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Sulfated Triterpenoid Saponins from the Leaves of *Bupleurum rotundifolium* L.

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Three sulfated tetraglycosides of oleane-type triterpenoids, rotundiosides A (**1**), B (**2**) and C (**3**), isolated from the leaves of *Bupleurum rotundifolium* L., were characterized as the 3β-sulfate ester of 16α-hydroxyolean-12-ene-28-oyl β-D-glucopyranosyl (1→6)-β-D-glucopyranosyl (1→2)-β-D-glucopyranosyl (1→6)-β-D-glucopyranoside, the 3β-sulfate ester of olean-12-ene-28-oyl β-D-glucopyranosyl (1→6)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl (1→6)-β-D-glucopyranoside and the 3β-sulfate ester of olean-12-ene-28-oyl β-D-glucopyranosyl (1→6)-β-D-glucopyranosyl (1→2)-β-D-glucopyranosyl (1→2)-β-D-glucopyranoside, respectively, on the basis of chemical and spectroscopic evidence.

Keywords——*Bupleurum rotundifolium*; Umbelliferae; rotundioside A; rotundioside B; rotundioside C; sulfated saponin; oleanolic acid; echinocystic acid

Previously, we reported the isolation and structural elucidation of two new saponins, rotundiosides E and F,[1] obtained from the leaves of *Bupleurum rotundifolium* L. (Umbelliferae). Further investigation on the leaves of the title plant led to the isolation of three new sulfated saponins. This paper deals with the structural elucidation of these new saponins, rotundiosides A (**1**), B (**2**) and C (**3**).

The leaves of *Bupleurum rotundifolium* were extracted with MeOH and the extract obtained was fractionated into two parts, one containing rotundiosides A, B, C (Fr. 1) and the other containing rotundiosides D, E, F, G (Fr. 2), as shown in Chart 1.

Fraction 1 was further separated by droplet counter-current chromatography (DCCC) followed by silica gel and reversed-phase chromatography to give pure rotundiosides A and B. The amounts of the two compounds obtained were insufficient to allow elucidation of the structures, so the mixture of rotundiosides A, B and C (Fr. 1) was also used for the structural establishment of rotundiosides A, B and C.

The acid hydrolysis of Fr. 1 followed by extraction with ether gave an ether-soluble portion and a water-soluble portion. From the ether-soluble portion, echinocystic acid (**4**) and oleanolic acid (**5**) were isolated and identified by comparison with authentic samples. The water-soluble portion showed the presence of glucose and sulfate group.[2] Acid hydrolyses of pure rotundiosides A and B gave **4** and **5**, respectively, suggesting that rotundiosides A and B are sulfated glycosides with echinocystic acid (**4**) and oleanolic acid (**5**) as the aglycones, respectively.

Alkaline hydrolysis of the mixture of rotundiosides A, B and C gave the aglycones **6** and **7**, showing that the sugar moieties were linked to the 28-carboxyl group in ester form.

The sulfate group was shown to be located at C-3, based on proton (^{1}H-) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) analyses. In the ^{1}H-NMR spectra of **6** and **7**, the H-3 signals appeared as dd peaks at 4.41 and 4.39 ppm, respectively, which could be ascribed to a methine proton attached to carbon bearing a sulfate group.[3] A comparison of the ^{13}C-NMR

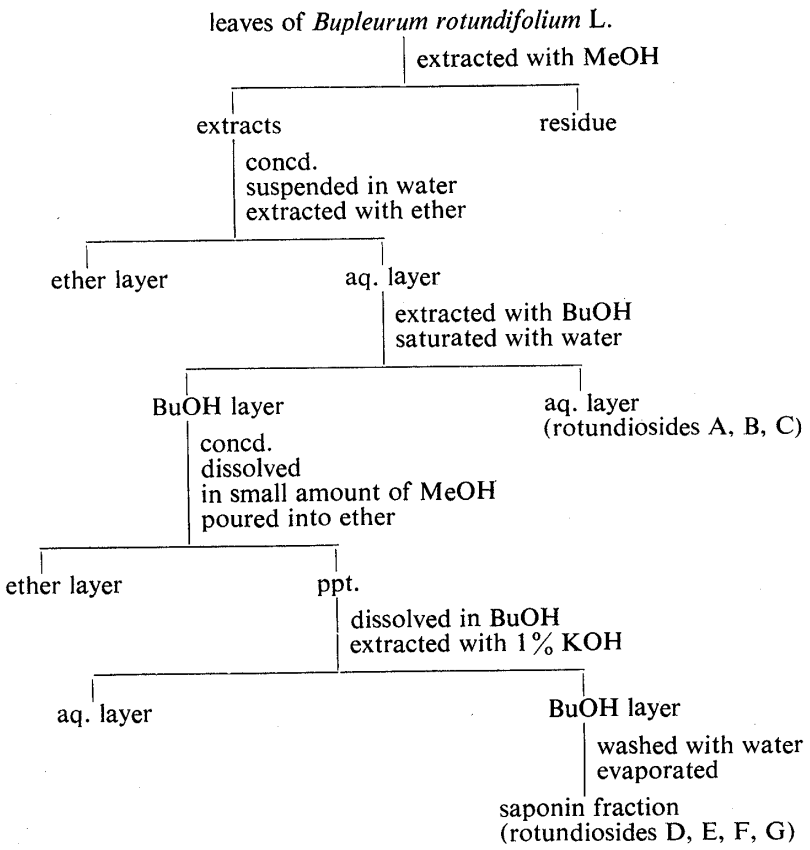

Chart 1

spectra of **6** and **7** with those of **4** and **5**, respectively, showed that the C-3 signal of **6** was shifted 7.6 ppm downfield and the C-3 signal of **7** was shifted 7.1 ppm downfield, supporting the presence of a sulfate group at C-3 (Table I).

This conclusion was confirmed by the syntheses of echinocystic acid 3-*O*-sulfate and oleanolic acid 3-*O*-sulfate by the following procedure: **4** and **5** were each dissolved in pyridine and then reacted with pyridine sulfur trioxide.[4] The reaction mixture was chromatographed on silica gel to give synthetic echinocystic acid 3-*O*-sulfate and oleanolic acid 3-*O*-sulfate, identical with **6** and **7** (Table I), respectively.

Partial hydrolysis of the mixture of rotundiosides A, B, C with snail enzyme followed by DCCC and silica gel chromatography gave three prosapogenins, RAS-1 (**8**), RAS-2 (**9**) and RBS-1 (**10**). Each of them (**8**—**10**) was shown to have a sulfate group at C-3 based on the comparison of the ^{13}C-NMR spectra of **8**—**10** with those of **6** and **7**. In particular, the chemical shifts of C-3 of **8** (85.4 ppm), **9** (85.0 ppm) and **10** (85.3 ppm) were in good agreement with those of **6** (85.6 ppm) and **7** (85.2 ppm), suggesting the presence of the sulfate group at C-3 in **8**—**10** (Tables I and II).

The 28-carboxyl carbon signals of **8**—**10** were shifted upfield by *ca.* 4 ppm, compared with those of **6** and **7**, and the signals of the anomeric carbons of glucoses in **8**—**10** were shifted upfield to *ca.* 96 ppm. These observations suggest the glucose to be linked in ester form to C-28.[5] Thus, the structures of **8** and **10** were deduced to be the 3β-sulfate ester of 16α-hydroxyolean-12-ene-28-oyl β-D-glucopyranoside and the 3β-sulfate ester of olean-12-ene-28-oyl β-D-glucopyranoside, respectively.

RAS-2 (**9**) was concluded to be a diglycoside of echinocystic acid 3-*O*-sulfate, based on analyses of the ^{13}C-NMR spectra (Table II). A downfield shift of the C-6 signal of glucose to 69.3 ppm was observed in the ^{13}C-NMR spectrum of **9**, indicating that the glucose′ (the

TABLE I. ^{13}C-NMR Spectral Data

	4	6	Echinocystic acid 3-*O*-sulfate	5	7	Oleanolic acid 3-*O*-sulfate
C- 1	38.9	38.6	38.7	39.0	38.9	38.8
C- 2	28.0	24.8	24.9	28.0	24.8	25.0
C- 3	78.0	85.6	85.7	78.1	85.2	84.9
C- 4	39.3	38.6	38.7	39.3	38.6	38.7
C- 5	55.8	56.0	56.1	55.8	56.2	56.2
C- 6	18.8	18.6	18.7	18.8	18.7	18.7
C- 7	33.3	33.3	33.3	33.3	33.3	33.2
C- 8	39.8	39.7	39.8	39.7	39.7	39.7
C- 9	47.2	47.0	47.1	48.1	47.9	47.9
C-10	37.3	37.0	37.1	37.4	37.1	37.1
C-11	23.7	23.7	23.8	23.8	23.8	23.8
C-12	122.4	122.5	122.5	122.5	122.6	122.4
C-13	144.9	145.0	145.0	144.7	144.8	144.7
C-14	42.0	42.0	42.1	42.2	42.1	42.1
C-15	36.0	36.0	36.1	28.3	28.3	28.3
C-16	74.6	74.6	74.7	23.8	23.8	23.8
C-17	48.8	48.8	48.9	46.6	46.7	46.6
C-18	41.3	41.4	41.4	42.0	42.0	41.9
C-19	47.2	47.0	47.1	46.5	46.6	46.4
C-20	30.9	30.9	31.0	30.9	30.9	30.9
C-21	36.0	36.0	36.1	34.2	34.2	34.2
C-22	32.7	32.4	32.6	33.3	33.3	33.2
C-23	28.7	28.5	28.6	28.8	28.7	28.6
C-24	16.5	17.0	17.0	16.5	17.1	17.1
C-25	15.6	15.5	15.5	15.5	15.4	15.4
C-26	17.4	17.3	17.4	17.4	17.4	17.3
C-27	27.1	27.2	27.3	26.2	26.2	26.2
C-28	179.8	180.2	180.1	180.0	180.7	180.0
C-29	33.5	33.3	33.3	33.3	33.3	33.1
C-30	24.7	24.8	24.9	23.8	23.8	23.8

δ in ppm from TMS; solvent, pyridine-d_5; measured at room temperature.

second glucose) was linked to C-6 of glucose (the first glucose counted from the aglycone). Therefore **9** was characterized as the 3β-sulfate ester of 16α-hydroxyolean-12-ene-28-oyl β-D-glucopyranosyl (1→6)-β-D-glucopyranoside.

It was very difficult to identify the prosapogenins with sulfate at C-3, because of their high polarity. In order to elucidate the whole structures of these saponins, the mixture of rotundiosides A, B and C was subjected to solvolysis to remove the sulfate group from the parent saponins,[6] giving a mixture of RA-4 (**11**), RB-4 (**12**) and RC-4 (**13**). Partial hydrolysis of the mixture of **11**—**13** with snail enzyme gave nine prosapogenins, RB-1 (**14**), RA-1 (**15**), RB-2 (**16**), RA-2 (**17**), RC-3 (**18**), RA-3 (**20**), RC-4 (**13**), RB-4 (**12**) and RA-4 (**11**). Further hydrolysis of RB-4 (**12**) furnished RB-3 (**19**). The assignments of ^{13}C-NMR data for these prosapogenins (especially the signals of the sugar moieties) are listed in Table II.

In the ^{13}C-NMR spectra of **15**, **17**, **20** and **11**, the carbon signals of the aglycones were coincident with those of echinocystic acid (**4**), therefore **15**, **17**, **20** and **11** were suggested to be the prosapogenins of rotundioside A (**1**). RA-1 (**15**) was shown to be echinocystic acid linked with a glucose through the 28-carboxyl group on the basis of the observation of an upfield shift of *ca.* 3.9 ppm (from 179.8 to 175.9 ppm) for the C-28 signal.

The application of the glycosidation shift rule[7] to the ^{13}C-NMR data of these

TABLE II. ^{13}C-NMR Spectral Data for Prosapogenins

		4[a]	**15**[b]	**17**[b]	**20**[c]	**11**[b]	**5**[a]	**14**[b]	**16**[b]	**19**[b]	**12**[b]	**18**[b]	**13**[b]	**8**[a]	**9**[a]	**10**[a]
Genin	C-3	78.0	78.7	78.4	78.6	78.5	78.1	78.4	78.4	78.4	78.3	78.3	78.2	85.4	85.0	85.3
	C-16	74.6	74.4	74.3	74.3	74.3								74.0	74.1	
	C-28	179.8	175.9	176.0	176.0	176.0	180.0	176.4	176.5	176.4	176.4	176.4	176.5	175.9	176.0	176.4
Glucose	1		95.8	95.6	95.7	95.8		95.8	95.8	95.6	95.6	93.8	93.6	95.6	95.9	95.8
	2		74.1	74.0	74.1	73.9		74.2	74.0	74.0	74.1	79.5	78.9	73.8	73.7	74.1
	3		78.7	78.1	77.9	78.1		78.9	78.2	78.1	78.2	77.3	76.5	78.8	78.3	78.7
	4		71.6	71.4	71.3	71.2		71.7	71.5	71.0	71.1	70.6	70.4	71.0	71.0	71.6
	5		78.4	77.9	77.9	77.1		78.3	77.9	78.1	77.1	77.8	77.9	78.3	77.8	78.4
	6		62.6	69.8	69.7	69.8		62.8	69.8	69.7	69.6	62.4	62.2	62.1	69.3	62.6
Glucose′	1			105.1	102.6	102.9			105.1	102.8	102.7	102.7	102.0		105.1	
	2			75.1	83.6	83.9			75.2	84.0	83.6	85.5	84.6		75.0	
	3			78.7	77.0	76.8			78.7	77.1	76.9	77.5	77.3		78.7	
	4			71.9	71.4	71.3			72.0	71.3	71.1	71.8	71.3		71.5	
	5			78.4	78.1	78.1			78.4	78.1	78.2	78.5	78.2		78.3	
	6			63.0	62.6	62.5			63.1	62.5	62.6	62.9	63.0		62.6	
Glucose″	1				105.5	105.0				105.9	105.4	106.2	104.8			
	2				75.9	75.1				76.2	75.2	76.6	74.8			
	3				78.5	78.1				78.6	78.2	79.0	78.2			
	4				71.4	71.3				71.3	71.4	72.7	71.9			
	5				78.5	78.1				78.6	78.0	79.0	77.6			
	6				62.7	69.8				62.5	69.9	63.6	69.5			
Glucose‴	1					105.7					105.7		104.8			
	2					76.0					76.1		76.1			
	3					78.5					78.6		78.2			
	4					71.3					72.0		72.6			
	5					78.5					78.3		78.2			
	6					62.5					63.1		63.6			

Chemical shifts were measured in pyridine-d_5 solution. *a*) At room temperature. *b*) At 70 °C. *c*) At 88 °C.

prosapogenins led to the determination of the linkage positions between sugars as follows: a comparison of the ^{13}C-NMR data of RA-2 (**17**) with those of RA-1 (**15**) showed that **17** was a glucoside of **15**, and the glucose′ (the second glucose counted from the aglycone) was shown to be linked to C-6 of glucose (the first glucose) based on the observation of a 7.2 ppm downfield shift of the C-6 signal of glucose on going from **15** to **17**. Similarly, RA-3 (**20**) was shown to be a glucoside of RA-2 and the glucose″ (the 3rd glucose) was suggested to be linked to C-2 of the glucose′ based on the 8.5 ppm downfield shift for the C-2 signal of the glucose′ on going from **17** (75.1 ppm) to **20** (83.6 ppm). Finally, RA-4 (**11**) was shown to be a tetraglycoside and was considered to be a glucoside of RA-3 (**20**). The glucose‴ (the terminal glucose) was determined to be linked to C-6 of the glucose″ based on the observation of a 7.1 ppm downfield shift for the C-6 signal of the glucose″ on going from **20** (62.7 ppm) to **11** (69.8 ppm).

The sugar linkage positions deduced by the analyses of the ^{13}C-NMR data as described above were well supported by the result of methylation analysis of rotundioside A, as follows. Permethylation of rotundioside A followed by silica gel column chromatography gave a permethylate of rotundioside A, which was methanolyzed, and the resulting methyl glucosides were analyzed by gas liquid chromatography (GLC). Methyl 3,4,6-tri-*O*-methyl glucopyranoside, methyl 2,3,4-tri-*O*-methyl glucopyranoside and methyl 2,3,4,6-tetra-*O*-methyl glucopyranoside were identified by comparison with authentic samples.

The linkages of these glucoses were considered to be β-glycosidic on the bases of the coupling constants for the anomeric protons (see Experimental) and the application of Klyne's rule[8] to the $[M]_D$ values calculated for the glucoses by use of the $[M]_D$ values of the prosapogenins listed in Table III.

TABLE III. Values of Molecular Rotation $[M]_D$

Compound	$[M]_D$	$-\Delta [M]_D$
Echinocystic acid (**4**)	+158°	−90°
RA-1 (**15**)	+68°	−88°
RA-2 (**17**)	−20°	−51°
RA-3 (**20**)	−71°	−68°
RA-4 (**11**)	−139°	

The following $[M]_D$ value was used: Methyl β-D-glucopyranoside −66°.

Thus, the structure of rotundioside A was established as the 3β-sulfate ester of 16α-hydroxyolean-12-ene-28-oyl β-D-glucopyranosyl (1→6)-β-D-glucopyranosyl (1→2)-β-D-glucopyranosyl (1→6)-β-D-glucopyranoside (Chart 2).

The prosapogenins **14**, **16**, **19** and **12** were shown to be glucosides of oleanolic acid based on the analyses of the ^{13}C-NMR data of these compounds, and therefore they were considered as the prosapogenins of rotundioside B. The ^{13}C-NMR signals for the glucoses of these prosapogenins were in agreement with those of corresponding prosapogenins of rotundioside A (*i.e.* **14** with **15**; **16** with **17**; **19** with **20**; **12** with **11**), suggesting the structure of the sugar moiety of rotundioside B to be identical with that of rotundioside A. This was further supported by methylation analysis of RB-4 (**12**). The same kinds of methyl fully or partially methylated glucopyranosides were identified as observed in the case of rotundioside A. In the ^{1}H-NMR spectrum of the permethylate of **12**, four anomeric proton signals were observed at 4.26 (1H, d, J=7 Hz), 4.37 (1H, d, J=8 Hz), 4.63 (1H, d, J=7 Hz), 5.20 (1H, d, J=9 Hz), suggesting β-glycosidic linkages of these glucoses.

Based on these observations, rotundioside B was deduced to be the 3β-sulfate ester of olean-12-ene-28-oyl β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl

(1→6)-β-D-glucopyranoside (Chart 2). This was further supported by the comparison of RB-1 (**14**) and RB-2 (**16**) with authentic samples synthesized by Ogihara *et al.*[9]

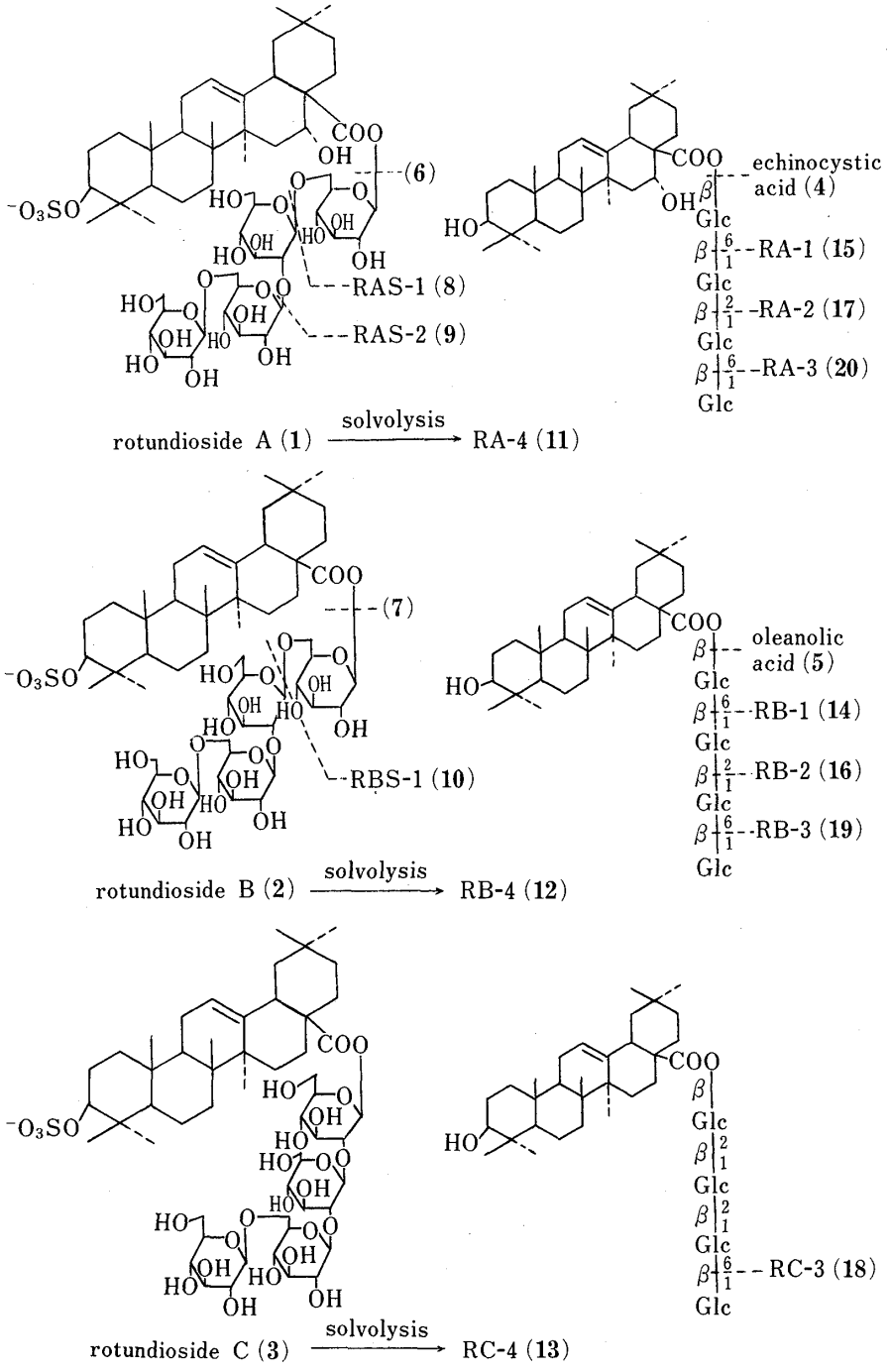

Chart 2

The minor prosapogenins, RC-4 (**13**), RC-3 (**18**) were suggested to be glycosides of oleanolic acid, and the sugar was shown to be linked to C-28 in ester form, according to the analyses of the ^{13}C-NMR data (Table II). The anomeric carbon signals of the glucoses in **13** and **18** were observed at 93.6 and 93.8, respectively, and were shifted upfield by *ca.* 2 ppm as compared with those of the prosapogenins of rotundiosides A and B. This observation suggests that **13** and **18** are different from the prosapogenins of rotundiosides A and B, and that the glucose′ was linked to C-2 of glucose in **13** and **18**, instead of C-6 of glucose in rotundiosides A and B.

Next, the ^{13}C-NMR spectrum of **18** showed the anomeric carbon signal of the glucose′ at 102.7 ppm, which was shifted upfield by *ca.* 3 ppm as compared with the ordinary methyl β-D-glucoside,[10] suggesting the glucose″ to be linked to C-2 of the glucose′. A comparison of ^{13}C-NMR spectrum of **13** with that of **18** showed that the signal of C-6 of the glucose″ was shifted from 63.6 ppm (in **18**) to 69.5 ppm (in **13**). This result suggests the glucose‴ to be linked to C-6 of the glucose″.

The structure of RC-4 (**13**) proposed on the basis of the spectral analyses described above was fully supported by the results of methylation analysis of **13** and **18** as follows: the permethylate of **18**, prepared according to Hakomori's method,[11] was methanolyzed, and methyl 3,4,6-tri-*O*-methyl glucopyranoside and methyl 2,3,4,6-tetra-*O*-methyl glucopyranoside (in a ratio of 2 : 1) were identified by GLC in comparison with authentic samples. By the same procedure, the methanolysis of the permethylate of **13** gave methyl 3,4,6-tri-*O*-methyl glucopyranoside, methyl 2,3,4-tri-*O*-methyl glucopyranoside and methyl 2,3,4,6-tetra-*O*-methyl glucopyranoside.

From these results, the structure of RC-4 was clearly established. Taking into account that RC-4 was obtained by desulfation of Fr. 1 (a mixture of rotundiosides A, B and C), RC-4 should be considered as a desulfated derivative of rotundioside C. Thus, rotundioside C was characterized as the 3β-sulfate ester of olean-12-ene-28-oyl β-D-glucopyranosyl (1→6)-β-D-glucopyranosyl (1→2)-β-D-glucopyranosyl (1→2)-β-D-glucopyranoside (**3**).

This is the first time that saponins with a sulfate group at C-3 have been isolated from the plant kingdom.

Experimental

All melting points were measured on a Yanagimoto microscope hot plate and are uncorrected. Optical rotations were taken with a Union PM-201 apparatus. ^{1}H-NMR spectra were measured on a JEOL JNM-MH-100 Spectrometer and ^{13}C-NMR spectra were measured on a JEOL JNM-FX-100 spectrometer with tetramethylsilane (TMS) as an internal standard; chemical shifts are given in δ (ppm). GLC were performed on a Shimadzu GC-6A gas chromatograph.

Isolation of Saponins——The dried leaves (2.2 kg) of *Bupleurum rotundifolium* were extracted with MeOH. The methanolic extract was concentrated and extracted with ether and *n*-BuOH successively to give the ether and *n*-BuOH extracts and the water-soluble portion. The water-soluble portion was chromatographed on silica gel with the lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10) to give the fraction containing rotundiosides A, B and C (Fr. 1).

Repeated separation of Fr. 1 by silica gel and reversed-phase chromatography followed by DCCC gave rotundioside A (**1**) (40 mg) and rotundioside B (**2**) (15 mg).

Rotundioside A, mp 232—237 °C, $[\alpha]_D^{23}$ −11.6° (c=0.78, MeOH).

Rotundioside B, mp 205—207 °C, $[\alpha]_D^{20}$ −1.6° (c=1.92, MeOH).

Acid Hydrolysis of the Mixture of Rotundiosides A, B and C——The mixture of rotundiosides A, B and C (2 g) was refluxed with dioxane (20 ml)–2 N H_2SO_4 (40 ml) for 3 h and the precipitate obtained was chromatographed on silica gel with $CHCl_3$–MeOH (30 : 1) to give compounds **4** (500 mg) and **5** (300 mg), which were identified as echinocystic acid and oleanolic acid, respectively, by comparison with authentic samples.

The filtrate was neutralized with ion-exchange resin (IR-45) and dried. The residue was trimethylsilylated and analyzed by GLC [2% OV-17 on Chromosorb W AW DMSS (80—100 mesh), column temp. 160 °C, N_2 flow rate 60 ml/min]. TMS–glucose was identified by comparison with an authentic sample.

Detection of Sulfate Group——The mixture (1 mg) of rotundiosides A, B, C was refluxed with 2 N hydrochloric acid for 2 h and then extracted with *n*-BuOH. The water layer was evaporated to dryness under a vacuum. The residue was dissolved in a little water and subjected to paper chromatography (Toyo filter paper No. 51A) with EtOH–H_2O (7 : 3). Then, the filter paper was air-dried and sprayed with a solution of $BaCl_2$ (100 mg) in 70% MeOH (50 ml). The paper was dried and sprayed with solution of sodium rhodizonate (10 mg) in 50% MeOH (50 ml). A positive reaction showed the presence of sulfate groups.

Alkaline Hydrolysis of the Mixture of Rotundiosides A, B and C——The mixture of rotundiosides A, B, C (3 g) was refluxed with 1 N KOH (40 ml) for 12 h and then neutralized with hydrochloric acid. The solution was extracted with *n*-BuOH to give the butanolic extract (550 mg), which was chromatographed on a silica gel column with the lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10) mixture, giving the aglycones (**6**) (173 mg) and (**7**) (112 mg).

6 was a white powder (MeOH–H_2O), mp 165—166 °C, $[\alpha]_D^{22}$ +23.2° (c=0.35, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 4.41 (1H, dd, J=10, 4 Hz, H-3), 5.64 (1H, br s, $W_{h/2}$=7 Hz, H-12), 5.21 (1H, br s, $W_{h/2}$=7 Hz, H-16). *Anal.* Calcd for $C_{30}H_{47}O_7S \cdot 0.5Ca \cdot 5H_2O$: C, 54.44; H, 8.68. Found: C, 54.36; H, 8.36.

7 was a white powder (MeOH–H_2O), mp 163—165 °C, $[\alpha]_D^{22}$ +54.0° (c=0.32, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 4.39 (1H, dd, J=10, 5 Hz, H-3), 5.47 (1H, br s, $W_{h/2}$=6 Hz, H-12). *Anal.* Calcd for $C_{30}H_{47}O_6S \cdot 0.5Ca \cdot 3.5H_2O$: C, 58.22; H, 8.79. Found: C, 58.34; H, 8.73.

Synthesis of Echinocystic Acid 3-*O*-Sulfate and Oleanolic Acid 3-*O*-Sulfate——a) Echinocystic acid (30 mg) in pyridine (5 ml) was stirred with sulfur trioxide–pyridine at room temperature for 1 h and then diluted with water. The solution was extracted with *n*-BuOH to give the butanolic extract, which was chromatographed on silica gel with the lower layer of $CHCl_3$–MeOH–H_2O (65 : 35 : 10). Echinocystic acid 3-*O*-sulfate was obtained as a white powder, mp 162—164 °C, $[\alpha]_D^{24}$ +25.2° (c=0.68, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 4.43 (1H, dd, J=10, 4 Hz, H-3), 5.62 (1H, br s, $W_{h/2}$=6 Hz, H-12), 5.23 (1H, br s, $W_{h/2}$=6 Hz, H-16).

b) Oleanolic acid (50 mg) was treated according to the same procedure as for echinocystic acid and then subjected to silica gel chromatography with $CHCl_3$–MeOH (4 : 1) mixture to give oleanolic acid 3-*O*-sulfate, a white powder, mp 161—163 °C. $[\alpha]_D^{22}$ +53.7° (c=0.75, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 4.47 (1H, dd, J=11, 4 Hz, H-3), 5.48 (1H, br s, $W_{h/2}$=6 Hz, H-12).

The synthetic echinocystic acid 3-*O*-sulfate and oleanolic acid 3-*O*-sulfate were identical with the aglycones obtained by alkaline hydrolysis.

Enzymatic Hydrolysis of the Mixture of Rotundiosides A, B, C——A solution of the mixture of rotundiosides A, B and C (4 g) and snail enzyme (10 ml) in NaOAc–HOAc buffer (pH 5.6, 50 ml) was stirred at room temperature for 6 d and then extracted with *n*-BuOH to give a butanolic extract (2.4 g) which was fractionated by DCCC to yield the following prosapogenins.

RAS-1 (**8**) (120 mg), colorless needles (MeOH–H_2O), mp 218 °C (dec.), $[\alpha]_D^{20}$ +9.2° (c=0.77, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 5.62 (1H, br s, H-12), 5.29 (1H, br s, H-16), 6.22 (1H, d, J=7 Hz, H-1 of glucose). *Anal.* Calcd for $C_{36}H_{57}O_{12}S \cdot 0.5Ca \cdot 2.5H_2O$: C, 55.51; H, 8.02. Found: C, 55.78; H, 8.35.

RAS-2 (**9**), a white powder, mp 220 °C, $[\alpha]_D^{23}$ −2.5° (c=0.41, MeOH).

RBS-1 (**10**), colorless needles (MeOH–H_2O), mp 214 °C (dec.), $[\alpha]_D^{20}$ +24.4° (c=1.03, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 6.26 (1H, d, J=7 Hz, H-1 of glucose).

Desulfation of the Mixture of Rotundiosides A, B, C——The mixture of rotundiosides A, B, C (4.6 g) was dissolved in pyridine (160 ml) and the solution was refluxed for 3 h. Removal of the solvent by evaporation under a vacuum gave a residue, which was dissolved in water and extracted with *n*-BuOH to give desulfated saponins (3.75 g).

Enzymatic Hydrolysis of Desulfated Saponins——A solution of the desulfated saponins (1.75 g) and snail enzyme (1 g) in NaOAc–HOAc buffer (pH 5.6, 50 ml) was stirred at room temperature for 7 d and then extracted with *n*-BuOH to give the butanolic extract (1.55 g), which was subjected to DCCC and silica gel chromatography to give the following prosapogenins:

RB-1 (**14**) (15 mg), RA-1 (**15**) (148 mg), RB-2 (**16**) (15 mg), RA-2 (**17**) (93 mg), RC-3 (**18**) (30 mg), RA-3 (**20**) (128 mg), RC-4 (**13**) (15 mg), RB-4 (**12**) (80 mg), RA-4 (**11**) (240 mg).

Another portion of the desulfated saponins (2 g) was repeatedly fractionated by DCCC to give RB-4 (**12**) (180 mg), which was dissolved in NaOAC–HOAc buffer containing snail enzyme 100 mg and stirred at room temperature for 24 h. The solution was then extracted with *n*-BuOH to give the butanolic extract, which was fractionated by DCCC to provide RB-3 (**19**) (7 mg) as colorless needles.

RB-1 (**14**), colorless needles (MeOH–H_2O), mp 228—230 °C, $[\alpha]_D^{20}$ +36.8° (c=0.76, MeOH). ^{1}H-NMR (pyridine-d_5, 70 °C) δ: 3.40 (1H, dd, J=9, 5 Hz, H-3), 5.44 (1H, br s, H-12), 6.10 (1H, d, J=7 Hz, H-1 of glucose). *Anal.* Calcd for $C_{36}H_{58}O_8 \cdot 1.5H_2O$: C, 66.95; H, 9.52. Found: C, 66.68; H, 9.48.

RA-1 (**15**), colorless needles, mp 226—229 °C, $[\alpha]_D^{22}$ +10.7° (c=0.38, MeOH). ^{1}H-NMR (pyridine-d_5, 70 °C) δ: 5.26 (1H, br s, $W_{h/2}$=8 Hz, H-16), 6.25 (1H, d, J=8 Hz, H-1 of glucose), 3.42 (1H, br s, H-3), 5.60 (1H, br s, $W_{h/2}$= 7 Hz, H-12). *Anal.* Calcd for $C_{36}H_{58}O_9 \cdot 0.5H_2O$: C, 67.15; H, 9.23. Found: C, 66.75; H, 9.11.

RB-2 (**16**), a white powder, mp 201—205 °C. ^{1}H-NMR (pyridine-d_5, 70 °C) δ: 3.37 (1H, dd, J=8, 5 Hz, H-3), 5.40 (1H, br s, H-12), 6.05 (1H, d, J=8 Hz, H-1 of glucose), 4.90 (1H, d, J=7 Hz, H-1 of glucose′).

RA-2 (**17**), colorless needles, mp 209—211 °C, $[\alpha]_D^{24}$ −2.5° (c=0.4, MeOH). ^{1}H-NMR (pyridine-d_5, 70 °C) δ: 3.46 (1H, dd, J=8, 4 Hz, H-3), 5.60 (1H, br s, H-12), 5.16 (1H, br s, H-16), 6.10 (1H, d, J=8 Hz, H-1 of glucose), 4.72 (1H, d, J=8 Hz, H-1 of glucose′). *Anal.* Calcd for $C_{42}H_{68}O_{14} \cdot 3H_2O$: C, 59.28; H, 8.76. Found: C, 59.27; H, 8.74.

RC-3 (**18**), a white powder, mp 198—203 °, $[\alpha]_D^{23}$ +11.8° (c=0.94, MeOH). ^{1}H-NMR (pyridine-d_5, 70 °C): 3.40 (1H, dd, J=8, 6 Hz, H-3), 5.54 (1H, br s, H-12), 6.10 (1H, d, J=8 Hz, H-1 of glucose), 5.14 (1H, d, J=7 Hz, H-1′ of glucose), 5.57 (1H, d, J=7 Hz, H-1″ of glucose). *Anal.* Calcd for $C_{48}H_{84}O_{21} \cdot 3H_2O$: C, 57.82; H, 8.49. Found: C, 57.95; H, 8.47.

RA-3 (**20**), colorless needles, mp 209—211 °C, $[\alpha]_D^{24}$ −7.4° (c=0.54, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 3.46 (1H, dd, J=9, 4 Hz, H-3), 5.62 (1H, br s, $W_{h/2}$=6 Hz, H-12), 5.20 (1H, br s, H-16), 6.22 (1H, d, J=8 Hz, H-1 of glucose), 4.88 (1H, d, J=8 Hz, H-1 of glucose′), 5.16 (1H, d, J=8 Hz, H-1 of glucose″). *Anal.* Calcd for $C_{48}H_{78}O_{19} \cdot 1.5H_2O$: C, 58.46; H, 8.28. Found: C, 58.43; H, 8.32.

RC-4 (**13**), a white powder, mp 209—212 °C, $[\alpha]_D^{21}$ +5.2° (c=0.58, MeOH). ^{1}H-NMR (pyridine-d_5) δ: 3.42 (1H, br s, H-3), 5.46 (1H, br s, H-12), 4.94 (1H, d, J=8 Hz, H-1 of glucose′), 5.31 (1H, d, J=8 Hz, H-1 of glucose″), 5.77 (1H, d, J=7 Hz, H-1 of glucose‴), 6.16 (1H, d, J=7 Hz, H-1 of glucose). *Anal.* Calcd for $C_{54}H_{88}O_{23}\cdot 3H_2O$: C, 55.95; H, 8.17. Found: C, 55.90; H, 8.15.

RB-4 (**12**), colorless needles (MeOH–H_2O), mp 210—212 °C, $[\alpha]_D^{24}$ +7.5° (c=0.27, MeOH). ^{1}H-NMR (pyridine-d_5, 73 °C) δ: 3.44 (1H, dd, J=9, 6 Hz, H-3), 5.43 (1H, br s, $W_{h/2}$=6 Hz, H-12), 6.12 (1H, d, J=7 Hz, H-1 of glucose), 4.83 (1H, d, J=7 Hz, H-1 of glucose′), 4.90 (1H, d, J=8 Hz, H-1 of glucose″), 5.12 (1H, d, J=7 Hz, H-1 of glucose‴). *Anal.* Calcd for $C_{54}H_{88}O_{23}\cdot H_2O$: 57.74; H, 8.08. Found: C, 57.74; H, 8.06.

RA-4 (**11**), a white powder, mp 210—213 °C, $[\alpha]_D^{22}$ −12.4° (c=0.49, MeOH). ^{1}H-NMR (pyridine-d_5, 70 °C) δ: 3.38 (1H, br s, H-3), 5.56 (1H, br s, H-12), 5.10 (1H, br s, H-16), 6.16 (1H, d, J=8 Hz, H-1 of glucose), 4.82 (1H, d, J=8 Hz, H-1 of glucose′), 4.85 (1H, d, J=8 Hz, H-1 of glucose″), 5.07 (1H, d, J=8 Hz, H-1 of glucose‴). *Anal.* Calcd for $C_{54}H_{88}O_{24}\cdot 2H_2O$: C, 56.05; H, 8.01. Found: C, 56.05; H, 8.32.

RB-3 (**19**), colorless needles, mp 256—257 °C, $[\alpha]_D^{20}$ +13.8° (c=0.15, MeOH). ^{1}H-NMR (pyridine-d_5, 70 °C) δ: 5.43 (1H, br s, H-12), 6.22 (1H, d, J=7 Hz, H-1 of glucose), 4.98 (1H, d, J=8 Hz, H-1 of glucose′), 5.06 (1H, d, J=9 Hz, H-1 of glucose″).

Methylation of Rotundioside A and RB-4 (12)——1) Rotundioside A (30 mg) was dissolved in dimethylformamide (4 ml) and refluxed with CH_3I (5 ml) and Ag_2O (0.5 g) for 16 h. The solution was filtered and the filtrate was repeatedly (3 times) subjected to methylation by Kuhn's method. The reaction mixture was evaporated to give a residue, which was chromatographed on silica gel with hexane–acetone (6 : 1) to give the permethylate of rotundioside A (2.4 mg). ^{1}H-NMR ($CDCl_3$) δ: 4.17 (1H, d, J=8 Hz, H-1 of glucose′), 4.37 (1H, d, J=8 Hz, H-1 of glucose″), 4.58 (1H, d, J=8 Hz, H-1 of glucose‴), 5.37 (1H, d, J=7 Hz, H-1 of glucose).

2) RB-4 (**12**) (50 mg) was methylated with the same procedure as used for rotundioside A, and the permethylate of RB-4 (2 mg) was obtained. ^{1}H-NMR ($CDCl_3$) δ: 4.26 (1H, d, J=7 Hz, H-1 of glucose′), 4.37 (1H, d, J=8 Hz, H-1 of glucose″), 4.63 (1H, d, J=7 Hz, H-1 of glucose‴), 5.20 (1H, d, J=9 Hz, H-1 of glucose).

Methanolyses of Rotundioside A Permethylate and RB-4 Permethylate——1) Rotundioside A permethylate (2.4 mg) was dissolved in 5% HCl–MeOH and refluxed for 5 h. Removal of the solvents gave a residue, which was analyzed by GLC [a) 10% diethylene glycol succinate (DEGS); b) 5% neopenthyl glycol succinate (NPGS) on Chromosorb W (60—80 mesh), column temp. 160 °C, N_2 flow rate 60 ml/min]. Methyl 3,4,6-tri-*O*-methyl glucopyranoside, methyl 2,3,4-tri-*O*-methyl glucopyranoside and methyl 2,3,4,6-tetra-*O*-methyl glucopyranoside were identified by comparison with authentic samples.

2) Methanolysis of RB-4 permethylate followed by GLC analysis showed the presence of the same kinds of methyl fully or partially methylated glucopyranosides as observed in the case of rotundioside A.

Methylation Analysis of RC-3 (18)——RC-3 (8 mg) was methylated according to Hakomori's method[11] to give RC-3 permethylate, which was methanolyzed and then analyzed by GLC (10% DEGS, column temp. 160 °C, N_2 flow rate 60 ml/min). Methyl 3,4,6-tri-*O*-methyl glucopyranoside and methyl 2,3,4,6-tetra-*O*-methyl glucopyranoside (in ratio 2 : 1) were identified by comparison with authentic samples.

Methylation Analysis of RC-4 (13)——RC-4 (5 mg) was methylated and then methanolyzed by the same procedure as described above. The methylated glucoses obtained were analyzed by GLC [5% XE-60 on Chromosorb W AW DMCS (80—100 mesh), column temp. 150 °C, N_2 flow rate 35 ml/min]. Methyl 3,4,6-tri-*O*-methyl glucopyranoside, methyl 2,3,4-tri-*O*-methyl glucopyranoside and 2,3,4,6-tetra-*O*-methyl glucopyranoside were identified by comparison with authentic samples.

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