Triterpene Saponins from *Bacopa monnieri* and Their Antidepressant Effects in Two Mice Models

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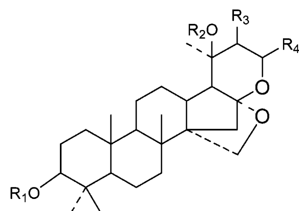
Three new triterpene glycosides, bacopasides VI−VIII (**1**−**3**), together with three known analogues, bacopaside I (**4**), bacopaside II (**5**), and bacopasaponsin C (**6**), were isolated from the whole plant of *Bacopa monnieri*. Compounds **4**, **5**, and **6** showed antidepressant activity when tested on forced swimming and tail suspension in mice, respectively.

Bacopa monnieri (L.) Wettst. (Scrophulariaceae), a perennial rambler, is distributed in Fujian, Taiwan, Guangdong, Yunnan, and Sichuan Provinces in China. In the Ayurvedic system of medicine of India, *B. monnieri* has been widely used to enhance memory development, learning, and intelligence for several centuries[1] and has been reported to possess several neuropsychopharmacological activities.[2] In addition, antiinflammatory, analgesic, and antipyretic properties have also been reported for the extract of this species.[3] The standardized methanolic extract of *B. monnieri* exhibited antidepressant activity in forced swimming and learned helplessness models of depression.[4] Phytochemical investigations have shown the presence of several saponins,[5,6] including bacoside A and its optical isomer, bacoside B.[7,8] Additional new saponins have also been reported, but no bioactivities have yet been reported.[9]

In continuation of our search for natural antidepressants, we recently investigated the constituents of *B. monnieri*. In this paper, we report the isolation and structural elucidation of three new triterpene glycosides, bacopasides VI−VIII (**1**−**3**), together with three known analogues, and their antidepressant activities.

Compound **1** was obtained as a white powder and showed a positive Liebermann-Burchard test. The positive HRESIMS displayed a quasimolecular ion $[M + 2Na]^+$ at *m/z* 892.3871, corresponding to the molecular formula $C_{41}H_{66}O_{16}Na_2S$. The ^1H NMR spectrum of **1** exhibited seven methyl singlets at δ_H 0.74, 0.78, 0.95, 0.97, 1.02, 1.61, and 1.69 and several resonances from an isobutenyl group at δ_H 5.30 (1H, d, $J = 8.0$ Hz), 1.69 (3H, s), and 1.61 (3H, s). The ^{13}C and DEPT spectra (Table 1) demonstrated a methine at δ_C 44.5, an oxygen-bearing methylene at δ_C 64.9, and a quaternary carbon at δ_C 109.1. Considering those saponins previously isolated from this species,[5−9] it was suggested that the aglycone of **1** is pseudojujubogenin. Two anomeric protons, δ_H 4.40 (1H, d, $J = 8.0$ Hz) and 4.16 (1H, d, $J = 7.0$ Hz), were observed in the ^1H NMR spectrum (Table 2) of **1**. Apart from the carbon resonances for the aglycone, 11 oxygen-bearing methines were observed in the ^{13}C NMR spectrum, which revealed the existence of a pentose and a hexose moiety. Acid hydrolysis of **1** and GC analysis of chiral derivatives of sugars in the acid hydrolysate afforded D-glucose and L-arabinose. When comparing the NMR data of **1** with those of the known bacopaside V,[10] the two compounds were found to be very similar except for a additional terminal glucose moiety in **1**. The ESIMS displayed a pseudomolecular ion peak $[M + H]^+$ at *m/z* 847 and a fragment ion peak at *m/z* 767 $[M + H - 180]^+$, indicating the loss of a sulfate group. Significantly, the downfield shift of C-6 of glucose from δ_C 62.3 in bacopaside V to 65.7 in **1** pointed to the sulfate group being located at the C-6 of glucose. The sugar sequence and glycosidic position were determined from the HMBC correlations between H-1 of glucose and C-3 (δ_C 82.9) of arabinopyranose and between H-1 of arabinopyranose and C-3 (δ_C 87.7) of the aglycone. From the above evidence, compound **1** was elucidated as 3-*O*-[6-*O*-sulfonyl-*β*-D-glucopyranosyl(1→3)]-α-L-arabinopyranosylpseudojujubogenin and named bacopaside VI.



	R1	R2	R3	R4
1		H		H
2		H	H	
3			H	
4		H		H
5		H		H
6		H		H

Compound **2** was obtained as white needles. The positive HRESIMS spectrum exhibited a quasimolecular ion peak $[M + Na]^+$ at *m/z* 921.4818, corresponding to the molecular formula $C_{46}H_{74}O_{17}Na$. The ^1H NMR spectrum of **2** exhibited seven methyl singlets at δ_H 0.69, 1.05, 1.06, 1.27, 1.37, 1.66, and 1.68, while the ^{13}C NMR spectrum of **2** (Table 1) gave a methylene at δ_C 45.1, a methine at δ_C 68.3, and a quaternary carbon at δ_C 110.2, implying

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Table 1. ^{13}C NMR Data of Compounds **1**−**5** in Pyridine-d_5 (125 MHz)

	1	**2**	**3**		**1**	**2**	**3**
1	38.0	38.4	38.4	3	82.9	83.2	88.4
2	25.8	26.4	26.4	4	67.3	68.2	70.0
3	87.7	88.3	88.8	5	65.7	65.4	78.1
4	39.4	39.5	39.4	6			62.4
5	55.2	55.8	55.8		Glc	Ara (f)	Ara (f)
6	17.5	17.9	17.9	1	104.3	110.0	109.6
7	35.8	35.7	35.7	2	73.8	83.5	83.5
8	36.6	37.2	37.1	3	75.9	77.8	77.4
9	52.0	52.7	52.5	4	69.8	84.6	84.5
10	36.7	36.9	36.7	5	74.8	61.7	61.8
11	21.0	21.4	21.3	6	65.7		
12	27.7	28.2	28.0			Glc	Glc
13	35.8	36.8	35.6	1		104.6	104.4
14	52.4	53.4	53.4	2		74.9	75.1
15	35.2	36.5	37.0	3		77.6	77.5
16	109.1	110.2	109.8	4		71.2	71.2
17	50.0	53.6	52.5	5		78.1	78.2
18	18.4	18.5	18.4	6		62.2	62.0
19	15.9	16.0	15.9				20-O-Ara
20	70.6	68.1	75.3	1			98.5
21	25.9	29.7	24.8	2			72.7
22	44.5	45.1	41.2	3			74.8
23	64.9	68.3	68.4	4			69.0
24	123.1	126.8	127.1	5			66.4
25	132.1	133.7	133.4				
26	26.3	25.2	25.4				
27	18.3	17.9	18.0				
28	27.4	27.4	27.4				
29	16.2	16.2	16.2				
30	64.7	65.5	65.6				
	Ara	Ara	Glc				
1	105.5	105.3	104.7				
2	70.1	76.7	78.8				

Table 2. ^{1}H NMR Data of the Sugar Moieties of **1**−**5** in Pyridine-d_5 (500 MHz)

	1 (mult. *J*, Hz)	**2** (mult. *J*, Hz)	**3** (mult. *J*, Hz)
C-3 sugar			
	Ara (p)	Ara(p)	Glc
1	4.16 (1H, d, 7.0)	4.77 (1H, d, 7.0)	4.87 (1H, d, 7.0)
2	3.50	4.49	4.11[a]
3	3.46	4.20	4.20
4	3.82	4.48	3.98
5	3.64	4.20	4.25[a]
	3.43	3.67	
6			4.48
			4.25[a]
	Glc	Glc	Ara (f)
1	4.40 (1H, d, 8.0)	5.14 (1H, d, 7.0)	6.27 (1H, d, 3.0)
2	3.07	3.96	5.03
3	3.17	4.22	3.86
4	3.27	4.20	4.68
5	3.07	3.94	4.34
6	4.02	4.50	
	3.77	4.32	
		Ara (f)	Glc
1		6.11 (1H, d, 2.0)	5.22 (1H, d, 7.0)
2		5.04	4.00
3		4.88	4.92
4		4.80	4.11[a]
5		4.34	4.22[a]
6			5.56
			20-O-Ara (p)
1			4.89 (1H, m)
2			4.39
3			4.02
4			4.30
5			4.22[a]

[a] Overlapped with other signals.

that the aglycone of **2** is jujubogenin. Three anomeric protons at δ_H 6.11 (1H, d, $J = 2.0$ Hz), 5.14 (1H, d, $J = 7.0$ Hz), and 4.77 (1H, d, $J = 8.0$ Hz) revealed the existence of three sugar moieties. On the basis of the analysis of 2D NMR experiments, including HMQC, HMBC, $^{1}H-^{1}H$ COSY, and TOCSY spectra, they were elucidated as arabinofuranose, glucose, and arabinopyranose, respectively. Their absolute configurations were determined as L, D, and L, respectively, by GC analysis of chiral derivatives of sugars in the acid hydrolysate. The linkage sequence of the sugar chain was determined by the HMBC correlations between H-1 of glucose and C-3 of arabinopyranose and between H-1 of arabinofuranose and C-2 of arabinopyranose. The HMBC correlation between H-1 of arabinpyranose and C-3 (δ_C 88.3) of the aglycone indicated that the sugar chain was attached to C-3 of the aglycone. Thus, compound **2** was determined as 3-*O*-{β-D-glucopyranosyl(1→3)-[α-L-arabinofuranosyl(1→2)]-α-L-arabinopyranosyl}jujubogenin and named bacopaside VII.

Compound **3**, a white powder, has the molecular formula $C_{52}H_{84}O_{22}$, deduced from a pseudomolecular ion peak $[M + Na]^+$ at *m/z* 1083.5322 in the positive HRESIMS. Detailed analysis of the ^{1}H and ^{13}C NMR spectra (Table 1) indicated that compound **3** shared the same aglycone as **2**. The ^{1}H NMR spectrum exhibited four anomeric protons at δ_H 4.87 (1H, d, $J = 4.9$ Hz), 4.89 (1H, m), 5.22 (1H, m), and 6.27 (1H, d, $J = 3.0$ Hz), and four corresponding anomeric carbons at δ_C 109.6, 104.7, 104.4, and 98.5 were found in the ^{13}C NMR spectrum, implying the presence of four sugar units. By comparison of the NMR data of **3** with those of the known bacoside A_3,[5] **3** was found to contain one more glucopyranose moiety. The additional glucopyranose was attached to C-20 from the HMBC correlation between H-1 of glucose and C-20 (δ_C 75.3) of the aglycone of **3**. All proton and carbon resonances were assigned through 2D NMR experiments. Compound **3** was thus identified as 3-*O*-{β-D-glucopyranosyl(1→3)[α-L-arabinofuranosyl(1→2)]-β-D-glucopyranosyl}-20-α-L-arabinopyranosyljujubogenin and named bacopaside VIII.

Compounds **2**, **4**, **5**, and **6** were evaluated for their antidepressant activities on a forced swimming test and a tail suspension test in mice. The immobility time of forced swimming mice exposed to compounds **4**, **5**, and **6** (50 mg/kg each) for five consecutive days was significantly reduced by 75% ($P < 0.01$), 55% ($P < 0.01$), and 63% ($P < 0.01$) (Table 3), respectively, while the immobility time of tail suspension mice exposed to compounds **4**, **5**, and **6** (50 mg/kg each) for five consecutive days was significantly reduced by 61% ($P < 0.01$), 38% ($P < 0.05$), and 47% ($P < 0.01$) (Table 3), respectively. Compound **2** showed no obvious reduction in the immobility time in both forced swimming and tail suspension models. The spontaneous activity test (Table 4) showed that compounds **2**, **4**, **5**, and **6** did not affect the spontaneous activity of mice. It was suggested that the mechanism of action of the antidepressant activity of compounds **4**, **5**, and **6** may not be due to stimulation or inhibition of the central nervous system.

Compounds **4**, **5**, and **6**, containing the same aglycone, exhibited antidepressant effects, while compound **2**, bearing a different aglycone moiety, showed no antidepressant activity. Therefore, it seems that the aglycone plays the crucial role for the antidepressant activity of those compounds. The sugar chain also could influence the antidepressant effect of those compounds, and the C-6 sulfate group of the terminal glucose of **1** could obviously improve the antidepressant activity.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. IR spectra were recorded on a Bruker Vector22 spectrometer with KBr pellets. NMR spectra were recorded on a Bruker DRX-500 spectrometer at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR. Chemical shifts are reported in ppm with TMS as internal standard. EIMS and HRESIMS were recorded on a Varian MAT-212 mass spectrometer and a Q-TOF micro mass spectrometer, respectively. Gas chromatography analysis was operated on an HP-5892 II with an FID detector, and an HP-20M (Carbowx 20M) capillary column (25 m × 0.32 mm × 0.3 μm) was used.

Table 3. Reduction of Immobility Time of Forced Swimming Mice and Tail Suspension in Mice Treated with Compounds **2**, **4**, **5**, and **6** (means ± SEM)

		forced swimming test		tail suspension test	
	n	floating time (s)	reduction (%)	floating time (s)	reduction (%)
control	10	124.4 ± 27.0		102.3 ± 29.4	
fluoxetine	10	24.9 ± 20.6**[a]	80	28.3 ± 23.6**	72
2	10	99.6 ± 35.2	20	94.5 ± 46.3	8
4	10	31.5 ± 22.3**	75	40.1 ± 18.9**	61
5	10	55.7 ± 31.9**	55	63.6 ± 34.4*	38
6	10	46.4 ± 25.3**	63	54.6 ± 27.1**	47

[a] Probability. *: $P < 0.05$, **: $P < 0.01$.

Table 4. Influence of the Investigated Compounds on the Spontaneous Motor Activity (means ± SEM)

	n	spontaneous motor activity (counts/6 min)	comparison with the control group
control	10	650.1 ± 98.5	
fluoxetine	10	671.3 ± 221.9	ns[a]
2	10	649.3 ± 167.1	ns
4	10	683.5 ± 128.9	ns
5	10	655.7 ± 131.9	ns
6	10	646.4 ± 205.3	ns

[a] ns: no significance.

Semipreparative HPLC was performed on a Waters liquid chromatograph 510 instrument with a PDA UV detector at 212 nm using an ODS column (Kromasil, 5 μm, 300 × 10 mm). Column chromatography was performed on silica gel (200−300 mesh, Yantai, China), silica gel H (10−40 μm, Yantai, China), macroporous resin (AB-8, Tianjin, China), RP silica gel (ODS, 25−40 μm, Merck), and Sephadex LH-20 (Pharmacia). TLC analysis was run on $HSGF_{254}$ precoated silica gel plates (10−40 μm, Yantai, China).

Plant Material. The whole plant of *B. monnieri* was collected in Zhangzhou, Fujian Province, and identified by Prof. Han-Chen Zheng, Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai. A voucher specimen (No. 0211-11) is deposited at the Herbarium of the School of Pharmacy, Second Military Medical University, Shanghai, China.

Extraction and Isolation. The dried whole plant of *B. monnieri* (8 kg) was extracted with MeOH at room temperature. The MeOH extract was partitioned with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH, respectively. The *n*-BuOH extract (380 g) was submitted to macroporous resin (1000 g) column chromatography and washed with H_2O (5.0 L), 10% EtOH (10.0 L), 30% EtOH (5.0 L), 50% EtOH (10.0 L), 70% EtOH (10.0 L), and 95% EtOH (5.0 L) (v/v), respectively. The combined 50% and 70% EtOH eluants afforded a saponin fraction (170 g), which was applied to column chromatography on silica gel (10 × 80 cm, 1000 g) with gradient $CHCl_3/CH_3OH$ (20:1, 3 L; 15:1, 2 L; 10:1, 2 L; 6:1, 2 L; 4:1, 2 L; 2:1, 2 L; 1:1, 2 L) as eluents. All subfractions were repeatedly subjected to Sephadex LH-20 (MeOH) and RP silica gel (ODS) column chromatography (H_2O/CH_3OH, 100:0 → 0:100) and finally purified by semipreparative HPLC (CH_3CN/H_2O, 45:55, flow rate of 3 mL/min) column chromatography to give **1** (72 mg), **2** (403 mg), **3** (86 mg), **4** (750 mg), **5** (650 mg), and **6** (510 mg).

Compound 1: white powder; $[\alpha]^{20}_{D}$ −26.3 (*c* 0.5, MeOH); ^{1}H NMR (DMSO-d_6, 500 MHz) δ_H 2.99 (1H, dd, 12.0, 4.0, H-3), 2.45 (1H, m, H-13), 0.95 (3H, s, H-18), 0.74 (3H, s, H-19), 1.02 (3H, s, H-21), 2.20 (1H, d, 11.0, H-22), 5.30 (1H, d, 10.0, H-24), 1.69 (3H, s, H-26), 1.61 (3H, s, H-27), 0.97 (3H, s, H-28), 0.78 (3H, s, H-29); ^{13}C NMR, see Tables 1 and 2; ESIMS *m/z* 847 $[M + H]^+$, 767 $[M - SO_3]^+$; HRESIMS *m/z* 892.3871 $[M + 2Na]^+$ (calcd for $C_{41}H_{66}O_{16}Na_2S$, 892.3867).

Compound 2: white needles from the mixed MeOH and H_2O; mp 250−252 °C, $[\alpha]^{20}_{D}$ −41.5 (*c* 0.5, MeOH); ^{1}H NMR (pyridine-d_5, 500 MHz) δ_H 3.23 (1H, dd, 4.0, 12.0, H-3), 2.81 (1H, m, H-13), 1.06 (3H, s, H-18), 0.69 (3H, s, H-21), 1.65 (1H, dd, 2.0, 14.0, H-22α), 1.74 (1H, dd, 2.0, 14.0, H-22β), 5.21 (1H, m, H-23), 5.52 (1H, d, 8.0, H-24), 1.68 (3H, s, H-26), 1.66 (3H, s, H-27), 1.27 (3H, s, H-28), 1.05 (3H, s, H-29); ^{13}C NMR, see Tables 1 and 2; ESIMS *m/z* 921 $[M + Na]^+$; HRESIMS *m/z* 921.4818 (calcd for $C_{46}H_{74}O_{17}Na$, 921.4824).

Compound 3: white powder; $[\alpha]^{20}_{D}$ −31.1 (*c* 0.5, MeOH); ^{1}H NMR (pyridine-d_5, 500 MHz) δ_H 3.25 (1H, dd, 4.0, 12.0, H-3), 3.00 (1H, m, H-13), 1.05 (3H, s, H-18), 0.63 (3H, s, H-19), 1.42 (3H, s, H-21), 4.31 (1H, brs, H-23), 5.51 (1H, d, 8.0, H-24), 1.81 (3H, s, H-26), 1.68 (3H, s, H-27), 1.27 (3H, s, H-28), 0.95 (3H, s, H-29); ^{13}C NMR, see Tables 1 and 2; ESIMS *m/z* 1083 $[M + Na]^+$; HRESIMS *m/z* 1083.5352 $[M + Na]^+$ (calcd for $C_{52}H_{84}O_{22}Na$, 1083.5352).

Acid Hydrolysis and GC Analysis of 1−5. Each compound (5 mg) was heated in 2.0 mol/L HCl−MeOH (1:1, v/v, 10 mL) at 90 °C for 4 h. The reaction mixture was evaporated to dryness and then partitioned between EtOAc and H_2O. The H_2O layer was neutralized by passing through an ion-exchange resin (Amberlite MB-3) column and concentrated to yield a sugar residue. The residue was treated with dry pyridine and L-cysteine methyl ester hydrochloride at 60 °C for 2 h with stirring and then concentrated to dryness. Trimethylsilylimidazole was added to the residue, and the mixture was heated for 1 h at 60 °C, followed by partition between *n*-hexane and water. The organic layer was analyzed by GC [HP-5892 II with an FID detector and an HP-20M (Carbowx 20M) capillary column (25 m × 0.32 mm × 0.3 μm)], and their retention times were compared with those of authentic sugars.[11] D-Glucose and L-arabinopyranose were detected from **1**, **2**, and **3**. D-Arabinofuranose was detected from **2** and **3**.

Drugs. All the drugs were freshly prepared each day. The tested compounds were suspended in 0.3% carboxymethylcellulose sodium (CMCNa) at a concentration of 2.5 mg/mL, while fluoxetine hydrochloride (Changzhou Huasheng Pharmaceuticals Co. Ltd.) was also suspended in 0.3% CMCNa at a concentration of 0.5 mg/mL. Fluoxetine hydrochloride and 0.3% CMCNa were applied as positive and normal controls, respectively.

Animals. Male ICR mice (18−22 g) from a breeding colony in the Experimental Animal Center of the Second Military Medical University, Shanghai, People's Republic of China, were housed with unlimited food and water in a room maintained with a 12:12 h light−dark cycle for a week before the experiment. The experiment was conducted in the light portion of the cycle, and all mice were gently handled daily. Mice were treated with saponins (50 mg/kg/20 mL) once daily for five consecutive days, while fluoxetine hydrochloride (10 mg/kg/20 mL) was administrated only once, on the fifth day. The control group received only 0.3% CMCNa once daily for 5 consecutive days. All substances were administered orally in a volume of 20 mL/kg.

Forced Swimming Test (FST). Compounds **2**, **4**, **5**, and **6** were tested for antidepressant activity using the FST initially described by Porsolt.[12,13] The tank (Plexiglas cylinder, 20 cm tall and 14 cm in diameter) was filled with H_2O (24 °C) to a height of 10 cm. The mouse was dropped into the water 1 h after administration on the fifth day and observed for 6 min. The first 2 min the animal was allowed to adjust to the new conditions. Over the next 4 min, the duration of the two types of motor activity, struggling and floating status, was recorded as immobility time with the observer unaware of administration.

Tail Suspension Test. The tail suspension test is the second method for assessing the antidepressant activity. The experiment was carried out according to the method described by Steru et al.[14] One hour after administration on the fifth day, the mouse was suspended by the tail from a lever (30 cm high) for 6 min with the movements of mouse being recorded by the observer. The total duration of the test (6 min) can be divided into agitation and immobility periods. The duration of immobility in the later 4 min represented the "behavior despair" status.

Spontaneous Motor Activity. The spontaneous motor activity of mouse was monitored via a little animal locomotor activity meter (Y-2, made in Second Military Medical University, China) 1 h after administration on the fifth day. The distance traveled in the open field (25 cm in height, 35 cm in diameter) was automatically recorded by the arithmometer of the Y-2. The mice were allowed to adjust to the

new conditions in the first 3 min, and then the spontaneous motor activity was recorded over the next 6 min.

The results were analyzed by one-way ANOVA (SPSS) followed by Dunnett's *t*-test. The data were presented as means ± SEM. All animal treatments were strictly in accordance with the National Institutes of Health Guide of the Care and Use of Laboratory Animals. The experiments were carried out under the approval of the Committee of Experimental Animal Administration of the University.

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