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Minor sulfated saikosaponins from the aerial parts of *Bupleurum rigidum* L.

Sandra Sánchez-Contreras[a], Ana María Díaz-Lanza[a,*], Carmen Bartolomé[b], Manuel Bernabé[c]

[a]*Laboratorio de Farmacognosia, Departamento de Fisiologia y Farmacologia, Facultad de Farmacia, Universidad de Alcalá de Henares, Carretera Madrid–Barcelona, KM 33,600, 28871 Alcalá de Henares, Madrid, Spain*

[b]*Departamento de Biología Vegetal, Facultad de Ciencias Biológicas, Universidad de Alcalá de Henares, Carretera Madrid–Barcelona, KM 33,600, 28871 Alcalá de Henares, Madrid, Spain*

[c]*Departamento de Química Orgánica Biológica, Instituto de Química Orgánica General, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain*

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Abstract

Five new sulfated saikosaponins (Sandrosaponins II–VI) were isolated as minor components from the aerial parts of *Bupleurum rigidum* L. Their structures have been established by 1D and 2D-NMR techniques, FABMS, and chemical methods. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Bupleurum rigidum*; Umbelliferae; Saponins; Saikosaponins

1. Introduction

Bupleuri Radix [root of *Bupleurum* spp.] is a well known crude drug used in Chinese traditional medicine for over 2000 years (Pistelli et al., 1996). Many species of the genus *Bupleurum* (Umbelliferae) have been shown to accumulate saikosaponins (Ding et al., 1986; Luo et al., 1993), compounds which are known to possess various biological properties such as anti-inflammatory and antihepatotoxic activities (Recio et al., 1995; Matsuda et al., 1997). We have previously reported on the isolation and characterization of two major saikosaponins (Buddlejasaponin IV and Sandrosaponin I) from *Bupleurum rigidum* (Sánchez-Contreras et al., 1998) as well as on their anti-inflammatory activities (Bermejo et al., 1998). The structure elucidation of five minor new sulfated saikosaponins obtained from an *n*-butanol extract of the aerial part of this species is presented herein.

2. Results and discussion

The MeOH extract of dried aerial parts of *B. rigidum* was partitioned between *n*-BuOH and water. The *n*-BuOH soluble fraction was chromatographed over a silica gel column, followed by repeated MPLC purification to give five compounds.

The molecular formula of compound **1** was established as $C_{48}H_{77}O_{22}SNa$ by HR-FABMS. The ^{1}H-NMR spectrum of **1** was typical of a saponin, showing signals for two coupled ethylenic protons (5.94 and 5.38 ppm, $J = 11.5$ Hz) and five methyl singlets in the region 0.5–1.5 ppm. Three doublets (4.86, 4.64, and 4.46 ppm) with coupling constants 7.8 Hz, characteristic of β-anomeric proton sugars, and multiplets in the zone of ring sugars were indicative of three different monosaccharides. A methyl doublet at 1.25 ppm (3H, $J = 6.4$ Hz) suggested the presence of a 6-deoxysugar.

* Corresponding author. Tel.: +34-91-885-46-42; fax: +34-91-885-46-79.

E-mail address: tfamdl@farma.alcala.es (A.M.Ana Marí. Díaz-Lanza).

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The ^{13}C-NMR spectrum of **1** contained signals for 48 carbons, two of them ethylenic (134.2 and 130.5 ppm), and three in the region of carbohydrate anomeric carbons (105.0, 104.8, and 103.4 ppm). The existence of only five methyl singlets and signals for five oxygen-bearing carbons more than those corresponding to the sugar moieties indicated that three methyl groups were oxidized and also that two hydroxyl additional groups were present in the aglycon moiety. The assignment of all the spectral signals was achieved through a combination of homonuclear proton–proton 2D (COSY, TOCSY) and proton–carbon 2D (HMQC and HMBC) heteronuclear experiments. The results are depicted in Tables 1–3. Comparison of COSY, TOCSY (mixing time = 85 ms), and HMQC spectra of the crowded region, 4.2–3.0 ppm, together with selective decoupling of proton connected with those of the high field zone (2.2–0.7 ppm), allowed to observe, in addition to the sugar atoms, three oxymethylene groups at 3.78–3.26 (C = 64.5 ppm), 3.90–3.05 (C = 73.0 ppm), and 3.24 (2H, C = 74.2 ppm), and also two oxymethyne groups, at 4.16 ppm (C = 65.3 ppm) and 3.60 ppm (C = 84.0 ppm). The direct proton–carbon connections obtained from the HMQC spectrum, together with the network of crosspeaks observed in the HMBC spectrum allowed to deduce their position and configuration. Thus, the occurrence in the HMBC spectrum of crosspeaks Me-24/C(73), Me-24/C(84), Me-24/C(44.5), and Me-24/C(47.7), allowed to assign the first CH_2OH to C-23 and the CH at 3.60 ppm to C-3. On the other hand, the presence in the HMBC of crosspeaks Me-30/C(74.2), Me-30/C-19, Me-30/C-21, led to the assignment of the signal at 3.24 ppm to the hydroxymethyl group at C-29. Analogously, crosspeaks among the proton at 3.05 ppm and carbons at 47.8 (C-17), 52.3 (C-18), 85.8 (C-13), and 65.4, allowed to deduce that the oxymethylene group at 3.90–3.05 ppm corresponded to C-28, and the proton at 4.16 ppm was linked to C-16. The existence of a 13,28-epoxy group was demonstrated by the presence of a crosspeak H-28/C-13 (85.8 ppm). The coupling constants of protons H-3 ($J = 10$, 5.9 Hz), and H-16 ($J = 11.7$, 4.8 Hz) indicated an axial disposition for both of them and hence, equatorial (β) arrangements

Table 1
^{1}H-NMR chemical shifts (δ) and proton–proton coupling constants (Hz) of the aglycon moieties of compounds **1–5**[a]

Atom	Compound				
	1	**2**	**3**	**4**	**5**
1	1.83–0.90	1.82–0.93	1.83–0.92	1.83–0.92	1.87–1.02
2	1.96–1.82	1.96–1.81	1.96–1.81	1.96–1.80	2.0–1.82
3	3.6 (*dd*, $J = 11.7$, 4.8)	3.61 (*dd*, $J = 11.7$, 4.8)	3.61 (*dd*, $J = 11.7$, 4.8)	3.61 (*dd*, $J = 11.7$, 4.9)	3.64
4	–	–	–	–	–
5	1.17	1.18	1.16	1.18	1.25
6	1.54–n.d.	152–n.d.	1.54–1.51	1.52–1.11	1.57–1.40
7	1.52–1.21	1.52–1.20	1.53–1.21	1.53–1.20	1.50–1.35
8	–	–	–	–	–
9	1.90	1.89	1.89	1.90	2.05
10	–	–	–	–	–
11	5.94 (*d*, $J = 11.5$)	5.93 (*d*, $J = 10.4$)	5.88	5.90 (*d*, $J = 11.5$)	5.58 (*d*, $J = 11.0$)
12	5.38 (*dd*, $J = 11.5$, 3.2)	5.42 (*dd*, $J = 10.4$, 3.0)	5.53 (*dd*, $J = 10.5$, 3.2)	5.52 (*dd*, $J = 11.5$, 3.2)	6.45 (*dd*, $J = 11.0$, 3.2)
13	–	–	–	–	–
14	–	–	–	–	–
15	1.59–1.44	1.58–1.43	1.59–1.42	1.58–1.42	1.94–1.43
16	4.16 (*dd*, $J = 10.0$, 5.9)	4.21 (*dd*, $J = 10.2$, 6.4)	4.18 (*dd*, $J = 10.3$, 5.9)	4.18 (*dd*, $J = 9.2$, 5.8)	4.05 (*t*, $J = 3.2$)
17	–	–	–	–	–
18	1.81	1.74 (*dd*, $J = 12.6$, 5.5)	1.90	1.96	–
19	185–1.23	1.62–1.58	2.18 (*dt*, $J = 12.2$, 3.2, 3.2)–1.52	2.10 (*dd*, $J = 11.7$, 3.5)–1.70	2.46 (*d*, $J = 14.7$)–1.79
20	–	–	–	–	–
21	1.57–1.13	1.40–1.28	2.03–1.26	1.96–1.41	1.64–1.25
22	2.10–1.28	2.03–1.21	2.05–1.36	2.10–1.41	2.03–1.63
23	3.78–3.26	3.78–3.25	3.77–3.27	3.78–3.26	3.78–3.28
24	0.72 (*s*)	0.72 (*s*)	0.72 (*s*)	0.72 (*s*)	0.73 (*s*)
25	0.94 (*s*)	0.94 (*s*)	0.94 (*s*)	0.94 (*s*)	0.95 (*s*)
26	1.09 (*s*)	1.08 (*s*)	1.08 (*s*)	1.09 (*s*)	0.74 (*s*)
27	1.05 (*s*)	1.06 (*s*)	1.05 (*s*)	1.06 (*s*)	1.25 (*s*)
28	3.9–3.05 (*d*, $J = 7.1$)	3.88–3.02 (*d*, $J = 7.1$)	3.86–2.95 (*d*, $J = 7.1$)	3.87–2.98 (*d*, $J = 7.3$)	3.74–3.30
29	3.24 (*b.s.*)	0.95 (*s*)	1.12 (*s*)	3.46 (*b.s.*)	3.27 (*s*)
30	0.9 (*s*)	3.52–3.37 (*d*, $J = 11.3$)	–	–	0.83 (*s*)

[a] Abbreviations: *s*, singlet; *d*, doublet; *b.s.*, broad singlet; *dd*, double doublet; *dt*, double triplet; *t*, triplet.

Table 2
^{13}C-NMR chemical shifts (δ) of the aglycon moieties of compounds **1**–**5**

Atom	Compound				
	1	**2**	**3**	**4**	**5**
1	39.3	39.3	39.4	39.3	39.1
2	26.4	26.4	26.4	26.4	26.5
3	84.2	84.2	84.5	84.4	84.4
4	44.4	44.4	44.4	44.4	44.4
5	48.0	48.1	48.4	48.2	48.3
6	18.2	18.8	18.3	18.2	18.9
7	32.1	32.1	32.3	32.2	32.9
8	43.0	43.0	42.9	42.9	42.5
9	53.9	53.9	54.0	54.0	54.8
10	37.0	37.0	37.1	37.1	37.3
11	134.2	134.1	133.3	133.5	127.1
12	130.5	130.6	131.2	131.0	126.7
13	85.8	85.8	82.6	85.8	137.7
14	46.5	47.4	47.1	46.6	41.9
15	36.0	36.1[a]	36.2	36.1	32.1
16	65.4	65.4	66.2	66.0	69.1
17	47.8	46.6	46.7	47.5	45.7
18	52.3	52.5	54.6	53.8	131.9
19	32.9	33.1	36.6	31.2	33.8
20	37.6	36.8[a]	47.1	52.8	38.4
21	29.6	30.8	32.9	27.4	30.2
22	25.5	25.8	28.1	27.5	24.0
23	64.4	64.4	65.0	64.6	64.5
24	12.6	12.6	12.6	12.6	12.7
25	18.8	18.2	18.8	18.8	18.9
26	20.2	20.2	20.2	20.2	17.6
27	21.2	21.3	21.1	21.1	22.1
28	73.3	73.3	73.6	73.6	65.2
29	74.6	28.7	29.7	71.9	74.1
30	19.7	66.0	184.0	182.0	20.6

[a] These values may be interchanged.

Table 3
Proton and carbon chemical shifts (δ) and proton–proton coupling constants (J, Hz), of the sugar moieties of compound **1**. Variations among chemical shifts of the rest of derivatives (**2**–**5**) were within $\pm$0.05 ppm (proton) and $\pm$0.3 ppm (carbon). For coupling constants the differences were $\pm$0.2 Hz[a]

Proton	Unit A	Unit B	Unit C
H-1	4.85 (*d*, $J = 7.8$)	4.64 (*d*, $J = 8.0$)	4.46 (*d*, $J = 7.8$)
H-2	3.12 (*dd*, $J = 7.8$, 9.5)	3.41 (*dd*, $J = 8.0$, 9.0)	3.92 (*dd*, $J = 7.8$, 9.8
H-3	3.34 (*dd*, $J = 9.0$, 9.5)	3.65 (*t*, $J = 9.0$)	3.78
H-4	3.13 (*dd*, $J = 9.0$, 9.2)	4.14 (*dd*, $J = 9.0$, 9.7)	3.87 (*dd*, $J = 3.4$, 1)
H-5	3.27 (*m*)	3.42 (*m*)	3.64 (*dq*, $J = 1$, 6.4)
H-6a	3.80 (*dd*, $J = 2.4$, 12.0)	3.85	1.26 (*d*, $J = 6.4$)
H-6b	3.54 (*dd*, $J = 7.1$, 12.0)	3.75	

Unit	C-1	C-2	C-3	C-4	C-5	C-6
A (β-Glcp)	103.4	76.4	78.3	72.4	78.3	63.6
B (β-Glcp)	105.0	75.3	76.9	77.5	76.4	62.2
C (β-Fucp)	104.8	**76.3**	**85.5**	72.7	71.4	16.9

[a] Abbreviations: *d*, doublet; *dd*, double doublet; *dq*, double quartet; *t*, triplet; *m*, multiplet. The underlined bold values mean glycosylation points.

of the hydroxyl groups at C-3 and C-16 were inferred for **1**.

Concerning the sugar moieties, they were labeled A–C, from low to high field, according to their anomeric protons. Measurement of most of the coupling constants (Table 3) led to the identification of A and B as glucose residues ($J_{2,3}$, $J_{3,4}$, and $J_{4,5} > 9$ Hz), and C as a fucose unit ($J_{2,3}$, $J_{3,4} > 9$ Hz, $J_{5,6} = 6.4$ Hz). One of the carbohydrate protons was strongly deshielded, suggesting that its geminal hydroxyl group was substituted. From the homonuclear 2D experiments and selective decoupling, the proton was identified as H-4 of unit B. As the presence of a sulfur atom was deduced from the MS spectrum, it was inferred that the hydroxyl at C-4 was substituted by a sulfate group. Acid hydrolysis of the compound followed by treatment with $BaCl_2$ gave a white precipitate of barium sulfate, confirming the existence of a sulfate group.

The ^{13}C-NMR chemical shifts values for the sugar units were deduced from HMQC and HMBC experiments. From these values (Table 3), it could be inferred that C-2 and C-3 of the fucose residue were shifted downfield, as compared to those obtained from model compounds (Bock et al., 1984), therefore, these were identified as the glycosylation sites. The HMBC experiment unequivocally demonstrated these conclusions, showing crosspeaks at H-1A/C-3C, H-1B/C-2C, and also H-1C/C-3 (aglycon). From all these data, the structure of compound **1** is proposed to be: 13,28-epoxy-3β,16β,23,29-tetrahydroxyolean-11-en-3-β-yl β-D-glucopyranosyl-(1 → 2)-[4-*O*-sulfo-β-D-glucopyranosyl-(1 → 3)-]β-D-fucopyranoside, and was given the trivial name of Sandrosaponin II.

The ^{1}H-NMR spectrum of compound **2** was very similar to that of **1**, with two ethylenic protons (5.93 and 5.42 ppm) and five methyl singlets. The chemical shifts of the anomeric doublets were identical to those found in the spectrum of **1**, and comparison of the pattern of the ring protons of the carbohydrate moieties for both **1** and **2** led to the conclusion that identical trisaccharide was present in both molecules. The values of the proton and carbon chemical shifts of both aglycon and sugar moieties, obtained by running similar homo and heteronuclear 2D experiments (Tables 1–3), suggested that we were leading with two similar compounds with opposite configuration at C-20.

Comparison of the values found for C-29 and C-30 in compounds **1** and **2** with those reported for related model compounds strongly supported our findings. Thus, C-29 (equatorial) hydroxymethyl groups show carbon chemical shift values around 67 ppm, and C-30 (axial) methyl groups values are found around 28 ppm. On the other hand, C-30 hydroxymethyl groups show chemical shifts around 75 ppm, while C-29 methyl groups values are about 28 ppm (see, for instance, González et al., 1986; Miyase and Matsushima, 1997). Therefore, the structure of this compound was assigned as being: 13,28-epoxy-3β,16β,23,30-tetrahydroxyolean-11-en-3β-yl β-D-glucopyranosyl-(1 → 2)[4-*O*-sulfo-β-D-glucopyranosyl-(1 → 3)-]β-D-fucopyranoside. The aglycon of this compound has been recently described as forming part of Clinoposaponin XIX, a saponin isolated from *Clinopodium* spp. (Miyase and Matsushima, 1997).

Compound **3** displayed a ^{1}H-NMR spectrum closely related to those of **1** and **2**, containing **2** ethylenic protons, five methyl singlets and a methyl doublet, together with three anomeric sugar protons, practically identical to those observed in compounds **1** and **2**. The ^{13}C-NMR spectrum showed 48 singlets, one of them at 184.5 ppm, indicative of the presence of a carboxyl group, while the number of oxygen-bearing carbons was only four more than those corresponding to sugar carbons.

The assignment of protons and carbons was achieved by using analogous ^{1}H–^{1}H homo- and ^{1}H–^{13}C- hetero-NMR techniques similar to those mentioned for compounds **1** and **2**, from which an identical sulfated trisaccharide was deduced to be present in the molecule. The carboxyl group of the aglycon moiety was found to be at C-30, as a crosspeak H-19ax/C=O was observed in the HMBC spectrum. Hence, the structure of compound **3** is proposed to be 13,28-epoxy-3β,16β,23-trihydroxyolean-11-en-3β-yl-30-oic acid β-D-glucopyranosyl-(1 → 2)-[4-*O*-sulfo-β-D-glucopyranosyl-(1 → 3)-]β-D-fucopyranoside.

Compound **4** gave a FABMS $m/z = 1067$. Its ^{1}H-NMR spectrum also contained two vicinal ethylenic protons, but only four methyl singlets. The number and pattern of the sugar protons led to the conclusion that the sulfated trisaccharide found in compounds **1**–**3** was also present here. The ^{13}C-NMR spectrum also contained 48 singlets, one of them at 182.0 ppm (carboxyl group). The oxygen-linked carbons were five more than those corresponding to the carbohydrate moiety, two of them with values imputable to hydroxymethyl groups. The HMBC spectrum showed crosspeaks CH_2OH/CO, CH_2OH/C-19, CH_2OH/C-21, and H-19ax/CO. All these data are in accordance with the structure depicted in Fig. 1.

The FABMS spectrum of compound **5** gave m/z 1037, identical to that found for compounds **1** and **2**. The ^{1}H-NMR spectrum showed two vicinal ethylenic protons (6.45 and 5.58 ppm), which are shifted downfield around 0.5 and 0.2 ppm, respectively, as compared with those observed in the preceding compounds, suggesting a conjugation of the double bond. Five methyl singlets were also observed at high field, in addition to a methyl doublet at 1.26 ppm which, together with the presence of three anomeric doublets

(*J* corresponding to β anomers) and the usual pattern of sugar ring protons demonstrated that the trisaccharide found in compounds **1**–**4** was also forming part of **5**.

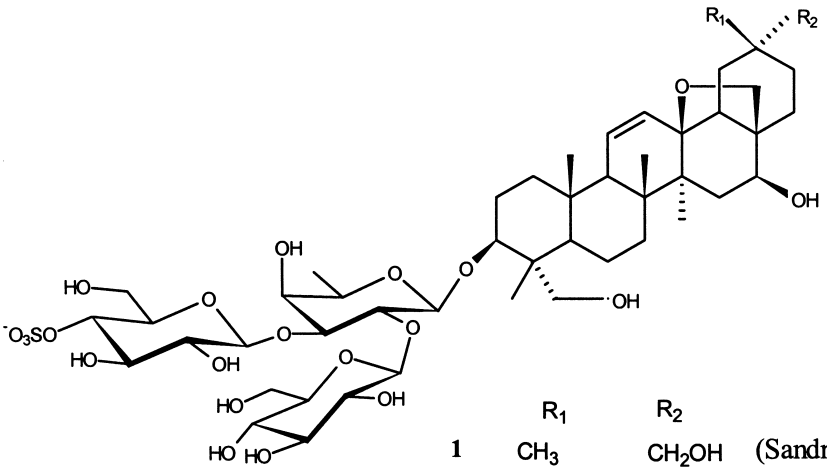
The ^{13}C-NMR spectrum was demonstrative of the existence of four ethylenic carbons (137.7, 131.9, 127.1, and 126.7 ppm). The absence of a 13,28-epoxy derivative was deduced from the upfield shifting of carbon 28, which now appeared at 65.2 ppm, indicating the opening of the ring.

Following analogous methodology than that used in the preceding compounds, we assigned all the protons and carbons of the molecule, from which the existence of two hydroxyl, at C-3 and C-16, and two hydroxymethyl groups at positions C-23 and C-28, were inferred. The configuration of the hydroxyl group at C-16 was α, as deduced from the coupling constants $J_{15\alpha,\,16} \cong J_{15\beta,\,16} \cong 3.2$ Hz, which indicated that H-16 was equatorial. The structure of **5** was then established as 3β,16α,23,28,29-pentahydroxy 11,13(18)-oleanedien-3β-yl-β-D-glucopyranosyl-(1 → 2)-[4-*O*-sulfo-β-D-glucopyranosyl-(1 → 3)-]β-D-fucopyranoside. An identical aglycon has been isolated from *B. scorzonerifolium* (Tan et al., 1966).

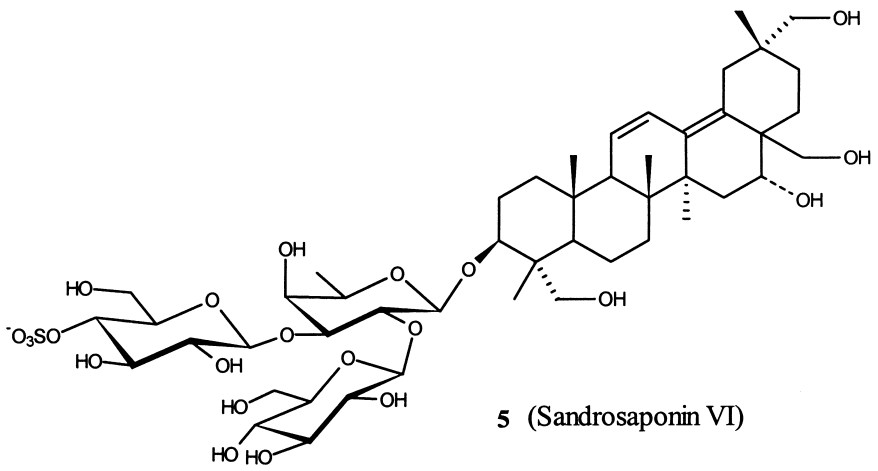
3. Experimental

3.1. General

IR spectra were recorded in KBr, on a Perkin Elmer 681 spectrometer. NMR spectra were recorded in



	R_1	R_2	
1	CH_3	CH_2OH	(Sandrosaponin II)
2	CH_2OH	CH_3	(Sandrosaponin V)
3	COOH	CH_3	(Sandrosaponin III)
4	COOH	CH_2OH	(Sandrosaponin IV)



5 (Sandrosaponin VI)

Fig. 1.

CD_3OD, on a Varian Unity 500 instrument at 25°C. Chemical shifts refer to the methanol-d_4 multiplet (^{1}H, 3.30 ppm; ^{13}C, 49.0 ppm). HR-FABMS were carried out in a VG AutoSpec (Fisons). Analytical TLC was carried out on Merck silica gel F_{254} aluminum sheets, eluted with *n*-BuOH–AcOH–AcOH-H_2O (4:1:5), and visualized with 1% vanillin in MeOH–H_2SO_4 (1:1). Carbohydrates were identified by chromatographic comparison with authentic samples of D-glucose and D-fucose.

3.2. Plant material

Bupleurum rígidum was collected in Guadalajara, Spain, in June 1995, and was identified by Dr. C. Bartolomé, Departamento de Biología Vegetal, Facultad de Ciencias, Universidad de Alcalá de Henares, Madrid, Spain. A voucher specimen has been deposited at the herbarium of the University of Alcalá.

3.3. Extraction and purification

The aerial parts of *B. rigidum* (900 g) were treated for 3 h at room temperature with $CHCl_3$ (9 l). Subsequently, the residue was extracted with 60% MeOH (5 l) for 24 h. After removal of the MeOH under vacuum, the resulting aqueous solution was extracted with *n*-BuOH saturated with H_2O (3 × 2 l). The *n*-BuOH extracts were concentrated to dryness under a vacuum, affording a saponin mixture (40 g, yield 4.4%), of which 7 g were chromatographed over a silica gel column, eluting with $CHCl_3$–MeOH–H_2O (80:20:1 → 55:37:7), to give 16 fractions. Four fractions 10, 12, 13 and 14, were separated and studied. All were chromatographed on silica gel using mixtures of MeOH–H_2O of increasing polarity as eluent.

Fraction 10 (0.125 g) afforded Sandrosaponin V (compound **2**, 4.2 mg), *fraction* 12 (0.48 g) afforded Sandrosaponin II (**1**, 24.4 mg), *fraction* 13 (0.721 g) gave Sandrosaponin III (**3**, 53.8 mg), and Sandrosaponin IV (**4**, 7.3 mg), and *fraction* 14 (0.32 g) afforded Sandrosaponin VI (**5**, 17.4 mg).

Sandrosaponin II: 13,28-epoxy-3β,16β,23,29-tetrahydroxyolean-11-en-3-β-yl β-D-glucopyranosyl-(1 → 2)-[4-*O*-sulfo-β-D-glucopyranosyl-(1 → 3)]β-D-fucopyranoside (**1**): amorphous powder, $[\alpha]_D$ + 47.8° (*c* 0.2 MeOH); IR: (KBr) cm^{-1} 3360, 2860, 2810, 1610, 1240, 1030, 950; ^{1}H-RMN (see Table 1); ^{13}C (see Table 2); HR-FABMS $[M]^+$ *m/z* 1083.4422, calc. for $C_{48}H_{77}O_{22}SNa$: 1083.4423.

Sandrosaponin III: 13,28-epoxy-3β,16β,23-trihydroxyolean-11-en-3β-yl-30-oic acid β-D-glucopyranosyl-(1 → 2)-[4-*O*-sulfo-β-D-glucopyranosyl-(1 → 3)]β-D-fucopyranoside. (**3**): amorphous powder, $[\alpha]_D$ + 53.5° (*c* 0.18 MeOH); IR: (KBr) cm^{-1} 3350, 2850, 2780, 1660, 1600, 1350, 1030, 940; ^{1}H-RMN (see Table 1); ^{13}C (see Table 2); FABMS $[M]^+$ *m/z* 1075, $C_{48}H_{75}O_{23}SNa$.

Sandrosaponin IV: 13,28-epoxy-3β,16β,23,29-tetrahydroxyolean-11-en-3β-yl 30-oic acid β-D-glucopyranosyl-(1 → 2)-[4-*O*-sulfo-β-D-glucopyranosyl-(1 → 3)-]β-D-fucopyranoside (**4**): amorphous powder, $[\alpha]_D$ + 48.5° (*c* 0.1 MeOH); IR: (KBr) cm^{-1} 3350, 2850, 2790, 1600, 1220, 1030, 950; ^{1}H-RMN (see Table 1); ^{13}C (see Table 2); FABMS $[M]^+$ *m/z* 1113, $C_{48}H_{75}O_{24}SNa_2$.

Sandrosaponin V: 13,28-epoxy-3β,16β,23,30-tetrahydroxyolean-11-en-3β-yl β-D-glucopyranosyl-(1 → 2)-[4-*O*-sulfo-β-D-glucopyranosyl-(1 → 3)-]β-D-fucopyranoside (**2**): amorphous powder, $[\alpha]_D$ + 30.6° (*c* 0.07 MeOH); IR: (KBr) cm^{-1} 3400, 2900, 2860, 1640, 1240, 1060, 970; ^{1}H-RMN (see Table 1); ^{13}C (see Table 2); FABMS $[M]^+$ *m/z* 1061 for $C_{48}H_{77}O_{22}SNa$.

Sandrosaponin VI: 3β,16α,23,28,29-pentahydroxy-11,13(18)-oleanedien-3β-yl β-D-glucopyranosyl-(1 → 2)-[4-*O*-sulfo-β-D-glucopyranosyl-(1 → 3)-]β-D-fucopyranoside (**5**): amorphous powder, $[\alpha]_D$ + 17.5° (*c* 0.14 MeOH); IR: (KBr) cm^{-1} 3350, 2880, 2800, 1600, 1230, 1030, 950; ^{1}H-RMN (see Table 1); ^{13}C (see Table 2); FABMS $[M]^+$ *m/z* 1078 for $C_{48}H_{77}O_{22}SCa$.

3.4. Acid hydrolysis of [1–5]

1–2 mg of each sample were refluxed with 10% HCl (4 ml) for 4 h. After extraction with ethyl ether, the aqueous solution was treated with $BaCl_2$ to give a white precipitate ($BaSO_4$), the determination was performed according to Akai et al. (1985). The rest of the aqueous layer was neutralized (10% *N*,*N*-dioctylmethylamine in $CHCl_3$) and concentrated under reduced pressure. The sugars were directly analyzed by TLC. Glucose and fucose were identified on comparison with authentic samples.

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