A new phenone from the roots of *Paeonia suffruticosa* Andrews

Liqin Ding\(^a\)#, Qingfei Zuo\(^a\)#, Dandan Li\(^a\), Xinchi Feng\(^b\), Xiumei Gao\(^a\), Feng Zhao\(^c\)# and Feng Qiu\(^a,\)\(^b\)#

\(^a\)Tianjin Key Laboratory of TCM Chemistry and Analysis, Institute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China; \(^b\)School of Chinese Materia Medica, Tianjin University of Traditional Chinese Medicine, Tianjin, China; \(^c\)School of Pharmacy, Key Laboratory of Molecular Pharmacology and Drug Evaluation (Yantai University), Ministry of Education, Collaborative Innovation Center of Advanced Drug Delivery System and Biotech Drugs in Universities of Shandong, Yantai University, Yantai, China

**ABSTRACT**

Thirteen phenones were obtained from the 70% ethanol extract of *Paeonia suffruticosa* Andrews. Their structures were determined on the basis of chemical methods and spectral data. Among them, compound 1 was identified as a new compound, and compounds 5 and 13 were obtained from genus *Paeonia* for the first time. The inhibitory effects of isolated compounds (1–12) on nitric oxide (NO) production in lipopolysaccharide-activated macrophages were evaluated, and NO production was suppressed significantly by compound 7.

**1. Introduction**

*Paeonia* is the single genus in the family Paeoniaceae, which consists of ca. 35 species distributed mainly in warm-temperate regions of Europe and Asia (Pan 1995). The roots of *P. suffruticosa*, *P. albiflora* and *P. lactiflora* are the most important crude drugs in traditional Chinese medicine, having been recorded in the Pharmacopoeia of the People’s Republic of China (2015). They have been used as anti-aggregation, anticoagulation (Ishida et al. 1985), anti-inflammatory (Duan et al. 2009), antioxidant (Rho et al. 2005; Lee et al. 2008), antitumor...
(Lee et al. 2002), anti-osteoporosis (Yen et al. 2007), analgesic and sedative agents (Hayashi et al. 1985), and as a remedy for cardiovascular, extravasated blood, stagnated blood and female genital diseases in oriental traditional medicine. It is well known that monoterpene compounds possessing a ‘cage-like’ pinane skeleton have been confirmed as the main biologically active constituents of *Paeonia* species (Duan et al. 2009; Ding et al. 2012a; Ding et al. 2012b; Zhu & Fang 2014). It is also reported that phenones, especially acetophenones, are the characteristic metabolites reported from *P. suffruticosa*, while present in *P. albiflora* and *P. lactiflora* in lower contents. However, most of the previous studies focused on monoterpene and monoterpene glycosides, instead of non-monoterpene part. In our investigation into constituents from the *Paeonia* genus, we obtained thirteen phenones, of which compound 1 as a new compound, and compounds 5 and 13 were reported from genus *Paeonia* for the first time from the root cortex of *P. suffruticosa* Andrews. They were elucidated as mudanoside C (1), paeonol (2) (Yasuda et al. 1999; Xiao et al. 2008), 1-(3-hydroxy-4-methoxyphenyl)ethanone (3) (Kong et al. 2010), 2, 3-dihydroxy-4-methoxyacetophenone (4) (Lin & Chern 1991), 3-O-β-d-glucopyranosyl-4-methoxyacetophenone (5) (Neish, 1957), paeonoside (6) (Yu et al. 1986), suffruticoside A (7), suffruticoside B (8), suffruticoside D (9) (Yoshikawa et al. 1992), paeonolide (10), apiopaeonoside (11) (Yu et al. 1986), mudanoside B (12) (Lin et al. 1998) and iriflophenone 2-O-β-d-glucopyranoside (13) (Lee et al. 2010) (see Figure 1) on the basis of spectroscopic analysis including MS, CD, 1D and 2D NMR and comparison with data published as well as physical and chemical properties. In the present research, we report the isolation and structural elucidation of the new compound and the inhibitory effects of compounds 1–12 on NO production in LPS-activated macrophages.

2. Results and discussion

Compound 1 was isolated as brown amorphous powder, which gave a positive reaction with FeCl₃–K₃[Fe(CN)₆] reagent, suggesting the presence of a phenolic hydroxyl (Shanta & Mathew 1970). Its molecular formula was determined as C₁₅H₂₀O₉ from its positive-ion HR-ESI-MS (367.1002 [M + Na]⁺, calcd. for C₁₅H₂₀O₉Na⁺, 367.1005). The ¹H NMR spectrum of 1 displayed a methyl group at δ_H 2.54 (3H, s), a methoxy group at δ_H 3.89 (3H, s), two para-coupled aromatic protons at δ_H 6.50 (1H, s), 7.64 (1H, s) and an anomeric proton at δ_H 4.78 (1H, d, J = 7.2 Hz). The ¹H and ¹³C NMR data as well as acid hydrolysis and GC comparison with an authentic sample indicated the presence of a β-glucopyranosyl moiety. The β-configuration of the glycosidic linkage was determined from the coupling constant of the anomeric proton. The ¹³C NMR spectra of compound 1 were similar to that of paeonol, except for the appearance of a glucosyl group, assignments confirmed with the help of 2D NMR (HSQC, HMBC and NOESY) experiments. The HMBC correlations from the methyl protons to C-1, C-6 and ketone carbonyl carbon, from the methoxy protons to C-4, and from H-1’ to C-5 supported the above deduction. The linked position of the glucosyl group was further established from the NOESY spectrum, in which H-6 (δ 7.64) exhibited a correlation with H-1’ (δ 4.78), indicating that the glucosyl moiety was located at C-5. On the basis of these data, the structure of 1 was elucidated as 2-hydroxy-4-methoxy-5-O-β-d-glucopyranosyl-acetophenone, named as mudanoside C.

Compounds 1–12 were evaluated for their inhibitory effects on NO production induced by LPS in macrophages (see Table 1). In addition, the cell viability in the present experiment was determined by the MTT method to find whether inhibition on NO production was due
to the cytotoxicity of the test compounds. As shown in Table 1, hydrocortisone (IC\textsubscript{50} 40.64 ± 3.22 μM) was used as positive control. It is pity that only compound 7 significantly suppressed NO production in LPS-activated macrophages.

3. Experimental

3.1. General experimental procedures

UV spectra were recorded on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Bruker IFS55 spectrometer. The \textsuperscript{1}H, \textsuperscript{13}C and 2D NMR spectra were performed on Bruker ARX-300 and ARX-600 spectrometers, using TMS as an internal standard. HR-ESI-MS spectra were obtained on an Agilent 6210 TOF mass spectrometer. Circular dichroism was measured on a MSO450 spectropolarimeter. Preparative HPLC was carried out on a LC-6AD liquid chromatograph with an YMC Pack ODS-A column (250 × 20 mm, 5 μm, 120 Å) and SPD-10A VP UV/VIS detector. Macroporous resin D101 was purchased from the Chemical Plant of Nankai.
University (Tianjin, China). Silica gel (200–300 mesh) used for column chromatography was supplied by Qingdao Marine Chemical Factory, Qingdao, P.R. China. ODS (40–75 μm) used for column chromatography was purchased from Merck (Darmstadt, Germany), and Sephadex LH-20 was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Mouse monocyte–macrophage RAW 264.7 cells (ATCC TIB-71) were purchased from the Chinese Academy of Science, People's Republic of China. RPMI 1640 medium, penicillin, streptomycin and foetal bovine serum were purchased from Invitrogen (New York).

3.2. Plant material

The roots of *P. suffruticosa* Andrews from Hebei Province of China were purchased from Liaoning Yaocai Co., Shenyang province, China, and were identified by Prof. Qishi Sun, Department of Pharmaceutical Botany, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University. A voucher specimen (Y20010062) was deposited in the herbarium of the Department of Natural Products Chemistry, Shenyang Pharmaceutical University.

3.3. Extraction and isolation

In continuation of our earlier investigation, the roots of *P. suffruticosa* Andrews (14 kg) were extracted with 70% EtOH (30 L × 3 times × 2 h). The combined extracts were concentrated under vacuum at 50 °C to afford a residue of 1.7 kg, which was suspended in H2O (8.0 L) and successively partitioned with cyclohexane (CHA, 24 L), ethyl acetate (EtOAc, 24 L) and n-butanol (n-BuOH, 24 L). The n-BuOH extract (450.0 g) was subjected to D-101 macroporous adsorptive resins (12 × 100 cm) to give 5 fractions (water, 20% ethanol, 40% ethanol, 60% ethanol, 95% ethanol), named B1-B5, respectively. The water fraction B1 (200.0 g) was isolated on repeated D-101 macroporous adsorptive resins with H2O, 10, 20, 30, 50 and 95% ethanol, and 6 fractions (B11-B16) were obtained. The 10% ethanol part B12 was chromatographed on Sephadex LH-20 column eluted with MeOH and then purified by Prep-HPLC (C18 column, 250 × 20 mm, 10 μm, YMC Co. Ltd.; flow rate, 8 mL/min; UV detector, 254 nm) eluted with MeOH/H2O (15:85) to afford compound 13 (tR 52 min, 13 mg). The 20% ethanol fraction B2 (85.0 g) was isolated on silica gel column chromatography (12 × 100 cm) with a CHCl3-MeOH gradient system (100:0 to 0:100) as eluent to give nine subfractions (B21-B29). Subfraction B24 (4 g) was further chromatographed on a C-18 reversed-phase open column (3 × 60 cm) and purified by PTLC (CHCl3-MeOH(5:1)) to obtain compound 5 (68 mg), purified by Prep-HPLC using MeOH-H2O (30:70, 254 nm) at a flow rate of 8 mL/min to give compound 6 (tR 45 min, 208 mg). Subfraction B26 (3 g) afforded compound 11 (tR 43 min, 41 mg) using the same methods with subfraction B24. Methanol precipitated compound 12 (80 mg) from subfraction B27 (10 g), and the supernatant was isolated on Sephadex LH-20 column and purified by Semi-HPLC (C18 column, 250 mm × 10 mm, 5 μm, YMC Co. Ltd.; flow rate, 3 mL/min; UV detector, 254 nm) eluted with MeOH/H2O (20:80) to yield compound 10 (tR 32 min, 41 mg). The 40% ethanol fraction B3 (150.0 g) was rechromatographed on silica gel column chromatography (10 × 100 cm), eluted with a gradient of CHCl3-MeOH to obtain nine subfractions (B31-B39). Subfraction B32 was purified by Sephadex LH-20 column eluted with CHCl3-MeOH(1:1) to get compound 4 (8 mg) as white crystals. Subfraction B33 was isolated by ODS open column chromatography, then purified by Prep-HPLC with MeOH-H2O.
(30:70, 8 mL/min, 254 nm) as eluent to give compound 1 (31 mg). In the same way, compounds 9 (53 mg), 7 (102 mg) and 8 (124 mg) were isolated from subfraction B36 (6 g) by C-18 reversed-phase open column chromatography and Prep-HPLC eluted with MeOH-H₂O (30:70, 8 mL/min, 254 nm).

Compound 2 (100 mg) as white needles was gained after recrystallising the CHA layer (195.0 g). The remainder was subjected to silica gel column chromatography (5 x 100 cm) with a gradient of petroleum ether–acetone (PE-CH₃COCH₃) to get ten fractions (C1-C10). Fraction C3 (3.5 g) was rechromatographed on silica gel column chromatography (3 x 100 cm), eluted with a gradient of PE-CH₃COCH₃. The PE-CH₃COCH₃(10:1) part was further subjected to Sephadex LH-20 column and purified by PTLC (PE-EtOAc(8:1)) to yield compound 3 (20 mg).

Mudanoside C (1): white, amorphous powder; UV(MeOH) λmax (log ε): 233 (3.88), 212 (3.88), 273 (3.80), 334 (3.52) nm; IR (KBr) νmax: 3495, 3392, 1635, 1514, 1443, 1382, 1269, 1075 cm⁻¹; HR-ESI-MS: m/z 367.1002 [M + Na]+ (calcd. for C₁₅H₂₀O₉Na+, 367.1005); 1H NMR (300 MHz, CD₃OD): 6.50 (1H, s, H-3), 7.64 (1H, s, H-6), 2.54 (3H, s, H-8), 3.89 (3H, s, OCH₃), 4.78 (1H, d, J = 7.2 Hz, H-1′), 3.46 (1H, m, H-2′), 3.42 (1H, m, H-3′), 3.34 (1H, t, J = 8.8 Hz, H-4′), 3.45 (1H, m, H-5′), 3.66 (1H, dd, J = 12.1, 6.5 Hz, H-6′), 3.91 (1H, dd, J = 12.1, 1.6 Hz, H-6′). 13C NMR (75 MHz, CD₃OD): 112.2 (C-1), 160.9 (C-2), 100.4 (C-3), 157.7 (C-4), 139.5 (C-5), 119.5 (C-6), 203.6 (C-7), 25.6 (C-8), 55.7 (OCH₃), 102.9 (C-1′), 74.0 (C-2′), 77.5 (C-3′), 70.6 (C-4′), 77.0 (C-5′), 61.7 (C-6′).

### 3.4. Acid hydrolysis of compound 1

Compound 1 (2 mg) was dissolved in 2 N HCl (2 mL) heated at 90 °C for 2 h and partitioned with cyclohexane and H₂O (each 1 mL x 3). The aqueous layer was evaporated to dryness to give residue, which was dissolved in anhydrous pyridine (2 mL), and L-cysteine methyl ester hydrochloride (2 mg) was mixed, and then warmed at 60 °C for 2 h. The trimethylsilylation reagent trimethylsilylimidazole (0.15 mL) was added, followed by warming at 60 °C for another 1 h. After drying the solution, the residue was partitioned between H₂O and cyclohexane. The cyclohexane layer was concentrated, then dissolved in 200 μL of cyclohexane and analysed by GC. The identification of the resulting glucose was carried out as described in the previous paper (Ding et al. 2012a).

### 3.5. Cell culture and nitrite determination

RAW 264.7 cells were harvested with trypsin and diluted to a suspension in fresh medium, seeded in 96-well plates at 1 x 10⁶ cells/mL, 200 μL/well and allowed to adhere for 2 h at 37 °C in 5% CO₂ in air. Then, the cells were treated with 1 μg/mL of LPS for 24 h with or without various concentrations of test compounds. DMSO was used as a solvent for the test compounds, which were applied at a final concentration of 0.2% (v/v) in cell culture supernatants. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using Griess reagent (Dirsch et al. 1998). Briefly, 100 μL of the culture supernatants from incubates was mixed with an equal volume of Griess reagent (a mixture with equal amount of 0.1% N-[1-naphthyl]ethylenediamine and 1% sulphanilamide in 5% H₃PO₄). The concentration of NO₂⁻ was calculated by using a prepared working line 0, 1, 2, 5, 10, 20, 50 and 100 μM sodium nitrite solutions, and the inhibitory rate on NO production induced by LPS was calculated by the NO₂⁻ levels as follows:
Experiments were performed in triplicate, and data were expressed as the mean ± SD of three independent experiments. After 100 μL of the culture supernatants was taken out for nitrite determination, cytotoxicity was evaluated by the MTT colorimetric assay (Qin et al. 2015). Briefly, an MTT solution was added at the final concentration of 200 μg/mL, and the cells were incubated for 4 h at 37 °C. The supernatant was removed, and 100 μL of DMSO was added to dissolve the formazan. The absorbance at 570 nm was measured by using a microplate reader. The LPS-treated cells were considered as having 100% of viable cells.

4. Conclusions

Previous phytochemical research on *P. suffruticosa* has led to the identification of many bioactive compounds; they were confirmed as monoterpenoids and their glycosides with a unique ‘cage-like’ skeleton, which occurred ubiquitously in all the species examined of the family Paeoniaceae (Yoshikawa et al. 1992; Lin et al. 1996; Okasaka et al. 2008; Duan et al. 2009). However, phenones are also characteristic and major constituents of *P. suffruticosa*, in which acetophenones are predominant. In this investigation, thirteen phenones were isolated from the roots of *P. suffruticosa* Andrews. Among them, compound 1 is a new compound, and compounds 5 and 13 were obtained from the genus *Paeonia* for the first time.

Compounds 1–12 were evaluated for their inhibitory effects on NO production induced by LPS in macrophages. As shown in Table 1, comparing with positive control hydrocortisone \((IC_{50} 40.64 ± 3.22 \mu M)\), it is pity that only compound 7 displayed strong inhibitory activities on NO production. Analogs displayed less or no inhibitory activity.

**Supplementary material**

Supplementary material relating to this article is available online, alongside Table S1 and Figures S1–S8.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was partly supported by National Natural Science Foundation of China under [grant number 81430095]; Ph. D. Programs Foundation of Ministry of Education of China under [grant number 20132134110006]; Applied Basic and Advanced Research Program of Tianjin [grant number 16JCYBJC27900].

\[
\text{Inhibitory rate (\%) = } 100 \times \frac{[\text{NO}_2^-]_{LPS} - [\text{NO}_2^-]_{LPS+\text{sample}}}{[\text{NO}_2^-]_{LPS} - [\text{NO}_2^-]_{\text{untreated}}}
\]
References

Ding LQ, Zhao F, Chen LX, Jiang ZH, Liu Y, Li ZM, Qiu F, Yao XS. 2012. New monoterpane glycosides from *Paeonia suffruticosa* Andrews and their inhibition on NO production in LPS-induced RAW 264.7 cells. Bioorg Med Chem Lett. 22:7243–7247. doi:10.1016/j.bmcl.2012.09.034.

Ding LQ, Jiang ZH, Liu Y, Chen LX, Zhao Q, Yao XS, Zhao F, Qiu F. 2012. Monoterpenoid inhibitors of NO production from *Paeonia suffruticosa*. Fitoterapia. 83:1598–1603. doi:10.1016/j.fitote.2012.09.008.

Dirsch VM, Stuppner H, Vollmar AM. 1998. The Griess assay: suitable for a bioguided fractionation of anti-inflammatory plant extracts. Planta Med. 64:423–426.

Duan WJ, Yang JY, Chen LX, Zhang LJ, Jiang ZH, Cai XD, Zhang X, Qiu F. 2009. Monoterpenes from *Paeonia albiflora* and their inhibitory activity on nitric oxide production by lipopolysaccharide-activated microglia. J Nat Prod. 72:1579–1584. doi:10.1021/np9001898.

Hayashi T, Shinbo T, Shimizu M, Arisawa M, Morita N, Kimura M, Matsuda S, Kikuchi T. 1985. *Paeonilactone-A*, -B, and -C, new monoterpenoids from paeony root. Tetrahedron Lett. 26:3699–3702. doi:10.1016/S0040-4039(00)89227-8.

Ishida H, Takamatsu M, Tsuji K, Kosuge T. 1985. Studies on active substances in the herbs used for oketsu (“stagnant blood”) in Chinese medicine. Ill. On the anticoagulative principles in curcumae rhizoma. Chem Pharm Bull. 33:1499–1502. pmid: 4042226.

Lee SM, Li ML, Tse YC, Leung SC, Lee MM, Tsui SK, Fung KP, Lee CY, Waye MM. 2002. *Paeoniae Radix*, a Chinese herbal extract, inhibit hepatoma cells growth by inducing apoptosis in a p53 independent pathway. Life Sci. 71:2267–2277. pmid: 12215374.

Lin HC, Ding HY, Wu YC. 1998. Two novel compounds from *Paeonia suffruticosa*. J Nat Pro. 61:343–346. doi:10.1021/np9704258.

Neish AC. 1957. Pungenin; a glucoside found in leaves of *Picea pungens* (Colorado spruce). Biochem Cell Biol. 35:161–167. doi:10.1139/o57-020.

Rho S, Chung HS, Kang M, Lee E, Cho C, Kim H, Park S, Kim HY, Hong M, Shin M, Bae H. 2005. Inhibition of production of reactive oxygen species and gene expression profile by treatment of ethanol extract of Moutan Cortex Radicis in oxidative stressed PC12 cells. Biol Pharm Bull. 28:661–666. doi:10.1248/bpb.28.661.
Yasuda T, Kon R, Nakazawa T, Ohsawa K. 1999. Metabolism of paeonol in rats. J. Nat. Prod. 62:1142–1144. doi:10.1021/np9804051.

Yen PH, Kiem PV, Nhiem NX, Tung NH, Quang TH, Minh CV, Kim JW, Choi M, Kim YH. 2007. A new monoterpenic glycoside from the roots of *Paeonia lactiflora* increases the differentiation of osteoblastic MC3T3-E1 cells. Arch. Pharm. Res. 30:1179–1185. doi:10.1007/BF02980258.

Yoshikawa M, Uchida E, Kawaguchi A, Kitagawa I, Yamahara J 1992. Galloyl-oxypaeoniflorin, suffruticosides A, B, C, and D, five new antioxidative glycosides, and suffruticoside E, a paeonol glycoside, from Chinese Moutan Cortex. Chem. Pharm. Bull. 40: 2248–2250. pmid:1423794.

Yu J, Lang HY, Xiao PG. 1986. A new compound, apiopaeonoside, isolated from the root of *Paeonia Suffruticosa*. Acta Pharm Sin. 21:191–197. pmid: 3788584

Zhu X, Fang ZH. 2014. New monoterpenic glycosides from the root cortex of *Paeonia suffruticosa* and their potential anti-inflammatory activity. Nat Prod Res. 28:301–305. doi:10.1080/14786419.2013.858340.