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NEW HEPATOPROTECTIVE SAPONINS, BUPLEUROSIDES III, VI, IX, AND XIII, FROM CHINESE BUPLEURI RADIX : STRUCTURE-REQUIREMENTS FOR THE CYTOPROTECTIVE ACTIVITY IN PRIMARY CULTURED RAT HEPATOCYTES

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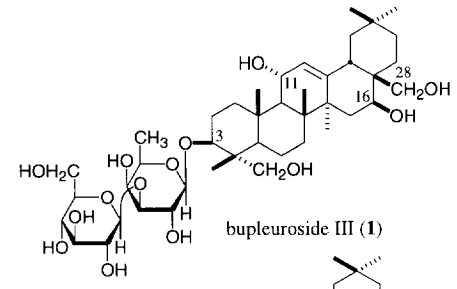
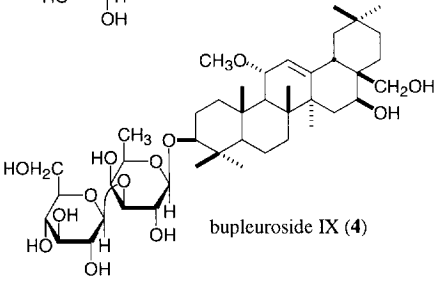
Abstract : New hepatoprotective saponins, bupleurosides III, VI, IX, and XIII, were isolated from Chinese Bupleuri Radix, the roots of *Bupleurum scorzonerifolium* WILLD., together with nine new and sixteen known saponins. The structures of bupleurosides III, VI, IX, and XIII were elucidated on the basis of chemical and physicochemical evidence. By examination of the structure requirements in saponins for the protective effect on the D-galactosamine-induced cytotoxicity in primary cultured rat hepatocytes, the 11-oxygen function, the 16β-hydroxyl group, and the 3-*O*-diglycoside moiety were found to be essential to exerting the hepatocytoprotective activity. © 1997 Elsevier Science Ltd.

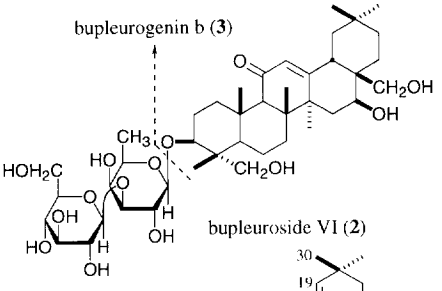
Bupleuri Radix, which is one of the most important components in Chinese traditional medicines, has been used as an antiinflammatory, antipyretic, and antihepatotoxic agent. Recently, due to the poor supply of Japanese Bupleuri Radix, Chinese Bupleuri Radix has been imported and commonly used in the traditional preparations. In regard to the bioactive constituents of Bupleuri Radix, many saikosaponins have been characterized from Japanese Bupleuri Radix, the roots of *Bupleurum falcatum* L.,[1] and, among them, saikosaponins a (**7**) and d (**8**) are reported to be antiinflammatory and antihepatotoxic principles.[2] In the course of our studies in search of bioactive principle from natural medicine,[3] we have found that the glycosidic fraction from a Chinese Bupleuri Radix, the roots of *B. scorzonerifolium* WILLD., showed potent protective effects on liver injury induced by D-galactosamine (D-GalN) and lipopolysaccharide (LPS) in mice and on the D-GalN-induced cytotoxicity in primary cultured rat hepatocytes. From the active glycosides fraction, new saponins called bupleurosides I—XIII were isolated together with sixteen known saponins. This communication deals with the structure elucidation of bupleurosides III (**1**), VI (**2**), IX (**4**), and XIII (**5**) with potent hepatoprotective effect and the structure-requirements of saponins for the protective effect on the D-GalN-induced cytotoxicity.

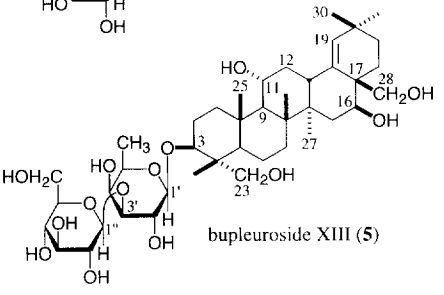
Isolation and Structure Elucidation of Bupleurosides (1, 2, 4, 5)

The MeOH extract of the Chinese Bupleuri Radix was partitioned into a AcOEt-water mixture and the water-soluble portion was further extracted with *n*-BuOH. The *n*-BuOH-soluble portion (so-called glycosidic fraction) with inhibitory effect on the hepatocytotoxicity was subjected to ordinary and reversed-phase silica gel column chromatography, and finally HPLC to give bupleurosides I (**6**, 0.0004%),[4] II (**16**, 0.0005%),[4] III (**1**, 0.0023%), IV (**17**, 0.0007%),[4] V (**11**, 0.0064%),[4] VI (**2**, 0.0013%), VII (**23**, 0.0005%),[4] VIII (**24**, 0.0004%),[4] IX (**4**, 0.0007%), X (**12**, 0.0003%),[4] XI (**25**, 0.0011%),[4] XII (**13**, 0.0011%),[4] and XIII (**5**, 0.0004%)[4] together with sixteen known saponins.









Bupleuroside III (**1**), mp 235-237 °C, $[\alpha]_D^{25}$ -4.4° (MeOH), $C_{42}H_{70}O_{14}$,[5] IR (KBr) : 3422, 1655, 1076 cm^{-1}, positive-ion FAB-MAS : *m/z* 821 $(M+Na)^+$, furnished saikosaponin b_3 (**18**) by MeOH treatment (reflux, 3 h), while mild aqueous acid treatment of saikosaponin a (**7**) yielded **1**. Comparison of the ^1H-NMR and ^{13}C-NMR (Table 1) data[6] for **1** with those for **18** led us to formulate the structure of bupleuroside III (**1**) as shown.

Bupleuroside VI (**2**), mp 193-196 °C, $[\alpha]_D^{25}$ +32.1° (MeOH), $C_{42}H_{68}O_{14}$,[5] positive-ion FAB-MAS : *m/z* 819 $(M+Na)^+$, showed absorption bands due to hydroxyl and enone functions at 3432, 1655, and 1075 cm^{-1} in its IR spectrum, while the enone absorption was observed at 247 nm in the UV spectrum. The carbon signals in the ^{13}C-NMR (Table 1)[6] of **2** were very similar to those of **1** and **18** , except for the signals due to the enone moiety. Acid hydrolysis of **2** with 5% H_2SO_4-dioxane liberated bupleurogenin b (**3**) together with D-fucose and D-glucose, which were identified by GLC analysis[7] of their trimethylsilyl thiazolidine derivatives. The sapogenol **3** was synthesized from a known triterpene 23-hydroxylongispinogenin acetate[8] by anodic oxidation (Pt, $NaClO_4$, CH_3CN-H_2O)[9] followed by deacetylation. Finally, **2** was derived to **18** by $NaBH_4$ reduction followed by the MeOH treatment. Consequently, the structure of bupleuroside VI (**2**) was characterized.

The carbon signals in the ^{13}C-NMR (Table 1)[6] of bupleuroside IX (**4**), mp 237-240 °C, $[\alpha]_D^{25}$ +23.8° (MeOH), $C_{43}H_{72}O_{13}$,[5] IR (KBr) : 3453, 1655, 1078 cm^{-1}, positive-ion FAB-MAS : *m/z* 819 $(M+Na)^+$, were superimposable on those of **18**, except for the signals due to the 23-methyl group. Acid treatment of saikosaponin e (**10**) with 10% AcOH-MeOH yielded **4**. This evidence led us to elucidate the structure of bupleuroside IX (**4**) as shown.

Bupleuroside XIII (**5**), mp 222-225 °C, $[\alpha]_D^{25}$ +4.2° (MeOH), $C_{42}H_{70}O_{14}$,[5] IR (KBr) : 3410, 1075 cm^{-1}, positive-ion FAB-MAS : *m/z* 821 $(M+Na)^+$, liberated D-fucose and D-glucose by the acid hydrolysis. The ^1H-NMR (pyridine-d_5) spectrum of **5** indicated the presence of the β-D-fucopyranosyl moiety [δ 1.41 (d, *J*=6.1 Hz, 6'-H_3), 4.98 (d, *J*=7.9 Hz, 1'-H)], the β-D-glucopyranosyl moiety [δ 5.35 (d, *J*=7.9 Hz, 1"-H)], three

Table 1. ^{13}C-NMR Data of Bupleurosides III (**1**), IV (**2**), IX (**4**), and XIII (**5**) and Saikosaponin b_3 (**18**)

	1	**2**	**4**	**5**	**18**		**1**	**2**	**4**	**5**	**18**
C-1	41.1	39.6	40.1	42.1	40.1	C-23	64.2	64.1	28.1	64.3	64.2
C-2	26.6	26.2	27.0	26.8	26.2	C-24	13.7	13.6	16.9	13.8	13.5
C-3	81.8	81.6	88.6	81.7	81.7	C-25	17.7	17.4	17.4	17.8	17.9
C-4	43.9	44.3	39.9	44.7	43.6	C-26	18.3	18.7	18.3	17.6	18.3
C-5	47.8	47.3	55.8	48.5	47.5	C-27	26.7	24.4	26.3	16.0	26.2
C-6	18.3	17.3	18.3	17.9	18.3	C-28	68.6	67.8	68.5	65.0	68.6
C-7	33.7	32.7	33.4	35.7	33.1	C-29	33.4	33.0	33.0	30.8	33.2
C-8	40.9	45.5	41.0	42.6	40.9	C-30	23.9	23.8	24.0	29.8	24.0
C-9	55.6	61.6	52.0	56.1	52.0	11-OMe			54.1		54.0
C-10	38.1	37.2	38.1	39.1	38.0	Fuc-1'	105.9	105.9	106.8	106.6	105.9
C-11	66.7	196.6	75.8	69.9	75.9	-2'	71.5	71.5	71.5	71.8	71.8
C-12	128.0	128.3	122.4	38.4	122.5	-3'	85.1	85.2	85.2	85.2	85.2
C-13	145.1	168.7	148.2	38.0	148.2	-4'	72.1	72.1	72.2	72.1	72.1
C-14	43.9	46.0	43.9	42.8	43.8	-5'	70.9	71.0	70.9	70.9	70.9
C-15	36.4	36.6	36.8	38.3	36.7	-6'	17.2	17.2	17.3	17.2	17.2
C-16	66.4	65.5	66.2	77.0	66.2	Glc-1"	106.5	106.5	106.7	106.0	106.6
C-17	43.8	41.2	43.6	44.3	43.6	-2"	75.7	75.7	75.9	75.8	75.7
C-18	43.8	43.9	44.0	138.8	43.9	-3"	78.3	78.3	78.7	78.4	78.3
C-19	46.4	45.3	43.9	133.3	46.9	-4"	71.8	71.8	71.7	71.6	71.7
C-20	30.9	31.0	31.1	32.2	31.1	-5"	78.7	78.6	78.4	78.8	78.7
C-21	34.1	34.0	34.2	34.4	34.2	-6"	62.6	62.7	62.7	62.6	62.6
C-22	25.9	25.3	25.9	28.1	25.8						

68MHz, pyridine-d_5

methines bearing a oxygen function [δ 4.06 (m, 16-H), 4.29 (m, 3-H), 4.23 (m, 11-H)] and a trisubstituted olefin [δ 5.25 (br s, 19-H)]. The position of the above mentioned functional groups was clarified by the HMBC experiment, which showed long-range correlations between the following proton and carbons : 1"-H and 3'-C; 1'-H and 3-C; 9-H, 12-H_2 and 11-C; 16-H and 17-C; 19-H and 18-C; 23-H_2 and 4-C; 28-H_2 and 17-C; 30-H_3 and 19-C. Furthermore, the ROESY experiment of **5** showed ROE correlations between the following protons : 11-H and 13-H, 25-H_3, 26-H_3; 16-H and 27-H_3. On the basis of this evidence, the structure of bupleuroside XIII (**5**) was determined.

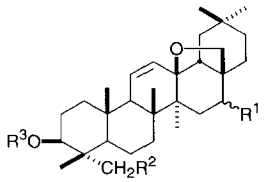
Bioassay Methods

In Vitro Experiment : The hepatocytoprotective effects of these saponins were determined by *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using primary cultured rat hepatocytes.[10] Detail protocol was described in footnote of Table 2 and inhibition (%) was obtained by next formula.

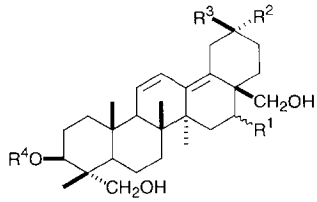
$$\text{Inhibition (\%)} = [(\text{O.D.}_{(\text{sample})}-\text{O.D.}_{(\text{control})}) / (\text{O.D.}_{(\text{normal})}-\text{O.D.}_{(\text{control})})] \times 100$$

In Vivo Experiment : The D-GalN/LPS-induced liver damage described by Tiegs *et al.*[11] was modified and used for this experiment. Detail protocol was described in footnote of Table 3.

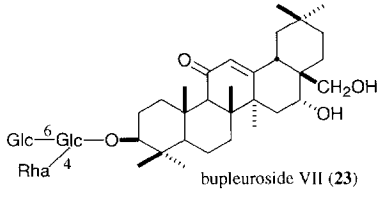
Statistical Analysis : Each value was expressed as the mean±S.E. and the statistical significance was determined by Dunnett's method.



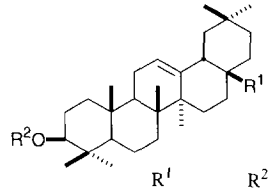
	R^1	R^2	R^3
bupleuroside I (**6**) :	β-OH	OH	—Fuc^{3}—Glc^{2}—Glc
saikosaponin a (**7**) :	β-OH	OH	—Fuc^{3}—Glc
saikosaponin d (**8**) :	α-OH	OH	—Fuc^{3}—Glc
saikosaponin c (**9**) :	β-OH	H	—Glc^{4}(6→1)Rha, Glc
saikosaponin e (**10**) :	β-OH	H	—Fuc^{3}—Glc



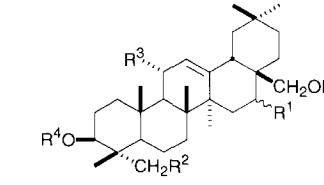
	R^1	R^2	R^3	R^4
bupleuroside V (**11**) :	α-OH	COOH	CH_3	—Fuc^{3}—Glc
bupleuroside X (**12**) :	α-OH	CH_3	CH_3	—Glc^{4}(6→1)Rha, Glc
bupleuroside XII (**13**) :	α-OH	CH_3	CH_2OH	—Glc^{4}(6→1)Rha, Glc
saikosaponin b_1 (**14**) :	β-OH	CH_3	CH_3	—Fuc^{3}—Glc
saikosaponin b_2 (**15**) :	α-OH	CH_3	CH_3	—Fuc^{3}—Glc



bupleuroside VII (**23**)



	R^1	R^2
bupleuroside VIII (**24**) :	COOH	—GlcA^{3}—Ara^{2}—Glc
bupleuroside XI (**25**) :	CH_2OH	—Glc^{4}(6→1)Rha, Glc



	R^1	R^2	R^3	R^4
bupleuroside II (**16**) :	α-OH	OH	OCH_3	—Fuc^{3}—Glc^{2}—Glc
bupleuroside IV (**17**) :	α-OH	OH	OH	—Fuc^{3}—Glc
saikosaponin b_3 (**18**) :	β-OH	OH	OCH_3	—Fuc^{3}—Glc
saikosaponin f (**19**) :	β-OH	H	H	—Glc^{4}(6→1)Rha, Glc
20 :	β-OH	H	OCH_3	—Glc^{4}(6→1)Rha, Glc
21 :	β-OH	OH	OCH_3	—Glc^{4}(6→1)Rha, Glc
22 :	β-OH	H	OH	—Glc^{4}(6→1)Rha, Glc

Fuc : β-D-fucopyranosyl; Glc : β-D-glucopyranosyl; Rha : α-L-rhamnopyranosyl; GlcA : β-D-glucopyranosiduronyl; Ara : α-L-arabinopyranosyl

Results and Discussion

The hepatocytoprotective effect of bupleurosides and known related saponins was examined by monitoring the inhibition of cytotoxicity induced by D-GalN in primary cultured rat hepatocytes. As shown in Table 2, bupleurosides III (**1**), VI (**2**), IX (**4**), and XIII (**5**) and saikosaponin b_3 (**18**) were found to exhibit the inhibitory activity. On the other hand, the saponins having the 13β,28-oxide function (**6**—**10**) and the 11, 13(18)-diene function (**11**—**15**) lacked the inhibitory activity. Bupleuroside IV (**17**) which was the 16-hydroxyl stereoisomer of **1**, showed no activity and also all saponins having the 16α-hydroxyl group (**8**, **11**—**13**, **15**, **16**, **23**) were found to lack the activity. These evidences led us to confirm that the 11-oxygen function and the 16β-hydroxyl group were essential to exerting the cytoprotective effect. Furthermore, since bupleurogenin b (**3**) and all triglycoside saponins (**6**, **9**, **12**, **13**, **16**, **19**—**25**) showed no activity, the

Table 2. Inhibitory Effects of Bupleurosides and Saikosaponins on D-GalN-induced Cytotoxicity in Primary Cultured Rat Hepatocytes

Sample	Inhibition (%)				
	0.3	1	3	10	30 (μg/ml)
Bupleuroside III (**1**)	6.8±0.4	16.7±1.8**	35.6±0.9**	43.1±4.6**	46.5±3.4**
Bupleuroside VI (**2**)	5.5±0.7	16.9±1.3**	44.5±2.3**	72.8±3.9**	90.9±5.7**
Bupleurogenin b (**3**)	0.6±0.4	0.6±1.0	0.0±0.3	0.4±1.2	4.9±1.0
Bupleuroside IX (**4**)	37.2±1.9**	64.9±4.5**	75.5±2.0**	73.3±1.0**	75.0±1.4**
Bupleuroside XIII (**5**)	15.8±1.1**	39.7±3.2**	59.4±4.5**	69.5±3.0**	64.7±2.4**
Bupleuroside I (**6**)	-5.4±2.7	-6.5±1.1	-7.8±1.6	-7.8±1.3	-12.2±0.7**
Saikosaponin a (**7**)	-3.6±2.5	-7.1±1.2	-11.6±0.4**	-12.7±0.3**	-14.8±0.5**
Saikosaponin d (**8**)	3.9±1.0	2.3±1.3	-4.2±0.1	-4.3±0.2	-5.3±0.3
Saikosaponin c (**9**)	-1.5±0.6	-0.7±0.4	-1.9±1.5	-1.0±0.9	-1.0±0.3
Saikosaponin e (**10**)	-12.1±4.6*	-17.1±1.4**	-26.9±0.8**	-29.2±0.6**	-30.6±10.3**
Bupleuroside V (**11**)	1.4±1.1	1.5±0.8	1.4±0.5	3.8±1.1	4.5±1.5
Bupleuroside X (**12**)	2.9±1.1	3.9±1.7	4.6±0.5	3.4±0.5	2.7±0.2
Bupleuroside XII (**13**)	0.1±1.3	0.3±0.5	-0.8±0.8	2.0±0.4	0.8±0.6
Saikosaponin b_1 (**14**)	0.1±1.1	-1.4±0.9	4.3±2.1	8.7±1.2	-5.1±1.6
Saikosaponin b_2 (**15**)	2.7±0.9	6.1±2.1	5.3±1.4	5.5±1.1	8.4±1.3
Bupleuroside II (**16**)	1.7±1.4	0.3±0.9	-0.4±1.0	-2.2±0.4	-2.9±0.8
Bupleuroside IV (**17**)	3.6±1.8	-4.0±2.0	-6.9±0.8	-8.3±0.8	-9.9±0.4
Saikosaponin b_3 (**18**)	8.7±3.2	13.9±4.1*	56.4±1.9**	70.4±2.0**	72.1±4.3**
Saikosaponin f (**19**)	3.1±2.3	0.2±0.8	-2.9±0.8	-2.9±1.4	-0.7±0.4
20	10.3±1.6	9.7±1.1	4.5±2.4	11.1±1.7	8.1±4.2
21	1.5±1.1	1.2±0.3	3.5±0.6	2.9±0.3	4.8±0.8
22	-2.3±1.7	-5.1±1.0	-3.4±1.3	-3.2±1.8	-6.5±1.4
Bupleuroside VII (**23**)	-0.6±0.7	2.5±1.8	4.3±1.1	2.2±1.3	3.2±1.2
Bupleuroside VIII (**24**)	1.5±1.0	0.1±1.0	-0.9±2.1	-1.4±1.2	-1.4±0.9
Bupleuroside XI (**25**)	-0.4±0.3	1.6±1.1	-1.0±0.1	0.3±0.8	-0.3±0.1

Hepatocytes were isolated from male Wistar rats (130—150g) by collagenase perfusion method. The cell suspension of 4×10^4 cells in 100 μl William's E medium containing calf serum (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), insulin (1 μM), and dexamethasone (1 μM) was inoculated in a 96-well tissue culture plate, and precultured for 4 h at 37°C under a 5% CO_2 atmosphere. The medium was exchanged with a fresh medium contained D-GalN (1 mM) and a test sample (0.3—30 μg/ml), and the hepatocytes were cultured for 44 h. The medium was exchanged with 100 μl of the medium, and 10 μl of MTT (5 mg/ml in PBS) solution was added to the medium. After 4 h culture, the medium was removed, and 100 μl of isopropanol containing 0.04N HCl was then added to dissolve the formazan produced in the cells. The optical density (O.D.) of the formazan solution was measured by microplate reader at 570 nm. Each value represents the mean±S.E. of 4 experiments (*p<0.05,**p<0.01).

common 3-*O*-diglycoside structure of **1**, **2**, **4**, and **5** was found to be important for the activity. In addition, *in vivo* experiment as shown in Table 3, bupleurosides III (**1**), VI (**2**), and XIII (**5**) also inhibited the D-GalN/LPS-induced liver injury in mice.

In this study, we made it clear that bupleurosides III (**1**), VI (**2**), IX (**4**), XIII (**5**), and saikosaponin b_3 (**18**) protected D-GalN-induced hepatocytotoxicity, and the structure-requirements for the activity were clarified.

Table 3. Inhibitory Effects of Bupleurosides III (**1**), VI (**2**), and XIII (**5**) on D-GalN/LPS-induced Hepatic Injury in Mice

	Dose (mg/kg, *i.p.*)	N	s-GPT (Karmen units)	s-GOT (Karmen units)
Normal	-	10	16±1**	51±4**
Control	-	14	8154±911	7755±1137
Bupleuroside III (**1**)	10	6	1483±632**	1261±445**
Bupleuroside VI (**2**)	10	10	7887±1640	7211±1470
	20	6	1530±397**	1404±329**
Bupleuroside XIII (**5**)	10	7	1265±343**	1209±273**
Hydrocortisone	20	10	202±86**	331±121**

Male ddY mice weighing about 25—27 g were used. After 20 h of fasting, a mixture of D-GalN and LPS from *Salmonella enteritidis* was injected intraperitoneally (*i.p.*) at a dose of 350 mg/kg and 10 μg/kg to produce liver injury. Each test sample was administered *i.p.* 1 h before D-GalN/LPS injection. Blood samples were collected 10 h after D-GalN/LPS injection, and serum GPT and GOT levels were determined by Reitman and Frankel's method. Each value represents the mean±S.E. (**$p<0.01$).

References and Notes

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4) a) The structures of bupleurosides I (**6**), II (**16**), IV (**17**), V (**11**), VII (**23**), VIII (**24**), X (**12**), XI (**25**), and XII (**13**) were elucidated on the basis of chemical and physicochemical evidence, and full characteristics will be presented in our full paper.

5) The molecular composition of the new compounds (**1**, **2**, **4**, **5**) given the chemical formula was determined by high-resolution FAB-MS measurement.

6) The ^{1}H-NMR and ^{13}C-NMR data were assigned with the aid of ^{1}H-^{1}H and ^{1}H-^{13}C COSY, DEPT, ^{1}H-^{1}H and ^{1}H-^{13}C HOHAHA, NOESY, and HMBC experiments.

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