Oleanene Glycosides of the Aerial Parts and Seeds of *Bupleurum falcatum* and the Aerial Parts of *Bupleurum rotundifolium*, and Their Evaluation as Anti-hepatitis Agents

Yuko NAKAHARA,[a] Masafumi OKAWA,[b] Junei KINJO,[b] and Toshihiro NOHARA*,[c]

[a] *Graduate School of Pharmaceutical Sciences, Faculty of Life Sciences, Kumamoto University; 5–1 Oe-honmachi, Kumamoto 862–0973, Japan:* [b] *Faculty of Pharmaceutical Sciences, Fukuoka University; 8–19–1 Nanakuma, Higashi-ku, Fukuoka 814–0180, Japan: and* [c] *Faculty of Pharmaceutical Sciences, Sojo University; 4–22–1 Ikeda, Kumamoto 860–0082, Japan.* Received May 25, 2011; accepted August 18, 2011; published online August 23, 2011

To facilitate effective resource utilization, we have investigated triterpene saponins such as saikosaponin from the aerial parts of *Bupleurum* (B.) *falcatum* L., which are commonly discarded. Seven oleanene saponins were isolated from this plant; they were classified as the 13,28-epoxy type, 12-ene type, 9(11),12-diene type, and 28-acid type on the basis of their structural characteristics. For comparison, we also examined the oleanene saponins of the seeds of *B. falcatum* and the aerial parts of *B. rotundifolium* L. to obtain seven saponins and one sapogenol from the former and thirteen oleanene saponins from the latter. Several compounds obtained from them were investigated for their hepatoprotective activity and hepatotoxicity. The 13,28-epoxy type saponins had hepatoprotectivity. Ursane type showed hepatotoxicity from middle concentration. The 11,13(18)-diene type saponins did not express hepatoprotective activity. The 28-acid type saponin which has a glucosyl carboxy group showed hepatoprotective action.

Key words *Bupleurum falcatum*; saikosaponin; hepatoprotective activity; *Bupleurum rotundifolium*

Bupleuri Radix appeared in 'Shen hong ben cao jing' as a drug harmless to humans and one of the most important Chinese crude drugs used as an antipyretic and anti-inflammatory. The occurrence of saikosaponins (saikosaponin a : c : d = 2 : 1 : 3) in this drug and their functions in peptide synthesis and detoxification in the liver are well known. Many earlier works were reported, as follows. Shibata *et al.* reported isolation of various saponins from Bupleuri Radix originating from *Bupleurum falcatum* cultivated in Japan and the liver-protecting activity of a mixture of the major saponin, saikosaponin a, with other saponins.[1)] Shimaoka *et al.* reinvestigated the structure of saikosaponin,[2)] and Tori *et al.* conducted a ^{13}C-NMR investigation of saikosapogenins.[3)] Ogihara and colleagues synthesized artificial derivatives of saikosaponins by using an acid or enzyme[4,5)] and examined their function of accelerating corticosteroid secretion.[6)] Mitsuhashi and colleagues isolated a new saikosaponin malonate from *B. scorzonerifolium*,[7)] and Yoshikawa *et al.* isolated a new saponin from *B. scorzonerifolium* and studied its liver-protecting components.[8,9)] Moreover, agricultural studies of the constituents of the seeds have been begun by Ono *et al.*,[10)] and studies of anti-proliferation of tumor cells have been initiated by Mihashi and colleagues.[11,12)]

In some areas in Kumamoto Prefecture, this plant is cultivated, and its aerial parts are discarded. We investigated the drug's constituents in conjunction with studies of the aerial parts of *B. rotundifolium*, which is cultivated for cut flowers, and the seeds of *B. falcatum*. We also examined the liver-protecting functions of the obtained specimens and elucidated the relationship between their structure and activity.

Harvested aerial parts (2.6 kg) of *B. falcatum* were extracted by refluxing with MeOH to give an extract (262.2 g), which was then separated by using the solvent partition method. Various chromatographies on Diaion HP-20, Sephadex LH-20, silica gel, and Chromatorex octadecylsilyl (ODS) silica were applied to provide BFA-5 (108 mg), BFA-6 (264 mg), BFA-8 (45 mg), BFA-11 (21 mg), BFA-13 (110 mg), BFA-17 (21 mg), BFA-20 (20 mg), BFA-22 (72 mg), BFA-25 (17 mg), BFA-27 (110 mg), BFA-28 (680 mg), and BFA-29 (20 mg).

BFA-8 (**1**), BFA-22 (**2**), BFA-5 (**3**), BFA-6 (**4**), and BFA-25 (**5**) were identified as saikosaponin a,[3)] saikosaponin c,[3)] saikosaponin f,[3)] saikosaponin b_2,[2)] and 4″-*O*-acetylsaikosaponin b_2,[13)] respectively, by fast atom bombardment-mass spectrometry (FAB-MS), ^{1}H- and ^{13}C-NMR spectra, and chemical reactions.

BFA-20 (**6**) was obtained as an amorphous powder having $[\alpha]_D$ −11.8° (MeOH). Positive high resolution (HR)-FAB-MS revealed a molecular formula of $C_{48}H_{78}O_{18}Na$ at *m*/*z* 965.5088 $[M+Na]^+$. The ^{1}H-NMR spectrum displayed signals due to six tertiary methyl groups at δ 0.90, 0.96, 1.00, 1.00, 1.06, and 1.66 (each 3H, s); one methyl pentosyl methyl group (3H, d, J=6.1 Hz, H_3-6) at δ 1.66; two hexosyl anomeric protons at δ 4.94 and 5.01 (each 1H, anomeric proton, d, J=7.9 Hz); one methyl pentosyl anomeric proton at δ 5.87 (1H, s); and two olefinic protons at δ 5.67 and 6.68 (1H, d, J=11.0 Hz and 1H, br d, J=8.5 Hz, respectively). The ^{13}C-NMR spectrum (Table 1) displayed a total of 48 carbon signals, of which 30 were attributable to saikogenin D comprised of BFA-6 (**4**). The remaining carbon signals arose from sugar moieties and coincided with those of the sugar moieties in BFA-22 (**2**), as listed in Table 1. Therefore, the structure of **6** was characterized as β-D-glucopyranosyl-(1→16)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl 3β,16α,23,28-tetrahydroxyolean-11,13(18)-diene.[4)]

BFA-17 (**7**) was obtained as an amorphous powder having $[\alpha]_D$ +45.9° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{36}H_{58}O_9Na$ at *m*/*z* 657.3982 $[M+Na]^+$. The ^{1}H-NMR spectrum displayed signals due to six tertiary methyl groups at δ 0.81, 0.97, 1.10, 1.21, 1.28, and 1.35 (each 3H, s); one olefinic proton at δ 5.44; and one hexosyl anomeric proton at δ 6.33 (1H, d, J=7.9 Hz). Since the ^{13}C-

* To whom correspondence should be addressed. e-mail: none@ph.sojo-u.ac.jp

© 2011 Pharmaceutical Society of Japan

Table 1. ^{13}C-Data for Compounds **1**—**7**

	1	**2**	**3**	**4**	**5**	**6**	**7**
C-1	38.6	38.4	38.8	38.4	38.4	38.3	38.6
2	26.0	26.4	26.5	26.1	26.1	25.9	26.3
3	81.6	89.0	89.0	81.7	81.7	82.1	78.9
4	43.7	39.6	39.5	43.7	43.7	43.6	39.4
5	47.3	55.2	55.7	47.3	47.4	47.3	55.7
6	17.2	18.4	18.5	18.8	18.3	18.3	18.5
7	31.5	31.8	33.4	31.9	31.9	31.9	32.4
8	42.2	42.1	40.1	41.1	41.9	41.1	39.9
9	53.0	52.8	47.0	54.0	54.0	53.9	48.0
10	36.2	36.3	36.7	36.5	36.5	36.3	36.0
11	132.2	132.1	24.1	126.2	126.3	126.2	23.7
12	131.1	131.1	122.6	126.2	126.2	126.2	123.0
13	84.0	84.0	143.9	136.0	136.0	136.1	144.0
14	45.6	45.6	43.8	41.8	41.1	41.9	42.2
15	36.1	36.0	36.7	32.3	32.3	32.3	33.1
16	64.0	64.0	66.6	67.6	67.7	67.6	23.7
17	46.9	47.0	41.0	45.3	45.3	45.3	47.0
18	52.1	52.1	44.4	133.0	133.1	133.0	41.3
19	37.7	37.8	47.0	39.0	39.0	39.0	41.7
20	31.5	31.6	31.1	32.6	32.6	32.6	35.7
21	34.6	34.7	34.3	35.4	35.5	35.5	29.5
22	25.7	25.7	26.5	24.4	24.4	24.4	32.4
23	64.0	27.9	28.2	64.1	64.8	64.3	28.2
24	13.0	16.3	17.0	13.1	24.1	13.1	17.0
25	18.7	18.1	15.6	18.2	18.9	18.5	15.5
26	20.0	19.9	17.0	17.2	17.3	17.2	17.5
27	20.8	20.9	27.1	21.9	21.9	21.9	26.2
28	73.0	72.6	69.0	64.7	64.7	64.7	1765.5
29	33.6	33.7	32.9	25.1	25.1	25.1	28.4
30	23.8	23.8	24.1	32.5	32.6	64.0	65.4
fuc-1	105.9			105.9	106.0		
2	71.5			71.5	71.6		
3	85.1			85.2	85.4		
4	71.7			71.8	72.1		
5	70.9			71.0	71.5		
6	17.2			17.2	17.4		
glcI-1	106.5	106.7	106.7	106.5	106.4	106.7	95.7
2	75.7	75.1	75.1	75.7	75.3	75.1	74.1
3	78.7	76.7	76.7	78.7	78.1	76.7	79.3
4	72.1	79.8	79.8	72.1	75.5	79.8	71.1
5	78.4	75.5	75.5	78.3	78.1	75.5	78.9
6	62.7	69.0	68.8	62.7	64.2	69.0	62.2
OCO$\underline{C}$H$_3$				20.1			
O$\underline{C}$OCH$_3$				170.8			
glcII-1		105.0	105.0			105.0	
2		74.7	74.8			74.7	
3		78.4	78.4			78.4	
4		71.4	71.4			71.4	
5		78.3	78.4			78.3	
6		62.5	62.6			62.5	
rha-1		102.9	102.9			102.9	
2		72.5	72.6			72.5	
3		72.5	72.5			72.5	
4		73.8	73.8			73.8	
5		70.5	70.4			70.5	
6		18.1	18.5			18.1	

NMR spectrum exhibited a total of 36 carbon signals that suggested the presence of one β-D-glucopyranosyl moiety, the remaining 30 carbon signals were regarded as attributable to a sapogenol moiety. Heteronuclear multiple bond coherence (HMBC) analysis identified six tertiary methyl groups and correlated an oxygen-bearing methylene at δ 65.4 with H_3-29; therefore, CH_2O is present at C-30. This sapogenol is identified as 3β,30-dihydroxyolean-12-ene-28-oic acid, or queretaroic acid.[14] HMBC from the glucosyl anomeric proton was observed at C-28 at δ 176.5. This glycoside was recognized as a new one.

Next, the seeds (2.6 kg) of *B. falcatum* were crushed with a mixer and extracted by refluxing with MeOH; the resulting extract (84.7 g) was then separated by using various chromatographies on Diaion HP-20, Sephadex LH-20, silica gel, and preparative thin layer chromatography to provide BFS-1 (30 mg), BFS-8 (8 mg), BFS-9 (13 mg), BFS-10 (31 mg), BFS-11 (34 mg), BFS-13 (28 mg), BFS-14 (15 mg), and BFS-16 (15 mg).

BFS-9 (**1**), BFS-1 (**2**), BFS-16 (**8**), BFS-11 (**10**), and BFS-13 (**4**) were identified as saikosaponin a,[3] saikosaponin c,[3] bupleuroside IX,[9] saikosaponin b_1,[2] and saikosaponin b_2, respectively, by FAB-MS and ^{1}H- and ^{13}C-NMR spectra.

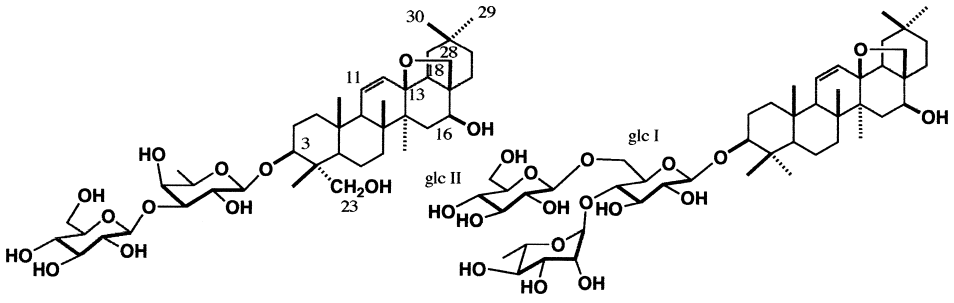
BFS-10 (**12**) was obtained as an amorphous powder having $[\alpha]_D$ +78.1° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{42}H_{68}O_{13}Na$ at *m/z* 803.4563 $[M+Na]^+$. The ^{1}H-NMR spectrum displayed signals due to six tertiary methyl groups at δ 0.89, 0.97, 1.00, 1.28, 1.29, and 1.30 (each 3H, s); one methyl pentosyl methyl group at δ 1.44 (3H, d, J=6.1 Hz, H_3-6); two anomeric protons at δ 4.97 (1H, d, J=7.9 Hz) and 5.33 (1H, d, J=7.9 Hz); and two olefinic protons at δ 5.65 (1H, d, J=6.1 Hz) and 5.76 (1H, d, J=6.1 Hz). The ^{13}C-NMR spectrum (Table 3) showed the presence of two trisubstituted double bonds at δ 116.0, 121.1, 145.3, and 154.9; their carbon chemical shifts and proton coupling constants indicated that they were located at the C-ring and homoannular diene, respectively. The respective carbon signals at δ 81.5, 66.8, 64.2, and 69.3 could be assigned to C-3, C-16, C-23, and C-28, respectively, and a β configuration was estimated for the hydroxyl group at C-16 by comparing its chemical shift with the reported one.[3] Therefore, sapogenol **27** was determined to be saikogenin H,[4] which was derived from saikosaponin a by treatment with 1 N H_2SO_4 followed by acid hydrolysis. The sugar moiety was assignable to the glucosyl-(1→3)-fucosyl residue. Consequently, the structure of BFS-10 (**12**) was determined to be 3-*O*-β-D-glucopyranosyl-(1→3)-β-D-fucopyranosyl 3β,16β,23,28-tetrahydroxyolean-9(11),12(13)-diene; this is identical to that of saikosaponin g, which has been artificially derived from saikosaponin a by treatment with 1 N H_2SO_4.[4]

BFS-8 (**9**) was obtained as an amorphous powder having $[\alpha]_D$ −15.0° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{30}H_{48}O_4Na$ at *m/z* 495.3446 $[M+Na]^+$. The ^{1}H-NMR spectrum displayed signals due to six tertiary methyl groups at δ 0.93, 1.01, 1.05, 1.07, 1.08, and 1.68 (each 3H, s), and two olefinic protons at δ 5.77 (1H, d, J=10.7 Hz) and 6.74 (1H, dd, J=2.8, 10.7 Hz). The ^{13}C-NMR spectrum (Table 3) showed a total of 30 carbon signals, including those of a disubstituted double bond and a tetrasubstituted double bond, and four oxygen-bearing carbon signals at δ 73.2, 67.6, 67.7, and 64.7, ascribable to C-3, C-16, C-23, and C-28, respectively. The configuration at C-16 in **9** was revealed to be identical with that of saikogenin D, which has been artificially derived from saikosaponin d by treatment with 1 N H_2SO_4 followed by acid hydrolysis with 2 N H_2SO_4.[4]

BFS-14 (**11**) was obtained as an amorphous powder having $[\alpha]_D$ −37.5° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{48}H_{78}O_{17}Na$ at *m/z* 949.5133

Table 2. Oleanene Glycosides Obtained from the Aerial Parts of *Bupleurum falcatum*

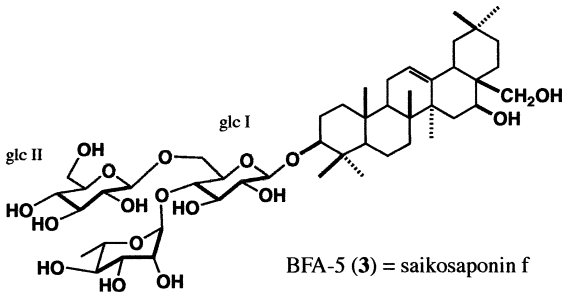
13,28-Epoxy type



BFA-8 (**1**) = saikosaponin a

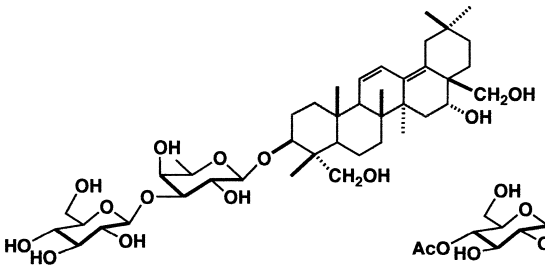
BFA-22 (**2**) = saikosaponin c

12-Ene type

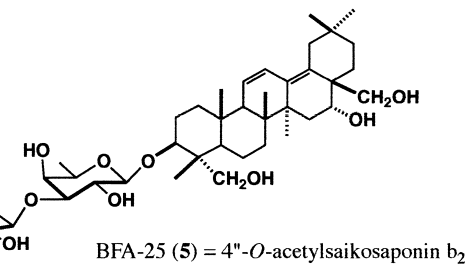


BFA-5 (**3**) = saikosaponin f

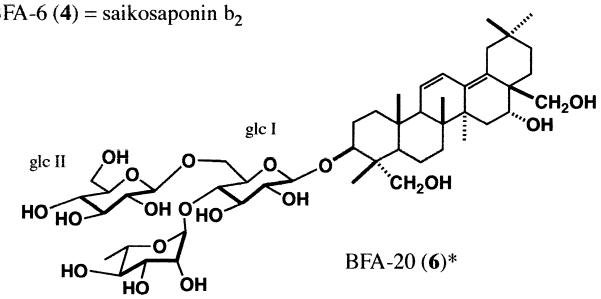
11,13(18)-Diene type



BFA-6 (**4**) = saikosaponin b_2

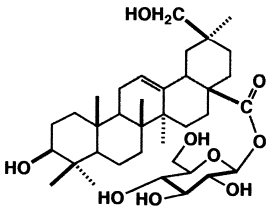


BFA-25 (**5**) = 4"-*O*-acetylsaikosaponin b_2



BFA-20 (**6**)*

28-Acid type



BFA-17 (**7**)*

$[M+Na]^+$. The ^{1}H-NMR spectrum displayed signals due to seven tertiary methyl groups at δ 0.83, 0.86, 0.90, 0.98, 0.99, 1.14, and 1.32 (each 3H, s); one methyl pentosyl methyl group (3H, d, J=6.1 Hz, H_3-6) at δ 1.67; three anomeric protons at δ 4.85 (1H, d, J=7.3 Hz), 4.98 (1H, d, J=7.9 Hz), and 5.87 (1H, br s); and two olefinic protons at δ 5.64 (1H, d, J=10.7 Hz) and 6.52 (1H, br d, J=10.7 Hz). The ^{13}C-NMR spectrum (Table 1) suggested the presence of a heteroannular diene and saikogenin C as a sapogenol, which is produced from saikosaponin c by treatment with intestinal bacteria. The sugar moiety was identical with glucosyl-(1→6)-[rhamnosyl-(1→4)]-glucosyl. HMBC analysis indicated that the sugar chain bonding connected with the C-3 of the sapogenol. Therefore, BFS-14 (**11**) was characterized as

saikosaponin h, naturally isolated as a genuine saponin for the first time.[4)]

The aerial parts (1.4 kg) of *B. rotundifolium* L. were extracted by refluxing with MeOH; the resulting extract (152 g) was then separated by using the solvent partition method. Various chromatographies on Diaion HP-20, silica gel, and Chromatorex ODS were applied to yield BRA-2 (19 mg), BRA-3 (19 mg), BRA-4 (35 mg), BRA-5 (60 mg), BRA-7 (14 mg), BRA-11 (36 mg), BRA-14 (69 mg), BRA-16 (12 mg), BRA-17 (14 mg), BRA-22 (22 mg), BRA-18 (38 mg), BRA-19 (6 mg), and BRA-20 (5 mg).

Table 3. ^{13}C-Data for Compounds **8**—**12**

	8	**9**	**10**	**11**	**12**
C-1	40.1	38.4	38.4	38.4	37.6
2	27.0	27.6	26.0	26.4	26.8
3	88.7	73.2	81.6	89.0	81.5
4	43.9	43.0	43.6	39.6	43.7
5	55.9	48.4	47.2	55.2	43.5
6	18.6	18.6	18.1	18.4	17.9
7	33.5	31.9	32.3	32.6	32.1
8	41.0	41.0	40.4	40.4	43.1
9	52.1	53.9	54.4	54.2	154.9
10	38.2	36.8	36.4	36.5	38.7
11	76.0	126.2	127.0	126.9	116.0
12	122.5	126.2	125.6	125.7	121.1
13	148.2	136.0	136.4	136.3	145.3
14	43.6	41.9	44.2	44.2	43.3
15	36.8	32.3	34.8	34.8	36.1
16	66.3	67.6	76.5	76.5	66.8
17	39.9	45.3	44.3	44.4	40.5
18	44.1	133.0	133.3	133.3	133.4
19	47.0	39.0	38.3	38.1	46.9
20	31.2	32.6	32.6	32.6	31.0
21	34.3	35.4	35.1	35.1	34.1
22	26.0	24.4	29.9	29.9	26.1
23	28.2	67.7	63.9	27.9	64.2
24	17.4	12.6	13.0	16.4	13.7
25	18.3	18.7	18.7	18.4	21.0
26	17.3	17.2	17.2	16.9	21.2
27	26.3	21.9	21.9	21.9	26.1
28	68.6	64.7	63.9	63.9	69.3
29	33.1	25.1	24.7	24.8	33.1
30	24.0	32.5	32.2	32.2	24.0
O$\underline{C}$H$_3$	54.1				
fuc-1	106.9		105.9		105.9
2	71.6		71.5		71.5
3	85.3		85.1		85.2
4	72.2		72.1		72.0
5	71.0		70.1		70.9
6	17.0		17.0		17.2
glcI-1	106.7		106.4	106.7	106.5
2	75.9		75.7	75.2	75.7
3	78.7		78.6	76.7	78.7
4	71.4		71.7	79.8	71.7
5	78.1		78.3	75.5	78.3
6	62.7		62.6	69.0	62.6
glcII-1				105.0	
2				74.8	
3				78.4	
4				71.5	
5				78.3	
6				62.6	
rha-1				102.9	
2				72.5	
3				72.6	
4				73.8	
5				70.5	
6				18.2	

BRA-3 (**13**), BRA-5 (**14**), BRA-2 (**15**), BRA-4 (**16**), and BRA-7 (**17**) were identified as rotundifolioside J,[11,12)] rotundifolioside I,[11,12)] rotundioside E,[15,16)] and rotundioside W,[11,12)] and rotundioside V,[11,12)] respectively.

BRA-22 (**18**) was obtained as an amorphous powder having $[\alpha]_D$ −22.5° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{48}H_{78}O_{17}Na$ at *m/z* 949.5143 $[M+Na]^+$. The ^{1}H-NMR spectrum displayed signals due to six tertiary methyl groups at δ 0.87, 0.91, 1.09, 1.25, 1.37, and 1.77 (each 3H, s); two methyl pentosyl methyl groups at δ 1.52 (3H, d, J=6.7 Hz, H$_3$-6) and 1.86 (3H, d, J=6.1 Hz, H$_3$-6); three anomeric protons at δ 4.79 (1H, d, J=7.3 Hz), 5.69 (1H, d, J=7.3 Hz), and 6.44 (1H, br s); and two olefinic protons at δ 5.70 (1H, d, J=7.9 Hz) and 6.77 (1H, br d, J=7.9 Hz). The ^{13}C-NMR spectrum (Table 5) showed a total of 48 carbon signals, of which 18 indicated the presence of a rhamnosyl-(1→2)-glucosyl-(1→2)-fucosyl moiety identical to that of BRA-3 (**13**). The remaining signals showed good agreement with those of rotundiogenin B in addition to those of C-20, C-29, and C-30. This means that in **18**, a hydroxymethyl is present at δ 73.6 at C-29 or C-30. HMBC analysis also supported this conclusion. By comparison with the 3β,16α,28,30-tetrahydroxyolean-11,13(18)-dine derivative[17)] isolated from *B. spinosum* and the 30-CH$_2$OH-isomer[18)] isolated from *B. rigidum*, **18** was determined to be a C-30-hydroxymethyl derivative. Therefore, its structure was characterized as 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranosyl 3β,16,28,30-tetrahydroxyolean-11,13(18)-diene.

BRA-11 (**19**) was obtained as an amorphous powder having $[\alpha]_D$ −41.8° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{48}H_{78}O_{17}Na$ at *m/z* 949.5139 $[M+Na]^+$. The ^{1}H-NMR spectrum displayed signals due to seven tertiary methyl groups at δ 0.87, 0.92, 1.09, 1.30, 1.34, 1.38, and 1.77 (each 3H, s); two methyl pentosyl methyl groups at δ 1.53 (3H, d, J=6.1 Hz, H$_3$-6) and 1.87 (3H, d, J=6.1 Hz, H$_3$-6); one oxygen-bearing methine proton at δ 3.33 (1H, br d, J=8.0 Hz); one oxygen-bearing methylene proton at δ 3.85 (1H, d, J=10.9 Hz) and 4.33 (overlapped); three anomeric protons at δ 4.80 (1H, d, J=6.7 Hz), 5.70 (1H, d, J=7.3 Hz), and 6.44 (1H, br s); and two olefinic protons at δ 5.73 (1H, d, J=10.4 Hz) and 6.77 (1H, br d, J=10.4 Hz). The ^{13}C-NMR spectrum (Table 5) showed a total of 48 carbon signals, of which 18 revealed the presence of a rhamnosyl-(1→2)-glucosyl-(1→2)-fucosyl moiety, as in BRA-22 (**18**). In the remaining signals due to the sapogenol

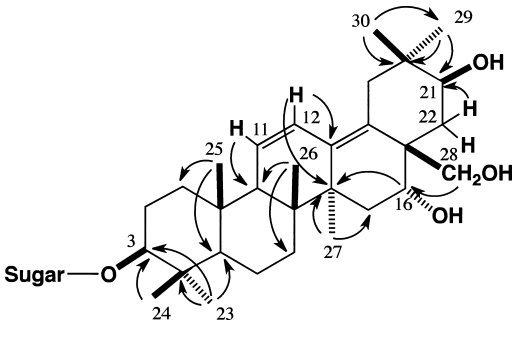
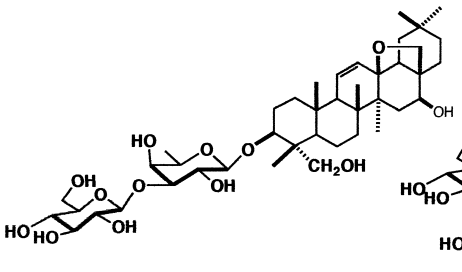


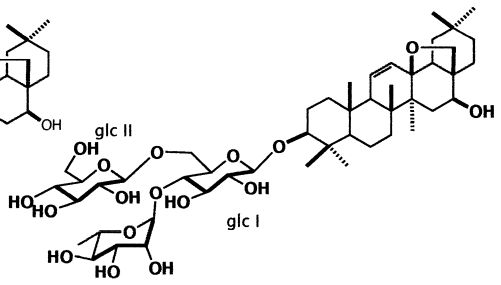
Fig. 1. HMBC of BRA-11 (**19**)

Table 4. Oleanene Glycosides Obtained from the Seeds of *Bupleurum falcatum*

13,28-Epoxy type

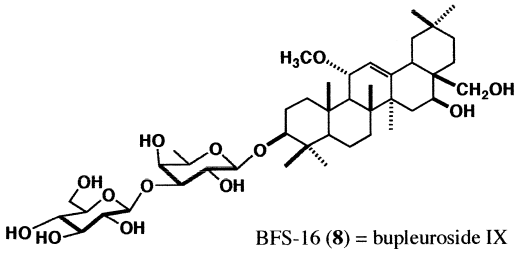


BFS-9 (**1**) = saikosaponin a



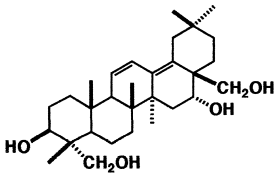
BFS-1 (**2**) = saikosaponin c

12-Ene type

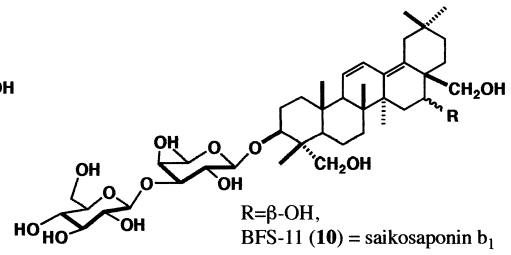


BFS-16 (**8**) = bupleuroside IX

9(11),12-Diene type

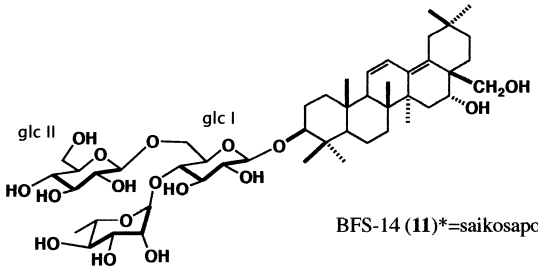


BFS-8 (**9**) *= saikogenin D



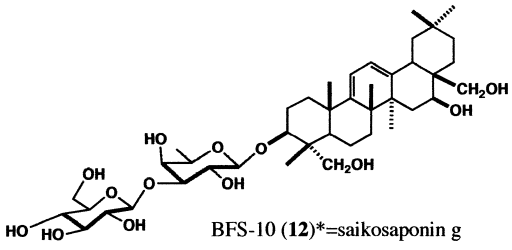
R=β-OH,
BFS-11 (**10**) = saikosaponin b_1

R=α-OH,
BFS=13 (**4**) = saikosaponin b_2



BFS-14 (**11**)*=saikosaponin h

28-Acid type



BFS-10 (**12**)*=saikosaponin g

moiety, three oxygen-bearing carbon signals were observed; the signals at δ 67.3 and 89.5 were assigned to carbons having an α-hydroxyl group at C-16 and a β-hydroxyl group at C-3, respectively. The remaining signal, at δ 73.7, was assigned to C-21 because HMBC analysis exhibited correlations from H_3-29 and H_3-30 to C-22, and from H_2-22 to C-22 (Fig. 1). Here, the configuration at the C-21 hydroxyl group was deduced to be β-equatorial because the signals due to H_3-29 and H_3-30 in BRA-11 (**19**) were shifted upward relative to those of rotundiogenin C, which has a C-21 hydroxyl group,[19] by the influence of the hydroxyl group. Therefore, the structure of BRA-11 (**19**) was characterized as 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranosyl 3β,16α,21β,28-tetrahydroxyolean-11,13(18)-diene, which indicates a new sapogenol.

BRA-16 (**20**) was obtained as an amorphous powder having $[\alpha]_D$ −1.5° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{47}H_{76}O_{17}Na$ at *m/z* 935.4973 $[M+Na]^+$. The ^{1}H-NMR spectrum showed signals due to seven tertiary methyl groups at δ 0.88, 0.94, 1.11, 1.30, 1.31, 1.34, and 1.76 (each 3H, s); one methyl pentosyl methyl groups at δ 1.59 (3H, d, J=6.1 Hz, H_3-6) and 1.87; one oxy-

Table 5. ^{13}C-Data for Compounds **13**—**19**

	13	**14**	**15**	**16**	**17**	**18**	**19**
C-1	38.7	38.7	38.4	38.5	38.4	38.4	38.4
2	26.6	26.6	26.6	26.6	26.0	26.6	26.6
3	89.5	88.8	89.5	88.8	84.6	89.6	89.5
4	39.9	39.8	40.0	39.8	43.7	39.9	39.9
5	55.6	55.5	55.6	55.5	48.4	55.6	55.5
6	18.0	18.0	18.6	18.6	18.5	18.7	18.6
7	32.0	32.0	32.6	32.6	32.4	32.6	32.5
8	42.0	42.0	42.0	41.1	41.9	42.0	41.1
9	52.7	52.6	53.8	53.8	54.0	53.9	53.8
10	36.4	36.3	36.6	36.6	36.6	36.6	36.6
11	134.2	134.1	126.3	126.2	126.3	126.4	126.5
12	129.1	129.1	126.2	126.3	126.2	126.1	126.4
13	85.1	85.1	136.0	135.1	136.1	136.4	136.3
14	44.1	44.1	41.1	41.9	41.1	41.1	31.7
15	35.6	35.5	31.9	31.9	31.9	32.1	67.3
16	77.1	76.9	67.7	67.7	67.7	68.0	47.7
17	46.4	46.4	45.3	45.3	45.3	45.9	47.8
18	61.5	61.5	133.1	133.1	133.1	133.0	132.2
19	38.5	38.3	39.0	39.1	39.1	34.0	39.3
20	41.7	41.7	32.6	32.6	32.6	38.3	38.0
21	33.7	33.7	35.5	35.5	35.5	30.3	73.7
22	35.4	35.4	24.5	24.5	24.5	24.3	33.1
23	28.1	17.9	28.1	28.0	65.6	28.2	28.1
24	16.2	16.3	16.3	16.4	12.9	16.3	16.3
25	17.4	18.3	18.3	18.4	18.9	18.3	18.3
26	19.0	19.4	17.4	17.3	17.3	17.4	17.2
27	16.4	16.4	21.9	21.9	21.9	22.0	21.9
28	77.7	77.7	64.8	64.8	64.8	65.1	65.7
29	18.9	18.9	25.1	25.1	25.1	21.1	29.2
30	19.8	19.8	32.6	32.6	32.6	73.6	18.4
fuc-1	105.3	105.3	105.3	105.2	103.7	105.3	105.3
2	77.2	80.6	77.4	80.6	81.2	77.6	77.3
3	76.3	75.5	76.2	75.5	75.6	76.3	76.2
4	72.8	72.6	72.9	72.6	72.6	72.9	72.9
5	70.9	71.2	70.8	71.2	71.2	70.9	70.8
6	19.4	17.3	17.2	17.2	17.3	17.2	17.4
glc-1	102.2	103.1	102.2	103.2	103.4	102.2	102.2
2	78.1	84.7	78.1	84.8	84.6	78.1	78.1
3	79.5	77.7	79.5	78.0	77.9	79.5	79.5
4	73.0	71.8	72.8	71.8	71.1	73.0	72.9
5	76.9	77.5	77.0	77.7	77.7	77.3	77.1
6	63.3	62.8	63.3	62.8	62.4	63.3	63.3
rha-1	101.9		101.9			101.9	101.9
2	72.5		72.5			72.5	72.8
3	72.8		72.8			72.8	72.5
4	74.4		74.3			74.4	74.3
5	69.5		69.5			69.5	69.5
6	18.2		19.0			19.0	19.0
xyl-1		106.5		106.6	106.5		
2		75.8		75.9	75.9		
3		78.0		77.5	77.6		
4		70.8		70.8	70.8		
5		67.4		67.4	67.5		

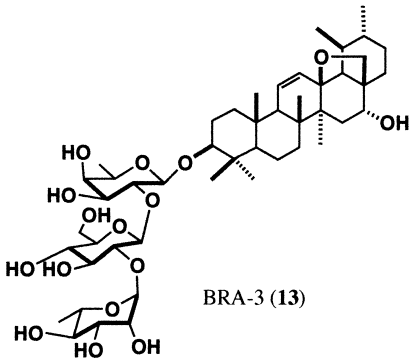
gen-bearing methine proton at δ 3.33 (1H, br d, J=8.0 Hz); one oxygenated methylene proton at δ 3.85 (1H, d, J=10.9 Hz), three anomeric protons at δ 4.83 (1H, d, J=7.9 Hz), 5.37 (1H, d, J=7.0 Hz), and 5.42 (1H, d, J=7.3 Hz); and two olefinic protons at δ 5.73 (1H, d, J=10.4 Hz) and 6.78 (1H, br d, J=10.4 Hz). The ^{13}C-NMR spectrum (Table 5) showed a total of 47 carbon signals, which indicated the presence of 3β,16α,21β,28-tetrahydroxyolean-11,13(18)-diene, as in BRA-11 (**19**), and a xylosyl-(1→2)-glucosyl-(1→2)-fucosyl moiety, as in BRA-5 (**14**),

Table 6. ^{13}C-Data for Compounds **20**—**25**

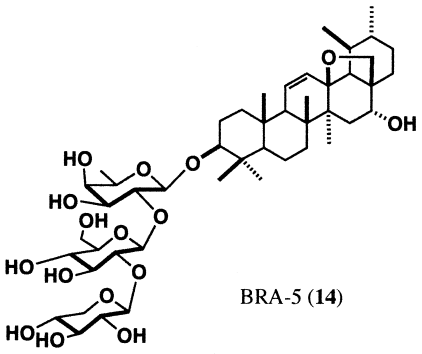
	20	**21**	**22**	**23**	**24**	**25**
C-1	38.4	38.5	38.7	38.9	38.8	38.8
2	26.6	26.6	26.4	26.4	26.4	26.4
3	88.8	89.5	89.8	89.9	89.7	89.7
4	39.8	39.9	39.6	40.0	39.6	39.6
5	55.5	55.6	56.0	56.2	56.0	56.0
6	18.6	18.6	18.5	18.4	18.5	18.5
7	32.6	32.4	32.3	32.3	32.1	31.6
8	41.1	42.3	40.0	40.1	40.0	40.1
9	53.8	54.0	48.0	48.2	47.1	47.1
10	36.6	36.7	36.9	37.0	37.0	36.9
11	126.4	126.0	23.8	23.8	23.8	23.8
12	126.4	128.0	122.5	122.6	122.6	122.1
13	136.3	137.0	144.6	144.5	144.4	145.0
14	41.9	40.9	42.1	42.1	42.0	41.9
15	31.7	32.3	29.3	29.4	35.9	36.0
16	67.3	68.4	23.0	22.9	74.1	73.9
17	47.8	46.0	47.0	47.0	49.1	49.1
18	132.2	131.0	41.7	41.9	41.3	41.3
19	39.4	39.3	40.2	46.2	47.2	47.3
20	38.0	32.6	30.7	30.7	30.8	30.8
21	73.7	217.1	34.0	34.0	36.1	36.6
22	33.2	44.3	33.2	33.5	33.4	33.5
23	28.0	28.2	28.6	28.6	28.3	28.5
24	16.4	16.3	16.8	16.7	16.8	16.8
25	18.4	18.4	15.4	15.5	15.6	15.6
26	17.2	17.4	17.4	17.5	17.5	17.5
27	21.9	22.2	26.2	26.2	27.2	27.2
28	65.8	69.5	176.5	176.5	175.5	175.9
29	29.3	26.1	23.7	23.7	24.6	24.6
30	18.4	24.6	33.1	33.2	33.2	33.2
fuc-1	105.0	105.3				
2	80.7	77.4				
3	75.5	76.3				
4	72.6	73.0				
5	71.2	70.9				
6	17.3	17.3				
glcI-1	103.2	102.2	105.2	105.2	105.0	105.2
2	84.8	78.1	78.5	78.5	78.3	78.4
3	78.0	79.5	79.5	78.8	78.9	78.8
4	71.8	72.8	70.1	70.1	72.5	72.6
5	77.7	77.0	77.9	77.9	77.8	77.8
6	62.8	63.3	62.8	62.8	62.6	62.8
glcII-1			93.4	93.5	95.8	93.8
2			78.4	78.4	74.2	78.2
3			79.4	79.2	79.4	79.3
4			78.8	79.5	78.8	79.9
5			79.2	79.4	79.3	79.3
6			61.9	61.9	62.1	61.8
glcIII-1			101.9	102.0	101.9	101.9
2			75.9	75.9	76.0	75.9
3			77.9	77.9	78.3	77.9
4			72.8	72.8	72.8	72.8
5			77.5	77.5	77.4	77.5
6			63.9	63.4	63.4	63.3
glcIV-1			104.6	105.2		105.1
2			75.9	76.7		75.8
3			79.5	79.6		79.4
4			72.0	72.1		71.5
5			78.2	78.6		78.2
6			62.8	62.8		62.7
glcV-1				102.4		
2				78.6		
3				79.2		
4				72.4		
5				77.1		
6				63.7		
xyl-1	106.6					
2	75.9					
3	77.4					
4	70.8					
5	67.4					
rha-1		101.9	101.8	101.8	101.9	101.8
2		72.6	72.4	71.7	71.8	71.9
3		72.8	72.6	72.7	72.3	72.8
4		74.3	74.4	74.4	74.4	74.3
5		69.6	69.5	69.5	69.5	69.5
6		19.1	19.1	19.1	18.9	19.0

Table 7. Oleanene Glycosides Obtained from the Aerial Parts of *Bupleurum rotundifolium*

13,28-Epoxy type

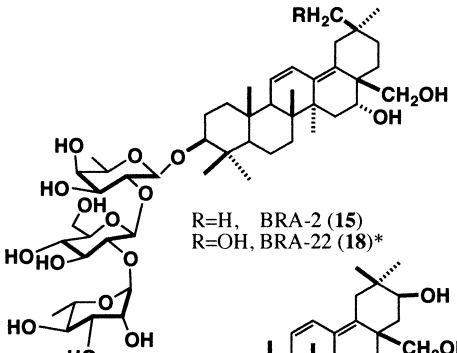


BRA-3 (**13**)

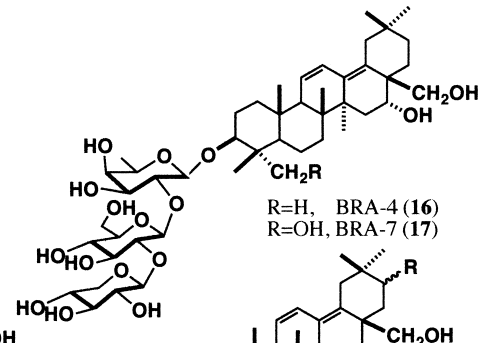


BRA-5 (**14**)

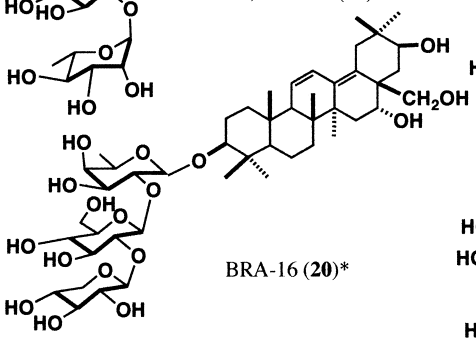
11,13(18)-Diene type



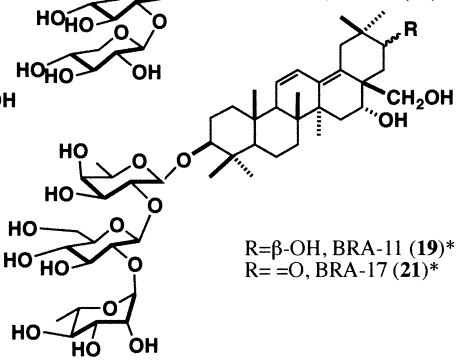
R=H, BRA-2 (**15**)
R=OH, BRA-22 (**18**)*



R=H, BRA-4 (**16**)
R=OH, BRA-7 (**17**)

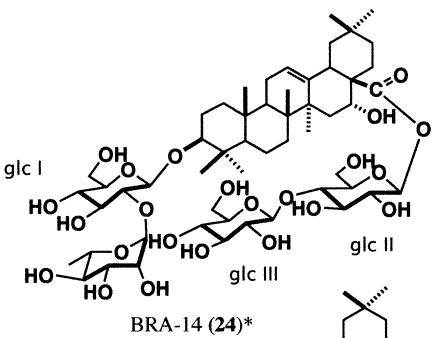


BRA-16 (**20**)*

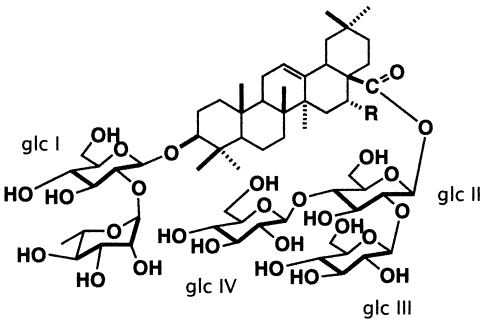


R=β-OH, BRA-11 (**19**)*
R= =O, BRA-17 (**21**)*

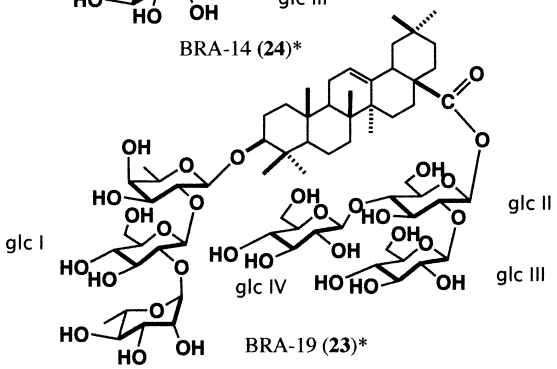
28-Acid type



BRA-14 (**24**)*



R=OH, BRA-18 (**25**)*
R=H, BRA-20 (**22**)*



BRA-19 (**23**)*

BRA-4 (**16**), and BRA-7 (**17**). Therefore, the structure of BRA-16 (**20**) was characterized as 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranosyl 3β,16α,21β,28-tetrahydroxyolean-11,13(18)-diene, which indicates a new sapogenol.

BRA-17 (**21**) was obtained as an amorphous powder having $[\alpha]_D$ +11.6° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{48}H_{76}O_{17}Na$ at *m/z* 947.4987 $[M+Na]^+$. The ^{1}H-NMR spectrum showed signals due to seven tertiary methyl groups at δ 0.83, 0.90, 1.10, 1.25, 1.32, 1.39, and 1.72 (each 3H, s); two methyl pentosyl methyl groups at δ 1.52 (3H, d, J=6.1 Hz, H_3-6) and 1.88 (3H, d, J=6.1 Hz, H_3-6); two methylene protons at δ 2.67 and 2.96 (2H, ABq, J=14.0 Hz) and 3.24 and 3.49 (2H, ABq, J=5.3 Hz); one oxygen-bearing methine proton at δ 3.34 (1H, m); one oxygen-bearing methylene proton at δ 3.88, 4.04 (2H, ABq, J=11.0 Hz); two olefinic protons at δ 5.77 (1H, d, J=11.0 Hz) and 6.71 (1H, br d, J=11.0 Hz); and three anomeric protons at δ 4.81 (1H, d, J=7.3 Hz), 5.72 (1H, d, J=7.9 Hz), and 6.44 (1H, br s).

The ^{13}C-NMR spectrum (Table 5) showed the presence of the same sugar moiety as in BRA-11 (**19**) and the occurrence of a carbonyl group at δ 217.1 in the sapogenol moiety. HMBC appeared from H_3-29, H_3-30, and H_2-22 to the carbon at δ 217.1; therefore, its carbonyl carbon should be located at C-21. Consequently, the structure of BRA-17 (**21**) was characterized as 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranosyl 3β,16α,28-trihydroxyolean-11,13(18)-diene-21-one.

BRA-20 (**22**) was obtained as an amorphous powder having $[\alpha]_D$ +34.0° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{60}H_{98}O_{27}Na$ at *m/z* 1273.6198 $[M+Na]^+$. The ^{1}H-NMR spectrum showed signals due to seven tertiary methyl groups at δ 0.83, 0.87, 0.89, 1.00, 1.24, 1.28, and 1.31 (each 3H, s); one methyl pentosyl methyl group at δ 1.79 (3H, d, J=6.1 Hz, H_3-6); one olefinic proton at δ 5.40 (1H, br s); and five anomeric protons at δ 5.01 (1H, d, J=6.1 Hz), 5.75 (1H, d, J=7.9 Hz), 5.85 (1H, d, J=7.3 Hz), 6.21 (1H, J=7.9 Hz), and 6.40 (1H, br s). HMBC was observed from the terminal rhamnosyl anomeric proton at δ 6.40 to the inner glucosyl C-2 at δ 78.5; from its inner glucosyl anomeric proton at δ 5.01 to the sapogenol C-3 at δ 89.8; from the terminal glucosyl anomeric proton at δ 5.85 to the inner glucosyl C-2 at δ 78.4; from another terminal glucosyl anomeric proton at δ 5.75 to the inner glucosyl C-4 at δ 78.8; and from its inner glucosyl anomeric proton at δ 6.21 to the sapogenol C-28 at δ 176.5. Consequently, the structure of BRA-20 (**22**) was characterized as 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl oleanolic acid 28-*O*-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside.

BRA-19 (**23**) was obtained as an amorphous powder having $[\alpha]_D$ +23.7° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{66}H_{108}O_{32}Na$ at *m/z* 1435.6725 $[M+Na]^+$, which corresponds to one mole of hexose more than BRA-20 (**22**). The ^{13}C-NMR spectrum showed the presence of oleanolic acid as a sapogenol, as in BRA-20 (**22**). HMBC similar to that of **22** revealed connectivity between the rhamnosyl-(1→2)-glucosyl-(1→2)-glucosyl-(1→C-3 of sapogenol) and glucosyl-(1→2)-[glucosyl(1→4)-glucosyl-(1→C-28 of sapogenol). Therefore, the structure of BRA-19 (**23**) was characterized as 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-β-D-glucopyranosyl-(1→2)-oleanolic acid 28-*O*-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside.

BRA-14 (**24**) was obtained as an amorphous powder having $[\alpha]_D$ +3.2° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{54}H_{88}O_{23}Na$ at *m/z* 1127.5669 $[M+Na]^+$. The ^{13}C-NMR spectrum showed a total of 54 carbon signals, of which 24 were assignable to the sugar moieties rhamnopyranosyl-(1→2)-glucosyl-(1→the C-3 of the sapogenol) and glucosyl-(1→4)-glucosyl-(1→the C-28 of sapogenol). The other 30 carbon signals include one hydroxy carbon at δ 74.1, which was identified as an α-hydroxy-bearing carbon at C-16 on oleanolic acid by HMBC. This sapogenol was identified as echinocystic acid.[20] Thus, the structure of BRA-14 (**24**) was characterized as 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl echinocystic acid β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

BRA-18 (**25**) was obtained as an amorphous powder hav-

Table 8. Hepatoprotective and Hepatotoxic Activity of 13,28-Epoxy Type Saponins

Substances	Dose (μM)	Protection (%)	Toxicity (%)
1 (Saikosaponin a)	10	13	96
	30	−8	317†
	90	−33†	492†
	200	−30†	455†
	500	−27†	390†
2 (Saikosaponin c)	10	13*	ND
	30	2	ND
	90	13*	ND
	200	20***	ND
	500	42***	ND
13	10	0	110
	30	−50†	605†
	90	−49†	705†
	200	−62†	647†
	500	−72†	638†
14	10	5	101
	30	0	120
	90	−67†	504†
	200	−77†	644†
	500	−93†	699†

Hepatoprotective effects of compounds **1**, **2**, **13** and **14** toward *in vitro* immunological liver injury on primary cultured rat hepatocytes. Significantly different from ref., effective $*p<0.05$, $***p<0.001$. Hepatotoxicity of compounds **1**, **2**, **13** and **14** in primary cultured rat hepatocytes. Significantly different from ref., toxic $\dagger p<0.001$.

Table 9. Hepatoprotective Activity of 12-Ene Type Saponins

Substances	Dose (μM)	Protection (%)	Toxicity (%)
3 (Saikosaponin f)	10	−11	111
	30	11	105
	90	7	114
	200	11	105
	500	45***	129
8 (Bupleuroside IX)	10	2	118
	30	8	136
	90	10	128†
	200	13	124†
	500	27	138†

Hepatoprotective effects of compounds **3** and **8** toward *in vitro* immunological liver injury on primary cultured rat hepatocytes. Significantly different from ref., effective $***p<0.001$. Significantly different from ref., toxic $\dagger p<0.001$.

Table 10. Hepatoprotective and Hepatotoxic Activity of 11,13(18)-Diene Type Saponins

Substances	Dose (μM)	Protection (%)	Toxicity (%)
4 (Saikosaponin b_2)	10	−74	94
	30	−59	88
	90	−50	98
	200	−28	94
	500	−108	154
10 (Saikosaponin b_1)	10	−10	117
	30	−7	110
	90	−23	144
	200	−3	144
	500	−269†	153
11 (Saikosaponin h)	10	−43	ND
	30	−18	ND
	90	−10	ND
	200	8	ND
	500	−10	ND
15 (Rotundioside E)	10	22	ND
	30	25	ND
	90	−4	ND
	200	−19	ND
	500	5	ND
16	10	−13	124
	30	−44	123
	90	−17	103
	200	40	118
	500	−21	135
17	10	−31	ND
	30	−37	ND
	90	−44	ND
	200	−16	ND
	500	−23	ND
18	10	−58	103
	30	−41	91
	90	−28	100
	200	−10	97
	500	41	93
19	10	0	ND
	30	14	ND
	90	9	ND
	200	21	ND
	500	32	ND
20	10	−43	ND
	30	−28	ND
	90	−21	ND
	200	−12	ND
	500	43	ND
21	10	−38	ND
	30	−48	ND
	90	−31	ND
	200	−3	ND
	500	27	ND

Hepatoprotective effects of compounds **4**, **10**, **11** and **15**—**21** toward *in vitro* immunological liver injury on primary cultured rat hepatocytes. Significantly different from ref. Hepatotoxicity of compounds **4**, **10**, **11** and **15**—**21** in primary cultured rat hepatocytes. Significantly different from ref., toxic † $p<0.001$.

ing $[\alpha]_D$ +2.7° (MeOH). Positive HR-FAB-MS revealed a molecular formula of $C_{60}H_{98}O_{28}Na$ at *m/z* 1289.6140 $[M+Na]^+$. The ^{13}C-NMR spectrum showed 60 carbon signals consisting of echinocystic acid and the same sugar moieties as in BRA-20 (**22**). Therefore, the structure of BRA-18 (**25**) was characterized as 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl echinocystic acid β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside.

We isolated a total of 25 oleanene saponins from the aerial parts of *B. falcatum* and *B. rotundifolium* and the seeds of *B. falcatum*. They are divided into four groups: the 13,28-epoxy type, the 12-ene type, the 9(11),12-diene type, and the 28-acid type, according to their structural characteristics.

Table 11. Hepatoprotective and Hepatotoxic Activity of 28-Acid Type Saponins

Substances	Dose (μM)	Protection (%)	Toxicity (%)
24	10	15	99
	30	33	98
	90	79***	83
	200	55***	89
	500	54**	114
25	10	−6	133
	30	6	125
	90	4	95
	200	0	112
	500	22	107

Hepatoprotective effects of compounds **24** and **25** toward *in vitro* immunological liver injury on primary cultured rat hepatocytes. Significantly different from ref., effective ** $p<0.01$, *** $p<0.001$. Hepatotoxicity of compounds **24** and **25** in primary cultured rat hepatocytes.

Next, some compounds obtained from them were investigated for their hepatoprotective activity and hepatotoxicity.

At the 13,28-epoxy type saponins, **2** (saikosaponin c) had weak hepatoprotectivity at 500 μM, but **1** (saikosaponin a) showed hapatotoxity from 30 μM. It was suggested that methyl group of the 23 position of **1** influenced activity.[21] But compound **3** of which an epoxy ring cleaved at C-13,28 of **1** has hepatoprotective activity at 500 μM. Ursane type (**13**, **14**) showed hepatotoxity from middle concentration. It was guessed that conformation of 29 and 30 position methyl group in ursane skelton damaged against cell membranes. On the other hands, 11,13(18)-diene type saponins did not express hepatoprotective activity and **4** and **10** showed the toxicity from middle concentration among them. The 28-acid type saponin (**24**, **25**) which has a glucosyl carboxy group showed hepatoprotective action. This result accorded with our previous paper.[22]

Experimental

General Procedure Optical rotations were measured with a JASCO P-1020 (l=0.5) automatic digital polarimeter. FAB-MS were obtained with a glycerol matrix in the positive ion mode using a JEOL JMS-DX300 and a JMS-DX 303 HF spectrometer. The ^{1}H- and ^{13}C-NMR spectra were measured in pyridine-d_5 with JOEL α-500 spectrometer, and chemical shifts are given on a δ (ppm) scale with tetramethylsilane (TMS) as the internal standard. Column chromatographies were carried out on a Diaion HP-20 (Mitsubishi Chemical Ind.), and silica gel 60 (230—400 mesh, Merck). TLC was performed on silica gel plates (Kieselgel 60 F_{254}, Merck) and PR C_{18} silica gel plates (Merck). The spots on TLC were visualized by UV light (254/366 nm) and sprayed with 10% H_2SO_4, followed by heating.

Extraction and Isolation of Respective Plants The aerial parts (2.6 kg) of *Bupleurum falcatum* L. cultivated at Kikka-cho, Kumamoto prefecture, was cutted and refluxed with MeOH for 10 h. Evaporation of MeOH gave the extractive (262.2 g), which was partitioned between *n*-hexane and 80% MeOH, and the methanolic layer was evaporated to give the residue. It was then chromatographed on Diaion HP-20 eluted with firstly water and gradiently mixed with MeOH to provide five fractions, fr. 1 (132.0 g), fr. 2 (48.7 g), fr.3 (25.6 g), fr. 4 (14.6 g), and fr. 5 (12.4 g). The fr. 4 eluted by 80% MeOH was subjected Sephadex LH-20 with MeOH to afford flavonoid and saponin faractions, from the latter of which was chromatographed on Chromatorex ODS with 60—100% MeOH to give BFA-5 (**3**, 108 mg), BFA-6 (**4**, 264 mg), BFA-8 (**1**, 45 mg), BFA-11 (21 mg), BFA-13 (110 mg), BFA-17 (**7**, 21 mg), BFA-20 (**6**, 20 mg), BFA-22 (**2**, 72 mg) . The fr. 5 eluate by 100% MeOH was also subjected to silica gel chromatography with $CHCl_3$–MeOH=15 : 1—$CHCl_3$–MeOH–water=9 : 1 : 0.1 to give BFA-25 (**5**, 17 mg).

The seeds (1.2 kg) of *Bupleurum falcatum* L. used for cultivating at Kikka-cho were smashed and extracted with MeOH for 1 h, and usual work-up gave the extractive (84.7 g), which was subjected to Diaion HP-20 with water–MeOH, gradiently, finally recovered with acetone) to provide seven fractions. The fr. 5 (23.2 g) eluted by 80% MeOH was chromatographed on silica gel with $CHCl_3$–MeOH–water=9 : 1 : 0.1—7 : 3 : 0.5 to afford fr. 5-1—fr. 5-8. The fr. 5-1 (3.0 g) was further purified by silicagel with $CHCl_3$–MeOH=50 : 1 and prepaartive TLC with $CHCl_3$–MeOH=15 : 1 to give BFS-8 (9, 8 mg). The fr. 5-4 (1.5 g) was chromatographed by HPLC to give BAS-9 (**1**, 13 mg), BAS-10 (**12**, 31 mg), and BAS-11 (**10**, 34 mg). From fr. 5-5 (14.6 g), fr. 5-6 (1.3 g), and fr. 5-8 (1.4 g), the respective BAS-13 (**4**, 28 mg), BAS-14 (**11**, 15 mg), and BAS-1 (**2**, 30 mg) were obtained by using HPLC with 80% MeOH. The fr. 6 (9.5 g) eluted by MeOH was chromatographed on silica gel with $CHCl_3$–MeOH–water=8 : 2 : 0.2 to give BAS-16 (**8**, 15 mg).

The aerial parts (1.4 kg) of *Bupleurum rotundifolium* L. cultivated at Oyano, Kumamoto prefecture, was cutted and refluxed with MeOH for 7 h. Evaporation of MeOH gave the extractive (152.1 g), which was defatted with *n*-hexane. The insoluble residue was passed through Diaion HP-20 eluted with water–MeOH, gradiently, to give five fractions. A part (2.3 g) of the fr. 4 (6.6 g) eluted by 80% MeOH was various chromatographed on silica gel with $CHCl_3$–MeOH–water=8 : 2 : 0.2, Chromatorex ODS with 50—100% MeOH, gradiently, and HPLC with 60% MeOH to give BRA-11 (**19**, 36 mg), BRA-14 (**24**, 69 mg), BRA-16 (**20**, 12 mg), BRA-17 (**21**, 11 mg), BRA-22 (**18**, 22 mg), BRA-18 (**25**, 38 mg), BRA-19 (**23**, 6 mg), and BRA-20 (**22**, 5 mg). From a part (6.5 g) of the fr. 5 (22.3 g) eluted by MeOH, BRA-2 (**15**, 19 mg), BRA-3 (**13**, 19 mg), BRA-4 (**16**, 35 mg), BRA-5 (**14**, 60 mg), BRA-7 (**17**, 14 mg) by using silica gel and ODS column chromatographies with $CHCl_3$–MeOH–water=8 : 2 : 0.2—7 : 3 : 0.5, and ODS with 60—100% MeOH, gradiently.

Identification of Component Monosaccharide of the Glycosides A glycoside (5 mg) was dissolved in 1 N HCl–MeOH (0.5 ml) and heated at 90 °C for 1 h. The acidic solution was neutralized with an ion exchanger resin (Amberlite IR-410) and concentrated *in vacuo*. The residue was trimethylsilyalted and checked by gas–liquid chromatography (GLC). Authentic sugar samples were treated in the same manner and t_R values were compared with those of the tetramethylsilyl derivatives of the metahnolysate of the glycoside.

The absolute configurations of the component monosaccharides were determined according to the method reported by Hara *et al.* Thus, a glycoside (5 mg) was hydrolyzed with 1 N HCl. After neutralization with Amberliet IR-410, the free sugars in the hydrolysate were converted into the thiazolidine derivatives and checked by GLC after trimethylsilylation. Authentic sugar samples were treated in the same manner and an unknown sugar was identified by comparison of its t_R value with those of the authentic sugar deriavtives.

Animals Male Wistar rats (6 weeks old, body weight 150—160 g) and a male New Zealand white rabbit (body weight 3 kg) were used.

Preparation of Primary Cultured Rat Hepatocytes Liver cells were isolated to a procedure developed by Berry and Friend.[23]

Preparation of Antiserum against Rat Hepatocytes The antiserum was prepared according to the method of Shiki *et al.*[24] An antibody to rat hepatocytes was raised in rabbits, first by injection of 1×10^8 cells, followed by four injections of 5×10^7 cells over a period of 4 weeks. The antiserum to the rat hepatocytes was prepared by the method of Harboe and Ingild.[25]

Determination of Hepatoprotective Activity of Compounds toward *in Vitro* Immunological Liver Injury One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to the above medium (300 μl) containing the antiserum against rat plasma membranes (80 μl/ml) and a dimethyl sulfoxide (DMSO) solution (4 μl) of the test samples [final concentration 0 (reference; Ref.), 10, 30, 90, 200, 500 μM]. Forty min after the antiserum was administered, the medium was withdrawn for determination of alanine aminotransferase (ALT). Control is the value of hepatocytes which were not administered the antiserum. The control was hepatocytes not treated with antiserum. The control value **1**, **2**, **13** and **14** was 6.48±1.4 (IU/l). The value of **1** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 27.50±4.1, 24.75±2.9, 29.25±3.0, 34.50±2.2, 33.75±1.0, 33.25±1.7 (IU/l), respectively. The value of **2** at 0 (ref.), 10, 30, 90, 200, 500 μM were 27.50±0.6, 24.75±1.7, 27.00±0.8, 24.75±1.0, 23.25±1.0, 18.75±0.5 (IU/l), respectively. The value of **13** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 26.25±4.9, 26.50±, 36.50±10.2, 36.25±3.1, 39.00±5.1, 41.00±1.5 (IU/l), respectively. The value of **14** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 26.00±4.9, 25.00±4.5, 26.00±1.0, 39.00±3.1, 41.00±5.1, 44.25±1.5 (IU/l), respectively. The control value **3** and **8** was 17.54±1.4 (IU/l). The value of **3** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 31.50±0.6, 33.00±0.5, 30.00±1.4, 30.50±1.0, 30.00±, 25.25±0.5 (IU/l), respectively. The value of **8** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 33.25±2.1, 33.00±2.1, 32.00±0.8, 31.75±1.5, 31.25±2.0, 29.00±1.2 (IU/l), respectively. The control value **4**, **10**, **11**, **15**, **16**, **17**, **18**, **19**, **20** and **21** was 9.17±1.4 (IU/l). The value of **4** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 17.25±1.4, 23.25±2.9, 22.00±1.7, 21.25±1.8, 19.50±3.3, 26.00±1.0 (IU/l), respectively. The value of **10** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 26.25±2.7, 28.00±15, 27.50±1.7, 30.25±3.3, 26.75±2.1, 72.25±3.0 (IU/l), respectively. The value of **11** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 19.00±2.4, 23.25±1.3, 20.75±2.6, 20.00±3.8, 18.25±1.5, 20.00±2.8 (IU/l), respectively. The value of **15** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 33.25±3.0, 28.00±6.1, 27.25±1.9, 34.25±5.3, 37.75±4.1, 32.00±1.8 (IU/l), respectively. The value of **16** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 21.00±2.2, 22.50±1.0, 26.25±4.2, 23.00±1.4, 16.25±0.5, 23.50±1.3 (IU/l), respectively. The value of **17** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 18.75±2.2, 21.75±1.7, 22.25±3.2, 23.00±1.8, 20.25±0.9, 21.00±1.8 (IU/l), respectively. The value of **18** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 19.00±1.8, 24.75±4.3, 23.00±3.4, 21.75±3.3, 20.00±1.2, 15.00±1.8 (IU/l), respectively. The value of **19** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 23.2±1.0, 23.25±0.5, 21.25±2.1, 22.00±1.2, 20.25±1.3, 18.75±3.1 (IU/l), respectively. The value of **20** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 19.75±3.0, 24.25±2.5, 22.75±2.2, 22.00±1.4, 21.00±3.0, 15.25±3.0 (IU/l), respectively. The value of **21** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 16.50±3.0, 19.25±1.7, 20.00±2.7, 18.75±1.3, 16.75±1.7, 14.50±1.7 (IU/l), respectively. The control value **24** and **25** was 17.54±1.4 (IU/l). The value of **24** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 38.75±2.5, 35.50±1.3, 31.75±1.7, 22.00±3.2, 27.00±2.2, 27.25±5.0 (IU/l), respectively. The value of **25** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 30.25±1.5, 29.50±0.6, 29.50±1.3, 29.75±1.3, 30.25±2.2, 27.50±1.3 (IU/l), respectively. Reference (ref.) value treated with the antiserum and not treated with the tested sample. The percent of protection is calculated as {1−(substance−control)/(ref.−control)}×100. The percent of protection of glycyrrhizin (positive control) was 32% at 500 μM.

Determination of Hepatotoxicity of Saponins toward Hepatocytes (without Anitiserum) In the same way as above, the cultured cells were exposed to the above-prepared medium (300 μl) containing the DMSO solution (4 μl) of the test samples [final concentration 0 (Ref.), 10, 30, 90, 200, 500 μM]. Forty minute after the test samples were administered, the medium was withdrawn for determination of ALT. The value of **1** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 24.80±2.1, 23.80±5.5, 78.50±5.0, 122.00±3.7, 112.80±1.9, 96.75±3.9 (IU/l), respectively. The value of **13** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 19.25±5.7, 21.25±4.8, 116.5±4.4, 135.80±13.7, 124.80±4.8, 122.80±5.3 (IU/l), respectively. The value of **14** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 20.50±3.7, 20.75±2.2, 24.50±0.6, 103.30±13.3, 132.00±7.0, 143.30±12.0 (IU/l), respectively. The value of **3** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 14.00±2.0, 15.50±0.6, 14.75±2.4, 16.00±0.9, 14.75±1.5, 18.00±1.6 (IU/l), respectively. The value of **8** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 12.50±2.1, 14.75±1.0, 17.00±2.8, 13.00±1.7, 15.50±1.3, 17.25±2.1 (IU/l), respectively. The value of **4** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 17.25±1.4, 23.25±2.9, 22.00±1.7, 21.25±1.8, 19.50±3.3, 26.00±1.0 (IU/l), respectively. The value of **10** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 26.25±2.7, 28.00±1.5, 27.50±1.7, 30.25±3.3, 26.75±3.0, 27.25±1.7 (IU/l), respectively. The value of **16** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 21.00±2.2, 22.50±1.0, 26.25±4.2, 23.00±1.4, 16.25±0.5, 23.5±1.7 (IU/l), respectively. The value of **18** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 19.00±1.8, 24.75±4.3, 23.00±3.4, 21.75±3.3, 20.00±1.2, 15.00±0.8 (IU/l), respectively. The percent of cytotoxicity is calculated as (sample/reference)×100. Reference is the value of hepatocytes which were not treated with the tested samples. The value of **24** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 20.75±2.5, 20.50±1.3, 20.25±1.7, 17.25±3.2, 18.50±2.2, 23.75±5.0 (IU/l), respectively. The value of **25** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 19.00±1.5, 25.25±0.6, 23.75±1.3, 18.00±1.3, 21.25±2.2, 20.25±1.3 (IU/l), respectively. The percent of cytotoxicity is calculated as (sample/reference)×100. Reference is the value of hepatocytes which were not treated with the tested samples. The percent of cytotoxicity is calculated as (sample/reference)×100. Reference is the value of hepatocytes which were not treated with the tested samples.

Instrument and Assay Method The activities of ALT were assayed by autoanalyzer COBAS MIRA (Roche) using commercial kits based on the ALT assay method.[26]

Statistical Analysis The data are shown as the mean±S.D. (n=4). After analysis of variances, Sheffe's test was employed to determine the significance of differences between reference and experimental samples.

References

1) Shibata S., Kitagawa I., Fujimoto H., *Chem. Pharm. Bull.*, **14**, 1023—1033 (1966).
2) Shimaoka A., Seo S., Minato H., *J. C. S.*, *Perkin 1*, **1975**, 2043—2048 (1975).
3) Tori K., Yoshimura Y., Seo S., Sakurai K., Ishii H., *Tetrahedron Lett.*, **46**, 4163—4166 (1976).
4) Shimizu K., Amagaya S., Ogihara Y., *Chem. Pharm. Bull.*, **33**, 3349—3355 (1985).
5) Nose M., Amagaya S., Takeda T., Ogihara Y., *Chem. Pharm. Bull.*, **37**, 1293—1296 (1989).
6) Nose M., Amagaya S., Ogihara Y., *Chem. Pharm. Bull.*, **37**, 2736—2740 (1989).
7) Ebita N., Nakajima K., Taguchi H., Mitsuhashi H., *Chem. Pharm. Bull.*, **38**, 1432—1434 (1990).
8) Yoshikawa M., Murakami T., Hirano K., Inadzuki M., Ninomiya K., Matsuda H., *Tetrahedron Lett.*, **38**, 7395—7398 (1989).
9) Matsuda H., Murakami T., Ninomiya K., Inadzuki M., Yoshikawa M., *Bioorg. Med. Chem. Lett.*, **7**, 2193—2198 (1997).
10) Ono M., Yoshida A., Ito Y., Nohara T., *Phytochemistry*, **51**, 819—823 (1999).
11) Fujioka T., Yoshida K., Fujii H., Nagao T., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **51**, 365—372 (2003).
12) Fujioka T., Yoshida K., Shibao H., Nagao T., Yoshida M., Matsunaga K., Takata J., Karube Y., Iwase Y., Okabe H., Mihashi K., Mihashi K., *Chem. Pharm. Bull.*, **54**, 1694—1704 (2006).
13) Ishi H., Nakamura M., *Chem. Pharm. Bull.*, **28**, 2367—2383 (1980).
14) Kircher H. W., *Phytochemistry*, **16**, 1078—1080 (1977).
15) Kobayashi Y., Takeda T., Ogihara Y., *Chem. Pharm. Bull.*, **29**, 2222—2229 (1981).
16) Navarro P., Ginder R. M., *Life Sci.*, **68**, 1199—1206 (2001).
17) Barrero A. F., Haïdour A., Sedqui A., Mansour A. I., Rodríguez-Garcia I., Löpez A., Muñoz-Dorado M., *Phytochemistry*, **54**, 741—745 (2000).
18) Sachez-Contreras S., Diaz-Lanza A. M., *Phytochemistry*, **54**, 783—789 (2000).
19) Kobaayshi Y., Ogihara Y., *Chem. Pharm. Bull.*, **29**, 2230—2236 (1981).
20) Akai E., Takeda T., Kobayashi Y., Chen Y., Ogihara Y., *Chem. Pharm. Bull.*, **33**, 4685—4690 (1985).
21) Kinjo J., Udayama M., Okawa M., Nohara T., *Biol. Pharm. Bull.*, **22**, 203—206 (1999).
22) Kinjo J., Okawa M., Udayama M., Sohno Y., Hirakawa T., Shii Y., Nohara T., *Chem. Pharm. Bull.*, **47**, 290—292 (1999).
23) Berry M. N., Friend D. S., *J. Cell Biol.*, **43**, 506—520 (1969).
24) Shiki Y., Shirai K., Saito Y., Yoshida S., Wakashin M., Kumagai A., *Wakan-Yaku Gakkaishi*, **1**, 11—14 (1984).
25) Harboe N., Ingild A., *Scand. J. Immunol.*, **2** (Suppl. 1), 161—164 (1973).
26) Heerspink W., Hafkenscheid J. C., Siepel H., van der Ven-Jongekrÿg J., Dijt C. C., *Enzyme*, **25**, 333—341 (1980).