Partial Hydrolysis of Soyasaponin I and the Hepatoprotective Effects of the Hydrolytic Products.[1] Study of the Structure–Hepatoprotective Relationship of Soyasapogenol B Analogs

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As a part of our studies of hepatoprotective drugs, we prepared some soyasapogenol B analogs from soyasaponin I. We examined the hepatoprotective effects of these analogs, using immunologically-induced liver injury, in primary cultured rat hepatocytes. Soyasaponin III and soyasapogenol B monoglucuronide were more effective than soyasaponin I. Both compounds were significantly effective even at 30 μM. The action of soyasapogenol B was almost equal to that of soyasaponin I, although glucuronic acid did not show any activity even at the highest dose (500 μM). When the two compounds were mixed, the hepatoprotective action did not change, compared with soyasapogenol B. Therefore, we concluded that the linkage between glucuronic acid and soyasapogenol B could enhance the hepatoprotective activity.

Key words soyasaponin; soyasapogenol B monoglucuronide; soyasapogenol B; hepatoprotective; immunologically-induced liver injury; primary cultured hepatocyte

Oleanene-type triterpene saponins are known to exhibit hepatoprotective activity. Among the oleanene saponins, glycyrrhizin[2] and saikosaponins[3] are the most well-known. Besides these compounds, some oleanolic acid-type saponins have also exhibited similar hepatoprotective activity.[2d,f,g,4]

Soyasaponin I (**1**)[5] is a representative saponin of leguminous plants and it exhibits a hepatoprotective action.[4d,6] As part of our studies of the chemical constituents of leguminous plants, we have obtained many analogous saponins together with **1** from various leguminous plants.[7] These saponins have been characterized as olean-12-ene-type triterpenes with a C-28 methyl group and a glucuronic acid moiety linked at C-3. Glycyrrhizin also belongs to this group (oleanene glucuronide). In the course of our studies of hepatoprotective drugs, we devised conditions for an *in vitro* assay using immunologically-induced liver injury on primary cultured rat hepatocytes and confirmed the hepatoprotective actions of **1** and the crude saponin of Puerariae Radix.[8] Furthermore, using the same procedure, we compared the hepatoprotective actions of soyasaponins I—IV, which were isolated from *Glycine soya*.[9] The order of potency was soyasaponin III > IV ≫ I > II. In particular, soyasaponin III was markedly effective even at 30 μM. Since soyasaponins I—IV have the same aglycone (soyasapogenol B), the differences in action seem to arise from differences in the sugar units linked at C-3. That is to say, the saponins having a disaccharide group (soyasaponins III and IV) are more potent than those with a trisaccharide group (soyasaponins I and II). Since partial hydolysis of **1** gives soyasapogenol B monoglucuronide together with soyasaponin III, we planned to further investigate the effects of the sugar moiety responsible for their action.

Herein, we describe the partial hydrolysis of soyasaponin I and the hepatoprotective activity of the hydrolytic products, discussing the structure–activity relationships of these analogs.

Results and Discussion

Soyasaponin I (**1**) was purified by column chromatography from commercially available soybean saponin. It was partially hydrolyzed with 0.5 N HCl to afford soyasaponin III (**2**) and soyasapogenol B monoglucuronide (SBMG, **3**); soyasapogenol B (**4**) was obtained by acid hydrolysis of **1** with 2 N HCl.

Next, we examined the hepatoprotective action of these analogs and **1** against immunologically-induced liver injury in primary cultured rat hepatocytes. In a previous

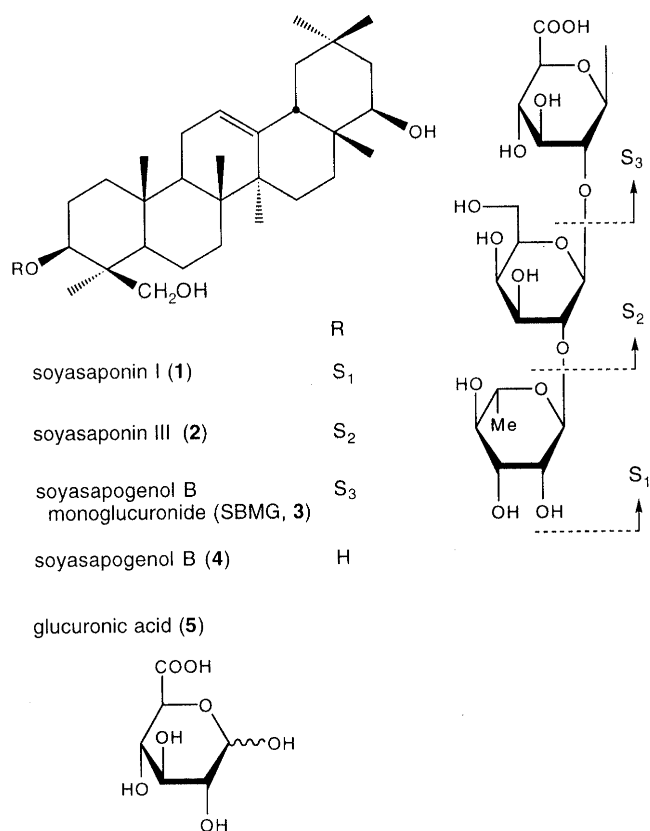

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Table 1. Hepatoprotective Activity of **1**—**5**

Substances	Dose (μM)	ALT I.U./l	Protection (%)
Control	—	13.17±1.5	—
Glycyrrhizin (Positive control)	0 (ref.)	102.75±3.4	—
	10	100.00±4.1	3
	30	105.50±1.0	−3
	90	102.25±4.8	1
	200	91.00±3.2[a]	13
	500	73.50±3.3[a]	33
1	0 (ref.)	103.25±2.2	—
	10	108.00±8.2	−5
	30	106.00±4.9	−3
	90	94.50±3.7	10
	200	87.75±4.6[a]	17
	500	51.50±1.7[a]	57
2	0 (ref.)	104.75±3.8	—
	10	104.25±4.7	1
	30	88.25±2.5[a]	18
	90	54.75±1.7[a]	55
	200	42.25±3.9[a]	68
	500	38.25±1.0[a]	73
3	0 (ref.)	95.00±0.8	—
	10	101.00±4.2	−7
	30	73.25±1.3[a]	27
	90	50.50±3.7[a]	54
	200	29.50±0.6[a]	80
	500	30.00±5.6[a]	79
4	0 (ref.)	102.75±2.5	—
	10	107.50±2.6	−5
	30	104.75±2.5	−2
	90	100.00±3.6	3
	200	95.50±3.0[b]	8
	500	75.25±2.6[a]	31
5	0 (ref.)	113.50±3.7	—
	10	116.50±3.3	−3
	30	123.25±1.7	−10
	90	114.25±5.9	−1
	200	121.25±6.1	−8
	500	117.00±6.1	−3
4+**5**	0 (ref.)	109.00±6.2	—
	10	110.25±3.9	−1
	30	110.00±2.9	−1
	90	102.50±3.4	7
	200	101.25±2.6	8
	500	78.50±5.2[a]	32

Hepatoprotective action of **1**—**5** on *in vitro* immunological liver injury in primary cultured rat hepatocytes. The control is the value for hepatocytes which were not treated with antiserum. The reference (ref.) was treated with antiserum and not with **1**—**5**. The percentage protection is calculated as [1−(substance−control)/(ref.−control)]×100. Significantly different from ref., effective *a*) $p < 0.01$, *b*) $p < 0.05$.

paper,[8] we reported that the activity of alanine aminotransferase (ALT) in the medium correlated well with the extent of hepatocyte damage induced by immunological liver injury. That is, the extracellular aspartate aminotransferase (AST) activity significantly increased up to 5 h, after treatment with antiserum at any of the doses studied. However, 60 min after treatment with any of the doses, most of the cells were stained by Trypan blue, which suggested that the majority of cells had died. However, since extracellular ALT activity reached a plateau following treatment with 40 μl/ml antiserum at 40 min, the ALT value seemed to reflect the liver injury accurately. Therefore, cell damage was evaluated using ALT activity.

The results of the hepatoprotective action of **1**—**3** are shown in Table 1. All these compounds exhibited higher activity than glycyrrhizin (positive control). Furthermore, the actions of **2** and **3** were more effective than that of **1**. Both (**2** and **3**) were effective even at 30 μM, although **3** had a slightly more potent effect than **2** at any dose.

Since SBMG exhibited potent hepatoprotective activity, the action of soyasapogenol B (**4**) and glucuronic acid (**5**) were also tested (Table 1). The action of **4** was almost comparable with that of **1** although the former was slightly less potent. In contrast, **5** did not exhibit any action even at the highest dose (500 μM). When the two compounds were mixed (Table 1), the hepatoprotective action did not change compared with **4**. Therefore, we concluded that the linkage between glucuronic acid and soyasapogenol B could enhance hepatoprotective activity.

The mechanism of this immunological liver injury is regarded as being caused by complement-mediated cell damage.[2b,c] In connection with this, Shinohara *et al.* reported the anti-complement action of soyasaponin I analogs.[10] The order of potency was soyasaponin III (1—2γ)>soyasaponin IV (3—5γ)≥SBMG (5γ)≫soyasaponin I (125γ)≥soyasaponin II (100—150γ). The order was very similiar to the order for the hepatoprotective activity obtained in our present and earlier experiments.[9] Shinohara *et al.* also demonstrated the anti-nephritic action of those analogs in an experimental *in vivo* model. Since some types of inflammation, including hepatitis and nephritis, are caused by excessive immunoreaction, it is possible that the oleanene glucuronides in the soybean and its products may play an important role in the suppression of such inflammation.

Experimental

The instruments and reagents used in this study were the same as those described in the previous paper.[8]

Preparation of Soyasaponin I (1) Saponin from soybeans (20 g) was purchased from Wako Pure Chem. Ind. Co. Ltd. and further purified by column chromatography using MCI gel CHP-20P, successively eluting with H_2O, 50% MeOH and MeOH. The MeOH eluate (14.5 g) was subjected to SiO_2 column chromatography using $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5) to give a fraction containing **1** (5.53 g). This fraction was further purified with Chromatorex ODS (30→65% MeOH) to give **1** (2.2 g), which was identical with an authentic sample.[5]

Preparation of Soyasaponin III (2) and Soyasapogenol B Monoglucuronide (3) A solution of **1** (410 mg) in 0.5 N HCl–dioxane (1 : 1, 60 ml) was heated at 100 °C for 1 h. The reaction mixture was diluted with H_2O and desalted on an MCI gel CHP-20P column, successively eluting with H_2O and MeOH. The MeOH eluate was evaporated *in vacuo*, and the residue (300 mg) was chromatographed over SiO_2 ($CHCl_3$–MeOH–H_2O = 7 : 3 : 0.5) to give **2** (180 mg) and **3** (59 mg).

Soyasaponin III (3)[2,8] White amorphous powder, $[\alpha]_D^{19}$ +19.0° [c=0.11, pyridine–H_2O (1 : 1)]. Time of flight (TOF) MS *m/z*: 819 $[M+Na]^+$. HPLC, conditions see ref. 11, (t_R, 34.4 min) and TLC, Kieselgel 60 F_{254} (Merck Art 5554); $CHCl_3$–MeOH–H_2O (6 : 4 : 1), *Rf* 0.54; *n*-BuOH–AcOH–H_2O (4 : 1 : 5, upper), *Rf* 0.37].

Soyasapogenol B Monoglucuronide (4)[12] $[\alpha]_D^{19}$ +24.3° [c=0.13, pyridine–H_2O (1 : 1)]. TOF MS *m/z*: 657 $[M+Na]^+$. ^{1}H-NMR (in pyridine-d_5) δ: 0.84 (3H, s, H-25), 1.00 (3H, s, H-26), 1.23 (3H, s, H-29), 1.29 (3H, s, H-28), 1.30 (3H, s, H-30), 1.31 (3H, s, H-27), 1.57 (3H, s, H-23), 2.41 (1H, br d, J=10.9 Hz, H-18), 5.17 (1H, d, J=7.6 Hz, glc A H-1), 5.31 (1H, br s, H-12). ^{13}C-NMR: 38.7, 26.9, 89.0, 44.4, 56.1, 19.2, 33.4, 40.0, 47.8, 36.6, 24.1, 122.5, 144.8, 42.4, 26.4, 28.7, 38.0, 45.3, 46.8, 30.9, 42.3, 75.6, 23.3, 63.3, 15.5, 17.1, 25.7, 21.2, 33.3, 28.7 (C-1—30), 106.5, 75.4, 78.1, 73.5, 78.1, 172.6 (glc A C-1—6).

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Preparation of Soyasapogenol B (4) A solution of **1** (100 mg) in 2 N HCl (10 ml) was refluxed for 2 h. The reaction mixture was filtered and the precipitate was washed with H_2O and dried *in vacuo*. The residue (45 mg) was chromatographed over SiO_2 (*n*-hexane–AcOEt = 1 : 1) to give **2** (35 mg) as an amorphous powder, which was identified by TLC of an authentic sample.[5,13] *Rf* 0.24 [$CHCl_3$–MeOH (19 : 1)], 0.42 [*n*-hexane–acetone (2 : 1)].

Preparation of Primary Cultured Rat Hepatocytes Liver cells were isolated according to a procedure developed by Berry and Friend.[14] The details are described in a previous paper.[8]

Preparation of Antiserum against Rat Liver Plasma Membranes Rat liver plasma membranes were prepared according to a procedure developed by Loten *et al.*[15] The details are described in a previous paper.[8]

Determination of Hepatoprotective Activity One day after the isolated rat hepatocytes were plated, the cultured cells were exposed to the above medium (300 μl) containing antiserum against rat plasma membrane (40 μl/ml) and a dimethyl sulfoxide (DMSO) solution (4 μl) of the tested compounds [final concentration 0 (reference), 10, 30, 90, 200, 500 μM]. Forty minutes after rat plasma membranes were treated with antiserum, the enzyme activity (ALT) in the medium was determined.

Instrument and Assay Method The ALT activity was assayed by autoanalyzer, COBAS MIRA (Roche) using commercial kits based on the ALT assay method.[16]

Statistical Analysis The data are shown as means $\pm$ S.D. ($n = 4$). After analysis of variance, Tukey's test was employed to determine the significance of differences between reference and experimental samples.

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