Antiproliferative Constituents from Umbelliferae Plants VI.[1,2] New Ursane-Type Saikosaponin Analogs from the Fruits of *Bupleurum rotundifolium*

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The MeOH extract of the fruits of *Bupleurum rotundifolium* showed inhibitory activity against human gastric adenocarcinoma (MK-1) cell growth (GI_{50}: 6.25 μg/ml). From this extract, 10 new ursane-type triterpene glycosides *viz.* three 3-*O*-triosides (called rotundifoliosides A, I, and J) of 13β,28-epoxy-3β,16α-dihydroxyurs-11-ene, two (G and H) of 13β,28-epoxy-3β,16α,23-trihydroxyurs-11-ene, two (E and F) of 13β,28-epoxy-3β,16α,21β-trihydroxyurs-11-ene, two (B and C) of 3β,11α,16α,28-tetrahydroxyurs-12-ene, and one (D) of 3β,11α,28-trihydroxy-15α,16α-epoxyurs-12-ene were isolated in addition to 16 new oleanane-type triterpene glycosides (rotundiosides J—Y), echinocystic acid 3-*O*-sulfate and 3 known oleanane-type triterpene glycosides (rotundiosides A, F and G). The isolation, structures and antiproliferative activity of the new ursane-type triterpene glycosides against MK-1, human uterus carcinoma (HeLa), and murine melanoma (B16F10) cell lines are described with some comments on the structural requirements for their activity.

Key words *Bupleurum rotundifolium*; Umbelliferae; antiproliferative activity; 13β,28-epoxyursane-type triterpene glycoside

Bupleuri radix, the dried root of *Bupleurum falcatum* L., is one of the most important crude drugs used in Chinese medicine, and its saponin constituents, saikosaponins, glycosides of oleanane-type triterpenes, have been well investigated. The saponin constituents in other *Bupleurum* species were also investigated in relation to saikosaponins. The saponins in the leaves of *Bupleurum rotundifolium* L. were investigated by Ogihara and his colleagues,[3—7] and seven saponins, rotundiosides A—G, were isolated and their structures established. Recently, Navarro *et al.*[8] reported the isolation and structures of two new rotundiosides (H and I) from the aerial parts of this plant. To our knowledge, however, the constituents of the fruits have not been investigated.

In the course of our search to identify the antiproliferative constituents in the Umbelliferae plants, we have found that the MeOH extract of the fruits of *B. rotundifolium* inhibits *in vitro* growth of human gastric adenocarcinoma (MK-1) cells, and this finding caused us to check the active constituents in the fruits.

The powdered fruits were extracted and fractionated checking the activity as shown in the Experimental section. By fractionation of the extract by column chromatography on silica gel and octadecyl silica gel (ODS), and further by preparative HPLC (ODS), we isolated a total of 30 saponins.

By preliminary NMR analysis, ten of them have proved to be new ursane-type triterpene glycosides (called rotundifoliosides A—J), sixteen to be new oleanane-type triterpene glycosides (rotundiosides J—Y), and others, echinocystic acid 3-*O*-sulfate, and rotundiosides A, F, and G, the known oleanane-type triterpene glycosides.

This paper deals with the characterization of the ten new ursane-type triterpene glycosides and their inhibitory activity against MK-1, human uterus carcinoma (HeLa) and murine melanoma (B16F10) cell growth.

Rotundifolioside I (**1**) was obtained as a white powder and positive-ion high resolution (HR) FAB-MS gave the molecular formula $C_{47}H_{76}O_{16}$, the same as that of rotundioside G (**2**). On acid hydrolysis, **1** gave D-fucose, D-glucose, and D-xylose. The fully methylated product of **1** gave methyl glycosides of 2,3,4-tri-*O*-methyl-D-xylopyranose, 3,4,6-tri-*O*-methyl-D-glucopyranose, and 3,4-di-*O*-methyl-D-fucopyranose on methanolysis, and the result from **2** was the same. The NMR spectra of the sugar moiety of **1** were superimposable on those of **2**, *viz.* β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranose. The ^{1}H-NMR spectrum (Table 1) of the aglycone moiety showed signals of five tertiary methyl groups, two secondary methyl groups, one disubstituted olefinic group, and one oxymethyl group linked to the quaternary carbons at both ends. The ^{13}C-NMR spectrum (Table 2) of the aglycone moiety exhibited the signals of five C–C bonded quaternary carbons, disubstituted olefinic carbons, an oxygen-bearing quaternary carbon, an oxymethyl carbon and two oxymethylene carbons. The above-mentioned spectral evidence suggests the aglycone is 13β,28-epoxyurs-3β,16α-dihydroxy-11-ene, the ursane-type analog of the aglycone of rotundioside G (**2**). The heteronuclear multiple bond connectivity (HMBC) spectrum clearly supported the above structure (Fig. 1). Therefore, the structure of **1** was established as 13β,28-epoxy-16α-hydroxyurs-11-en-3β-yl β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside.

Rotundifolioside J (**3**), $C_{48}H_{78}O_{16}$, gave D-fucose, D-glucose, and L-rhamnose on acid hydrolysis. The NMR spectra of the sugar moiety of **3** were the same as those of rotundioside F (**4**), and the NMR spectra of the aglycone moiety were the same as those of **1**. Therefore, the structure of **3** was determined to be 13β,28-epoxy-16α-hydroxyurs-11-en-3β-yl α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside.

Rotundifolioside A (**5**), $C_{47}H_{76}O_{17}$, was obtained in very low yield, and therefore, the structure was determined only on the basis of the spectral data. The NMR spectra of the aglycone moiety of **5** were the same as those of **1**. Comparison of the NMR spectra of the sugar moieties of **1** and **5** re-

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Table 1. ^{1}H-NMR Data (δ, J in Hz) for Compounds **1**, **3**, **5**—**12** (Pyridine-d_5, 500 MHz)

	1	**3**	**5**	**6**	**7**
H-1	*ca.* 0.93	*ca.* 0.92, 1.77	*ca.* 0.86	*ca.* 1.01	*ca.* 0.91
	1.79 (br d, 13.0)		1.69 (br d, 13.0)	1.84 (br d, 13.0)	1.73 (br d, 13.0)
H-2	*ca.* 1.92, 2.20	*ca.* 1.96, 2.18	1.89 (m)	*ca.* 2.07	*ca.* 2.00
			2.28 (m)	2.31 (m)	2.35 (m)
H-3	3.30 (dd, 4.5, 11.5)	3.30 (dd, 5.0, 12.0)	3.34 (dd, 4.5, 11.5)	4.07 (dd, 4.5, 11.5)	*ca.* 4.10
H-5	0.80 (br d, 7.0)	0.80 (br d, 10.5)	0.77 (br dd, 3.5, 10.0)	*ca.* 1.53	*ca.* 1.50
H-6	*ca.* 1.25, 1.52	*ca.* 1.25, 1.53	*ca.* 1.50	*ca.* 1.60, 1.80	*ca.* 1.60, 1.79
H-7	*ca.* 1.27, 1.50	*ca.* 1.27, 1.50	*ca.* 1.25, 1.50	*ca.* 1.24, 1.61	*ca.* 1.24, 1.60
H-9	2.07 (br s)	2.08 (br s)	2.03 (br s)	2.18 (br s)	2.13 (br s)
H-11	5.79 (dd, 2.5, 10.5)	5.80 (dd, 2.5, 10.5)	5.79 (dd, 3.5, 10.5)	5.79 (dd, 3.0, 10.5)	5.79 (dd, 3.0, 10.0)
H-12	5.85 (d, 10.5)	5.84 (d, 10.5)	5.81 (d, 10.5)	5.89 (d, 10.5)	5.84 (d, 10.0)
H-15	*ca.* 1.53	*ca.* 1.56	1.47 (dd, 5.0, 13.5)	*ca.* 1.44	*ca.* 1.45
	2.17 (dd, 4.5, 14.0)	2.18 (dd, 5.0, 14.0)	2.17 (dd, 5.0, 13.5)	2.15 (dd, 5.0, 14.0)	*ca.* 2.15
H-16	*ca.* 4.21	*ca.* 4.24	4.22 (br dd, 5.0)	*ca.* 4.19	*ca.* 4.20
H-18	*ca.* 1.50	*ca.* 1.48	*ca.* 1.50	*ca.* 1.49	*ca.* 1.45
H-19	2.64 (m)	2.63 (m)	2.66 (m)	2.63 (m)	2.63 (m)
H-20	*ca.* 1.00	*ca.* 0.95	*ca.* 1.00	*ca.* 1.01	*ca.* 0.98
H-21	*ca.* 1.53	*ca.* 1.52	*ca.* 1.55	*ca.* 1.55	*ca.* 1.50
	2.45 (m)	2.45 (m)	2.47 (m)	2.44 (m)	2.45 (m)
H-22	*ca.* 1.45, 2.02	*ca.* 1.44, 2.03	*ca.* 1.50, 2.02	*ca.* 1.44, 2.01	*ca.* 1.45, 2.00
H-23	1.29 (s)	1.34 (s)	1.28 (s)	*ca.* 3.78, 4.28	3.78 (d, 12.0)
					ca. 4.33
H-24	1.10 (s)	1.09 (s)	1.12 (s)	1.10 (s)	1.12 (s)
H-25	0.93 (s)	0.90 (s)	0.91 (s)	1.01 (s)	0.98 (s)
H-26	1.30 (s)	1.28 (s)	1.29 (s)	1.32 (s)	1.33 (s)
H-27	1.75 (s)	1.75 (s)	1.75 (s)	1.70 (s)	1.69 (s)
H-28	3.25 (d, 7.0)	3.26 (d, 7.0)	3.25 (d, 7.0)	3.25 (d, 7.0)	3.24 (d, 7.0)
	3.55 (d, 7.0)	3.55 (d, 7.0)	3.55 (d, 7.0)	3.55 (d, 7.0)	3.54 (d, 7.0)
H-29	1.16 (d, 6.0)	1.14 (d, 6.5)	1.17 (d, 6.5)	1.14 (d, 6.0)	1.14 (d, 6.0)
H-30	1.01 (d, 5.5)	1.00 (d, 6.0)	1.01 (d, 6.0)	0.99 (d, 6.0)	*ca.* 0.98
Fuc 1	4.75 (d, 7.5)	4.76 (d, 7.5)		4.94 (d, 7.5)	
2	4.49 (dd, 7.5, 9.5)	4.65 (dd, 7.5, 9.0)		4.47 (dd, 7.5, 9.0)	
3	4.22 (dd, 3.0, 9.5)	4.40 (dd, 3.5, 9.0)		*ca.* 4.18	
4	4.00 (br d, 3.0)	3.89 (br d, 3.5)		*ca.* 3.97	
5	3.80 (br q, 6.5)	3.80 (br q, 6.5)		3.70 (br q, 6.0)	
6	1.55 (d, 6.5)	1.50 (d, 6.5)		1.52 (d, 6.0)	
Glc 1			4.93 (d, 8.0)		5.10 (d, 8.0)
2			4.10 (dd, 8.0, 9.0)		4.06 (dd, 8.0, 9.0)
3			*ca.* 4.33		*ca.* 4.26
4			4.10 (t, 9.0)		*ca.* 4.09[a]
5			3.93 (ddd, 3.0, 5.5, 9.0)		3.83 m
6			*ca.* 4.35		*ca.* 4.33[b]
			4.55 (br d, 12.0)		4.49 (br d, 11.0)[b]
Glc 1	5.40 (d, 7.5)	5.61 (d, 7.5)	5.50 (d, 8.0)	5.40 (d, 7.5)	5.49 (d, 8.0)
2	*ca.* 4.15	4.24 (dd, 7.5, 9.0)	4.18 (dd, 8.0, 9.0)	*ca.* 4.18	*ca.* 4.18
3	*ca.* 4.14	4.17 (t, 9.0)	4.28 (t, 9.0)	*ca.* 4.18	*ca.* 4.26
4	*ca.* 4.14	3.95 (t, 9.0)	4.19 (t, 9.0)	4.21 (t, 9.0)	*ca.* 4.26[a]
5	3.66 (ddd, 3.0, 5.0, 11.5)	3.68 (ddd, 3.0, 6.5, 9.0)	3.85 (ddd, 3.0, 5.0, 9.0)	3.63 (ddd, 3.5, 6.0, 9.0)	3.83 m
6	4.27 (dd, 5.0, 11.5)	4.15 (dd, 6.5, 11.5)	*ca.* 4.35	*ca.* 4.31	4.37 (br d, 11.0)[b]
	4.34 (dd, 3.0, 11.5)	4.38 (dd, 3.0, 11.5)	4.47 (dd, 3.0, 12.0)		4.43 (br d, 11.0)[b]
Xyl 1	5.37 (d, 7.0)		5.38 (d, 7.0)	5.36 (d, 7.0)	5.33 (d, 7.0)
2	*ca.* 4.09		*ca.* 4.09	*ca.* 4.11	*ca.* 4.06
3	*ca.* 4.13		*ca.* 4.10	*ca.* 4.19	*ca.* 4.08
4	*ca.* 4.19		*ca.* 4.11	*ca.* 4.19	*ca.* 4.08
5	3.72 (dd, 10.5, 10.5)		3.69 (m)	3.76 (dd, 9.5, 11.5)	3.69 (m)
	4.43 (dd, 5.0, 10.5)		*ca.* 4.35	4.43 (dd, 5.0, 11.5)	*ca.* 4.33
Rha 1		6.33 (br s)			
2		*ca.* 4.77			
3		*ca.* 4.77			
4		4.36 (t, 9.5)			
5		5.05 (dq, 9.5, 6.0)			
6		1.83 (d, 6.0)			

Table 1. (Continued)

	8	9	10	11	12
H-1	*ca.* 0.90	*ca.* 0.95	*ca.* 1.63	*ca.* 1.66	*ca.* 1.70
	1.79 (br d, 14.0)	1.80 (br d, 14.0)	2.82 (br d, 14.0)	2.83 (br d, 14.0)	2.51 ddd (3.0, 3.0, 14.0)
H-2	1.93 (br q, 14.0)	1.92 (br q, 14.0)	1.98 (m)	1.98 (m)	1.98 (m)
	2.23 (m)	2.21 (m)	2.25 (m)	2.24 (m)	2.28 (m)
H-3	3.31 (dd, 4.5, 11.5)	3.32 (dd, 4.0, 12.0)	3.38 (dd, 4.5, 12.0)	3.40 (dd, 4.5, 11.5)	3.39 (dd, 4.5, 13.0)
H-5	0.79 (m)	0.84 (br d, 10.5)	0.96 (m)	*ca.* 1.02	1.03 (br d, 10.5)
H-6	*ca.* 1.25, 1.50	*ca.* 1.58	*ca.* 1.35, 1.55	*ca.* 1.38, 1.60	*ca.* 1.50, 1.63
H-7	*ca.* 1.25, 1.50	*ca.* 1.30, 1.53	*ca.* 1.35, 1.66	*ca.* 1.38, 1.75	*ca.* 1.60, 1.72
H-9	2.07 (br s)	2.11 (br s)	2.02 (d, 9.0)	2.04 (d, 9.0)	1.96 (d, 8.0)
H-11	5.80 (dd, 3.0, 10.0)	5.80 (dd, 3.0, 11.0)	*ca.* 4.51	4.50 (m)	*ca.* 4.36
H-12	5.87 (d, 10.0)	5.87 (br d, 11.0)	5.67 (d, 3.0)	5.66 (d, 3.5)	5.67 (d, 4.0)
H-15	*ca.* 1.32	*ca.* 1.55	*ca.* 1.66	*ca.* 1.66	3.09 (d, 4.0)
	2.18 (dd, 5.0, 14.0)	2.19 (dd, 5.0, 14.0)	2.28 (dd, 4.0, 15.0)	2.29 (dd, 4.0, 15.0)	
H-16	*ca.* 4.22	4.24 (br s)	4.68 (m)	4.68 (m)	3.38 (d, 4.0)
H-18	1.64 (d, 12.0)	1.63 (br d, 12.0)	1.85 (d, 11.0)	1.85 (d, 11.0)	1.80 (d, 11.0)
H-19	2.82 (m)	2.82 (m)	*ca.* 2.46	*ca.* 2.47	2.10 (m)
H-20	*ca.* 1.20	*ca.* 1.20	*ca.* 1.12	*ca.* 1.12	*ca.* 0.95
H-21	4.79 (ddd, 5.0, 13.0, 13.0)	4.78 (ddd, 5.0, 13.0, 13.0)	*ca.* 1.70	*ca.* 1.72	*ca.* 1.60, 1.80
			2.34 (m)	2.35 (m)	
H-22	1.74 (dd, 13.0, 13.0)	1.74 (dd, 13.0, 13.0)	*ca.* 2.05, 2.46	*ca.* 2.05, 2.47	*ca.* 2.00
	2.58 (dd, 5.0, 13.0)	2.58 (dd, 5.0, 13.0)			2.36 (ddd, 3.0, 3.0, 13.5)
H-23	1.29 (s)	1.36 (s)	1.33 (s)	1.40 (s)	1.35 (s)
H-24	1.10 (s)	1.09 (s)	1.16 (s)[c)]	1.14 (s)[d)]	1.17 (s)
H-25	0.92 (s)	0.92 (s)	1.19 (s)[c)]	1.18 (s)[d)]	1.08 (s)
H-26	1.29 (s)	1.30 (s)	1.12 (s)	1.12 (s)	1.12 (s)
H-27	1.75 (s)	1.76 (s)	1.86 (s)	1.87 (s)	1.61 (s)
H-28	3.37 (d, 7.0)	3.37 (d, 7.0)	3.55 (d, 10.0)	3.56 (d, 11.0)	3.76 (d, 10.5)
	3.59 (d, 7.0)	3.59 (d, 7.0)	3.80 (d, 10.0)	3.81 (d, 11.0)	3.88 (d, 10.5)
H-29	1.22 (d, 6.5)	1.21 (d, 6.5)	1.14 (d, 6.5)	1.13 (d, 6.5)	0.97 (d, 6.5)
H-30	1.40 (d, 6.5)	1.40 (d, 6.5)	1.03 (d, 6.5)	1.03 (d, 6.5)	0.97 (d, 5.0)
Fuc 1	4.79 (d, 7.5)	4.79 (d, 7.5)	4.74 (d, 8.0)	4.75 (d, 8.0)	4.76 (d, 8.0)
2	4.51 (dd, 7.5, 9.0)	4.62 (dd, 7.5, 9.0)	4.49 (dd, 8.0, 9.0)	4.59 (dd, 8.0, 9.0)	4.49 (dd, 8.0, 9.5)
3	*ca.* 4.22	4.39 (dd, 3.5, 9.0)	*ca.* 4.22	4.36 (dd, 3.0, 9.0)	*ca.* 4.23
4	4.02 (br d, 3.0)	3.83 (br d, 3.5)	3.98 (br d, 3.0)	3.78 (br d, 3.0)	3.99 (br d, 3.5)
5	3.82 (br q, 6.0)	3.76 (br q, 6.5)	3.76 (br q, 6.5)	3.70 (br q, 6.0)	3.77 (br q, 6.0)
6	1.56 (d, 6.0)	1.50 (d, 6.5)	1.50 (d, 6.5)	1.45 (d, 6.0)	1.50 (d, 6.0)
Glc 1					
2					
3					
4					
5					
6					
Glc 1	5.42 (d, 7.5)	5.66 (d, 8.0)	5.42 (d, 7.5)	5.67 (d, 8.0)	5.42 (d, 7.5)
2	*ca.* 4.15	4.28 (dd, 8.0, 9.0)	*ca.* 4.15	4.29 (dd, 8.0, 9.0)	*ca.* 4.20
3	*ca.* 4.22	4.20 (t, 9.0)	4.20 (t, 9.0)	4.20 (t, 9.0)	*ca.* 4.20
4	*ca.* 4.15	4.03 (t, 9.0)	4.16 (t, 9.0)	4.05 (t, 9.0)	*ca.* 4.20
5	3.68 (ddd, 3.0, 5.0, 9.0)	3.62 (ddd, 3.5, 6.0, 9.0)	3.68 (ddd, 3.5, 5.0, 9.0)	3.60 (ddd, 3.0, 6.0, 9.0)	3.68 (ddd, 3.0, 5.0, 9.0)
6	4.28 (dd, 5.0, 11.5)	4.18 (dd, 6.0, 11.5)	4.29 (dd, 5.0, 11.5)	4.19 (dd, 5.0, 12.0)	4.31 (dd, 5.0, 11.5)
	4.37 (dd, 3.0, 11.5)	4.28 (dd, 3.5, 11.5)	4.36 (dd, 3.5, 11.5)	4.28 (dd, 3.0, 12.0)	4.37 (dd, 3.0, 11.5)
Xyl 1	5.39 (d, 7.0)		5.39 (d, 7.0)		5.40 (d, 7.5)
2	4.12 (dd, 8.0, 9.0)		4.11 (dd, 7.0, 8.5)		4.11 (dd, 7.5, 9.0)
3	4.14 (t, 9.0)		4.14 (t, 8.5)		4.14 (dd, 9.0, 10.0)
4	*ca.* 4.20		*ca.* 4.20		*ca.* 4.21
5	3.74 (dd, 10.5, 11.5)		3.73 (dd, 9.0, 11.5)		3.72 (dd, 8.0, 12.0)
	4.45 (dd, 5.0, 11.5)		4.44 (dd, 5.0, 11.5)		4.45 (dd, 5.0, 12.0)
Rha 1		6.39 (br s)		6.42 (br s)	
2		*ca.* 4.76		*ca.* 4.75	
3		*ca.* 4.76		*ca.* 4.75	
4		4.36 (t, 9.5)		*ca.* 4.34	
5		5.07 (dq, 9.5, 6.0)		5.06 (dq, 9.5, 6.0)	
6		1.84 (d, 6.0)		1.82 (d, 6.0)	

a, *b*, *c*, *d*) Assignments may be interchanged. Abbreviations: Fuc, D-fucose; Glc, D-glucose; Xyl, D-xylose; Rha, L-rhamnose, all in a pyranose form.

Table 2. ^{13}C-NMR Data (δ) for Compounds **1**, **3**, **5**—**12** (Pyridine-d_5, 125 MHz)

	1	**3**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	38.68	38.85	38.55	38.53	38.44	38.66	38.66	41.71	41.65	40.97
2	26.54	26.63	26.49	25.81	25.82	26.54	26.51	27.06	26.98	26.88
3	88.77	89.98	88.96	82.99	83.18	88.81	89.56	88.86	89.51	88.80
4	39.78	40.07	39.69	43.59	43.59	39.79	39.89	40.03	40.08	39.91
5	55.51	55.72	55.35	48.37	48.23	55.49	55.57	56.46	56.51	56.10
6	17.94	18.09	17.89	17.76	17.75	17.93	17.94	18.59	18.59	18.76
7	31.99	32.13	31.94	31.73	31.71	31.98	31.96	34.49	34.44	33.26
8	41.97	42.13	41.92	41.95	41.93	42.01	42.04	43.42	43.42	43.57
9	52.61	52.80	52.52	52.71	52.66	52.61	52.62	54.66	54.69	56.32
10	36.33	36.50	36.24	36.25	36.19	36.32	36.37	38.38	38.40	38.57
11	134.11	134.21	134.06	134.06	134.03	133.83	133.83	67.43	67.42	66.04
12	129.01	129.30	128.96	129.02	129.03	129.28	129.32	131.33	131.34	130.93
13	85.06	85.34	85.03	85.02	85.05	84.97	84.98	140.32	140.31	138.53
14	44.08	44.24	44.06	44.05	44.06	44.09	44.10	42.40	42.37	42.65
15	35.51	35.57	35.48	35.52	35.51	35.25	35.25	35.76	35.77	55.40
16	76.89	77.04	76.86	76.86	76.86	76.06	76.07	73.91	73.93	62.41
17	46.35	46.50	46.32	46.31	46.31	48.03	48.02	42.00	41.98	39.03
18	61.52	61.67	61.49	61.48	61.50	60.97	60.98	53.96	53.93	53.68
19	38.50	38.67	38.47	38.43	38.44	37.01	37.02	39.38	39.36	39.16
20	41.71	41.86	41.68	41.67	41.69	49.71	49.69	39.90	39.88	39.61
21	33.64	33.79	33.61	33.61	33.61	72.55	72.94	33.42	33.38	31.86
22	35.32	35.45	35.30	35.30	35.31	45.11	45.11	34.17	34.13	34.81
23	27.87	28.19	27.82	65.61	65.50	27.87	28.06	28.41	28.59	28.19
24	16.23	16.23	16.22	12.70	12.75	16.23	16.14	17.07	16.97[f]	16.83
25	18.21	18.25	18.15	18.67	18.63	18.19	18.12	17.07	17.00[f]	17.33
26	19.35	19.52	19.31	19.37	19.37	19.36	19.37	18.36[e]	18.36[g]	20.44
27	16.33	16.50	16.32	16.24	16.27	16.35	16.33	24.66	24.63	20.98
28	77.64	77.81	77.60	77.63	77.61	77.31	77.31	70.97	70.92	68.33
29	18.78	18.96	18.73	18.71	18.71	19.01	19.02	18.40[e]	18.37[g]	21.22
30	19.73	19.89	19.69	19.66	19.66	15.58	15.58	21.70	21.67	18.17
Fuc 1	104.91	105.56		103.59		104.93	105.28	104.81	105.04	104.77
2	80.57	76.91		81.22		80.47	78.16	80.49	77.96	80.58
3	75.44	76.20		75.46		75.47	76.16	75.47	76.19	75.44
4	72.53	73.14		72.49		72.46	72.83	72.54	72.87	72.51
5	71.11	71.08		70.99		71.14	70.86	71.05	70.72	71.04
6	17.23	17.43		17.20		17.23	17.32	17.17	17.24	17.16
Glc 1			104.72		103.51					
2			82.96		83.49					
3			78.62		78.65					
4			71.11		70.81[b]					
5			78.17		78.13[c]					
6			62.95		62.79[d]					
Glc 1	103.11	102.17	103.17	103.33	103.43	103.08	101.87	103.05	101.80	103.14
2	84.61	78.55	84.53	84.52	84.56	84.64	79.43	84.62	79.43	84.66
3	77.59	79.50	77.87	77.63	77.83	77.94	77.14	77.94	77.42	77.58
4	71.81	72.89	71.83	71.12	71.13[b]	71.81	72.73	71.80	72.79	71.84
5	77.34	77.68	77.60	77.46	77.67[c]	77.44	77.23	77.37	76.93	77.29
6	62.83	63.49	62.90	62.26	62.44[d]	62.83	63.32	62.83	63.28	62.87
Xyl 1	106.44		106.39	106.45	106.45	106.46		106.44		106.48
2	75.75		75.86	75.81	75.90	75.77		75.75		75.77
3	77.93		77.69	77.88	77.72	77.61		77.60		77.92
4	70.70		70.66	70.69	70.63[b]	70.73		70.72		70.70
5	67.31		67.34	67.39	67.40	67.33		67.31		67.32
Rha 1		102.02					102.14		102.13	
2		72.56[a]					72.46		72.71	
3		72.77[a]					72.46		72.43	
4		74.44					74.34		74.31	
5		69.53					69.45		69.44	
6		19.05					18.95		18.91	

a, *b*, *c*, *d*, *e*, *f*, *g*) Assignments may be interchanged.

vealed that the fucopyranosyl group in **1** is replaced by a hexopyranosyl group in **5**. The signals due to the terminal β-D-xylopyranosyl group were consistent with those in **1**. Two sets of the oxymethylene proton signals in the spectrum of **5**, one at δ 3.85 (ddd, J=3.0, 5.0, 9.0 Hz) and the other at δ 3.93 (ddd, J=3.0, 5.5, 9.0 Hz), are reasonably assigned to H-5 of two glucopyranosyl groups. These spectral findings indicated that the β-D-fucopyranosyl group in **1** is replaced by a glucopyranosyl group to give **5**. From this NMR spectral evidence, the sugar structure was suggested to be β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranose. Based on these results, we propose that the structure of **5** is 13β,28-epoxy-16α-hydroxyurs-11-en-3β-yl β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

Rotundifolioside H (**6**), a white powder, $C_{47}H_{76}O_{17}$, gave the same sugars as **1**. The NMR spectra clearly indicated that the sugar moiety is the same as that of **1**, thus indicating the molecular formula of the aglycone to be $C_{30}H_{48}O_4$, having one more oxygen atom than the molecular formula of the aglycone of **1**. The ^{1}H-NMR spectrum showed decrease of one tertiary methyl group from **1** and addition of a hydroxymethyl group. When the ^{13}C-NMR spectra of **1** and **6** were compared, the spectrum of **6** showed upfield shifts of C-3 (Δ 5.78 ppm), C-5 (Δ 7.14 ppm), and C-24 (Δ 3.53 ppm), and downfield shifts of C-4 (Δ 3.81 ppm) and C-23 (Δ 37.74 ppm). These spectral data indicated that the hydroxymethyl group in **6** was located at C-23, thus: 13β,28-epoxy-16α,23-dihydroxyurs-11-en-3β-yl β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside.

The NMR spectra of the aglycone moiety of rotundifolioside G (**7**), $C_{47}H_{76}O_{18}$, were the same as that of **6**, and the spectra of the sugar moiety were superimposable on those of **5**. Therefore, the structure of **7** was concluded to be 13β,28-epoxy-16α,23-dihydroxyurs-11-en-3β-yl β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

The molecular formula of rotundifolioside E (**8**), $C_{47}H_{76}O_{17}$, has one more oxygen than **1**, while the NMR spectra of the sugar moiety of **8** were almost the same as those of **1**, indicating the additional oxygen atom should be in the aglycone moiety. The general NMR spectral feature of the aglycone moiety was similar to that of **1**, however, the methylene carbon signal (δ 33.64) assignable to C-21 of **1** was shifted down to δ 72.55 in **8**. Furthermore, an upfield shift of C-30 (Δ 4.15 ppm) and downfield shifts of C-20 (Δ 8.00 ppm) and C-22 (Δ 9.79 ppm) were observed compared to those of **1**, undoubtedly indicating the presence of a hydroxy group at C-21 of **1**. The HMBC spectrum (Fig. 3) also supported the presence of a hydroxy group at C-21. Its configuration was determined to be β from the coupling constants: $J_{H20,H21}$ (13.0 Hz), $J_{H21,H22eq}$ (5.0 Hz), and $J_{H21,H22ax}$ (13.0 Hz). From these data, **8** was concluded to be 13β,28-epoxy-16α,21β-dihydroxyurs-11-en-3β-yl β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside.

The molecular formula of rotundifolioside F (**9**), $C_{48}H_{78}O_{17}$, has one additional carbon and two more proton atoms than **8**. The ^{13}C-NMR spectrum of the aglycone moiety of **9** was the same as that of **8**, thus suggesting that the additional carbon and two proton atoms must be in the sugar moiety. The NMR spectrum of the sugar moiety was almost the same as that of **3**. These results have led to the conclusion that **9** is 13β,28-epoxy-16α,21β-dihydroxyurs-11-en-3β-yl α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside.

Rotundifolioside B (**10**) has a molecular formula, $C_{47}H_{78}O_{17}$, which has one additional oxygen and two proton atoms than **1**. Judging from the NMR spectra, the structure

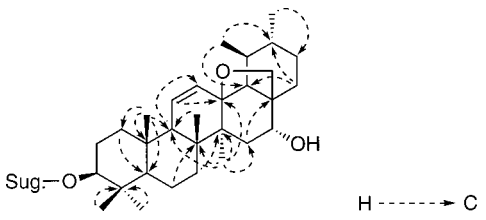

Fig. 1. ^{1}H–^{13}C HMBC Correlations in **1**

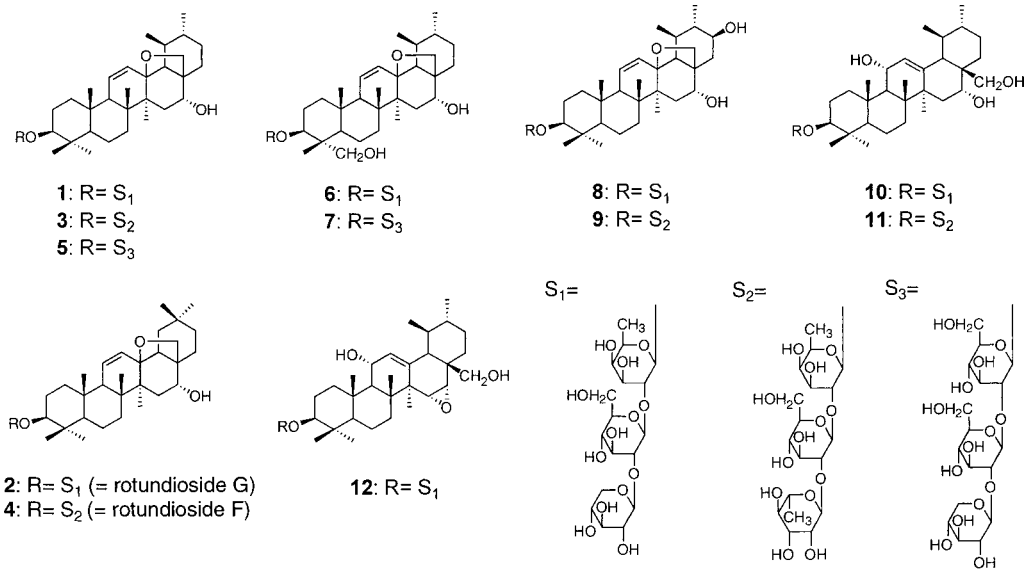

Fig. 2. Structures

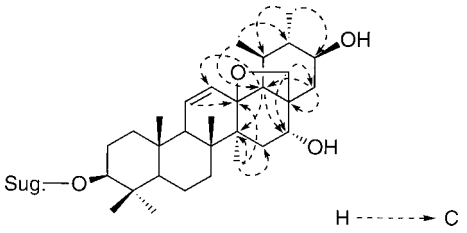

Fig. 3. ^{1}H–^{13}C HMBC Correlations in D/E rings of **8**

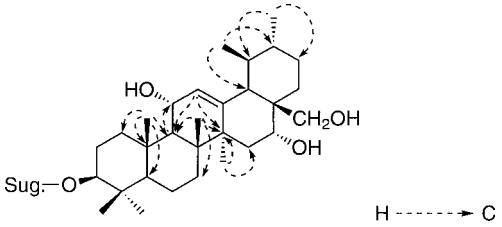

Fig. 4. ^{1}H–^{13}C HMBC Correlations in **10**

of the sugar moiety is the same as that of **1**. The ^{1}H-NMR spectrum of the aglycone moiety of **10** showed signals of five tertiary methyl groups and two secondary methyl groups, indicating that the aglycone is also an ursane-type triterpene, however, differing from **1**, **10** has a trisubstituted double bond (δ 5.67, d, J=3.0 Hz) and an oxymethyl group (δ 3.55, d, J=10.0 Hz; δ 3.80, d, J=10.0 Hz). The ^{13}C-NMR spectrum of the aglycone moiety of **10** exhibited the signals of five C–C bonded quaternary carbons (δ 38.38, 40.03, 42.00, 42.40, 43.42), trisubstituted olefinic carbons (δ 131.33, 140.32), and an oxymethyl carbon (δ 70.97), and three oxymethylene carbons (δ 88.86, 67.43, 73.91); however, the signals due to an oxygen-bearing quaternary carbon and a hydroxymethyl carbon characteristic of the 13β,28-epoxide ring were not observed. The ^{1}H- and ^{13}C-NMR data suggested that **10** is a triglycoside of an ursane-type triterpene which was derived from **1** by hydrolytic opening of the 13β,28-epoxy ring, migration of the 11,12-double bond to 12,13, and then attachment of a hydroxyl group to the C-11 carbanion. The structure was confirmed by analysis of the HMBC spectrum (Fig. 4). The configuration of the hydroxyl group at C-11 was determined to be α from the coupling constant of $J_{H9,H11}$ (9.0 Hz).

From the above spectral evidence, the structure of **10** was established as 11α,16α,28-trihydroxyurs-12-en-3β-yl β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside.

Rotundifolioside C (**11**) has a molecular formula $C_{48}H_{80}O_{17}$, having one additional carbon and two proton atoms than **10**. The NMR spectra of the aglycone moiety of **11** were the same as those of **10**, and the sugar moiety showed the same NMR spectra as those of **3**. Thus, the structure of **11** was established as 11α,16α,28-trihydroxyurs-12-en-3β-yl α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside.

Rotundifolioside D (**12**) has a molecular formula of $C_{47}H_{76}O_{17}$, two proton atoms less than that of **10**. The NMR spectra of the sugar moiety of **12** were the same as those of **10**, and the NMR spectra of the aglycone moiety resembled those of **10**. A comparison of the ^{13}C-NMR spectrum of **12** with that of **10** showed the downfield shift of C-15 (Δ 19.64 ppm) and upfield shift of C-16 (Δ 11.50 ppm). These spectral findings clearly indicated the presence of a 15,16 epoxy ring. In rotating frame nuclear Overhauser enhancement and exchange spectroscopy (ROESY) spectrum of **12**, the correlations between H-15 and H-26, and H-16 and H-22 were observed, indicating the configurations of H-15 and H-16 to be β. Thus, the structure of **12** was established as 15α,16α-epoxy-11α,28-dihydroxyurs-12-en-3β-yl β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside.

Table 3. Antiproliferative Activities (GI_{50}, μM) against MK-1, HeLa, and B16F10 Cell Lines *in Vitro*

Compound	MK-1	HeLa	B16F10
1	20	37	18
3	16	21	11
5	48	71	31
6	18	31	18
7	84	>100 (μg/ml)	46
8	>100 (μg/ml)	>100 (μg/ml)	>100 (μg/ml)
9	>100 (μg/ml)	>100 (μg/ml)	>100 (μg/ml)
10	>100 (μg/ml)	>100 (μg/ml)	>100 (μg/ml)
11	>100 (μg/ml)	>100 (μg/ml)	>100 (μg/ml)
12	>100 (μg/ml)	>100 (μg/ml)	>100 (μg/ml)

Rotundifoliosides A—J are all new ursane-type triterpene triosides, among which rotundifoliosides A (**5**), E (**8**), F (**9**), G (**7**), H (**6**), I (**1**), J (**3**) are the first ones having the 13β,28-epoxy ring system corresponding to the oleanane-type saikosaponins isolated from Bupleuri radix.

The antiproliferative activity of the new ursane-type triterpene glycosides against MK-1, HeLa, and B16F10 cell lines was examined by MTT assay.[9] The 50% growth inhibition (GI_{50}) values are listed in Table 3. All active glycosides have the 13β,28-epoxy ring system, and the glycosides of other aglycones are practically inactive. Rotundifoliosides E (**8**) and F (**9**) are inactive although they have the 13β,28-epoxy group. We cannot at present provide now any reasonable explanation for this loss of activation. The presence of a β-hydroxyl group at C-21 might have an overwhelming influence on the decrease of activity. Among the active compounds, rotundifoliosides A (**5**) and G (**7**) were less active than the others. Compounds **5** and **7** have a β-D-glucopyranosyl group directly linked to the aglycone instead of a β-D-fucopyranosyl group in the more active compounds. It is possible that the β-D-fucopyranosyl group plays some role in the antiproliferative activity.

The structures and antiproliferative activity of sixteen new glycosides of the oleanane-type triterpenes will be reported in the near future.

Experimental

All the instruments and materials used were the same as those shown in the previous paper.[10] ^{1}H- (500 MHz) and ^{13}C- (125 MHz) NMR spectra were measured in pyridine-d_5. Chemical shifts are expressed on the δ scale with tetramethylsilane as an internal standard. The signal assignment was based on comparison with data reported for compounds having similar structures, and confirmed with the aid of NMR spectral techniques (decoupling difference spectrum, NOE difference spectrum, ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY, HMQC, phase-sensitive NOESY, ROESY, long-range ^{1}H–^{13}C COSY, and HMBC).

Extraction and Preliminary Fractionation *Bupleurum rotundifolium*

was cultivated in the medicinal plant garden of Fukuoka University, and the fruits were collected and air-dried. The powder of the fruits (600 g) was extracted first with benzene (2 l) and then with MeOH (2 l). The benzene solution was concentrated *in vacuo* to give an oil (9.8 g). The MeOH solution was concentrated *in vacuo*, the extract was suspended in water and the suspension was passed through a column of Diaion HP-20. The H_2O eluate was concentrated *in vacuo* to give a yellow syrup (BRF-W; 2.0 g). The column was then washed with MeOH and the eluate was concentrated *in vacuo* to give a brown powder (BRF-M; 25.4 g). The 50% growth inhibition (GI_{50}, μg/ml) values of the benzene extract, BRF-W, and BRF-M against MK-1 cells were >50, >50, and 32, respectively.

Fractionation of BRF-M, and Isolation of the Constituents BRF-M (25.4 g) was chromatographed on YMC gel ODS-A using MeOH–H_2O (1 : 2→1 : 0) as the eluting solvent to separate into 14 fractions: frs. 1 (0.2 g), 2 (0.2 g), 3 (0.6 g), 4 (0.9 g), 5 (0.8 g), 6 (1.2 g), 7 (1.6 g), 8 (0.6 g), 9 (3.2 g), 10 (3.1 g), 11 (4.1 g), 12 (4.9 g), 13 (2.1 g), and 14 (1.3 g).

Fraction 3 was further chromatographed on silica gel using $CHCl_3$–MeOH–H_2O (8 : 1 : 0.1→6 : 4 : 1) to give 2 fractions (frs. 3-1, -2). Fraction 3-1 was chromatographed on YMC gel ODS-A [MeOH–H_2O (2 : 1→1 : 0)], Sephadex LH-20 (EtOH) and then subjected to preparative HPLC on an ODS column (50% MeOH) to give echinocystic acid 3-*O*-sulfate (25 mg). Fraction 3-2 was chromatographed on Sephadex LH-20 (EtOH) and subjected to preparative HPLC on an ODS column (40% MeOH) to give rotundiosides A (49 mg) and J (75 mg).

Fraction 4 was further chromatographed on silica gel [$CHCl_3$–MeOH–H_2O (7 : 3 : 0.5→6 : 4 : 1)] to give rotundioside K (370 mg).

Fraction 6 was further chromatographed on silica gel [$CHCl_3$–MeOH–H_2O (8 : 1 : 0.1→6 : 4 : 1)], YMC gel ODS-A [MeOH–H_2O (1 : 1→1 : 0)], and then subjected to preparative HPLC on an ODS column [80% MeOH] to give rotundifolioside A (**5**, 1.8 mg).

Fraction 7 was further chromatographed on silica gel [$CHCl_3$–MeOH–H_2O (8 : 1 : 0.1→6 : 4 : 1)] and YMC gel ODS-A [MeOH–H_2O (1 : 1→1 : 0)] to give rotundifoliosides B (**10**, 118 mg) and C (**11**, 8.4 mg).

Fraction 8 was chromatographed on silica gel [$CHCl_3$–MeOH–H_2O (8 : 1 : 0.1→6 : 4 : 1)] and separated into 2 fractions (frs. 8-1, -2). Fraction 8-1 was further separated into 2 subfractions (frs. 8-1-1, 8-1-2) by chromatography on YMC gel ODS-A [MeOH–H_2O (1 : 1→1 : 0)]. Fraction 8-1-1 was subjected to preparative HPLC on an ODS column [20% isopropanol] to give rotundioside L (33 mg) and rotundifolioside D (**12**, 12 mg). Fraction 8-1-2 was subjected to preparative HPLC on an ODS column [60% MeOH] to give rotundifolioside E (**8**, 10 mg). Fraction 8-2 was chromatographed on YMC gel ODS-A [MeOH–H_2O (1 : 1→1 : 0)] and then subjected to preparative HPLC on an ODS column [60% MeOH] to give rotundioside M (20 mg) and rotundifolioside F (**9**, 16 mg).

Fraction 9 was chromatographed on silica gel [$CHCl_3$–MeOH–H_2O (8 : 1 : 0.1→6 : 4 : 1)] and separated into 2 fractions (frs. 9-1, -2). Fraction 9-1 was subjected to preparative HPLC on an ODS column [70% MeOH] to give rotundioside N (4.5 mg). Fraction 9-2 was subjected to preparative HPLC on an ODS column (80% MeOH) to give rotundioside O (1.4 mg).

Fraction 10 was separated into 2 fractions (frs. 10-1, -2) by chromatography on silica gel [$CHCl_3$–MeOH–H_2O (8 : 1 : 0.1→6 : 4 : 1)]; fr. 10-1 was chromatographed on YMC gel ODS-A [MeOH–H_2O (1 : 1→1 : 0)] and then subjected to preparative HPLC on an ODS column [75% MeOH] to give rotundiosides P (90 mg) and Q (6 mg). Fraction 10-2 was chromatographed on YMC gel ODS-A using MeOH–H_2O (1 : 1→1 : 0) and then subjected to preparative HPLC on an ODS column [75% MeOH] to give rotundiosides R (8 mg) and S (26 mg), and rotundifolioside G (**7**, 9 mg).

Fraction 11 was separated into 2 fractions (frs. 11-1, -2) by silica gel chromatography [$CHCl_3$–MeOH–H_2O (8 : 1 : 0.1→6 : 4 : 1)]. From fr. 11-1, rotundioside T (132 mg) and rotundifolioside H (**6**, 14 mg) were isolated by preparative HPLC on an ODS column [80% MeOH]. Fraction 11-2 gave rotundiosides U (70 mg) and V (18 mg) when subjected to preparative HPLC on an ODS column [80% MeOH].

From fr. 12, rotundioside W (6.6 mg) was obtained by chromatography on silica gel [$CHCl_3$–MeOH–H_2O (8 : 1 : 0.1→6 : 4 : 1)], YMC gel ODS-A [MeOH–H_2O (2 : 1→1 : 0)], and preparative HPLC on an ODS column [80% MeOH].

Fraction 13 was further chromatographed on YMC gel ODS-A [MeOH–H_2O (2 : 1→1 : 0)] and 2 fractions separated (frs. 13-1, -2). Fraction 13-1 was subjected to preparative HPLC on an ODS column (80% MeOH) to give rotundiosides X (30 mg) and Y (210 mg), while fr. 13-2 was further separated into 2 fractions (frs. 13-2-1, -2) by silica gel chromatography [$CHCl_3$–MeOH–H_2O (8 : 1 : 0.1→6 : 4 : 1)]. Both fractions were separately subjected to preparative HPLC on an ODS column (80% MeOH) to give rotundifolioside I (**1**, 280 mg) and rotundioside G (**2**, 7 mg) from fr. 13-2-1, and rotundioside F (**4**, 93 mg) and rotundifolioside J (**3**, 48 mg) from fr. 13-2-2.

All compounds were obtained as white powders, rotations ($[\alpha]_D^{24}$ in pyridine), HR-FAB-MS data (*m*/*z*) of rotundifoliosides are shown below. ^{1}H- and ^{13}C-NMR data are listed in Tables 1 and 2, respectively. The data of oleanane-type triterpene glycosides will be shown in the next paper.

Rotundifolioside I (**1**): −10.1° (*c*=0.99). $C_{47}H_{76}O_{16}Na$, calcd.: 919.5031, observed: 919.5027.

Rotundifolioside J (**3**): +31.3° (*c*=1.00). $C_{48}H_{78}O_{16}Na$, calcd.: 933.5188, observed: 933.5193.

Rotundifolioside H (**5**): −9.4° (*c*=0.64). $C_{47}H_{76}O_{17}Na$, calcd.: 935.4980, observed: 935.4988.

Rotundifolioside A (**6**): −62.0° (*c*=0.18). $C_{47}H_{76}O_{17}Na$, calcd.: 935.4980, observed: 935.4985.

Rotundifolioside G (**7**): +3.8° (*c*=0.90). $C_{47}H_{76}O_{18}Na$, calcd.: 951.4929, observed: 951.4928.

Rotundifolioside E (**8**): −16.8° (*c*=0.59). $C_{47}H_{76}O_{17}Na$, calcd.: 935.4980, observed: 935.4987.

Rotundifolioside F (**9**): −12.9° (*c*=0.47). $C_{48}H_{78}O_{17}Na$, calcd.: 949.5137, observed: 949.5117.

Rotundifolioside B (**10**): −60.5° (*c*=0.62). $C_{47}H_{78}O_{17}Na$, calcd.: 937.5137, observed: 937.5160.

Rotundifolioside C (**11**): −93.0° (*c*=0.29). $C_{48}H_{80}O_{17}Na$, calcd.: 951.5293, observed: 951.5280.

Rotundifolioside D (**12**): −40.4° (*c*=1.11). $C_{47}H_{76}O_{17}Na$, calcd.: 935.4980, observed: 935.4952.

Identification of Component Monosaccharides 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 exchange resin (Amberlite IR-410) and concentrated *in vacuo*. The residue was trimethylsilylated 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 methanolysate of the glycoside.

The absolute configurations of the component monosaccharides were determined according to the method reported by Hara *et al.*[11] Thus, a glycoside (5 mg) was hydrolyzed with 1 N HCl. After neutralization with Amberlite 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 derivatives.

GC-CI-MS Analysis of the Methylated Sugars Derived from the Fully Methylated Glycosides Compound **1** (10 mg) was fully methylated according to the method reported by Hakomori,[12] and the product was purified by column chromatography on silica gel. The permethylate was dissolved in 1 N HCl–MeOH (1 ml) and the solution was refluxed for 3 h. After neutralization with Ag_2CO_3, the product was acetylated with Ac_2O–pyridine (1 : 1) (0.2 ml) at room temperature. The solvent was blown off by an N_2 stream and the residue was checked by gas-liquid chromatography-chemical ionization mass spectrometry (GC-CI-MS). Methyl glycosides of 2,3,4-tri-*O*-methyl-D-xylopyranose, 3,4,6-tri-*O*-methyl-2-*O*-acetyl-D-glucopyranose, and 3,4-di-*O*-methyl-2-*O*-acetyl-D-fucopyranose were identified by comparison of t_R values and CI-MS patterns with those of authentic samples.

Measurement of the Antiproliferative Activities against Tumor Cell Lines MK-1, HeLa and B16F10 were used as tumor cell lines. Cellular growth was evaluated using the MTT-microculture tetrazolium assay described by Mosmann.[9] MK-1 cells were provided by Prof. M. Katano, Faculty of Medicine, Kyushu University, and HeLa and B16F10 cells were supplied by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku Umiversity.

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