Structures of Three New Oleanene Glucuronides Isolated from *Lathyrus palustris* var. *pilosus* and Hepatoprotective Activity[1)]

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Three new saponins, named palustrosides I, II and III, together with azukisaponins II, V and soyasapogenol B monoglucuronide, were isolated from the aerial parts of *Lathylus palustris* L. var. *pilosus* LEDEB. The structures of palustrosides I, II and III were identified as 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosides of soyasapogenol E, abrisapogenol E, and bredemolic acid 28-*O*-β-D-glucopyranoside, respectively, by spectroscopic and chemical methods. As part of our studies on hepatoprotective drugs, we also examined the hepatoprotective effects of these saponins towards immunologically induced liver injury in primary cultured rat hepatocytes. The activity of the disaccharide group was greater than that of the trisaccharide group. This information regarding the structure–activity relationships substantiated previously obtained data. Structure–hepatoprotective relationships for the sapogenol moiety suggested that the hydroxyl group at C-30 reduces the hepatoprotective effect. On the other hand, the carbonyl group at C-22 may be equivalent to a hydroxyl group at C-22 in terms of hepatoprotective action. Oleanolic acid-type saponins also exhibited hepatoprotective action.

Key words *Lathylus palustris* var. *pilosus*; Leguminosae; triterpenoidal saponin; oleanene glucuronide; hepatoprotective activity; primary cultured rat hepatocyte

Lathylus palustris L. var. *pilosus* LEDEB. (Leguminosae) is used not only as forage, but also as a folk remedy against nephritic disease.[2)] Although some flavonoids have been isolated from this plant,[3)] there are no reports concerning saponins. As part of our studies on the chemical constituents of leguminous plants,[1)] we investigated oleanene-type triterpene glucuronides (oleanene glucuronides) in the aerial parts of the titled plant. Furthermore, in a series of studies on hepatoprotective drugs,[4)] we examined the structure-hepatoprotective relationships of the obtained oleanene glucuronides. Herein, we describe the structural elucidation of three new oleanene glucuronides and their hepatoprotective activities.

A methanolic extract of the aerial parts of *L. palustris* var. *pilosus* was separated by Sephadex LH-20 column chromatography to afford the total saponin fraction after several solvent partitions. After repeated silica gel chromatography of the total saponin fraction, saponins **1**—**6** were obtained. Saponins **4**—**6** were identified as azukisaponins II,[5)] V,[5b,6)] and soyasapogenol B monoglucuronide,[7)] respectively.

Palustroside I (**1**) showed an $[M-H]^-$ ion peak at *m/z* 793 in the negative ion FAB-MS. Exact measurement under high-resolution (HR) conditions indicated that the composition is $C_{42}H_{65}O_{14}$ at *m/z* 793.4380 $[M-H]^-$ in the negative FAB-MS. Acid hydrolysis of **1** gave soyasapogenol E,[8)] glucuronic acid (glcA) and glucose (glc). The absolute configurations of the sugars were determined to be D-form, according to the procedure developed by Hara *et al.*[9)] In the ^{13}C-NMR spectrum of **1** (Tables 1, 2), the signals for the aglycone moiety were in good agreement with those of dehydrosoyasaponin I.[10)] Since the sugar signals were identical with those of **4**, the structure of **1** was elucidated to be 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl soyasapogenol E.

Palustroside II (**2**) furnished abrisapogenol E,[11)] D-glcA and D-glc in the manner described above. In the negative and HR/negative FAB-MS, **2** showed a peak at *m/z* 811 due to $[M-H]^-$ and at *m/z* 811.4484 $[M-H]^-$ ($C_{42}H_{67}O_{15}$), respectively. In the ^{13}C-NMR spectrum (Tables 1, 2), the signals due to the sugar moiety of **2** were in agreement with those of **1**. On the other hand, the signals due to the aglycone moiety were superimposable on those of wistariasaponin B_2.[11b)] Consequently, the structure of **2** was concluded to be 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl abrisapogenol E.

Palustroside III (**3**) showed an $[M-H]^-$ ion peak at *m/z* 971 in the negative FAB-MS. The HR/negative FAB-MS study indicated that the composition is $C_{48}H_{75}O_{20}$ at *m/z* 971.4830 $[M-H]^-$. Acid hydrolysis of **3** furnished the same sugar units as **1** and **2**. However, the obtained sapogenol was completely different from those of **1** and **2**. In the ^{13}C-NMR spectrum of **3**, the signals for the A–B rings of sapogenol and the sugar signals linked at C-3 were superimposable on those of **1** and **2**, whereas the remaining signals were identical with those of a 28-*O*-β-D-glucopyranosyl hederagenin derivative.[12)] Therefore, the sapogenol of **3** was assumed to be bredemolic acid,[13)] which has been isolated from *Bredemeyera floribunda*. Since the ^{13}C-NMR signals for the aglycone moiety of **3** were consistent with the reported data,[13b)] except for C-2—4 due to glycosylation,[14)] the structure was characterized as 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl bredemolic acid 28-*O*-β-D-glucopyranoside.

Next, we compared the hepatoprotective actions of these new compounds (**1**—**3**) with the two known ones (**4**, **5**). In a previous paper,[4a)] we reported that the activity of alanine aminotransferase (ALT) in the medium was in good agreement with the extent of hepatocyte damage induced by immunological liver injury. Therefore, cell damage was evaluated by means of ALT activity. Hepatoprotective activity is summarized in Table 3. All tested compounds exhibited protective activity. In particular, the activity of the disaccharide group of compounds (**1**—**4**) was greater than that of the trisaccharide group (**5**). This information substantiated previously obtained structure–activity relationship data.[4d)] Furthermore, we have already reported that the hepatoprotective activity of **6** was comparable with that of a disaccharide (soy-

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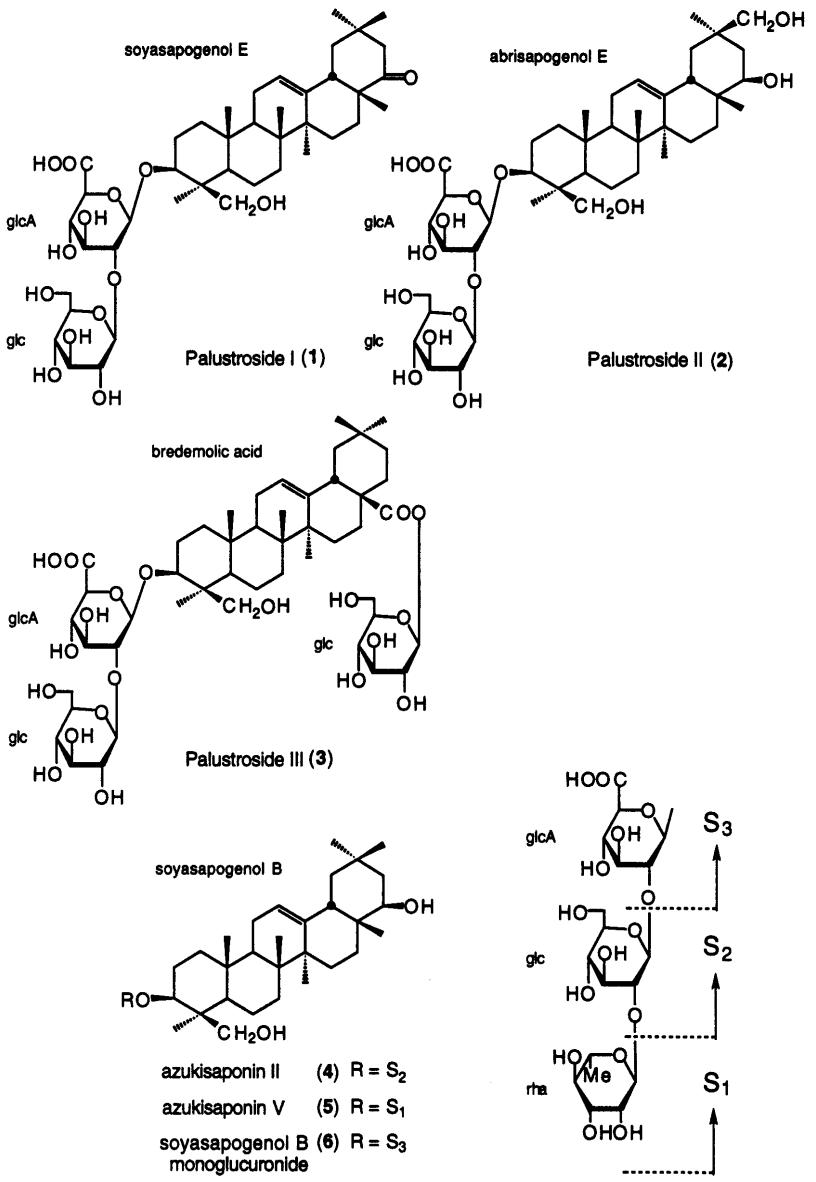

asaponin III).[4b] When the actions of **1** and **4** were compared, they were very similar at any dosage. Therefore, the carbonyl group at C-22 must be equivalent to the hydroxyl group at C-22 in terms of hepatoprotective effect. In contrast, the action of the hydroxyl derivative (**2**) at C-30 of **4** was depressed compared with that of **4**. Since we reported a similar effect for the hydroxyl group at C-29,[4e] the hydroxymethyl group at C-20 seems to reduce the hepatoprotective action, regardless of configuration. On the other hand, oleanolic acid-type saponin (**3**) which has a glucosyl carboxy group at C-28 also exhibited hepatoprotective action, although it was weaker than **1**, **2** and **4**, which have a methyl group at C-28.

Experimental

The instruments and reagents used in this study were the same as those described in the previous papers.[4a,15]

Extraction and Isolation The aerial parts (1.2 kg) of *L. palustris* var. *pilosus* collected in the medicinal garden of Hokkaido University were extracted with MeOH, and the extract (50 g) was partitioned between EtOAc and 40% MeOH after partition with *n*-hexane and 80% MeOH. The 40% MeOH layer (39 g) was separated by Sephadex LH-20 column chromatography to give the total saponin fraction. After silica gel column chromatography using $CHCl_3$: MeOH : H_2O=7 : 3 : 0.5→6 : 4 : 1 to afford fractions 1 to 6, these fractions were separated by silica gel (1-BuOH : AcOH : H_2O=4 : 1 : 5, upper) to provide compounds **1** (0.001%), **2** (0.0007%), **3** (0.0007%), **4** (0.009%), **5** (0.003%) and **6** (0.002%), respectively. Azukisaponins II[5] (**4**), V[5b,6] (**5**) and soyasapogenol B monoglucuronide[7] (**6**) were identified by comparison of their physical data {$[\alpha]_D$, FAB-MS, ^{1}H-NMR and ^{13}C-NMR (Tables 1, 2)} with reported values.

Compound 1 (Palustroside I) A white powder, $[\alpha]_D^{25}$ −9.8° (c=0.52, pyridine : H_2O=1 : 1), HR negative ion FAB-MS *m/z*: 793.4380 ($C_{42}H_{65}O_{14}$, Calcd for 793.4374). Negative ion FAB-MS *m/z*: 793 $[M-H]^-$, 631 $[M-H-glc]^-$. ^{1}H-NMR (in pyridine-d_5): 0.72, 0.86, 0.96, 1.17, 1.22, 1.27, 1.38 (each 3H, s, *tert*-Me×7), 5.24 (1H, s, H-12), 5.59 (1H, d, J=7 Hz, glc H-1). ^{13}C-NMR: Tables 1 and 2.

Identification of Sugars Present in 1—3 A small sample of **1** was hydrolyzed in 2 N HCl/H_2O at 90 °C for 2 h. After filtration of the mixture, the filtrate was neutralized with 2 N NaOH/H_2O. After desalting, the sugar mixture was subjected to TLC analysis [TLC, Kieselgel 60 F_{254} (Merck Art 5554), $CHCl_3$: MeOH : H_2O=6 : 4 : 1, *Rf*s: 0.07 (glcA), 0.41 (glc)]. According to the above method, the sugars for **2** and **3** were also identified as being composed of the same units.

D, L Determination of Sugars Present in 1—3 The absolute configuration of glucuronic acid was determined after $NaBH_4$ reduction, according to Tanaka *et al.*[16] A sample of **1** (1 mg) was methylated in ethereal CH_2N_2. To a methanolic solution of this methylated sample was added $NaBH_4$ (*ca.* 5 mg), and the mixture kept at room temperature for 30 min. The reaction mixture was worked up with MCI gel CHP 20P. The MeOH eluate was evaporated and heated in 2 N HCl/H_2O at 90 °C for 2 h. The hydrolysate was subjected to MCI gel CHP 20P and Amberlite IRA-400 to give a sugar fraction.

Table 1. ^{13}C-NMR Data for Compounds **1**—**6** (Aglycone Moieties)

	1	2	3	4	5	6
C-1	38.5	38.7	38.6	38.7	38.6	38.7
2	26.6	26.5	26.3	26.3	26.5	26.4
3	90.7	91.0	91.1	91.6	90.8	89.0
4	43.8	43.7	43.6	43.7	43.7	44.4
5	56.1	56.3	56.2	56.3	56.2	56.1
6	18.5	18.7	18.6	18.6	18.3	18.9
7	33.0	33.4	33.4	33.4	33.2	33.4
8	39.8	39.9	39.8	39.9	40.0	40.0
9	47.8	47.8	47.8	47.8	47.7	47.8
10	36.4	36.4	36.5	36.4	36.5	36.6
11	24.0	24.0	23.3	24.0	24.1	24.1
12	123.0	123.0	122.8	122.6	122.4	122.5
13	141.8	144.4	144.2	144.6	144.9	144.8
14	42.0	42.4	42.1	42.4	42.4	42.4
15	26.0	26.3	28.2	26.4	26.5	26.9
16	27.3	19.0	23.3	28.9	28.7	28.7
17	47.8	38.1	47.2	38.0	38.0	38.0
18	47.5	45.5	41.7	45.6	45.3	45.3
19	46.7	42.0	46.3	46.8	46.8	46.8
20	34.1	35.9	30.8	30.8	31.0	30.9
21	50.9	38.3	34.0	41.9	42.4	42.3
22	215.6	75.3	32.5	75.7	75.6	75.6
23	22.5	22.7	22.6	22.8	22.7	23.3
24	63.3	63.4	63.3	63.3	63.4	63.3
25	15.6	15.7	15.4	15.7	15.8	15.5
26	16.7	17.1	17.3	17.1	17.0	17.1
27	25.5	25.6	26.1	25.5	25.8	25.7
28	20.9	21.0	177.2	20.9	21.2	21.2
29	31.8	28.0	33.2	32.9	33.4	33.3
30	25.3	70.1	23.7	28.8	28.7	28.7

Chemical shifts (δ: ppm) were measured in pyridine-d_5.

Table 2. ^{13}C-NMR Data for Compounds **1**—**6** (Sugar Moieties)

	1	2	3	4	5	6
glcA C-1	105.1	104.5	104.3	104.9	105.0	106.5
2	81.1	80.8	80.5	78.4[a]	81.7	75.4
3	76.1[a]	75.3[a]	75.3[a]	75.7	76.1[a]	78.1[a]
4	72.9	73.1	73.0	73.9	72.9	73.5
5	78.2[b]	78.0[b]	77.9[b]	78.3[a]	78.2[b]	78.1[a]
6	172.4	175.9	174.0	175.4	174.8	172.4
glc C-1	104.8	104.2	104.0	101.8	104.3	
2	75.8[a]	75.4[a]	75.3[a]	78.7[a]	75.8[a]	
3	78.4[b]	77.8[b]	77.9[b]	15.9	78.4[b]	
4	69.9	70.1	70.1	70.0	69.9	
5	78.2[b]	78.0[b]	77.9[b]	77.7[a]	78.2[b]	
6	61.6	61.7	61.6	61.4	61.2	
rha C-1				101.9		
2				72.1		
3				72.2		
4				74.0		
5				69.4		
6				18.7		
28-glc C-1			95.6			
2			73.7			
3			78.1[b]			
4			70.8			
5			78.9[b]			
6			61.8			

a, *b*) In each vertical column, these may be interchanged.

This fraction was dissolved in pyridine (0.1 ml) and added to a pyridine solution (0.2 ml) of L-cysteine methyl ester hydrochloride (0.1 mol/l) and warmed at 60 °C for 2 h. The solvent was evaporated under an N_2 stream and dried *in vacuo*. The remaining syrup was trimethylsilylated with trimethylsilylimidazole (0.1 ml) at 60 °C for 1 h. After addition of *n*-hexane and H_2O, the *n*-hexane layer was removed and checked by GC. The retention time (t_R) of the peak was 13.4 min (D-glc).

Identification of the Sapogenol of 1 A small sample of **1** was hydrolyzed in 2 N HCl/H_2O at 90 °C for 2 h. After filtration of the mixture, the precipitate was identified as soyasapogenol E[8,10] by TLC. *Rf*s: 0.37 [$CHCl_3$: MeOH (19 : 1)], 0.35 [*n*-hexane : acetone (3 : 1)].

Compound 2 (Palustroside II) A white powder, $[\alpha]_D^{25}$ +0.50° (*c*=0.68, pyridine), HR negative ion FAB-MS *m/z*: 811.4484 ($C_{42}H_{67}O_{15}$, Calcd for 811.4480). Negative ion FAB-MS *m/z*: 811 $[M-H]^-$, 649 $[M-H-glc]^-$. ^{1}H-NMR (in pyridine-d_5): 0.69, 0.91, 1.18, 1.21, 1.25, 1.35 (each 3H, s, *tert*-Me×6), 5.35 (1H, s, H-12), 5.48 (1H, d, *J*=7 Hz, glc H-1). ^{13}C-NMR: Tables 1 and 2.

Identification of the Sapogenol of 2 A small sample of **2** was hydrolyzed in the same manner. The precipitate was identified as abrisapogenol E[11] by TLC. *Rf*s: 0.20 [$CHCl_3$: MeOH (19 : 1)], 0.15 [*n*-hexane : acetone (3 : 1)].

Compound 3 (Palustroside III) A white powder, $[\alpha]_D^{25}$ −4.6° (*c*=0.52, pyridine : H_2O=1 : 1), HR negative ion FAB-MS *m/z*: 971.4830 ($C_{48}H_{75}O_{20}$, Calcd for 971.4851). Negative ion FAB-MS *m/z*: 971 $[M-H]^-$, 809 $[M-H-glc]^-$. ^{1}H-NMR (in pyridine-d_5): 0.62, 0.90, 0.95, 0.99, 1.24, 1.31 (each 3H, s, *tert*-Me×6), 5.41 (1H, s, H-12), 5.47 (1H, d, *J*=7 Hz, glc H-1), 6.18 (1H, d, *J*=9 Hz, 28-glc H-1). ^{13}C-NMR: Tables 1 and 2.

Preparation of Primary Cultured Rat Hepatocytes Liver cells were isolated according to the procedure developed by Berry and Friend.[17] The detailed procedure was described in the previous papers.[4a,c]

Preparation of Antiserum against Rat Hepatocytes The antiserum was prepared according to the method of Shiki *et al.*[18] An antibody to the 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.[19]

Table 3. Hepatoprotective Activity of Compounds **1**—**5**

Substance	Dose (μM)	ALT	
		IU/l	Protection (%)
Control	—	6.33±1.2	—
Palustroside I (**1**)	0 (ref.)	62.75±1.7	—
	10	60.00±3.2	5
	30	58.75±1.3	7
	90	36.75±2.4*	46
	200	14.00±2.4*	86
	500	16.00±1.4*	83
Palustroside II (**2**)	0 (ref.)	70.25±3.6	—
	10	68.25±2.5	3
	30	69.75±2.8	1
	90	64.75±2.1	9
	200	35.50±2.6*	54
	500	18.25±1.7*	81
Palustroside III (**3**)	0 (ref.)	66.25±2.3	—
	10	65.00±1.8	2
	30	66.00±2.4	0
	90	64.75±2.2	3
	200	50.75±1.7*	26
	500	29.00±1.4*	62
Azukisaponin II (**4**)	0 (ref.)	69.75±4.6	—
	10	68.00±3.3	3
	30	64.75±2.2	8
	90	42.50±1.7*	43
	200	13.25±2.1*	89
	500	12.75±2.2*	90
Azukisaponin V (**5**)	0 (ref.)	62.25±4.4	—
	10	60.75±2.6	3
	30	59.75±4.5	4
	90	60.00±3.4	4
	200	59.75±4.6	4
	500	42.75±2.1*	35

Hepatoprotective actions of compounds **1**—**5** toward *in vitro* immunological liver injury in primary cultured rat hepatocytes. Control is the value of hepatocytes which were not treated with the antiserum. Reference (ref.) value treated with the antiserum and not treated with the tested samples. The percent of protection is calculated as {1−(substance−control)/(ref.−control)}×100. Significantly different from ref., effective * $p<0.001$.

Determination of Hepatoprotective Activity One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to the above-prepared medium (300 μl) containing the antiserum against rat plasma membranes (80 μl/ml) and a dimethyl sulfoxide (DMSO) solution (4 μl) of the tested saponins or positive controls (glycyrrhizin and soyasaponin I) [final concentration 0 (reference); 10; 30; 90; 200; 500 μM]. At forty minutes after the antiserum was administered, the medium was withdrawn for determination of ALT. Control is the value of hepatocytes which were not administered the antiserum. The control value was 6.33±1.2 (IU/l). The percent of protection is calculated as {1−(sample−control)/(reference−control)}× 100. The protection percentage for glycyrrhizin and soyasaponin I (positive controls) were 32 and 42% at 500 μM, respectively.

Instrument and Assay Method The activities of ALT were assayed by autoanalyzer COBAS MIRA (Roche) using commercial kits based on the ALT assay method.[20]

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

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