Hepatoprotective and Hepatotoxic Actions of Oleanolic Acid-Type Triterpenoidal Glucuronides on Rat Primary Hepatocyte Cultures[1)]

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The protective effects of oleanolic acid-type saponins and their derivatives on *in vitro* immunological liver injury of primary cultured rat hepatocytes were studied. A known antihepatotoxic saponin (chikusetsusaponin IVa, 1) showed hepatoprotective activity in this model. Although a rhamnosyl derivative (2) of 1 similarly showed hepatoprotective activity, its prosapogenin (5) did not show any hepatoprotective activity. On the contrary, 5 exhibited cytotoxicity toward liver cells. In the absence of antiserum, monodesmosyl saponins showed hepatotoxicity, while the bisdesmosyl saponins except for 1, did not show such hepatotoxicity. In order to clarify the effects of the sugar residues at C-3 and C-28 responsible for hepatoprotective and hepatotoxic actions, oleanolic acid 3-*O*-glucuronide (2a) and oleanolic acid 28-*O*-glucoside (2b) were prepared and tested. 2b showed neither hepatoprotective action nor hepatotoxicity. In contrast, 2a was effective at 90 μM on hepatoprotection, although it showed strong hepatotoxicity. Oleanolic acid (2c) itself showed both hepatoprotective action and weak hepatotoxicity. Therefore, the hepatoprotective activity of these types of saponins could represent a balance between hepatoprotective action and hepatotoxicity.

Key words hepatoprotective activity; hepatotoxicity; primary cultured rat hepatocyte; immunological liver injury; triterpenoidal saponin; oleanolic acid

Some oleanane-type triterpenoidal saponins are known to exhibit hepatoprotective action. Among them, glycyrrhizin[2)] and saikosaponins[3)] are the most well-known. Besides these compounds, some oleanolic acid-type saponins have also exhibited similar hepatoprotective activity.[2b,2d,2e,4)] In a series of studies on hepatoprotective drugs, we elucidated the conditions for an *in vitro* assay method using immunological liver injury of rat primary hepatocytes cultures.[5)] We reported the preventive effect of a characteristic oleanane-type triterpenoidal glucuronide in Leguminosae which is defined as an olean-12-ene skeleton with a C-28 methyl group and a glucuronic acid moiety linked at C-3.[6)] In the preceding paper,[6d)] we also confirmed the hepatoprotective action of an oleanolic acid-type glucuronide.

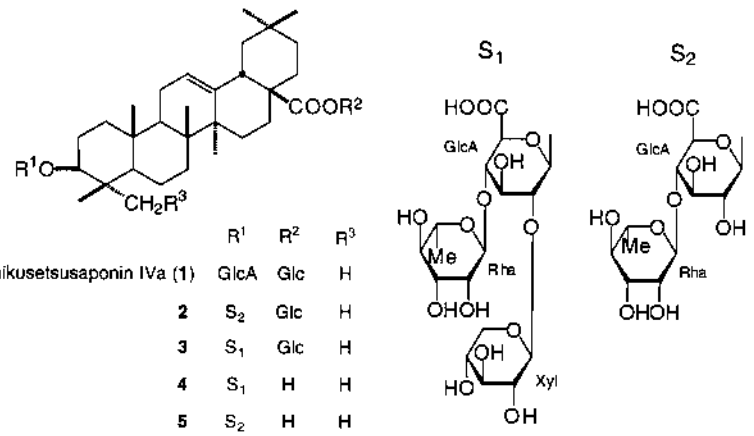
We have isolated six oleanolic acid-type glucuronides (**1**—**6**) from *Dumasia truncata*.[7)] As a part of continuing study, we examined the preventive effects of **1**—**6** using the above method. Furthermore, since one of the saponins showed strong cytotoxicity in these experiments, we also examined the cytotoxicity of **1**—**6** toward hepatocytes without antiserum. Moreover, from the standpoint of the structure–hepatoprotective and –hepatotoxic relationships for the carboxyl group at C-28, oleanolic acid 3-*O*-glucuronide (**2a**) and oleanolic acid 28-*O*-glucoside (**2b**) were prepared and tested.

Herein, we present not only the hepatoptotective actions of oleanolic acid derivatives but also their cytotoxicity toward liver cells, discussing the structure–activity relationships.

Experimental

Instruments and Reagents The instruments and reagents used in this study were the same as those described in the previous papers.[5,7)]

Chemicals The saponins **1**—**6** which had been already isolated from *Dumasia truncata*[7)] were used as the test samples. All other chemicals were



	R^1	R^2	R^3
Chikusetsusaponin IVa (1)	GlcA	Glc	H
2	S_2	Glc	H
3	S_1	Glc	H
4	S_1	H	H
5	S_2	H	H
6	S_2	Glc	OH
Oleanolic acid 3-*O*-glucuronide (2a)	GlcA	H	H
Oleanolic acid 28-*O*-glucoside (2b)	H	Glc	H
Oleanolic acid (2c)	H	H	H

GlcA : β-D-glucopyranosiduronic acid
Glc : β-D-glucopyranosyl

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obtained from commercial sources.[5)]

Oleanolic Acid 3-*O*-glucuronide (**2a**)[8)] and Oleanolic Acid 28-*O*-glucoside (**2b**)[9)]: A solution of **2** (300 mg) in 0.5 N HCl in dioxane–H_2O (1 : 1) was stirred at r.t. for 14 d. The reaction mixture was subjected to Diaion HP-20 column chromatography using H_2O and MeOH. The MeOH eluate was further separated by slica gel column chromatography ($CHCl_3$–MeOH–H_2O) to give **2a** (6 mg) and **2b** (4 mg), which were identified by comparison of their physical data with reported values.

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

Preparation of Primary Cultured Rat Hepatocytes Liver cells were isolated according to a procedure developed by Berry and Friend.[10)] The detailed procedure was described in the previous paper.[5,11)]

Preparation of Antiserum against the Rat Hepatocytes The antiserum was prepared according to the method of Shiki *et al.*[12)] 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.[13)]

Determination of Hepatoprotective Activity of Saponins toward *in Vitro* Immunological Liver Injury 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 dimethyl sulfoxide (DMSO) solution (4 μl) of the test samples or glycyrrhizin [final concentration 0 [reference (Ref.)]; 10; 30; 90; 200; 500 μM]. Forty minutes after the antiserum was administered, the medium was withdrawn for determination of ALT (Alanine aminotransferase). Control is the value of hepatocytes which were not administered the antiserum. The control value for **1**—**6** was 7.25±1.8 (IU/l). The values of **1** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 39.25±1.6, 39.50±2.9, 38.75±5.0, 36.25±2.2, 36.00±2.2, 32.75±3.3 (IU/l), respectively. The values of **2** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 44.75±3.5, 42.75±3.5, 44.75±4.1, 45.50±2.4, 45.50±2.4, 33.50±2.5 (IU/l), respectively. The values of **3** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 35.25±2.9, 36.50±1.3, 36.25±1.5, 34.00±1.4, 27.25±1.9, 22.00±3.4 (IU/l), respectively. The values of **4** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 35.50±1.7, 33.25±1.7, 32.75±3.1, 32.50±2.5, 35.25±1.5, 29.25±1.3 (IU/l), respectively. The values of **5** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 31.00±2.2, 32.50±1.7, 31.00±1.2, 33.00±1.8, 32.00±0.8, 101.80±1.7 (IU/l), respectively. The values of **6** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 35.25±2.5, 35.00±1.8, 35.75±1.7, 34.75±1.0, 33.25±2.2, 30.00±1.8 (IU/l), respectively. The control value for **2a**—**c** was 3.50±1.0 (IU/l). The values of **2a** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 37.50±3.7, 29.50±3.7, 30.25±3.2, 24.25±3.6, 29.50±3.0, 37.00±2.4 (IU/l), respectively. The values of **2b** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 39.50±3.1, 32.25±1.3, 40.75±2.2, 40.25±3.4, 44.50±1.7, 47.50±2.4 (IU/l), respectively. The values of **2c** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 42.25±1.7, 36.75±4.3, 36.25±5.4, 35.50±3.7, 38.75±2.6, 30.50±3.7 (IU/l), respectively. The percent of protection is calculated as {1−(sample-control)/(reference-control)}×100. Reference is the value of hepatocytes which were administered the antiserum and not treated with the test samples. The percent of protection of glycyrrhizin and soyasaponin I (positive controls) was 33% and 44% at 500 μM, respectively.

Determination of Hepatotoxicity of Saponins toward Hepatocytes (without Antiserum) In the same way as above, the cultured cells were exposed to the above-prepared medium (300 μl) containing the DMSO solution (4 μl) of the test samples [final concentration 0 (Ref.); 10; 30; 90; 200; 500 μM]. Forty minutes after the test samples were administered, the medium was withdrawn for determination of ALT. The values of **1** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 22.25±3.8, 23.25±5.1, 23.50±4.7, 23.25±5.7, 29.75±1.9, 90.75±2.8 (IU/l), respectively. The values of **2** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 16.50±1.2, 18.25±2.4, 19.00±2.5, 17.50±3.4, 17.00±2.1, 16.50±3.7 (IU/l), respectively. The values of **3** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 21.00±2.6, 20.25±3.3, 23.75±5.3, 20.25±3.0, 19.75±3.4, 21.50±2.6 (IU/l), respectively. The values of **4** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 16.75±1.2, 16.75±2.4, 17.75±2.5, 16.00±3.4, 19.50±2.1, 29.50±3.7 (IU/l), respectively. The values of **5** at 0 (Ref.), 10, 30, 90, 2200, 500 μM were 24.75±2.5, 27.25±3.0, 29.25±1.5, 84.00±2.4, 94.75±3.9, 92.25±1.7 (IU/l), respectively. The values of **6** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 26.50±2.4, 23.75±3.4, 24.50±2.6, 24.00±4.1, 22.00±2.8, 21.25±2.1 (IU/l), respectively. The values of **2a** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 5.75±1.3, 8.50±2.4, 9.00±2.2, 54.00±2.8, 119.30±1.0, 113.30±2.8 (IU/l), respectively. The values of **2b** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 5.00±0.8, 5.50±0.6, 5.25±0.5, 6.50±0.6, 5.50±0.6, 6.50±0.6 (IU/l), respectively. The values of **2c** at 0 (Ref.), 10, 30, 90, 200, 500 μM were 6.00±0.8, 13.75±1.7, 18.00±2.2, 18.25±1.0, 18.75±3.3, 16.00±4.1 (IU/l), respectively. The percent of cytotoxicity is calculated as (sample/reference)×100. Reference is the value of hepatocytes which were not treated with the tested samples.

Instrument and Assay Method The activities of ALT were assayed by autoanalyzer COBAS MIRA (Roche) using commercial kits based on the ALT assay method.[14)]

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

Results and Discussion

In a previous paper,[5)] we reported that the activity of ALT in the medium was in good agreement with the extent of hepatocytes damage induced by immunological liver injury. Therefore, the cell damage was evaluated by means of ALT

Table 1. Hepatoprotective and Hepatotoxic Activity of Compounds **1**— **6**, **2a**, **2b** and **2c**

Substances	Dose (μM)	Protection (%)[a)]	Cytotoxicity (%)[b)]
1	10	−1	104
	30	−2	106
	90	9	104
	200	10	134
	500	20*	408††
2	10	5	111
	30	0	105
	90	−2	106
	200	−2	103
	500	30*	100
3	10	−4	96
	30	−4	113
	90	4	96
	200	29**	94
	500	47***	102
4	10	8	100
	30	10	106
	90	11	96
	200	1	116
	500	22*	176†
5	10	−6	110
	30	0	118
	90	−8	339††
	200	−4	383††
	500	−298†	373††
6	10	1	90
	30	−2	92
	90	2	91
	200	7	83
	500	19*	80
2a	10	24	148
	30	21	157
	90	39***	939††
	200	24	2075††
	500	1	1970††
2b	10	20	110
	30	−3	105
	90	−2	130
	200	−14	110
	500	−22	130
2c	10	14	229††
	30	15	300††
	90	17	304††
	200	9	313††
	500	30*	267††

a) Hepatoprotective activity of compounds **1**—**6**, **2a**, **2b** and **2c** toward *in vitro* immunological liver injury on primary cultured rat hepatocytes. Significantly different from Reference, effective $*p<0.05$, $**p<0.01$, $***p<0.001$, toxic $\dagger p<0.001$. *b*) Hepatotoxicity of compounds **1**—**6**, **2a**, **2b** and **2c** in primary cultured rat hepatocytes. Significantly different from Reference, toxic $\dagger p<0.01$, $\dagger\dagger p<0.001$.

activity. The results of hepatoprotective action of **1**—**6**, which were isolated from *Dumasia truncata*, are shown in Table 1.

A known antihepatotoxic saponin (**1**)[4b] also showed hepatoprotective activity in this model. Although both a rhamnosyl derivative (**2**) and a rhamnosyl xylosyl derivative (**3**) of **1** similarly showed hepatoprotective activity, the levels of activity depended upon the number of sugar molecules linked at C-3. In a similar manner, the hepatoprotective activity of saponin **4**, which was a prosapogenin of **3**, was less effective than that of **3**. Furthermore, not only did **5** lack hepatoprotective activity, but also it showed strong hepatotoxicity. Since Saito *et al*. also reported a similar result,[4e] monodesmosyl saponin would be less effective than the bisdesmosyl saponin in an *in vitro* model. On the other hand, the action of the hydroxyl derivative (**6**) at C-23 of **2** was slightly depressed compared with that of **2**. Since we reported a similar effect for the hydroxyl group at C-24,[11] the hydroxymethyl group at C-4 seems to reduce the hepatoprotective action, regardless of configuration.

Since **5** showed strong cyototoxicity at the highest dose, the cytotoxicity of **1**—**6** toward liver cells was also examined without antiserum (Table 1). Both monodesmosyl saponins (**4**, **5**) showed hepatotoxicity. In contrast, the bisdesmosyl saponins (**2**, **3**, **6**), except for **1**, did not show any hepatotoxicity. When the hepatotoxicity of **4** and **5** was compared, **5** was much more toxic than **4**. Since **1** also showed some hepatotoxicity, even though it was bisdesmoside, the hepatotoxicity might depend on the number of sugar molecules. In order to clarify the effects of the sugar residues at C-3 and C-28 responsible for hepatoprotective and hepatotoxic actions, oleanolic acid 3-*O*-glucuronide (**2a**) and oleanolic acid 28-*O*-glucoside (**2b**) were prepared and tested (Table 1).

Oleanolic acid 28-*O*-glucoside (**2b**) showed neither hepatoprotective action nor hepatotoxicity. In contrast, oleanolic acid 3-*O*-glucuronide (**2a**) was slightly effective at 90 μM on hepatoprotection, although it showed the strongest hepatotoxicity at higher dose. Similarly to the results of Hikino *et al*.,[4a] oleanolic acid (**2c**) itself showed both hepatoprotective activity and weak hepatotoxicity. Since some oleanolic acid-type saponins were known to exhibit preventive effects toward *in vivo* liver injury model,[2b,2d,4c] the hepatoprotective activity of these types of saponins could represent a balance between hepatoprotective action and hepatotoxicity.

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