

A New Monoterpene Glycoside and Antibacterial Monoterpene Glycosides from *Paeonia suffruticosa*

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(Received April 5, 2006)

Antibacterial activity-guided fractionation of the CHCl₃-MeOH (1:1) extract of *Paeonia suffruticosa* root bark furnished three monoterpene glycosides, 6-O-vanillyloxypaeoniflorin (1), mudanpioside-H (2), and galloyl-oxypaeoniflorin (3). Of the isolated compounds, compound 1 is a new compound. All isolated compounds showed broad, but moderate, antibacterial activity with minimum inhibitory concentration (MIC) values in the range of 100 to 500 µg/mL against eighteen pathogenic microorganisms of concern for public health or zoonosis.

Key words: *Paeonia suffruticosa*, 6-O-Vanillyloxypaeoniflorin, Monoterpene glycoside, mudanpioside-H, Galloyl-oxypaeoniflorin, Antibacterial

INTRODUCTION

The discovery and development of antibiotics are among the most significant advances in medicine in the 20th century. Nevertheless, within recent years, infections have increased to a great extent, and antibiotic resistance is becoming an ever-increasing therapeutic problem. Therefore, to ensure that effective drugs will be available in the future, it is necessary to develop new antibacterial agents. Natural products from higher plants may offer a new source of antimicrobial agents, possibly with novel mechanisms of action (Barbour *et al.*, 2004). In the course of screening for antibacterial compounds from herbal medicines, the CHCl₃-MeOH (1:1) extract of *Paeonia suffruticosa* root bark exhibited promising antibacterial activity against various pathogens that are of concern for public health or zoonosis.

Paeonia suffruticosa Andr. (Paeoniaceae) is widely distributed in Asia. The root bark of this plant has been used in traditional Chinese medicine for the treatment of

diseases, including hypertension and allergic rhinitis, and also as an antimicrobial or anti-inflammatory (Zhu, 1998). Previous phytochemical and pharmacological studies of this plant have led to the isolation of paeonol derivatives (Lin *et al.*, 1991), monoterpene glycosides (Ding *et al.*, 1999; Kitagawa *et al.*, 1979; Murakami *et al.*, 1996; Tani *et al.*, 1980), gallic acid glycosides (Takechi *et al.*, 1982), flavonoids (Wang *et al.*, 2005), and triterpenoids (Lin *et al.*, 1998); vasodilatory and anti-inflammatory effects (Kang *et al.*, 2005), acaricidal activity (Kim *et al.*, 2004), and anti-septic activity (Li *et al.*, 2004) have also been described for this plant. In this paper, we describe the isolation and structural elucidation of a new antibacterial monoterpene glycoside and two known antibacterial monoterpene glycosides from the root bark of *P. suffruticosa*.

MATERIALS AND METHODS

General experimental procedures

Melting points were determined with a Yanaco MP-S3 micro melting point apparatus and are not corrected. Optical rotation was measured on a JASCO DIP-370 digital polarimeter. UV spectra were obtained on a Milton Roy 3000 spectrophotometer. ¹H-, ¹³C-NMR, and two-

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dimensional (2D)-NMR spectra were taken on a JEOL JNM-ECP 500 (^1H , 500 MHz; ^{13}C , 125 MHz) spectrometer. FAB-MS and ESI-MS spectra were measured on a JMS-HX110/110A Tandem Mass spectrometer (JEOL) and an API-2000 spectrometer, respectively. TLC was carried out on silica gel 60 F₂₅₄ and RP-18 F₂₅₄ plates (Merck, Germany). Column chromatography was performed over silica gel 60 (230–400 mesh, Merck) and Sephadex LH-20 (Pharmacia, Sweden).

Plant material

The root bark of *Paeonia suffruticosa* was purchased in March 2004 at the University Oriental herbal drugstore, Iksan, Korea, and identified by Dr. Kyu-Kwan Jang, Botanical Garden, Wonkwang University. A voucher specimen (No. WP04-131) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea).

Extraction and isolation

Dried and pulverized *P. suffruticosa* root bark (2 kg) was extracted twice with CHCl_3 -MeOH (1:1, 2 L) at room temperature for two days, and the extract was concentrated *in vacuo* to give a dried extract (216.95 g). The MeOH extract was dissolved in 60% aqueous MeOH (1 L) and partitioned with CHCl_3 (800 mL x 2). The 60% aqueous methanolic fraction was then evaporated, and the resulting extract was subsequently dissolved in distilled water (1 L) then partitioned with EtOAc (800 mL x 2). The EtOAc-soluble fraction (15.09 g) was chromatographed on a silica gel column using CHCl_3 -MeOH (8:1→1:1, gradient) to obtain five fractions (Fr. E1~5). Fraction E3 (1.1 g) was purified by Sephadex LH-20 column chromatography (CHCl_3 : MeOH, 6:1) to yield compounds **1** (50 mg) and **2** (60 mg). Fraction E4 (2.3 g) was subjected to chromatography on a Sephadex LH-20 column, and eluted with CHCl_3 -MeOH (4:1) to yield seven subfractions (Fr. E41~47). Fr. E45 (70 mg) was chromatographed on Sep-Pak C₁₈ cartridge with CH_3CN (20% in H_2O) followed by MeOH (100%) to give compound **3** (21.7 mg).

6-O-vanillyloxypaeoniflorin (1)

Amorphous powder; m.p. 142–145°C; $[\alpha]_{\text{D}}^{25}$: -6.0° (c 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 201 (4.56), 262 (4.21) nm; HRFABMS m/z 669.5927 (calcd for $\text{C}_{31}\text{H}_{34}\text{O}_{15}\text{Na}$, 669.5917); ^1H - and ^{13}C -NMR data, see Table I.

Mudanpioside-H (2)

Amorphous powder; m.p. 158–161°C; (-)-ESI-MS m/z 615 [M-H]; ^1H -NMR (Pyridin- d_5 , 500 MHz) δ : 1.67 (3H, s, H-10), 2.28 and 2.44 (each 1H, d, $J = 12.8$ Hz, H-3), 2.23 (1H, d, $J = 8.2$ Hz, H-6a), 2.85 (1H, dd, $J = 8.2, 6.4$ Hz, H-6b), 3.06 (1H, d, $J = 6.4$ Hz, H-5), 5.01 and 5.16 (each

Table I. ^1H -, ^{13}C -NMR, and 2D NMR Data for Compound **1** (CD_3OD , 500 MHz)

position	δ_{C} (ppm)	mult.	δ_{H} (ppm)	mult. (J/Hz)	HMBC (H→C)
1	88.7				
2	86.0				
3	44.6		2.31 d (12.0)		4
			2.50 d (12.0)		
4	105.8				
5	43.7		3.08 d (6.4)		1, 4, 7, 8
6	22.9		2.33 d (10.5)		1, 2, 4, 5
			2.92 dd (10.5, 6.4)		
7	71.4				
8	60.7		5.04 d (12.0)		1, 5, 7, 9
			5.19 d (12.0)		
9	101.6		5.95 s		2, 4, 7, 8
10	19.7		1.71 s		1, 2, 3
1'	100.2		5.18 d (7.3)		1
2'	74.8		4.06 t (8.7)		1', 3'
3'	78.1		4.26 t (8.7)		2', 4'
4'	71.8		4.10 t (8.7)		3', 5'
5'	75.2		4.14 dd (8.7, 7.3)		3', 4'
6'	64.9		4.97 dd (11.4, 7.3)		4', 7''
			5.27 d (11.4)		
1''	121.2				
2''	132.3		8.16 d (8.7)		4'', 6'', 7''
3''	116.0		7.06 d (8.7)		1'', 5''
4''	163.6				
5''	116.0		7.06 d (8.7)		1'', 3''
6''	132.3		8.16 d (8.7)		2'', 4'', 7''
7''	166.5				
1'''	121.5				
2'''	113.3		7.94 d (1.3)		4''', 6''', 7'''
3'''	148.3				
4'''	153.3				
5'''	116.2		7.25 d (8.2)		1''', 3'''
6'''	124.7		8.02 dd (8.2, 1.3)		2''', 4''', 7'''
7'''	166.5				
-OCH ₃	55.8		3.80 s		3'''

1H, d, $J = 12.0$ Hz, H-8), 5.92 (1H, s, H-9), 4.05 (1H, t, $J = 7.8$ Hz, H-2'), 4.09 (1H, t, $J = 7.8$ Hz, H-4'), 4.10 (1H, dd, $J = 7.8, 5.1$ Hz, H-5'), 4.24 (1H, t, $J = 7.8$ Hz, H-3'), 4.96 (1H, dd, $J = 11.2, 5.1$ Hz, H-6'a), 5.14 (1H, d, $J = 7.8$ Hz, H-1'), 5.19 (1H, d, $J = 11.2$ Hz, H-6'b), 7.06 (2H, d, $J = 8.7$ Hz, H-3'', H-5''), 8.13 (2H, d, $J = 8.7$ Hz, H-2'', H-6''), 7.18 (2H, d, $J = 8.7$ Hz, H-3''', H-5'''), 8.27 (2H, d, $J = 8.7$ Hz, H-2''', H-6'''); ^{13}C -NMR (Pyridin- d_5 , 500 MHz) δ : 88.8 (C-1), 85.9 (C-2), 44.6 (C-3), 105.9 (C-4), 43.7 (C-5), 22.7 (C-6), 71.7 (C-7), 60.7 (C-8), 101.6 (C-9), 19.7 (C-10), 100.2 (C-1'), 74.8 (C-2'), 78.1 (C-3'), 71.4 (C-4'), 75.1 (C-5'), 64.6 (C-6'), 121.2 (C-1''), 132.3 (C-2''), 116.0 (C-3''), 163.5 (C-4''), 116.0 (C-5''), 132.3 (C-6''), 166.4 (C-7''), 121.5 (C-1'''), 132.3 (C-2'''), 116.1 (C-3'''), 163.6 (C-4'''), 116.1 (C-5'''), 132.3 (C-6'''), 166.6 (C-7''').

Galloyl-oxypaeoniflorin (3)

Amorphous powder; m.p. 174–176°C; (-)-ESI-MS m/z 647 [M-H]⁻; ^1H -NMR (CD_3OD , 500 MHz) δ : 1.24 (3H, s, H-10), 1.69 and 1.90 (each 1H, d, $J = 12.4$ Hz, H-3), 1.73 (1H, d, $J = 10.5$ Hz, H-6a), 2.43 (1H, dd, $J = 10.5, 6.4$ Hz, H-6b), 2.51 (1H, d, $J = 6.4$ Hz, H-5), 4.64 (2H, br s, H-8),

5.36 (1H, s, H-9), 3.24 (1H, t, $J = 7.8$ Hz, H-2'), 3.28 (1H, t, $J = 7.8$ Hz, H-4'), 3.29 (1H, dd, $J = 7.8, 6.8$ Hz, H-5'), 3.54 (1H, t, $J = 7.8$ Hz, H-3'), 4.44 (1H, dd, $J = 12.0, 6.8$ Hz, H-6'a), 4.53 (1H, d, $J = 7.8$ Hz, H-1'), 4.50 (1H, d, $J = 12.0$ Hz, H-6'b), 6.82 (2H, d, $J = 8.7$ Hz, H-3, H-5''), 7.88 (2H, d, $J = 8.7$ Hz, H-2'', H-6''), 7.07 (2H, s, H-2''', H-6'''); ^{13}C -NMR (Pyridin- d_5 , 500 MHz) δ : 88.0 (C-1), 85.9 (C-2), 43.1 (C-3), 105.0 (C-4), 42.5 (C-5), 21.6 (C-6), 70.7 (C-7), 59.8 (C-8), 100.9 (C-9), 18.2 (C-10), 98.7 (C-1'), 73.6 (C-2'), 76.5 (C-3'), 70.7 (C-4'), 73.9 (C-5'), 63.4 (C-6'), 120.7 (C-1''), 131.7 (C-2''), 114.9 (C-3''), 162.4 (C-4''), 114.9 (C-5''), 131.7 (C-6''), 166.8 (C-7''), 120.0 (C-1'''), 108.8 (C-2'''), 145.3 (C-3'''), 138.6 (C-4'''), 145.3 (C-5'''), 108.8 (C-6'''), 166.8 (C-7''').

Bacterial strains and culture medium

E. coli O157 (ATCC 43890), *E. coli* O157 (ATCC 35150), MRSA (ATCC 700698), and *Shigella dysenteriae* (ATCC 49557) were obtained from the American Type Culture Collection. Local isolates of *E. coli* K88 (E-126), *E. coli* K99 (E-125), *E. coli* O157 (from cattle), MRSA 6 (from chicken), MRSA 104 (from human), *Salmonella enteritidis* (Sal-36), *S. gallinarum*, *S. paratyphi* A (JOL 381), *S. typhi* (JOL 380), *S. typhimurium* (Sal-13), *Shigella dysenteriae* (JOL 377), *S. flexneri* (JOL 378), *Vibrio cholerae* O1 (JOL 375), and *V. cholerae* O139 (JOL 376) were provided by the National Veterinary Research and Quarantine Service, Republic of Korea. Bacterial strains were suspended in Tryptic soy broth (TSB, Difco, USA) and incubated at 37°C for 20 h. Müller-Hinton agar (MHA, Difco, U.S.A.) was used for the agar diffusion method and MIC.

Disk diffusion method for the Determination of antibacterial activity

Sterile filter paper discs (6 mm) were soaked with 5 μL

of extract residue diluted in the corresponding extractive solvent (200 mg/mL), so that each disc was impregnated with 1.0 mg of residue; the discs were then allowed to dry before being placed on the top layer of an agar plate. The plates were then incubated for 18 h at 37°C. The antibacterial activity was evaluated by measuring the diameter (mm) of the inhibition zone. Each experiment was performed in duplicate, and the mean of the diameters of the inhibition zones was calculated.

Determination of minimum inhibitory concentration (MIC) of isolated compounds

The serial microplate dilution method of Eloff (1998) was used to screen the plant extracts for antibacterial activity. By measuring reduction of tetrazolium violet, this method allows the determination of the minimal inhibitory concentration (MIC) of each plant extract against each bacterial species. The bacterial cultures were incubated in TSB overnight at 37 °C, and a 1% dilution of each culture in fresh TSB was prepared prior to use in the microdilution assay. Two-fold serial dilutions of plant extract (100 μL) were prepared in 96-well microtitre plates, and 100 μL of bacterial culture were added to each well. The plates were incubated overnight at 37°C, and bacterial growth was detected by then adding 40 μL *p*-iodonitrotetrazolium violet (INT) (Sigma) to each well. After incubation at 37°C for 1 h, INT is reduced to a red formazan by biologically active organisms, in this case, the dividing bacteria. Bacterial growth was shown to be inhibited when the solution in the well remained clear. The lowest concentration of extract in such a well was considered the minimal inhibitory concentration (MIC).

RESULTS AND DISCUSSION

Antibacterial activity of the CHCl_3 -MeOH (1:1) extract of

Table II. Antibacterial activity of the CHCl_3 -MeOH (1:1) extract of *P. suffruticosa* root bark and its fractions^a

Strain	Origin	CHCl_3 -MeOH (1:1) extract	CHCl_3 soluble Fr.	EtOAc soluble Fr.	Aqueous Fr.
<i>E. coli</i> O157	ATCC 43890	11 ^b	8	10	ND ^c
<i>E. coli</i> O157	Cattle	ND	ND	10	ND
MRSA 6	Chicken	11	7	11	ND
MRSA 104	Human	13	7	12	ND
<i>Salmonella paratyphi</i> A	JOL 381	ND	ND	13	ND
<i>S. typhi</i>	JOL 380	ND	ND	11	ND
<i>Shigella dysenteriae</i>	JOL 377	11	8	14	ND
<i>S. flexneri</i>	JOL 378	16	13	15	ND
<i>Vibrio cholerae</i> O1	JOL 375	12	7	15	7
<i>V. cholerae</i> O1	JOL 376	16	11	16	ND

^a Values are the means of duplicates at the concentration of 1 mg/mL. ^b Inhibitory zone diameters are in mm. ^c No detected antibacterial activity at the concentration of 1 mg/mL.

P. suffruticosa root bark and its fractions against ten pathogenic microorganisms was evaluated. As shown in Table II, the EtOAc-soluble fraction of the extract exhibited the most promising antibacterial activity with inhibition zones of 10-16 mm against all of the tested bacterial species. Repeated column chromatography of the EtOAc-soluble antibacterial fraction of *P. suffruticosa* root bark on silica gel and Sephadex LH-20 gel yielded three compounds (compounds 1-3). The chemical structures of compounds 2 and 3 were identified as mudanpioside-H (Ding *et al.*, 1999) and galloyl-oxypaeoniflorin (Yoshikawa *et al.*, 1992), respectively, by comparing the optical rotation, ¹H-NMR, ¹³C-NMR, and MS data with those reported in literature.

Compound 1 was obtained as an amorphous powder with the negative optical rotation ($[\alpha]_{23}^D, -6.0^\circ$). High-resolution fast atom bombardment mass spectrometry (HRFABMS) established the molecular formula as C₃₁H₃₄O₁₅, showing a [M + Na]⁺ ion at *m/z* 669.5927 (calcd for C₃₁H₃₄O₁₅Na, 669.5917). The ¹H-NMR spectrum of 1 showed signals for two methylene protons at δ 2.31, 2.50 (each, d, *J* = 12.0 Hz), and δ 2.33 (d, *J* = 10.5 Hz), 2.92 (dd, *J* = 10.5, 6.4 Hz), a methylene bearing an acyloxy functionality at δ 5.04 and 5.19 (each, d, *J* = 12.0 Hz), a methine proton at δ 3.08 (d, *J* = 6.4 Hz), an acetalic proton at δ 5.95 (s) and a methyl at δ 1.71 (s); all of these were assigned to a monoterpene moiety (Lin *et al.*, 1996).

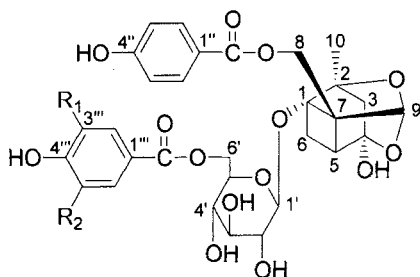
In addition, the glucose moiety appeared at δ 5.18 (d, *J* = 7.3 Hz), 4.06 (t, *J* = 8.7 Hz), 4.26 (t, *J* = 8.7 Hz), 4.10 (t, *J* = 8.7 Hz), 4.14 (dd, *J* = 8.7, 7.3 Hz), and 4.97 (dd, *J* = 11.4, 7.3 Hz), and methylene protons at δ 5.27 (d, *J* = 11.4 Hz) and 5.18 (d, *J* = 7.3 Hz). The remaining signals disclosed the presence of a 4-hydroxybenzoyloxy unit at δ 7.06 (d, *J* = 8.7 Hz) and 8.16 (d, *J* = 8.7 Hz), and for a 4-hydroxy-3-methoxybenzoyloxy unit at δ 7.94 (d, *J* = 1.3 Hz), 7.25 (d, *J* = 8.2 Hz), 8.02 (dd, *J* = 8.2, 1.3 Hz) and 3.80 (s). This observation was further supported by the ¹³C-NMR spectrometric assignments. The locations of each moiety of monoterpene, glucose, 4-hydroxybenzoyloxy, and 4-hydroxy-3-methoxybenzoyloxy esterifying unit were confirmed by HMBC spectrum, in which long-range correlations were observed between H-8 and C-7'. This phenomenon proved the attachment of the 4-hydroxybenzoyloxy group to C-8 of the monoterpene, of the anomeric proton H-1' to C-1 of monoterpene, and of the other 4-hydroxy-3-methoxybenzoyloxy to C-6' of glucose (Tanaka *et al.*, 2000; Wu *et al.*, 2002) (Table I). The assignments of the other signals were confirmed by HMQC and HMBC experiments. Based on the above spectral analysis, the structure of compound 1 was established as 6'-*O*-vanillyloxypaeoniflorin.

The antibacterial activity of compounds 1-3 was assessed, and all of these compounds showed broad, but moderate, antibacterial activity against eighteen pathogenic

Table III. Antibacterial activity (MIC, $\mu\text{g/mL}$) of compounds 1-3

Strain	Origin	1	2	3	Kanamycin A	Penicillin G
<i>E. coli</i> O157	ATCC 43890	200	100	200	10	50
<i>E. coli</i> O157	ATCC 35150	300	200	100	10	50
<i>E. coli</i> O157	Cattle	200	100	100	ND ^a	ND
<i>E. coli</i> K88	E-126	200	200	200	50	100
<i>E. coli</i> K99	E-125	200	200	200	50	50
MRSA	ATCC 700698	200	300	300	ND	100
MRSA 6	Chicken	300	300	300	50	ND
MRSA 104	Human	200	300	300	ND	ND
<i>Salmonella enteritidis</i>	Sal-36	200	200	200	50	50
<i>S. gallinarum</i>	-	200	100	100	50	10
<i>S. paratyphi</i> A	JOL 381	200	100	100	ND	ND
<i>S. typhi</i>	JOL 380	200	200	200	50	ND
<i>S. typhimurium</i>	Sal-13	200	200	100	50	10
<i>Shigella dysenteriae</i>	ATCC 49557	500	300	300	ND	ND
<i>S. dysenteriae</i>	JOL 377	200	100	100	ND	ND
<i>S. flexneri</i>	JOL 378	200	100	100	10	ND
<i>Vibrio cholerae</i> O1	JOL 375	100	200	300	ND	ND
<i>V. cholerae</i> O139	JOL 376	100	100	100	50	1

^a No detected antibacterial activity at the concentration of 100 $\mu\text{g/mL}$.



	R ₁	R ₂
1	OCH ₃	H
2	H	H
3	OH	OH

Fig. 1. Chemical structures of compounds 1-3 isolated from *P. suffruticosa*

microorganisms that are of concern for public health or zoonosis (Table III). The MIC values of compounds 1-3 for all tested bacteria were found to be in the range of 100 to 500 µg/mL. Kanamycin A and penicillin G were used as positive controls. Although these positive controls showed potent antibacterial activity, with MIC values of 1 - 100 µg/mL, some of the pathogenic strains were not affected by kanamycin A and/or penicillin G, even at a concentration of 1 mg/mL (Table III).

Each of the bacteria used in this study is a major cause of serious infections in public health or zoonosis, as described briefly below. *Escherichia coli* O157 is a concern to public health on a global scale (Mead and Griffin, 1998) and is found in a wide variety of foodstuffs. Both *E. coli* K88 and K99 strains are major causes of diarrhea in piglets (Jin *et al.*, 1998). The methicillin-resistant *Staphylococcus aureus* (MRSA) strains represent a serious cause of nosocomial infections in many countries (Tiemersma *et al.*, 2004). The genus *Salmonella* is a typical member of the family Enterobacteriaceae and consists of gram-negative, nonspore-forming bacilli, and remains the primary cause of food poisoning worldwide (Mead *et al.*, 1999). *Shigella flexneri* is a gram-negative bacterium which causes the most communicable of bacterial dysenteries, shigellosis (Jennison and Verma, 2004). The facultative human pathogen *Vibrio cholerae*, which can be isolated from estuarine and aquatic environments, is well recognized as the causative agent of the human intestinal disease cholera (Riedl and Klose, 2002).

In conclusion, three monoterpene glycosides, 6'-*O*-vanillyloxypaeoniflorin (1), mudanpioside-H (2) and galloyloxypaeoniflorin (3), were isolated from the root bark of *P. suffruticosa*, and all of these compounds showed moderate antibacterial activities against various pathogens that are of concern for public health or zoonosis.

ACKNOWLEDGEMENTS

This work was supported by grant No. RTI05-03-02 from the Regional Technology Innovation Program of the Ministry of Commerce, Industry and Energy (MOCIE).

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