (phenazine ethosulfate, PES). The MTS tetrazolium compound (Owen's reagent) is bioreduced by live cells into a colored formazan product that is soluble in tissue culture medium. The assay was performed by adding 10 μL/well of commercial solution (Cell Titer 96 AQ_{ueous} one solution cell proliferation assay, Promega), and the absorbance of formazan dye produts was measured at 490 nm 3 h later using a microplate reader (Brorad, Richmond, Ml).

Results were calculated as follows:

$$\text{lymphocyte proliferation (\%)} = \frac{\text{DO(spleen cells + saponin} \pm \text{mitogen)} - \text{DO(medium)}}{\text{DO(spleen cells} \pm \text{mitogen)} - \text{DO(medium)}} \times 100$$

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