Saponins Isolated from *Allium chinense* G. Don and Antitumor-promoting Activities of Isoliquiritigenin and Laxogenin from the Same Drug[1)]

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Received November 1, 1999; accepted February 2, 2000

Investigation of the Chinese crude drug "Xiebai," the bulbs of *Allium chinense* G. Don (*Liliaceae*), led to the isolation of 2 saponins, xiebai-saponin I (laxogenin 3-*O*-β-xylopyranosyl (1→4)-[α-arabinopyranosyl (1→6)]-β-glucopyranoside) (1) and laxogenin 3-*O*-α-arabinopyranosyl (1→6)-β-glucopyranoside (2), and the aglycone, laxogenin (3), together with 2 chalcones, isoliquiritigenin (4) and isoliquiritigenin-4-*O*-glucoside (5), and β-sitosterol glucoside (6). Compounds 1—5 were tested *in vitro* for their inhibitory effect on the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated ^{32}Pi-incorporation into phospholipids of HeLa cells. In addition to this, laxogenin (3) was proven to have an antitumor-promoting activity in a two-stage lung carcinogenesis experiment.

Key words *Allium chinense*; xiebai-saponin I; isoliquiritigenin; antitumor-promoting activity; laxogenin; *Liliaceae*

Allium chinense G. Don has been cultivated since ancient times, and the bulbs, named "Rakkyo" in Japan, are widely used as pickles and spices. The bulbs of both *Allium chinense* G. Don and *A. macrostemon* Bunge are also main sources of a Chinese traditional medicine "Xiebai", which is used for the treatment for chest pain, stenocardia, and heart asthma. We were the first to report on the chemical constituents of the Chinese crude drug "Xiebai" and the pharmacological activities of 3 kinds of Chinese prescriptions containing "Xiebai" against anti-human platelet aggregation.[2)] Afterwards, Okuyama and Yao *et al.* isolated a number of spirostan and furostan oligoglycosides and sulfur-containing compounds from *A. chinense* and *A. macrostemon*.[3,4)] Along this line, we have examined the antitumor-promoting constituents of *Allium* plants on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-enhanced phospholipid metabolism and isolated laxogenin and N-*p*-coumaroyl tyramine from *A. chinense* as the active principles.[5)] In the present study, we tried to isolate the more active constituents of *A. chinense* by biological-active directed fractionation using a ^{32}Pi-incorporation assay.

The fractions which showed potential activity in the ^{32}Pi-incorporation assay were purified repeatedly, and they gave a new saponin (**1**) and a known one (**2**), laxogenin (**3**) as their aglycone, and 2 chalcones (**4** and **5**) as active constituents. A new compound, xiebai-saponin I (**1**), has the molecular formula $C_{43}H_{68}O_{17}$, as determined by FAB-MS at *m/z* 856 and the quasi molecular ion peak at *m/z* 857 $(M+H)^+$ in the chemical ionization mass spectrum (CI-MS). Investigation of the NMR spectra (see Table 1) suggested that **1** and **2** are glycosides of **3**. From chemical shifts of C_{23-26}, these compounds were ascribable to the (25*R*)-spirostan type. The bands of absorption at 982, 918, 900, and 864 cm^{-1} in the IR spectrum supported the presence of (25*R*)-spirostan. In the NMR spectra of **1**, 3 anomeric protons and carbons were recognized at δ 4.92, 5.02, 5.47, and 102.07, 105.14, 105.73, respectively. All the carbons of the aglycone were assigned in comparison with those of **3** and **2** by distortionless enhancement by polarization transfer (DEPT) and ^{1}H–^{13}C correlation spectroscopy (COSY) spectra. Detailed analysis of the ^{1}H- and ^{13}C-NMR spectra with the aid of ^{1}H detected heteronuclear multiplet bond connectivity (HMBC), double quantum filtered correlation spectroscopy (DQF-COSY) and heteronuclear single quantum coherence (HSQC) experiments and correlations of HSQC-homonuclear Hartmann-Hahn (HOHAHA) method enabled us to make complete assignments on each sugar proton and carbon signal, respectively. One of the anomeric protons at δ 4.92, which is ascribed to that of glucose, correlates with the carbon at δ 76.99 (C_3) in the HMBC method. The carbon signals at δ 79.85 corresponding to C_4 and at δ 68.1 corresponding to C_6 of the glucose moiety are correlated with each anomeric proton at δ 5.47, owing to xylose, and at δ 5.02 owing to arabinose, by the DQF-COSY and HSQC methods. On acid hydrolysis, **1** afforded an aglycone, **3**, and glucose, xylose and arabinose as the sugar components (detected by TLC). Consequently, the structure of **1** was established as laxogenin 3-*O*-β-xylopyranosyl-(1→4)-[α-arabinopyranosyl(1→6)]-β-glucopyranoside. So far, Xiebai-saponin I (**1**) has not been found in any other natural sources. However, Matsuura *et al.* reported that **1** was ob-

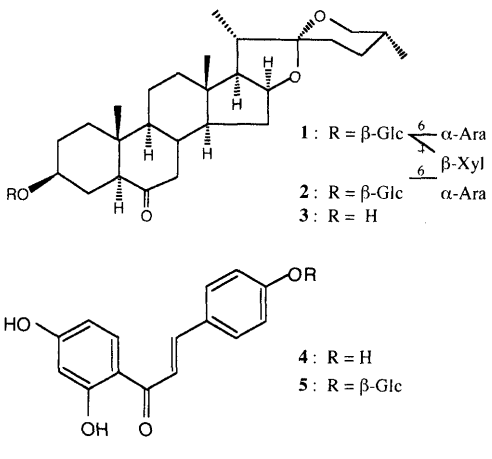


Fig. 1. Structures of Compounds **1**—**5**

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Table 1. ^{13}C-NMR Spectral Data of Xiebai-saponin I (**1**) and the Related Compounds **2** and **3**

	Laxogenin (**3**)	**2**	**1**		**2**	**1**
	Aglycone part				Sugar part	
C-1	36.62	36.82	36.64	Glc-1	102.17	102.07
C-2	30.02	29.58	29.45	-2	75.15	74.86
C-3	70.57	76.85	76.98	-3	78.49	78.49
C-4	30.67	27.02	26.94	-4	72.29	79.82
C-5	56.49	56.48	56.44	-5	76.96	75.00
C-6	210.54	209.48	209.52	-6	69.72	68.10
C-7	46.75	46.74	46.74			
C-8	37.35	37.39	37.34	Ara-1	105.32	105.70
C-9	53.88	53.73	53.67	-2	71.93	72.56
C-10	40.92	41.09	41.08	-3	74.35	74.55
C-11	21.37	21.54	21.55	-4	68.96	69.85
C-12	39.51	39.64	39.62	-5	66.92	67.33
C-13	40.92	40.84	40.84			
C-14	56.78	56.48	56.41	Xyl-1	—	105.73
C-15	31.57	31.82	31.82	-2	—	74.55
C-16	80.44	80.84	80.84	-3	—	76.32
C-17	62.03	62.87	62.82	-4	—	71.09
C-18	16.42	16.44	16.46	-5	—	67.37
C-19	13.22	13.10	13.06			
C-20	41.59	41.99	41.96			
C-21	14.46	14.93	14.99			
C-22	109.32	109.23	109.25			
C-23	31.32	31.82	31.77			
C-24	28.73	29.23	29.22			
C-25	30.26	30.58	30.58			
C-26	66.88	66.29	66.88			
C-27	17.12	17.28	17.30			

1 and **2** were measured in pyridine-d_5 and **3** in $CDCl_3$: δ values in ppm. Multiplicities of carbon signals were determined by the DEPT method. The sugar part was determined by the HMBC and HSQC-HOHAHA methods.

Table 2. Inhibitory Effect of Each Compound Isolated from *A. chinense* on the TPA-Enhanced ^{32}Pi-Incorporation into Phospholipids of HeLa Cells

Sample name	Concentration	Inhibition
Xiebai-saponin I (**1**)	50 μg/ml	#
	25 μg/ml	14.0%
	10 μg/ml	8.3%
Laxogenin diglycoside (**2**)	50 μg/ml	#
	25 μg/ml	28.9%
	10 μg/ml	7.8%
Laxogenin (**3**)	100 μg/ml	82.1%*
Isoliquiritigenin (**4**)	50 μg/ml	100.0%
	25 μg/ml	95.3%
	10 μg/ml	43.3%
Isoliquiritin (**5**)	50 μg/ml	−2.2%
	25 μg/ml	7.6%
	10 μg/ml	7.6%

TPA: 50 nM. HeLa cells were treated with various concentrations of each compound. After 1 h, ^{32}Pi (7.4 kBq/culture) was added with or without TPA (50 nM). Incubation was continued for 4 h, then radioactivity incorporated into phospholipid fraction was measured. Data, expressed as percentages on TPA-enhanced ^{32}Pi incorporation, are mean values of duplicate experiments. # This sample could not be evaluated because it showed strong cytotoxicity to HeLa cells. *: Data taken from Ref. 5.

Table 3. Inhibitory Effect of Laxogenin (**3**) Isolated from *A. chinense* on the Lung Tumor Formation Induced by Glycerol in 4-NQO-Initiated Mice

Condition	Incidence (%)	Tumors per mouse		Inhibition (%)
Control 1 (5% glycerol)	12/12 (100.0)	7.9		
Control 2+HCO60	11/11 (100.0)	7.8	**	0.0
+HCO60+laxogenin	11/12 (90.9)	1.9		75.6

For initiation, 4-NQO dissolved in a mixture of olive oil and cholesterol (20 : 1) was given by a single s.c. injection on the first experimental day (10 mg/kg body weight). Glycerol, as tumor promoter, was dissolved in drinking water at a concentration of 5% and given *ad libirum* from experimental week 5 to 30. Laxogenin was dissolved in 5% glycerol at a concentration of 12.5 ppm during tumor promotion. **: $p>0.01$ (t-test, compared with control 2).

tained by acid hydrolysis of chinenoside I isolated from *A. chinense* G. Don.[6] No antitumor property of this compound was previously reported.

The latter compounds **2**—**6** were characterized by comparison of their spectral data with those in the literature.[6—10]

Incorporation of ^{32}Pi into the phospholipids of HeLa cells was assayed by the same method described previously.[11] As noted in Table 2, chalcone **4** exhibited potential activity comparable to that of **5**, which is a glycoside of **4**. Yamamoto *et al.* reported the antitumor-promoting effect of **4** *in vitro* and *in vivo*.[12] Moreover, various biological activities of compound **4** were also reported, *e.g.* an anti-platelet aggregation effect, inhibition of the aldose reductase, and c-adenosine 5′-diphosphate (ADP) phosphodiesterase.[9,13] **3** showed remarkable activity at a high dose level, while two saponins, **1** and **2**, showed strong cytotoxicity to HeLa cells at the same dose. These results suggest that cytotoxicity might be caused by the structures of the sugar chain in these compounds.

We examined the antitumor-promoting activity of **3** in the mouse lung tumor formation induced by 4-nitroquinoline-1-oxide (4-NQO) as an initiator and by glycerol as a promoter. Polyoxyethylene hydrogenated castor oil (HCO-60), a surface-active agent, was used to dissolve **3**, while it did not affect tumorigenesis in this experiment. As shown in Table 3, oral administration of **3** reduced the average number of tumors per mouse. The tumors per mouse in control 2 group were 7.8, and those in the group treated with **3** were 1.9. The inhibitory effect of **3** was 75.6% ($p<0.01$, t-test) compared with that in the control 2 group at 25 weeks of promotion.

MATERIALS AND METHODS

Plant Material The Chinese crude drug "Xiebai," the bulbs of *Allium chinense* G. Don, were purchased from Mikuni Co., Ltd. (Osaka, Japan).

Extraction and Isolation The materials (20 kg) were extracted twice with hot water. A small volume of the aqueous solution was lyophilized to yield a water-extract (12 kg). The aqueous solution was partitioned into AcOEt- and then *n*-butanol-soluble fractions, which were evaporated *in vacuo*, and gave corresponding portions (198 g, 1.1 kg), respectively. The AcOEt-soluble part (45.5 g) was chromatographed subsequently on silica gel to give 10 fractions (fr. 1—10). Each fraction was monitored on the TPA-stimulated ^{32}Pi-incorporation into the phospholipids of HeLa cells. Those fractions which showed potential activity in this assay were purified repeatedly to give a new (**1**) and a known (**2**) saponin, laxogenin (**3**) as their aglycone, and 2 chalcones (**4** and **5**) as the active constituents. Fraction 8 (24.6 g) was separated by silica gel column chromatography into 11 fractions (fr. 12—22). Then fraction 18 was subjected to a Sephadex LH-20

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column (MeOH, detection of eluates by TLC), which gave two saponins, **1** (463 mg) and **2** (229 mg). Moreover, separation of fraction 16 by the same condition gave **3** (695 mg) as their aglycone. Compounds **1** and **2** were recrystallized from MeOH. **3** was purified by HPLC (silica gel (20×250 mm), *n*-hexane : AcOEt=1 : 4, 8.5 ml/min, t_R 14 min).

Fraction 5 (11.6 g) was followed by silica gel column chromatography and a Sephadex LH-20 column (MeOH, detection by TLC), and that gave chalcone **5** (126 mg). **5** was recrystallized from MeOH–water. **4** was isolated from fraction 2 (9.1 g) by a silica gel column.

Fraction 9 (6.8 g) was separated by a silica gel column, which gave β-sitosterol glucoside (**6**, 112 mg).

Compound 1 (Xiebai-saponin I) (**1**): Colorless needles; mp 265.0—266.0 °C; FAB-MS at *m/z* 879 (M^++Na), 857 (M^++H), 431 (aglycone+H); EI-MS at *m/z* 857 (M^++H). IR ν cm^{-1}: 3440 (OH), 2948, 1708 (C=O), 982, 918, 900, 864. ^{1}H-NMR: δ4.91 (1H, d, J=7.59 Hz, anomeric-H), 5.02 (1H, d, J=7.26 Hz, anomeric-H), 5.47 (1H, d, J=7.92, anomeric-H). ^{13}C-NMR spectrum was shown in Table 1.

Compound 2 (**2**): Colorless needles; mp 265.0—266.0 °C; FAB-MS (Pos.) at *m/z* 725 (M^++1). IR ν cm^{-1}: 3440 (OH), 2948, 1708 (C=O), 982, 918, 900, 864. ^{1}H-NMR: δ4.93 (1H, d, J=7.59 Hz, anomeric-H), 4.94 (1H, d, J=7.26 Hz, anomeric-H). **2** was identified as laxogenin-3-*O*-α-arabinopyranosyl(1→6)-β-glucopyranoside isolated from *Smilax sieboldii* (*Liliaceae*) by comparison of its physical and spectroscopic data.[7]

Compound 3 (**3**) (laxogenin): Colorless needles; mp 214.0—216.5 °C; EI-MS spectrum *m/z* 430 (M^+). **2** was identified as laxogenin isolated from the same material by comparison of its physical and spectroscopic data.[6]

Compound 4 (**4**): Yellow powder; mp 202.0—203.0 °C; **4** afforded the molecular weight at *m/z* 256 in EI-MS spectrum. ^{1}H- and ^{13}C-NMR spectra of **4** suggested a chalcone skeleton (see Table 1). In comparing the spectral data with those values reported,[8] **4** was identified as isoliquiritigenin.

Compound 5 (**5**): Yellow powder; mp 140.0—142.0 °C; On acid hydrolysis, **5** gave **4** as an aglycone and glucose. **5** was identified as isoliquiritin by comparison of its physical and spectroscopic data with the reported values.[9]

Compound 6 (**6**): White powder; mp 284.0—286.0 °C; EI-MS spectrum *m/z* 576 (M^+), 414 (aglycone). **6** was identified as β-sitosterol glucoside by comparison of its physical and spectroscopic data with the previous report.[10]

TPA-Enhanced ^{32}Pi-Incorporation into the Phospholipid of Cultured Cells Incorporation of ^{32}Pi into phospholipids of HeLa cells was assayed by the same method described previously.[11]

Two-Stage Mouse Lung Carcinogenesis Experiments This experiment was performed by the same method as indicated in our previous papers.[13] 1.25 mg of laxogenin (**3**: 2.91 μmol) was dissolved in 5% 100 ml of glycerol solution with HCO-60. Mice in the control group were given 5% glycerol without (control 1) or with (control 2) HCO-60 as a surface-active agent. Each experimental group consisted of 15 mice.

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