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Phytochemistry 65 (2004) 969–974
PHYTOCHEMISTRY
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Flavone glucosides with immunomodulatory activity from the leaves of *Pleioblastus amarus*

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Received 29 July 2003; received in revised form 22 October 2003

Abstract

Three flavone glucosides, pleiosides A–C, were isolated from the leaves of *Pleioblastus amarus*, along with two known flavones: tricin and tricetin 3,5-dimethoxy-7-*O*-β-D-glucopyranoside. Their structures were elucidated by extensive spectral studies. Pleiosides A–C were found to inhibit the proliferation of murine T and significantly stimulate the proliferation of murine B lymphocytes in vitro.

Keywords: *Pleioblastus amarus*; Gramineae; Flavone glucosides; Immunomodulatory activity

1. Introduction

Pleioblastus amarus (Gramineae) occurs widely in south China. The dried tender leaves of the plant are used in Chinese herbal medicine as antipyretic and diuretic agents (State Administration of Traditional Chinese Medicine, 1999). Previous chemical work on the plant resulted in the isolation of several known flavonoids rutin, quercetin 7-*O*-glucoside) (Xiao et al., 2001). As part of our studies on Chinese medicinal plants, we have investigated the chemical constituents of the leaves of *Pleioblastus amarus*. The present paper deals with the isolation, structure elucidation and in vitro immunomodulatory activity of three new flavone glucosides (**1**–**3**). Additionally, we isolated two known flavones (**4**–**5**) which were obtained from the plant for the first time.

2. Results and discussion

Compound **1**, obtained as a yellow powder, has the molecular formula $C_{24}H_{26}O_{12}$ determined by HRE-SIMS (*m*/*z* 506.1323 $[M]^+$, calc. 506.1322). It showed positive reactions with HCl/Mg and α-naphthol agents. The UV spectrum of **1** displayed maximum absorptions at 271 and 327 nm, indicating that **1** is a flavone derivative (Agrawal, 1989). The IR spectrum showed the presence of hydroxyl (3403 cm^{-1}), conjugated and 5-OH chelated ketone (1623 cm^{-1}) and aromatic rings (1504 cm^{-1}). Hydrolysis of **1** with β-glucosidase afforded glucose as detected by co-TLC, suggesting that **1** is a flavone glucoside with a 5-hydroxyl group.

The sugar moiety of **1** identified as glucose was confirmed by analyses the ^1H and ^{13}C NMR spectroscopic data (Table 1). The signals of the flavone moiety arise from three methoxyl (δ 3.88, 3.89, 3.89, each 3H, *s*) and five aromatic protons, which were assignable to H-6 and H-8 (δ 6.39, 6.87, each 1H, *d*, $J = 1.1$ Hz), H-3 (δ 7.12, 1H, *s*) and equivalent H-2′ and H-6′ (δ 7.37, 2H, *s*). The ^{13}C NMR spectrum of **1** (Table 1) showed the presence of a glucose unit (δ 101.9, 74.0, 76.5, 69.8, 77.4 and 60.7) and a 3′,5′,7-trimethoxyflavone, which included three methoxyl, five methine and ten quaternary carbons. The unambiguous assignments of all protons and carbons were made by ^1H–^1H COSY, HMQC and HMBC (Table 1) analyses.

The glucosylated position in **1** was deduced by HMBC experiments (Fig. 1). In the HMBC spectrum, the correlations of H-2′, H-6′ (δ 7.37) and H-1″ of

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doi:10.1016/j.phytochem.2003.11.013

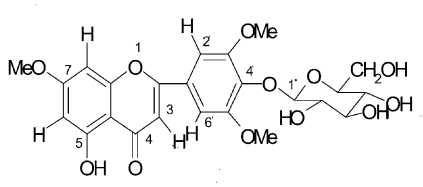
glucose (δ 5.16) with C-4′ (δ 137.7) were observed, suggesting glucosylation at C-4′. Furthermore, the characteristic fragment derived from flavone as $[A_1+H]^+$ at m/z 167.0294 $[C_8H_6O_4+H]^+$ indicated one hydroxyl and one methoxyl group in ring A (Chibber and Sharma, 1979), which confirmed glucosylation at C-4′ of ring B. The β-glucosyl linkage was deduced from the coupling constant of the anomeric proton at δ 5.16 ($J=7.4$ Hz). On the basis of above evidence, **1** was determined as tricin 7-methyl ether-4′-*O*-β-D-glucopyranoside, a new flavone glucoside named pleioside A.

Compound **2** was obtained as a yellow powder. A molecular formula of $C_{24}H_{26}O_{12}$, (as also noted for **1**), was determined from its HRESIMS (m/z 506.1318 $[M]^+$, calc. 506.1322). Hydrolysis of **2** with β-glucosidase afforded glucose by co-TLC detection. The UV spectrum of **2** exhibited characteristic absorption bands of a flavone moiety at 265 and 342 nm. The IR spectrum showed absorptions at 3419, 1643 and 1515 cm^{-1}, indicating the presence of hydroxyl and carbonyl groups and an aromatic system. Furthermore, the carbonyl absorption at 1643 cm^{-1} revealed the lack of chelation with free 5-hydroxyl group in flavones.

Analysis of the ^1H and ^{13}C NMR spectra (Table 1) showed that signals of **2** were very similar to that of **1**. The differences are: (a) the 5-OH signal of **2** was absent in the ^1H NMR prediction; (b) the C-4 signal was shifted upfield from δ 182.0 in **1** to δ 177.1 in **2**, indicating that the 5-OH group was glucosylated. The assignment of all protons and carbons were made by ^1H–^1H COSY, HMQC and HMBC experiments (Table 1). The HMBC correlation of the C-5 signal at δ 158.2 and the H-1″ signal of glucose at δ 4.76 confirmed glucosylation at the C_5-OH functionality (Fig. 1). The β-glucosyl linkage was deduced from coupling constant of anomeric proton at δ 4.76 ($J=7.4$ Hz). Therefore, **2** was determined as tricin 7-methyl ether 5-*O*-β-D-glucopyranoside, a new flavone glucoside named pleioside B.

Compound **3** was obtained as a yellow amorphous powder and its molecular formula, $C_{27}H_{30}O_{14}$, was established from HRESIMS (m/z 578.1537 $[M]^+$, calc. 578.1533). The UV spectrum of **3** showed absorptions at λ_{max} 272 and 334 nm, indicating the presence of a flavone skeleton. The IR spectrum showed functionalities absorptions corresponding to functionalities at 3430 (OH), 1625 (conjugated and 5-OH chelated carbonyl) and 1496 (aromatic ring) cm^{-1}, respectively. Hydrolysis of **3** with β-glucosidase yielded glucose as detected by co-TLC, suggesting that **3** is a flavone glucoside.

The ^1H NMR spectra of **3** (Table 1) showed signals of a glucose and a flavone moiety. Based on the NMR analysis of **3**, the five aromatic proton signals of flavone moiety can be assigned for H-6 and H-8 (δ 6.82, 6.55, each 1H, *d*, $J=2.0$ Hz), H-2′ (δ 7.50, 1H, *d*, $J=2.4$ Hz),



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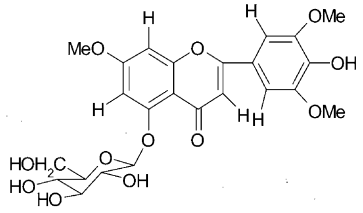

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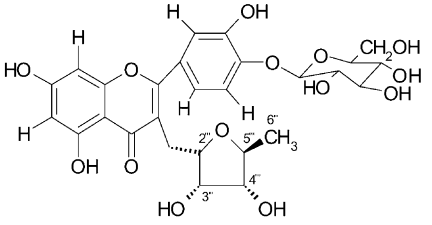

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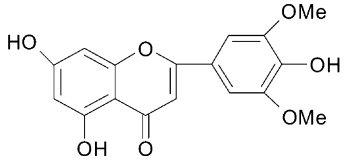

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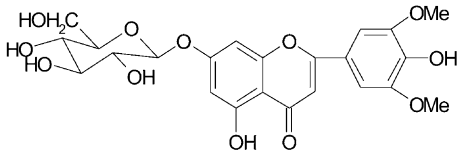

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H-5′ (δ 7.22, 1H, *d*, *J* = 8.4 Hz) and H-6′ (δ 7.52, 1H, *dd*, *J* = 8.4, 2.4 Hz), indicating that **3** is a 3′,4′,5,7-tetrahydroxyflavone. The HMBC correlation between H-2′, H-6′, H-1″ and C-4′ revealed the glucosylation at C-4′. Except for 3′, 5, 7-trihydroxy-4′-*O*-glucosyl substitution, an additional part comprised by a monocyclic unit of $C_6H_9O_3$ was attached to the flavone at C-3. The ^1H NMR spectrum showed signals for one methyl (δ 1.06, 3H, *d*, *J* = 4.7 Hz, H-6‴, one methylene (δ 1.46, 1H, *d*, *J* = 13.8 Hz; δ2.19, 1H, *dd*, *J* = 13.8, 11.7 Hz) and four oxygenated methines (δ 5.30, 1H, *dd*, *J* = 11.7, 2.7 Hz, H-2‴; δ 4.01, 1H, *m*, H-3‴; δ 3.22, 1H, *d*, *J* = 3.2 Hz, H-4‴ and δ 3.82, 1H, *m*, H-5‴). Further studies on the HMBC (Fig. 1), HMQC, ^1H–^1H COSY spectra of **3** revealed that the additional monocyclic unit should be a 2‴-methylene-3‴,4‴-dihydroxy-5‴-methyl tetrahydrofuran. The ROESY spectrum of **3** (Fig. 2) showed a correlation between H-2‴and H-3‴, H-3‴and H-4‴, H-3‴ and H-6‴, and H-4‴and H-6‴, indicating that H-2‴, H-3‴, H-4‴and C-6‴ were at one side and C-1‴, H-5‴ and two hydroxyl groups were at the opposite side of the tetrahydrofuran ring. Therefore, **3** was determined as luteolin 3-(2‴-methylene-3‴,4‴-dihydroxy-5‴-methyltetrahydrofuran) 4′-*O*-β-D-glucopyranoside, a new flavone derivative named pleioside C.

The remaining two compounds were identified as tricin (**4**) (Harborne J.B., 1994) and tricetin-3′, 5′-dimethoxy-7-*O*-β-D-glucopyranoside (**5**) (Williams and Harborne, 1973).

The new compounds were subjected for evaluation of their immunomodulatory activity in vitro. It was found that compound **1** inhibited significantly ($P < 0.01$) the proliferation of murine T cells and stimulated significantly ($P < 0.01$) the proliferation of murine B lymphocytes in vitro at concentrations of 4×10^{-5} M, **2** and **3** stimulated significantly ($P < 0.01$) the proliferation of murine B lymphocytes at concentrations of 4×10^{-6} M (Table 2).

3. Experimental

3.1. General

IR spectra were recorded as KBr pellets on a Perkin-Elmer 599B spectrophotometer. UV spectra were measured on a Shimadzu UV-3000 spectrophotometer in absolute MeOH. MS were determined on a Varian Mat-711 mass spectrometer. NMR spectra were measured on a Brüker AM-400 spectrometer with TMS as int. standard and DMSO as solvent. 2D-NMR spectra were performed on a Brüker DRX 500 spectrometer. Optical rotations were recorded with a Jasco Dip-181 spectropolarimeter. Silica gel (200–300 mesh and H60) and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden) were used for cc and silica gel GF_{254} for TLC (Qingdao Marine Chemical Co.). β-Glucosidase was manufactured by Lizhu Dongfeng Bio-Tech Co. Ltd., Shanghai, People's Republic of China.

3.2. Plant material

The leaves of *Pleioblastus amarus* were supplied by Shanghai jahwa United Co., Ltd., Shanghai, People's Republic of China, in September 2001. The materials were authenticated by MS. Yao in Shanghai jahwa

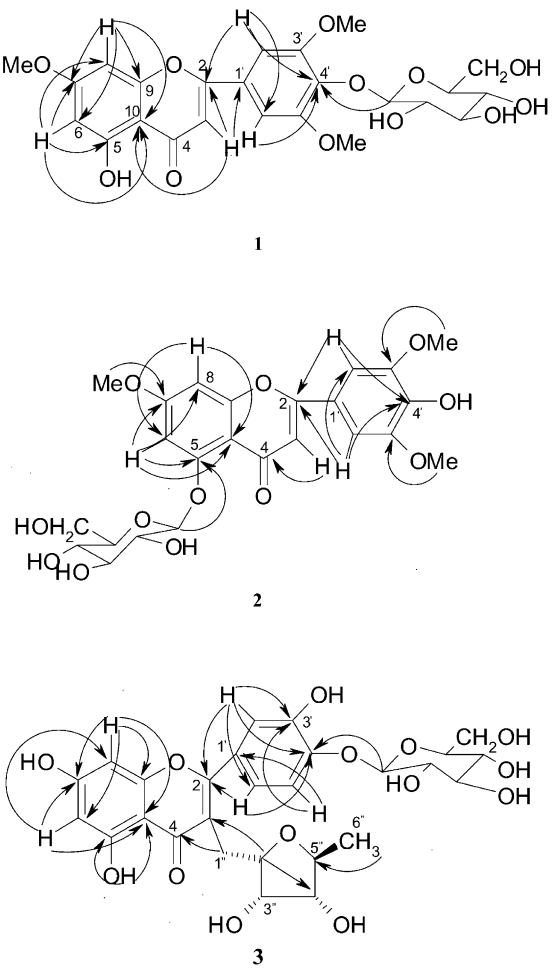

Fig. 1. Key correlations (H→C) observed in the HMBC spectra of **1**–**3**.

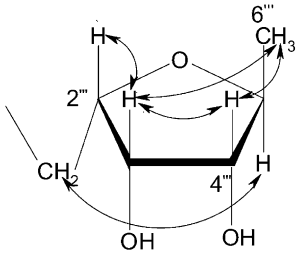

Fig. 2. Significant ROESY correlations of compound **3**.

Table 1
^{1}H NMR(400 MHz) and ^{13}C NMR (100 MHz) spectral data of **1–3** in DMSO-d_6 (ppm, J in Hz)

C	**1**		**2**		**3**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2		163.2		161.3		162.6
3	7.12 *s*	104.8	6.90 *s*	106.5		110.5
4		182.0		177.1		182.1
5		161.1		158.2		157.3
6	6.39 *d* (1.1)	98.1	6.89 *d* (2.5)	103.5	6.82 *d* (2.0)	103.9
7		165.3		163.6		163.4
8	6.87 *d* (1.1)	92.9	7.13 *d* (2.5)	96.7	6.55 *d* (2.0)	94.8
9		157.4		158.5		156.2
10		104.8		109.3		103.6
1′		125.6		120.3		124.6
2′	7.37 *s*	105.0	7.32 *s*	104.2	7.50 *d* (2.4)	113.6
3′		152.9		148.2		146.9
4′		137.7		139.5		148.6
5′		152.9		148.2	7.22 *d* (8.4)	116.0
6′	7.37 *s*	105.0	7.32 *s*	104.2	7.52*dd* (8.4,2.4)	118.6
7-OCH_3	3.88 *s*	56.1	3.91 *s*	56.2		
3′,5′-OCH_3	3.89 *s*	56.8	3.85 *s*	56.4		
5-OH	12.87 *s*				12.51 *s*	
1″	5.16 *d* (7.4)	101.9	4.76 *d* (7.4)	104.2	4.87 *d* (7.1)	101.2
2″	3.47 *m*	74.0	3.38 *m*	73.6	3.57 *m*	73.2
3″	3.40 *m*	76.5	3.32 *m*	75.8	3.52 *m*	75.8
4″	3.41 *m*	69.8	3.18 *m*	69.9	3.41 *m*	69.8
5″	3.19 *m*	77.4	3.38 *m*	77.6	3.47 *m*	77.3
6″	3.66 *dd* (12.0,4.8)	60.7	3.50 *dd* (11.5, 6.0)	60.9	3.73 *dd* (12.0, 6.0)	60.7
	3.77 *dd* (12.0,2.4)		3.72 *dd* (11.5, 2.0)		3.94 *dd* (12.0, 6.0)	
1‴					1.46 *d* (13.8)	31.4
					2.19 *dd* (13.8,11.7)	
2‴					5.30 *dd*,(11.7,2.7)	67.4
3‴					4.01 *m*	70.7
4‴					3.22 *d* (3.2)	68.7
5‴					3.82 *m*	66.5
6‴					1.06 *d* (4.7)	17.1

Table 2
Effect of compounds **1–3** on murine lymphocyte proliferation induced by concanavalin A (ConA) (5 μg/ml) or lipopolysaccharide (LPS) (10 μg/ml)

Compounds	Concentration (M)	[^{3}H] TdR incorporation$\times 10^{-3}$ (cpm)			
		ConA-induced T cell proliferation		LPS-induced B cell proliferation	
Negative control		1.82±0.19		1.81±0.35	
Positive control (ConA or LPS)		107.24±3.45		28.91±1.99	
1	4×10^{-7}	100.68±1.13		30.97±1.46	
	4×10^{-6}	97.13±2.78		34.35±1.96	↑
	4×10^{-5}	90.18±2.59	↓↓	40.87±2.65	↑↑
2	4×10^{-7}	104.32±4.01		32.34±2.12	
	4×10^{-6}	108.12±0.24		35.65±1.05	↑↑
	4×10^{-5}	100.72±1.09		32.33±0.95	
3	4×10^{-7}	98.72±5.23		29.80±2.34	
	4×10^{-6}	95.06±7.28		34.30±2.27	↑↑
	4×10^{-5}	89.22±2.37	↓	36.49±2.06	↑

Results are represented as mean±S.D. based on three independent experiments. ($n=3$; ↑, ↓ $P<0.05$; ↑↑, ↓↓$P<0.01$ compared with control group).

United Co., Ltd., where a specimen (No. 010910) is deposited.

3.3. Extraction and isolation

The air-dried and powdered leaves of *Pleioblastus amarus* (10 kg) were exhaustively extracted with 95% EtOH (5 L×3) at room temp. The extract was concentrated in vacuo to yield an EtOH extract (200 g), which was then suspended in distilled water and partitioned successively with petroleum ether, EtOAc and BuOH. The EtOAc extract was concentrated in vacuo to give a residue (16 g), which was applied to a silica gel column, eluted with petroleum ether containing increasing amounts of Me_2CO to afford five fractions (A–E). Fraction E was subjected to repeated silica gel cc eluted with $CHCl_3$–MeOH (30:1) to yield compound **1** (12 mg). The BuOH extract portion (50 g) was applied to a silica gel column eluted with $CHCl_3$–MeOH (25:1–1:1) to afford eight fractions (A–H). Fraction B was subjected to repeated silica gel cc eluted with $CHCl_3$–MeOH (25:1) followed by Sephadex LH-20 (MeOH) chromatography to give compound **2** (14 mg). By the same method, fraction H afforded compound **3** (40 mg), fraction D afforded **4** (12 mg), and fraction G afforded compound **5** (25 mg).

3.4. Hydrolysis of flavonoid glucosides

Compound **1** (3 mg) was dissolved in H_2O (8 ml) and incubated with almond β-glucosidase (20 mg) for 24 h at 37 °C. The flavonoid was dissolved in MeOH to separate it from the sugar moiety. The sugar moiety was dissolved in H_2O and analyzed by silica gel HPTLC (Merck) developed with Me_2CO–2 mM NaOAc (17:3, v/v). Sugar was detected by spraying with 0.2% naphthoresorcinol in Me_2CO–3N H_3PO_4 (5:1, v/v) and then heated at 105 °C for 5 min. D-Glucopyranose (Chemprosa Holding AG) was used as standard. The same method to identify the sugar moiety was used for compounds **2** and **3**.

3.5. Pleioside A (*1*)

Yellow amorphous powder. $[\alpha]_D^{20}-15^\circ$ (Pyridine; *c* 0.1). UV (MeOH) λ_{max}: 271, 327 nm. IR (KBr) ν_{max} cm^{-1}: 3403 (OH), 1623 (>C=O), 1504 (C=C) cm^{-1}. ^1H NMR spectral data (400 MHz, DMSO-d_6) and ^{13}C NMR spectral data (100 MHz, DMSO-d_6) are shown in Table 1. HRESIMS *m/z* 506.1323 $[M]^+$ (calc. for $C_{24}H_{26}O_{12}$, 506.1322).

3.6. Pleioside B (*2*)

Yellow amorphous powder. $[\alpha]_D^{20}-209^\circ$ (Pyridine; *c* 0.1). UV (MeOH) λ_{max}: 265, 342 nm. IR (KBr) ν_{max} cm^{-1}: 3419 (OH), 1643 (>C=O), 1515 (C=C) cm^{-1}. ^1H NMR spectral data (400 MHz, DMSO-d_6) and ^{13}C NMR spectral data (100 MHz, DMSO-d_6) are shown in Table 1. HRESIMS *m/z* 506.1318 $[M]^+$ (calc. for $C_{24}H_{26}O_{12}$ 506.1322).

3.7. Pleioside C (*3*)

Yellow amorphous powder. $[\alpha]_D^{20}$ +33.5° (Pyridine; *c* 0.4). UV (MeOH) λ_{max}: 272, 334 nm. IR (KBr) ν_{max} cm^{-1}: 3430 (OH), 1625 (>C=O), 1496 (C=C) cm^{-1}. ^1H NMR spectral data (400 MHz, DMSO-d_6) and ^{13}C NMR spectral data (100 MHz, DMSO-d_6) are shown in Table 1. HRESIMS *m/z* 578.1537 $[M]^+$ (calc. for $C_{27}H_{30}O_{14}$ 578.1533).

3.8. Lymphocyte proliferation test

The prepared spleen cells of mice (4×10^6) were seeded into each well of a 96-well microplate and various concentrations of compounds **1–3** and 5μg/ml of concanavalin A (Con A, from *Canavalia ensiformis* Type III, Sigma) or lipopolysaccharide (LPS, from *Escherichia coli*, Sigma) were added alone or in combination. The plates were cultured at 37 °C with 5% CO_2 in a humidified atmosphere for 48 h. For the last 6 h, each well was pulsed with 0.25 μ*Ci*/well ^3H-TdR (thymidine, [methyl-^3H], ICN Pharmaceuticals, Inc., Irvine, CA). The cells were harvested and the radioactivity incorporated was counted by a liquid scintillation counter. All counts/min values shown were the mean of triplicate sample+SD. Statistical analysis was carried out by Student *t*-test. ConA or LPS was used as a positive control (Li et al., 1990; Xiang and Li, 1993).

Acknowledgements

We thank the National Center for Drug Screening, Shanghai, and Department of Pharmacology, Shanghai Institute of Materia Medica, People's Republic of China for assistance with the bioassay.

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