Four New Cycloartane Glycosides from *Aquilegia vulgaris* and Their Immunosuppressive Activities in Mouse Allogeneic Mixed Lymphocyte Reaction

Makiko NISHIDA,[a] Hitoshi YOSHIMITSU,*,[a] Masafumi OKAWA,[b] and Toshihiro NOHARA[b]

[a] *Faculty of Engineering, Kyushu Kyoritsu University; 1–8 Jiyugaoka Yahata-nishi-ku, Kitakyushu 807–8585, Japan: and*
[b] *Faculty of Pharmaceutical Sciences, Kumamoto University; 5–1 Oe-honmachi, Kumamoto 862–0973, Japan.*
Received February 12, 2003; accepted March 12, 2003

Four new cycloartane glycosides, named aquilegiosides C—F, were isolated from the dried aerial parts of *Aquilegia vulgaris*. Their structures were determined by two dimensional (2D) NMR spectroscopic analysis and chemical evidence. Aquilegiosides C—F suppressed the proliferation of lymphocytes in mouse allogeneic mixed lymphocyte reaction with IC_{50}, ranging from 3.7×10^{-5} to 2.2×10^{-4} M.

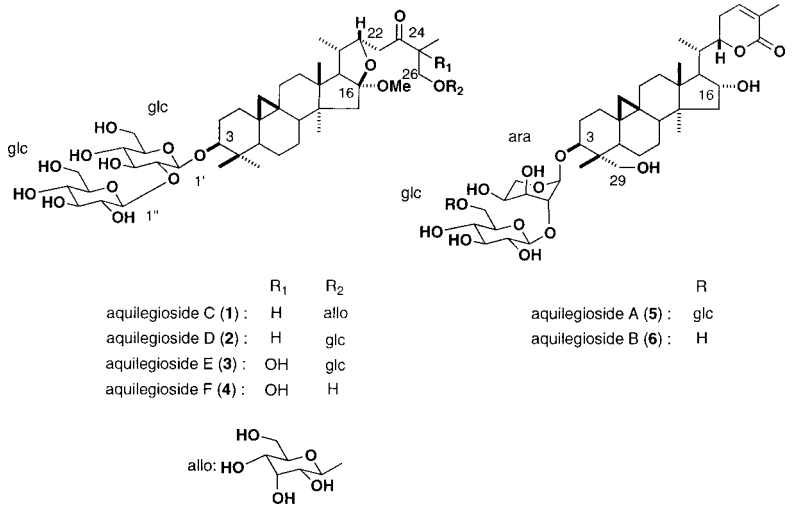
Key words *Aquilegia vulgaris*; cycloartane glycoside; immunosuppressive activity; aquilegioside; Ranunculaceae

Aquilegia vulgaris L. (Japanese name, seiyouodamaki) is cultivated as a garden plant. We previously reported on the structural elucidation of two cycloartane glycosides, aquilegiosides A and B, from *A. flabellata* Sieb. *et* ZUCC. var. *flabellata* (Japanese name, odamaki).[1)] During our investigation on the chemical constituents in the Ranunculaceous plant, we have now isolated four new cycloartane glycosides, aquilegiosides C (**1**), D (**2**), E (**3**) and F (**4**), as well as two known ones, aquilegiosides A (**5**) and B (**6**), from the dried aerial parts of *A. vulgaris*. This paper describes their structural elucidation. Immunosuppressive activities of the isolated cycloartane glycosides in mouse allogeneic mixed lymphocyte reaction are also discussed.

Results and Discussion

The methanolic extract of the air-dried aerial parts of *A. vulgaris* was partitioned into a chloroform-water solvent system. The water-soluble portion was separated by MCI gel CHP20P, octadecyl silica gel (ODS) and silica gel column chromatographies and finally HPLC to give aquilegiosides C (**1**), D (**2**), E (**3**) and F (**4**), together with aquilegiosides A (**5**) and B (**6**).

The molecular formula of aquilegioside C (**1**) was determined as $C_{49}H_{80}O_{20}$ by high resolution (HR)-FAB-MS showing an $[C_{49}H_{80}O_{20}Na]^+$ ion at *m/z* 1011.5156. The IR spectra showed a hydroxyl band (3464 cm^{-1}) and a carbonyl band (1718 cm^{-1}). The ^{1}H-NMR spectra displayed one cyclopropane methylene at δ 0.13 (d, J=4.3 Hz) and 0.52 (d, J=4.3 Hz), four quaternary methyls at δ 1.10, 1.16, 1.17 and 1.34, two secondary methyls at δ 1.13 (J=7.3 Hz) and 1.16 (J=6.7 Hz), three anomeric protons at δ 4.94 (d, J=7.3 Hz), 5.31 (d, J=7.9 Hz) and 5.39 (d, J=7.3 Hz). The above data indicated that **1** was a cycloartane triglycoside derivative. Besides, the chemical shifts of the aglycone moiety except for the signals owing to the side-chain moiety and the D-ring in the ^{13}C-NMR spectra of **1** showed coincidence with those of squarroside I.[2)] A sequence of connectives through a secondary methyl proton at δ 1.13, a methine proton at δ 2.44 (m), an oxygen-bearing methine proton at δ 5.05 (ddd, J=5.5, 7.9, 7.2 Hz) and methylene protons at δ 2.99 (1H, dd, J=5.5, 17.7 Hz) and 3.19 (1H, dd, J=7.9, 17.7 Hz), in turn, was observed in the ^{1}H–^{1}H correlation spectroscopy (COSY), and their signals could be assigned to the H-21, H-20, H-22 and H-23, respectively. Further, the H-20 coupled with a methine proton at δ 2.42 (br s, H-17). The long-range correlations between δ 3.19 (H-23) and δ 211.6 (C-24); a secondary methyl proton at δ 1.16 and the carbon signals at δ 47.2 (CH), 71.6 (CH_2), 211.6 (C-24) indicated that secondary methyl and hydroxy-methyl groups were located at the terminal on the side chain. In addition, the long-range



* To whom correspondence should be addressed. e-mail: Fyoshimit@kyukyo-u.ac.jp

correlation cross peaks between δ 2.42 (H-17) and δ 119.7 (C-16); δ 5.05 (H-22) and δ 119.7 (C-16); the methoxy proton at δ 3.36 and δ 119.7 (C-16) resulted in the five-membered acetal ring between C-16 and C-22 (Fig. 1). The nuclear overhauser and exchange spectroscopy (NOESY) and nuclear overhauser effect difference spectra (NOEDS) suggested the stereo configuration for the structure of **1** to be as shown in Fig. 2. At the present time, the configuration at C-25 is uncertain. On acid hydrolysis, **1** afforded D-glucose and D-allose, whose structures were confirmed by the ^{1}H-NMR coupling pattern (Table 1) and specific rotations using chiral detection in the HPLC analysis. The NMR data could be assigned with the aid of ^{1}H–^{1}H COSY, heteronuclear multiple quantum coherence (HMQC), total correlation spectroscopy (TOCSY) and heteronuclear multiple bond correlation spectroscopy (HMBC) experiments. In the HMBC experiment, the anomeric proton signals at δ 4.94 (H-1′), 5.39 (H-1″) and 5.31 (H-1‴) showed long-range correlations with the carbon signals at δ 88.7 (C-3), 83.4 (C-2′) and 71.6 (C-26), respectively (Fig. 3). From the above evidence, the structure of **1** was concluded to be 26-*O*-β-D-allopyranosyl (16*S*,20*S*,22*S*)-16β,22-epoxy-16α-methoxy-3β,26-dihydroxy-cycloartan-24-one 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

In the ^{1}H- and ^{13}C-NMR data (Tables 1, 2) of aquilegioside D (**2**) ($C_{49}H_{80}O_{20}$), the signals due to the aglycone moiety and the sugar moiety, attached to the C-3 hydroxyl group, were in good agreement with those of **1**, although the signals due to the sugar moiety attached to the C-26 hydroxyl group were not identical. On acid hydrolysis, **2** afforded D-glucose and L-arabinose together with several unidentified artificial sapogenols. The above data indicated a glucosyl unit, attached to the C-26 hydroxyl group, in **2**. The NMR data of **2**, which could be assigned with the aid of ^{1}H–^{1}H COSY, HMQC, TOCSY and HMBC techniques, showed signals due to the trisaccharide moiety consisted of three glucopyranosyl moieties [δ 4.88 (d, J=7.9 Hz, H-1‴), 4.95 (d, J=7.3 Hz, H-1′) and 5.39 (d, J=7.3 Hz, H-1″)]. The HMBC experiment of **2** showed the same result as that of **1** except for the long-range correlation between H-1‴ of the glucopyranosyl moiety and C-26 of the aglycone moiety. From the data presented above, the structure of **2** was concluded to be 26-*O*-β-D-glucopyranosyl (16*S*,20*S*,22*S*)-16β,22-epoxy-16α-methoxy-3β,26-dihydroxy-cycloartan-24-one 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

The molecular formula of aquilegioside E (**3**) was determined as $C_{49}H_{80}O_{21}$ by HR-FAB-MS showing an $[C_{49}H_{80}O_{21}Na]^+$ ion at m/z 1027.5081. The ^{1}H-NMR spectra displayed one cyclopropane methylene at δ 0.13 (d, J=3.7 Hz) and 0.50 (d, J=3.7 Hz), five quaternary methyls at δ 1.09, 1.17, 1.19, 1.35 and 1.56, a secondary methyl at δ 1.18 (J=7.3 Hz), three anomeric protons at δ 4.93 (d, J=7.9 Hz), 4.97 (d, J=7.3 Hz) and 5.41 (d, J=7.3 Hz). The ^{1}H-NMR data are indicative of **3** being a cycloartane triglycoside closely related to **2**. On acid hydrolysis, **3** afforded D-glucose together with several unidentified artificial sapogenols. In a comparative study of the ^{1}H-NMR data of **3** with that of **2**, **3** showed the quaternary methyl at δ 1.56 instead of the secondary methyl at δ 1.19 (d, J=6.7 Hz) in **2**. Furthermore, a sequence of connectives through a secondary methyl proton at δ 1.18 (J=7.3 Hz, H-21), a methine proton at δ 2.53 (m, H-20), a hydroxy-methine proton at δ 5.22 (ddd, J=6.7, 6.7, 7.3 Hz, H-22) and methylene protons at δ 3.52 (1H, dd, J=6.7, 18.3 Hz, H-23) and 3.60 (1H, dd, J=6.7, 18.3 Hz, H-23), in turn, was observed in the ^{1}H–^{1}H COSY. Meanwhile, the molecular formula $C_{49}H_{80}O_{21}$ was higher by O_1 than that of **2**. The above data indicated the presence of an additional hydroxyl group, linked to the C-25, in **3**. The long-range correlations between the quaternary methyl proton at δ 1.56 and the carbon signals at δ 77.2 (CH_2, C-26), 79.8 (C, C-25) and 214.3 (C, C-24) elucidated the terminal structure on the side chain. Moreover, the long-range correlations were observed between δ 2.44 (H-17) and δ 119.6 (C-16), between δ 5.22 (H-22) and δ 119.6 (C-16) and between the methoxy proton at δ 3.36 and δ 119.6 (C-16). In addition, the NOESY and NOEDS of **3** showed the same result as that of **1**. At the present time, the configuration at C-25 is uncertain. The NMR data could be assigned with the aid of ^{1}H–^{1}H COSY, HMQC, TOCSY and HMBC experiments. In the HMBC experiment, the anomeric proton signals at δ 4.97 (H-1′), 5.41 (H-1″) and 4.93 (H-1‴) showed long-range correlations with the carbon signals at δ 88.7 (C-3), 83.6 (C-2′) and 77.2 (C-26), respectively. Consequently, the structure of **3** was determined to be 26-*O*-β-D-glucopyranosyl (16*S*,20*S*,2*S*)-16β,22-epoxy-16α-methoxy-3β,25,26-trihydroxy-cycloartan-24-one 3-*O*-β-D-

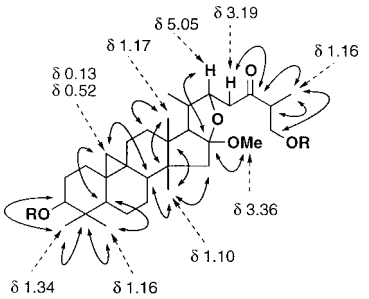


Fig. 1. HMBC Correlations of **1**

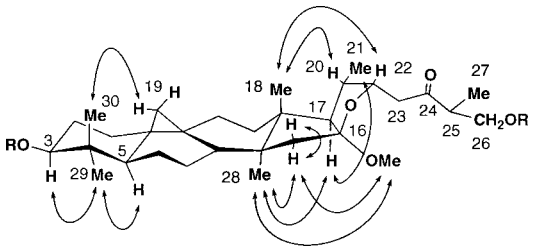


Fig. 2. NOEDS and NOESY Correlations of **1**

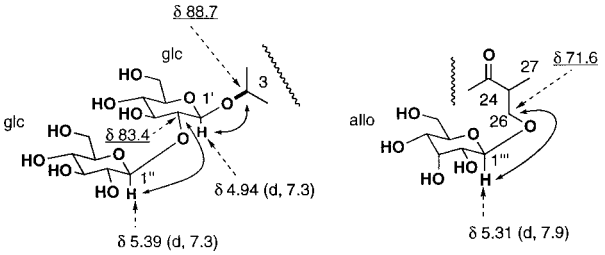


Fig. 3. ^{1}H–^{13}C Long-Range Correlation of the Saccharide Moieties of **1**

J values (Hz) in the ^{1}H-NMR spectrum are given in parentheses. Underlined values indicate ^{13}C-NMR chemical shifts.

Table 1. ^{1}H-NMR Chemical Shifts of **1**—**4** (Pyridine-d_5)

	1	**2**	**3**	**4**
H- 1	1.06, 1.41	1.07, 1.41	1.07, 1.41	1.07, 1.40
2	1.83, 2.37	1.84, 2.38	1.86, 2.39	1.86, 2.38
3	3.45 dd (4.3, 11.6)	3.45 dd (4.9, 11.6)	3.46 dd (4.3, 11.5)	3.46 dd (4.3, 11.6)
5	1.22	1.22	1.21	1.23
6	0.70, 1.51	0.69, 1.50	0.68, 1.50	0.69, 1.51
7	1.01, 1.22	0.99, 1.21	1.02, 1.22	1.01, 1.23
8	1.55	1.53	1.54	1.54
11	1.08, 1.91	1.08, 1.90	1.08, 1.91	1.08, 1.90
12	1.39, 1.53	1.38, 1.52	1.40, 1.53	1.40, 1.53
15	1.63 d (13.4)	1.63 d (13.4)	1.65 d (12.8)	1.65 d (13.4)
	1.93 d (13.4)	1.93 d (13.4)	1.93 d (12.8)	1.94 d (13.4)
17	2.42 br s	2.41 br s	2.44 br s	2.45 br s
18	1.17 s	1.17 s	1.19 s	1.18 s
19	0.13 d (4.3)	0.13 d (4.3)	0.13 d (3.7)	0.13 d (3.7)
	0.52 d (4.3)	0.51 d (4.3)	0.50 d (3.7)	0.52 d (3.7)
20	2.44 m	2.41 m	2.53 m	2.57 m
21	1.13 d (7.3)	1.14 d (7.3)	1.18 d (7.3)	1.19 d (7.3)
22	5.05 ddd (5.5, 7.9, 7.2)	5.05 ddd (5.5, 7.2, 7.9)	5.22 ddd (6.7, 6.7, 7.3)	5.22 ddd (6.7, 6.7, 7.3)
23	2.99 dd (5.5, 17.7)	3.00 dd (5.5, 17.1)	3.52 dd (6.7, 18.3)	3.51 dd (6.7, 18.3)
	3.19 dd (7.9, 17.7)	3.21 dd (7.9, 17.1)	3.60 dd (6.7, 18.3)	3.56 dd (6.7, 18.3)
25	3.13	3.17		
26	3.78 dd (5.5, 9.8)	3.87 dd (5.5, 9.8)	4.21 d (11.0)	4.04 d (11.0)
	4.41 dd (7.3, 9.8)	4.43 dd (7.3, 9.8)	4.31 d (11.0)	4.49 d (11.0)
27	1.16 d (6.7)	1.19 d (6.7)	1.56 s	1.62 s
28	1.10 s	1.10 s	1.09 s	1.10 s
29	1.34 s	1.34 s	1.35 s	1.35 s
30	1.16 s	1.16 s	1.17 s	1.17 s
OMe	3.36 s	3.36 s	3.36 s	3.36 s
	glc	glc	glc	glc
H- 1′	4.94 d (7.3)	4.95 d (7.3)	4.97 d (7.3)	4.97 d (7.3)
2′	4.28 dd (7.3, 9.2)	4.26 dd (7.3, 9.2)	4.27 dd (7.3, 9.2)	4.27 dd (7.3, 9.2)
3′	4.32 dd (9.2, 9.2)	4.33 dd (9.2, 9.2)	4.37 dd (9.2, 9.2)	4.37 dd (9.2, 9.2)
4′	4.17 dd (9.2, 9.2)	4.17 dd (9.2, 9.2)	4.19 dd (9.2, 9.2)	4.20 dd (9.2, 9.2)
5′	3.90 m	3.90 m	3.90 m	3.91 m
6′	4.35 dd (4.9, 11.6)	4.34 br d (10.4)	4.32 br d (10.6)	4.34 br d (10.4)
	4.53 dd (2.4, 11.6)	4.54 br d (10.3)	4.63 br d (10.8)	4.55 br d (10.4)
	glc	glc	glc	glc
H- 1″	5.39 d (7.3)	5.39 d (7.3)	5.41 d (7.3)	5.41 d (7.3)
2″	4.13 dd (7.3, 9.2)	4.14 dd (7.3, 9.2)	4.15 dd (7.3, 9.2)	4.16 dd (7.3, 9.2)
3″	4.28 dd (9.2, 9.2)	4.26 dd (9.2, 9.2)	4.27 dd (9.2, 9.2)	4.27 dd (9.2, 9.2)
4″	4.33 dd (9.2, 9.2)	4.32 dd (9.2, 9.2)	4.37 dd (9.2, 9.2)	4.37 dd (9.2, 9.2)
5″	3.92 m	3.95 m	3.96 m	3.95 m
6″	4.44 dd (4.3, 11.0)	4.47 dd (4.3, 11.0)	4.48 br d (10.4)	4.47 br d (10.5)
	4.51 dd (3.0, 11.0)	4.51 br d (10.5)	4.52 dd (3.1, 11.5)	4.53 br d (10.4)
	allo	glc	glc	
H- 1‴	5.31 d (7.9)	4.88 d (7.9)	4.93 d (7.9)	
2‴	3.39 dd (3.1, 7.9)	4.00 dd (7.9, 9.2)	4.00 dd (7.9, 9.2)	
3‴	4.70 dd (2.5, 3.1)	4.21 dd (9.2, 9.2)	4.22 dd (9.2, 9.2)	
4‴	4.20 dd (3.1, 9.8)	4.25 dd (9.2, 9.2)	4.26 dd (9.2, 9.2)	
5‴	4.44 m	3.95 m	3.96 m	
6‴	4.35 dd (4.9, 11.6)	4.37 dd (5.5, 11.5)	4.39 dd (5.5, 11.6)	
	4.50 dd (2.4, 11.6)	4.55 br d (10.4)	4.57 br d (11.0)	

Coupling constants (J in Hz) are given in parentheses.

glucopyranosyl-(1→2)-β-D-glucopyranoside.

The molecular formula of aquilegioside F (**4**) was determined as $C_{43}H_{70}O_{16}$ by HR-FAB-MS showing an $[C_{43}H_{70}O_{16}Na]^+$ ion at m/z 865.4553. In the ^{1}H- and ^{13}C-NMR data (Tables 1 and 2) of **4**, the signals due to the aglycone moiety except for the terminal moiety on the side chain and the sugar moiety, attached to the C-3 hydroxyl group, were in good agreement with those of **3**. On acid hydrolysis, **4** afforded D-glucose together with several unidentified artificial sapogenols. Meanwhile, the molecular formula $C_{43}H_{70}O_{16}$ was lower by $C_6H_{10}O_5$ than that of **3**. The above data suggested that **4** lost a glucosyl unit, attached to the C-26 hydroxyl group, from **3**. The NMR data of **4**, which could be assigned with the aid of ^{1}H–^{1}H COSY, HMQC and HMBC techniques, showed signals due to the disaccharide moiety consisted of two glucopyranosyl moieties [δ 4.97 (d, J=7.3 Hz, H-1′) and 5.41 (d, J=7.3 Hz, H-1″)]. In the HMBC experiment, the anomeric proton signals at δ 4.97 (H-1′) and 5.41 (H-1″) showed long-range correlations with the carbon signals at δ 88.7 (C-3) and 83.6 (C-2′), respectively. Thus, the structure of **4** was determined to be (16*S*,20*S*,22*S*)-16β,22-epoxy-16α-methoxy-3β,25,26-trihydroxy-cycloartan-24-one 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

Table 2. ^{13}C-NMR Data for **1**—**4** (Pyridine-d_5)

	1	**2**	**3**	**4**
C- 1	31.9	31.9	32.0	32.0
2	29.9	29.8	29.9	29.9
3	88.7	88.6	88.7	88.7
4	41.3	41.2	41.3	41.3
5	47.5	47.5	47.6	47.6
6	20.8	20.7	20.8	20.8
7	26.2	26.1	26.2	26.2
8	47.4	47.3	47.4	47.4
9	19.5	19.4	19.5	19.5
10	26.9	26.8	26.9	26.9
11	26.5	26.4	26.5	26.5
12	31.0	30.9	31.0	31.0
13	44.3	44.2	44.3	44.3
14	49.4	49.3	49.4	49.4
15	45.1	45.1	45.3	45.3
16	119.7	119.7	119.6	119.6
17	69.0	69.0	69.1	69.2
18	19.4	19.3	19.4	19.4
19	30.3	30.3	30.4	30.4
20	34.5	34.4	34.5	34.6
21	18.2	18.1	18.2	18.1
22	82.2	82.1	82.2	82.2
23	45.4	45.4	41.8	42.0
24	211.6	211.5	214.3	215.0
25	47.2	47.1	79.8	81.0
26	71.6	71.6	77.2	69.2
27	13.7	13.7	23.1	22.8
28	19.5	19.4	19.4	19.5
29	25.8	25.7	25.8	25.8
30	15.3	15.3	15.4	15.3
OMe	50.1	50.1	50.2	50.1
	glc	glc	glc	glc
C- 1′	104.9	104.8	105.0	105.0
2′	83.4	83.3	83.6	83.6
3′	78.3	78.3	78.4	78.4
4′	71.6	71.6	71.6	71.6
5′	78.0	77.9	78.0	78.0
6′	62.8	62.7	62.8	62.8
	glc	glc	glc	glc
C- 1″	106.0	105.9	106.2	106.2
2″	77.1	77.0	77.2	77.2
3″	78.2	78.2	78.3	78.3
4″	71.7	71.7	71.7	71.7
5″	78.0	77.9	78.0	78.0
6″	62.8	62.7	62.8	62.8
	allo	glc	glc	
C- 1‴	102.3	104.7	105.6	
2‴	72.2	74.9	75.0	
3‴	73.1	78.5	78.8	
4‴	69.0	71.5	71.6	
5‴	76.0	78.5	78.5	
6‴	63.0	62.7	62.7	

Aquilegiosides A (**5**), B (**6**), C (**1**), D (**2**), E (**3**) and F (**4**) were evaluated for their immunosuppressive activity in mouse allogeneic mixed lymphocyte reaction.[3] Aquilegiosides C—F (**1**—**4**) suppressed the proliferation of lymphocytes and the 50% inhibitory concentrations (IC_{50}) were calculated from the dose-dependent curve. These IC_{50} were listed in Table 3. Consequently, we found that the cycloartane glycosides (**1**—**4**), possessing a five-membered acetal ring, had potent suppressive effect against lymphocytes in mice spleen, while the cycloartane glycosides (**5**, **6**) did not show this effect. These results suggested that a five-membered acetal ring of a cycloartane glycoside is essential for expression of the immunosuppressive effect. Moreover, the presence of an additional hydroxyl group linked to the C-25 and the loss of a glucosyl unit attached to the hydroxyl group at C-26 tended to increase the immunosuppressive effect.

Table 3. Inhibitory Effects on Mouse Allogeneic Mixed Lymphocyte Reaction of **1**—**6**

Compounds	IC_{50} (μg/ml)	IC_{50} (μM)
Cyclosporine A	0.05	0.04
Aquilegioside A (**5**)	>1000	
Aquilegioside B (**6**)	>1000	
Aquilegioside C (**1**)	225	227
Aquilegioside D (**2**)	154	155
Aquilegioside E (**3**)	73	72
Aquilegioside F (**4**)	31	37

On the other hand, Hemmi *et al.* reported that cimicifugoside, possessing a five-membered hemiacetal ring, had a suppressive effect on proliferation of mouse lymphocytes stimulated with mitogens such as concanavalin A and lipopolysaccharide.[5]

Experimental

Optical rotations were taken with a JASCO DIP-1000 automatic digital polarimeter. The NMR spectra was measured with a JEOL alpha 500 NMR spectrometer and chemical shifts are given on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard. The IR spectra was measured with a JEOL JIR-6500W spectrometer. The HR-FAB-MS was recorded with a JEOL HX-110 spectrometer. HPLC was carried out using a TSK gel-120A (7.8 mm i.d.×30 cm) column with a Tosoh CCPM pump, Tosoh RI-8010 detector and JASCO OR-2090 detector. Absorbance spectra was recorded on a Bio-Rad Microplate Reader Model 550. TLC was performed on pre-coated Kieselgel 60F_{254} (Merck), and detection was achieved by spraying with 10% H_2SO_4 followed by heating. Column chromatography was carried out on Kieselgel (230—400 mesh, Merck), Sephadex-LH20 (Pharmacia Find Chem. Co. Ltd.) and MCI gel CHP20P (Mitsubishi Chemical Ind.)

Plant Material The plant seeds defined as the seed of *A. vulgaris* were provided by Sakata Seed Corp., Kanagawa, Japan. The plant seeds were cultivated at the Botanical Garden of Kumamoto University.

Extraction and Isolation The dried aerial parts of *A. vulgaris* (1.3 kg) were extracted with MeOH at room temperature for six months, and the extract (308 g) was partitioned in chloroform and water (1 : 1). The water-soluble portion (221 g) was subjected to MCI gel CHP20P column chromatography with MeOH–H_2O (50→60→70→80→90%) to afford ten fractions (fr. 1—fr. 10). Fraction 5 (3 g) was further separated by ODS column chromatography with MeOH–H_2O (40→50→60→70%) to afford two fractions (fr. 11—fr. 12). Fraction 11 was subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5), followed by HPLC with MeOH–H_2O (13 : 9), to furnish aquilegioside E (**3**) (8 mg). Fraction 6 (843 mg) was further separated by ODS column chromatography with MeOH–H_2O (40→50→60→70%) to afford three fractions (fr. 13—fr. 15). Fraction 14 was subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5), followed by HPLC with MeOH–H_2O (13 : 9), to furnish aquilegioside D (**2**) (20 mg). Fraction 15 was subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5), followed by HPLC with MeOH–H_2O (13 : 9), to furnish aquilegioside C (**1**) (21 mg). Fraction 7 (606 mg) was further separated by ODS column chromatography with MeOH–H_2O (40→50→60→70%) to afford two fractions (fr. 16—fr. 17). Fraction 16 was subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5), followed by HPLC with MeOH–H_2O (7 : 3), to furnish aquilegioside F (**4**) (7 mg). Fraction 17 was subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5), followed by HPLC with MeOH–H_2O (13 : 9), to furnish aquilegioside A (**5**) (45 mg). Fraction 10 was subjected to silica gel column chromatography with $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5), followed by HPLC with MeOH–H_2O (7 : 3), to furnish aquilegioside B (**6**) (27 mg).

Aquilegioside C (**1**): A white powder, $[\alpha]_D^{25}$ −28.3° (c=1.08, pyridine). Pos. FAB-MS (m/z): 1011 $[M+Na]^+$. HR-FAB-MS (m/z): 1011.5156 $[M+Na]^+$ (Calcd for $C_{49}H_{80}O_{20}Na$ 1011.5141). IR (KBr) cm^{-1}: 3464 (OH), 1718 (C=O). ^{1}H- and ^{13}C-NMR (pyridine-d_5): Tables 1 and 2.

Aquilegioside D (**2**): A white powder, $[\alpha]_D^{25}$ −31.7° (c=1.07, pyridine).

Pos. FAB-MS (*m/z*): 1011 $[M+Na]^+$. HR-FAB-MS (*m/z*): 1011.5149 $[M+Na]^+$ (Calcd for $C_{49}H_{80}O_{20}Na$ 1011.5141). ^1H- and ^{13}C-NMR (pyridine-d_5): Tables 1 and 2.

Aquilegioside E (**3**): A white powder, $[\alpha]_D^{25}$ −8.6° (*c*=0.43, pyridine). Pos. FAB-MS (*m/z*): 1027 $[M+Na]^+$. HR FAB-MS (*m/z*): 1027.5081 $[M+Na]^+$ (Calcd for $C_{49}H_{80}O_{21}Na$ 1027.5090). ^1H- and ^{13}C-NMR (pyridine-d_5): Tables 1 and 2.

Aquilegioside F (**4**): A white powder, $[\alpha]_D^{25}$ −2.5° (*c*=0.35, pyridine). Pos. FAB-MS (*m/z*): 865 $[M+Na]^+$. HR-FAB-MS (*m/z*): 865.4553 $[M+Na]^+$ (Calcd for $C_{43}H_{70}O_{16}Na$ 865.4562). ^1H- and ^{13}C-NMR (pyridine-d_5): Tables 1 and 2.

Sugar Analysis A solution of each compound (**1**, **2**, **3** or **4**) (1 mg) in 2 N HCl–dioxane (1 : 1, 2 ml) was heated at 100 °C for 1 h. The reaction mixture was diluted with H_2O and evaporated to remove dioxane. The solution was neutralized with Amberlite MB-3 and passed through a SEP-PAK C_{18} cartridge to give a sugar fraction. The sugar fraction was concentrated to dryness *in vacuo* to give a residue, which was dissolved in CH_3CN–H_2O (3 : 1, 250 ml). The sugar fraction was analyzed by HPLC under the following conditions: column, Shodex RS-Pac DC-613 (6.0 mm i.d.×150 mm, Showa Denko, Tokyo, Japan); solvent, CH_3CN–H_2O (3 : 1); flow rate, 1.0 ml/min; column temperature, 70 °C; detection, refractive index (RI) and optical rotation (OR). t_R (min) of sugars were as follow. **1**: D-allose 7.0 (+), D-glucose 7.4 (+). **2**: D-glucose 7.4 (+). **3**: D-glucose 7.4 (+). **4**: D-glucose 7.4 (+). [reference: D-allose 7.0 (positive optical rotation: +), D-glucose 7.4 (positive optical rotation: +)].

Biological Assays Animals: Male BALB/cAnNCrj and C57BL/6NCrj mice were purchased from Japan Charles River Laboratories, Kanagawa, Japan. All animals were used at 5 weeks of age.

Cell Culture Medium: RPMI-1640 (Life Technologies, Inc., Rockville, MD, U.S.A.) was supplemented with 2 mM L-glutamine, penicillin at 100 U/ml, streptomycin at 100 μg/ml, 25 nM HEPES and $NaHCO_3$ at 2 mg/ml. Fetal bovine serum (Life Technologies, Inc., Rockville, MD, U.S.A.) was heat-inactivated at 56 °C for 30 min and added to the medium as indicated.

Mouse Allogeneic Mixed Lymphocyte Reaction: Mouse allogeneic mixed lymphocyte reaction was carried out by culturing BALB/c mouse spleen cells (4×10^5 cells, responder) and an equal number of C57BL/6 mouse spleen cells treated with mitomycin C at 40 μg/ml for 30 min at 37 °C (stimulator) in 200 μl of RPMI-1640 medium containing 5×10^{-5} M 2-mercaptoethanol, 10% fetal bovine serum and a variable amount of test substance. The cells were placed in a 96-well flat-bottomed microtest plate (No. 3072 Falcon, Becton Dickinson, Lincoln Park, NJ) and cultured for 4 d at 37 °C in an atmosphere of 5% CO_2. After 4 d, 10 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8)[4] solution was added to each well and cultured for 1 d at 37 °C in an atmosphere of 5% CO_2. The absorbance at a wavelength of 450 nm was measured with a microplate reader. The results were expressed as IC_{50} values. WST-8 assay was performed using a cell counting kit-8 (Dojindo Laboratories).

Acknowledgements We are grateful to Mr. K. Takeda and Mr. T. Iriguti of Kumamoto University for measurement of NMR spectra. We are grateful to Prof. H. Okabe and Dr. T. Nagao in Department of Pharmaceutical Sciences, Fukuoka University, for measurements of HR-FAB-MS.

References

1) Yoshimitsu H., Nishida M., Hashimoto F., Nohara T., *Phytochemistry*, **51**, 449—452 (1999).
2) Yoshimitsu H., Nishida M., Qian Z.-Z., Lei Z.-H., Nohara T., *Chem. Pharm. Bull.*, **48**, 828—831 (2000).
3) Bach F. H., Widner M. B., Bach M. L., Klein J., *J. Exp. Med.*, **136**, 1430—1444 (1972).
4) Ishiyama M., Miyazono Y., Sasamoto K., Ohkura Y., Ueno K., *Talanta*, **44**, 1299—1305 (1997).
5) Hemmi H., Ishida N., *J. Pharmacobio-Dyn.*, **3**, 643—648 (1980).