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ISOBENZOFURANONE DERIVATIVES FROM THE ROOTS OF *PHLOMIS BETONICOIDES* AND THEIR BIOACTIVITY

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Abstract – Three new (**1-3**) together with three known (**4-6**) isobenzofuranone derivatives, were isolated from the roots of *Phlomis betonicoides*. Their structures were elucidated by spectroscopic methods, including extensive 1D- and 2D-NMR techniques. Compounds **1-6** were evaluated for their anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activity. The results showed that compounds **1-6** showed weak inhibition with diameter of inhibition zone of (IZD) of 9.2 ± 1.2 , 8.6 ± 1.5 , 9.8 ± 1.6 , 11.4 ± 1.2 , 8.2 ± 1.0 and, 11.6 ± 1.3 mm, respectively. Compounds **1-6** were also tested for the antioxidant activity, and they showed good antioxidant activity with an IC₅₀ value of 4.6 ± 0.8 , 4.4 ± 0.6 , 4.0 ± 0.6 , 4.2 ± 0.7 , 4.8 ± 0.6 , and 4.1 ± 0.5 $\mu\text{g/mL}$, respectively.

Phlomis betonicoides Diels is an annual herb plant belong to the family Lamiaceae, *Phlomis* Linn. It is of 7.5-14 cm in height, grown in meadow slopes, alpine meadows and river banks at cold plateau in Southwest China.¹ In China, its root has been used as traditional Chinese medicine by Tibetan people for treatment of bloating (food poisoning), stomachache, diarrhea and cold. It is also a good medicine for strengthening the stomach.²

The previous phytochemical studies of the roots of *P. betonicoides* have shown the presence of diterpene,^{3,4} shanzhiside esters,³⁻⁵ isoflavones,⁶⁻⁷ isobenzofuranone,⁸ and the homologous. The isobenzofuranone derivatives are an important class of secondary metabolites displaying a variety of biological effects, such as antioxidant, antimicrobial, antiviral, antiplatelet, cytotoxic activity and antiarrhythmic effects.⁸⁻¹¹ In our ongoing research on biologically active metabolites from Chinese herbal

medicine, we investigated the chemical constituents of the roots of *P. betonicoides* collected in Xichang Prefecture, Sichuan Province. This leads to the isolation of three new (**1-3**) together with three known (**4-6**) isobenzofuranone derivatives. The structures of **1-6** were elucidated by spectroscopic methods including extensive 1D- and 2D-NMR techniques. Compounds **1-6** were also evaluated for the anti-methicillin-resistant *Staphylococcus aureus* activity (anti-MRSA) and antioxidant activity.

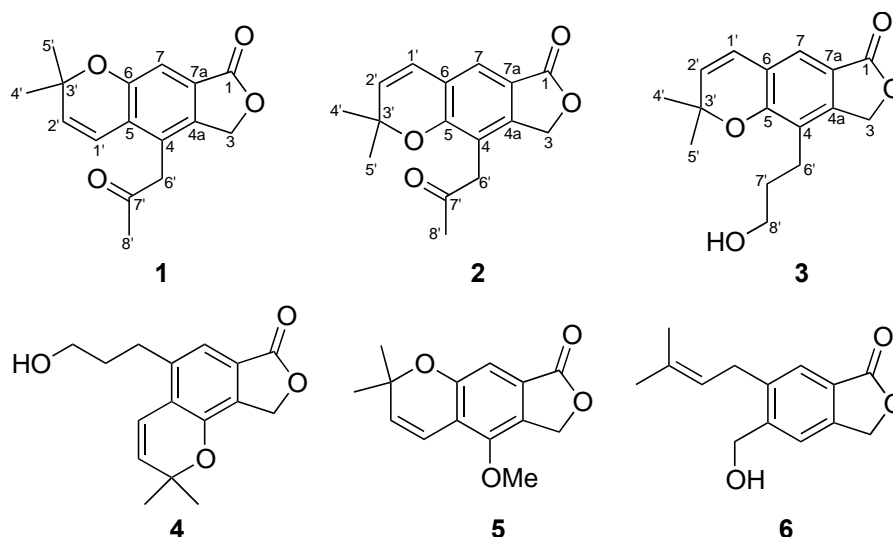


Figure 1. The isobenzofuranone derivatives from the roots of *Phlomis betonicoides*

A 95% aq. methanol extract prepared from the roots of *P. betonicoides* was subjected repeatedly to column chromatography on silica gel, MCI-GEL, Sephadex LH-20, RP-18 and preparative HPLC to afford three new isobenzofuranone derivatives, 2,2-dimethyl-5-(2-oxopropyl)-2*H*-furo[3,4-*g*]chromen-8(6*H*)-one (**1**), 2,2-dimethyl-9-(2-oxopropyl)-2*H*-furo[3,4-*g*]chromen-6(8*H*)-one (**2**), and 9-(3-hydroxypropyl)-2,2-dimethyl-2*H*-furo[3,4-*g*]chromen-6(8*H*)-one (**3**), together with three known isobenzofuranone derivatives (**4-6**). The structures of compounds **1-6** were shown in Figure 1, and the ^1H and ^{13}C NMR spectroscopic data of **1-3** were listed in Table 1. The known compounds, compared with the literature, were identified as 5-(3-hydroxypropyl)-2,2-dimethyl-2*H*-furo[3,4-*h*]chromen-7(9*H*)-one (**4**),⁸ 5-methoxy-2,2-dimethyl-2*H*-furo[3,4-*g*]chromen-8(6*H*)-one (**5**),¹² and 5-hydroxymethyl-6-prenylisobenzofuran-1(3*H*)-one (**6**).¹³

Compound **1** was obtained as a pale-yellow gum. The molecular formula $\text{C}_{16}\text{H}_{16}\text{O}_4$ was deduced from a pseudomolecular ion $[\text{M}+\text{Na}]^+$ at m/z 295.0952 in the HR-ESI-MS (calcd 295.0946 for $\text{C}_{16}\text{H}_{16}\text{NaO}_4$), indicating the presence of nine degrees of unsaturation in the molecule. The IR spectrum showed characteristic absorption bands for

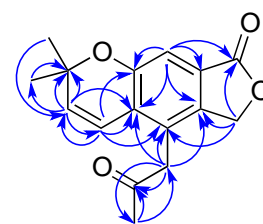


Figure 2. The HMBC (↷) correlations of **1**

saturated methylene (2962 cm⁻¹), carbonyl groups (1755 and 1682 cm⁻¹), and aromatic rings (1615, 1541, 1440 cm⁻¹), and the UV spectra showed absorption maxima at 210, 283 and 322 nm indicating the existence of a conjugated aromatic system. The structure of **1** was further elucidated by interpretation of NMR data (Table 1), including DEPT, HMBC and HSQC data. The ¹H and ¹³C NMR spectrum of **1** along with analysis of the DEPT spectra displayed 16 carbon signals and 16 proton signals, respectively, corresponding to a 1,2,3,4,5-penta-substituted phenyl ring (C-4~C-7, C-4a, and C-7a; H-7), a (2-oxopropyl group (-CH₂COCH₃, C-6'~C-8'; H₂-6' and H₃-8'),¹⁴ a 2,2-dimethyl-2*H*-pyran moiety (-CH=CH-C(CH₃)₂-O-; C-1'~C-5'; H-1', H-2', and H₆-4',5'),¹⁵ an ester carbonyl group (C-1), and an oxygenated methylene (C-3, H₂-3). On the basis of the molecular formula, in addition to four degrees of unsaturations for aromatic ring, two degree of unsaturations for 2,2-dimethyl-2*H*-pyran moiety, one degree of unsaturation for carbonyl group, and one degree of unsaturation for 2-oxopropyl group, the still one ring was needed to meet the required nine degrees of unsaturation. The HMBC correlations (Figure 2) from H₂-3 to C-1, C-4, C-4a, C-7a, and from H-7 to C-1, C-4a, C-7a suggested that an isobenzofuran-1(3*H*)-one moiety was formed between aromatic ring, C-1, and C-3, and compound **1** should be a isobenzofuranone derivative.^{8,12}

Table 1. ¹H and ¹³C NMR data for compounds **1-3** (500 and 125 MHz, in CDCl₃)

No.	Compound 1		Compound 2		Compound 3	
	δ_C	δ_H (m, <i>J</i> , Hz)	δ_C	δ_H (m, <i>J</i> , Hz)	δ_C	δ_H (m, <i>J</i> , Hz)
1	169.0 s		169.4 s		169.8 s	
3	71.4 t	5.37 s	71.9 t	5.33 s	71.7 t	5.34 s
4	132.9 s		127.2 s		125.2 s	
5	126.6 s		158.9 s		156.9 s	
6	152.7 s		116.3 s		115.4 s	
7	113.2 d	7.45 s	124.3 d	6.89 s	123.9 d	6.92 s
4a	140.7 s		147.3 s		146.4 s	
7a	125.6 s		120.5 s		119.9 s	
1'	115.3 d	6.67 d (9.8)	118.3 d	6.69 d (9.8)	118.5 d	6.66 d (9.8)
2'	128.2 d	5.82 d (9.8)	128.9 d	5.84 d (9.8)	128.7 d	5.81 d (9.8)
3'	75.6 s		75.9 s		76.0 s	
4',5'	26.2 q	1.67 s	26.4 q	1.64 s	26.5 q	1.65 s
6'	44.6 t	3.44 s	42.6 t	3.43 s	20.6 t	2.68 t (7.8)
7'	205.2 s		205.6 s		37.7 t	1.90 m
8'	30.2 q	2.23 s	30.5 q	2.17 s	63.2 t	3.56 t (6.6)

Since the nucleus of compound was determined, the additional carbons (2,2-dimethyl-2*H*-pyran moiety, and 2-oxopropyl group) were accounted for the remaining substituents. Long-range correlations from H-1' to C-4, C-5, and C-6, from H-2' to C-5 were observed. This led us to conclude that the

2,2-dimethyl-2*H*-pyran moiety was fused in an angular manner at C-5 and C-6, and C-1' was linked to C-5. The 2-oxopropyl group located at C-4 was confirmed by the HMBC correlations of H-6' with C-4, C-5, and C-4a. Accordingly, the structure of **1** was determined, and gives the systematic name of 2,2-dimethyl-5-(2-oxopropyl)-2*H*-furo[3,4-*g*]chromen-8(6*H*)-one.

Compound **2** was also assigned the molecular formula of C₁₆H₁₆O₄ as supported by the HRESIMS (m/z 295.0949 [M+Na]⁺). The ¹H and ¹³C NMR spectroscopic data of **2** were similar to those of compound **1**. The obvious chemical shift differences resulted from the substituents positions variation on the isobenzofuranone skeleton. The 2,2-dimethyl-2*H*-pyran moiety located at C-5 and C-6, and C-1' linked to C-6, were supported by the HMBC correlations from H-1' to C-5, C-6, and C-7, and from H-2' to C-6. The 2-oxopropyl group located at C-4 was confirmed by the HMBC correlations of H-6' with C-4, C-5, and C-4a. Thus, the structure of 2,2-dimethyl-9-(2-oxopropyl)-2*H*-furo[3,4-*g*]chromen-6(8*H*)-one (**2**) was established as shown.

9-(3-Hydroxypropyl)-2,2-dimethyl-2*H*-furo[3,4-*g*]chromen-6(8*H*)-one (**3**) was also isolated as a yellow gum, and it gave a pseudomolecular ion peak at m/z 297.1110 [M+Na]⁺, consistent with a molecular formula of C₁₆H₁₈O₄. Its ¹H and ¹³C NMR spectroscopic data were also similar to those of **2**. The marked differences between them were due to the inexistence of a 2-oxopropyl group signals, and appearance of a 3-hydroxypropyl group signals (-CH₂CH₂CH₂OH, C-6'~C-8'; H₂-6'~H₂-8')¹⁶ in compound **3**. These change indicated that the 2-oxopropyl group in **2** was replaced by a 3-hydroxypropyl group in compound **3**. The HMBC correlations from H-6' to C-4, C-5, and C-4a, and from H-7' to C-4, supported 3-hydroxypropyl group located at C-4. In addition, the 2,2-dimethyl-2*H*-pyran moiety located at C-5 and C-6, and C-1' linked to C-6, were also elucidated by the HMBC correlations from H-1' to C-5, C-6, and C-7, and from H-2' to C-6. The structure of **3** was therefore determined.

The biosynthetic logic of compounds **1-3** should be considered as the isobenzofuranone derivative precursor reacted with isopentenyl pyrophosphate (IPP) under an isopentenyl transferase. The IPP could be transferred into different carbon position of isobenzofuranone backbone and formed C-5 and C-6 isopentenyl substituted products, respectively.

Since certain isobenzofuranone derivatives exhibit potential antibacterial activity,^{9,17,18} compounds **1-6** were screened for anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activity according to arbitrary criterion with diameter of inhibition zone (IZD)¹⁹ as follow: very weak inhibition (with IZD of 6-8 mm), weak inhibition (with IZD of 8-12 mm), good inhibition (with IZD of 12-16 mm), and strong inhibition (with IZD of >16 mm) activities, respectively. The IZD of the positive control (Vancomycin) was 32±1.2 mm and the negative control to zero. The results revealed that compound **1-6** showed weak inhibition with IZD of 9.2±1.2, 8.6±1.5, 9.8±1.6, 11.4±1.2, 8.2±1.0 and, 11.6±1.3 mm, respectively.

Since certain isobenzofuranone derivatives exhibit potential antioxidant activity,²⁰⁻²² compounds **1-6** were

also tested for their antioxidant activity by the 2',7'-dichlorofluorescein diacetate (DCFH) method reported previously, with vitamin C (IC_{50} value of 4.2 ± 0.6) as positive control.²³ The results revealed that compounds **1-6** showed good antioxidant activity with an IC_{50} value of 4.6 ± 0.8 , 4.4 ± 0.6 , 4.0 ± 0.6 , 4.2 ± 0.7 , 4.8 ± 0.6 , and 4.1 ± 0.5 $\mu\text{g/mL}$, respectively.

General Experimental Procedures. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectra. 1D- and 2D-NMR spectroscopic data were recorded on a DRX-500 NMR spectrometer with TMS as internal standard. Chemical shifts (δ) are expressed in ppm with reference to the TMS signal. HRESIMS was performed on a VG Autospec-3000 spectrometer. Semi-preparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with Zorbax PrepHT GF (21.2 mm \times 25 cm) or Venusil MP C₁₈ (20 mm \times 25 cm) columns. Column chromatography was performed using silica gel (200-300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40-63 μm , Merck, Darmstadt, Germany), Sephadex LH-20 (Sigma-Aldrich, Inc, USA), or MCI gel (75-150 μm , Mitsubishi Chemical Corporation, Tokyo, Japan). Column fractions were monitored by TLC visualized by spraying with 5% H₂SO₄ in EtOH and heating.

Plant Material. The root of *Phlomis betonicoides* Diels were collected in Xichang Prefecture, Sichuan Province, and harvested in October 2017. The species was identified by Prof. Y. J. Chen. A voucher specimen (YNNI 16-9-88) was deposited in the herbarium of the Yunnan Minzu University.

Extraction and Isolation. The air-dried samples (4.8 kg) were crushed to 30-50 mesh, and the powders were extracted with 95% MeOH (4 \times 8 L) at room temperature and filtered. The filtrate was evaporated under reduced pressure, and the crude extract (485 g) was applied to a silica gel (150-200 mesh) column eluted with chloroform-methanol (CHCl₃-MeOH) gradients (20:1, 9:1, 8:2, 7:3, 6:4, 5:5) to afford six fractions (A-F). Further separation of fraction B (9:1, 48.9 g) by silica gel column chromatography, eluted with CHCl₃-acetone (1:0-1:2), yielded subfractions B1-B7. Subfraction B2 (9:1, 10.22 g) was loaded on another silica gel column using petroleum ether-ethyl acetate (EtOAc) elution, and then separated semi-preparative HPLC (62% MeOH-H₂O, flow rate 20 mL/min) to afford **1** (13.6 mg) and **2** (14.4 mg). Subfraction B3 (8:2, 12.8 g) was separated on the other silica gel column eluted by petroleum ether-EtOAc, followed by semi-preparative HPLC (58% MeOH-H₂O, flow rate 20 mL/min) to give **3** (16.2 mg), **4** (12.5 mg), **5** (10.9 mg) and **6** (13.1 mg).

Anti-MRSA Agar Disc Diffusion Assay. The MRSA strain ZR11 was clinical isolated from infectious samples of critically ill patients in the Clinical Laboratory of the First People's Hospital of Yunnan Province, and confirmed by standard cefoxitin disk diffusion test following CLSI standard procedures.¹⁹ The anti-MRSA activity of the compounds was evaluated via the disc diffusion method. The ZR11 strain was inoculated in Mueller Hinton Broth and were incubated at 37 °C for 24 h. The turbidity of bacterial

suspension was adjusted to 0.5 McFarland standard which equals to 1.5×10^8 colony-forming units (CFU)/mL. Sterile filter paper discs (6 mm) were impregnated with 20 mL (50 mg) of each compound and placed on inoculated Mueller Hinton agar containing bacterial suspension which adjusted to 0.5 McFarland standard. The commercial available discs containing 30 mg Vancomycin were used as positive control whereas discs without samples (5% DMSO) acted as negative control. The inhibition zones including the diameter of the disc (mm) were measured and compared after incubation at 37 °C for 24 h. The tests were carried out in triplicate for each sample.

Antioxidant Assays. The antioxidant activity was tested by 2',7'-dichlorofluorescein diacetate (DCFH) method reported previously.²³ Myelomonocytic HL-60 cells (1×10^6 cells/mL, ATCC) were suspended in RPMI 1640 medium with 10% FBS and antibiotics at 37 °C in 5% CO₂: 95% air. 125 μ L of the cell suspension was added to each well of a 96-well plate. After treatment with a different concentration of the test material for 30 min, the cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min. Then the cells were incubated for 15 min after the addition of 5 μ g/mL DCFH-DA (Molecular Probes). DCFH-DA is a non-fluorescent probe that diffuses into cells. Cytoplasmic esterases hydrolyse DCFH-DA to 2',7'-dichlorofluorescein (DCFH), and the reactive oxygen species (ROS) generated within HL-60 cells oxidize DCFH to the fluorescent dye 2',7'-dichlorofluorescein (DCF). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalyzed oxidation of DCFH in HL-60 cells was measured by PMA trede control incubations with and without the test materials. The levels of DCF were measured using a CytoFluor 2350, fluorescence measurement system (Millipore) with an excitation wavelength at 485 nm (bandwidth 20 nm) and emission at 530 nm (bandwidth 25 nm).

2,2-Dimethyl-5-(2-oxopropyl)-2H-furo[3,4-g]chromen-8(6H)-one (1): Obtained as pale-yellow gum; UV (MeOH), λ_{\max} (log ϵ) 322 (3.18), 283 (3.56), 210 (3.87) nm; IR (KBr) λ_{\max} 3079, 2962, 1755, 1682, 1615, 1541, 1440, 1364, 1162, 1055, 904, 836 cm^{-1} ; ¹H NMR and ¹³C NMR data (CDCl₃, 500 and 125 MHz, respectively), Table 1; ESIMS (positive ion mode) m/z 295 [M+Na]⁺; HRESIMS (positive ion mode) m/z 295.0952 [M+Na]⁺ (calcd 295.0946 for C₁₆H₁₆NaO₄).

2,2-Dimethyl-9-(2-oxopropyl)-2H-furo[3,4-g]chromen-6(8H)-one (2): Obtained as pale-yellow gum; UV (MeOH), λ_{\max} (log ϵ) 325 (3.22), 286 (3.59), 210 (3.85) nm; IR (KBr) λ_{\max} 3066, 2957, 1753, 1678, 1614, 1545, 1442, 1360, 1157, 1050, 886, 807 cm^{-1} ; ¹H NMR and ¹³C NMR data (CDCl₃, 500 and 125 MHz, respectively), Table 1; ESIMS (positive ion mode) m/z 295 [M+Na]⁺; HRESIMS (positive ion mode) m/z 295.0949 [M+Na]⁺ (calcd 295.0946 for C₁₆H₁₆NaO₄).

9-(3-Hydroxypropyl)-2,2-dimethyl-2H-furo[3,4-g]chromen-6(8H)-one (3): Obtained as pale-yellow gum; UV (MeOH), λ_{\max} (log ϵ) 314 (3.12), 275 (3.52), 210 (3.81) nm; IR (KBr) λ_{\max} 3410, 3052, 2945, 1680, 1612, 1550, 1438, 1364, 1146, 1039, 910, 842 cm^{-1} ; ¹H NMR and ¹³C NMR data (CDCl₃, 500 and

125 MHz, respectively), Table 1; ESIMS (positive ion mode) m/z 297 $[M+Na]^+$; HRESIMS (positive ion mode) m/z 297.1110 $[M+Na]^+$ (calcd 297.1103 for $C_{16}H_{18}NaO_4$).

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