HETEROCYCLES, Vol. 94, No. 8, 2017, pp. 1582 - 1588. © 2017 The Japan Institute of Heterocyclic Chemistry
Received, 9th May, 2017, Accepted, 14th June, 2017, Published online, 23rd June, 2017
DOI: 10.3987/COM-17-13739

THREE NEW ISOFLAVONES FROM THE ROOT OF *PUERARIA LOBATA* AND THEIR BIOACTIVITIES

Feng-Mei Zhang,[1,2] Jing Li,[2] Qi-Li Mi,[2] Shi-Yun Tang,[2] Ye-Kun Yang,[1,2] Jian-Hua Chen,[2] Chun-Bo Liu,[2] Qin-Peng Shen,[2] Zhi-Hua Liu,[2] Guang-Yu Yang,[2] Qiu-Fen Hu,[2] Zhong-Tao Ding,[1,*] and Xue-Mei Li[2,*]

[1] School of Chemical Science and Technology, Yunnan University, Kunming 650091, P.R. China. [2] Key Laboratory of Tobacco Chemistry of Yunnan Province, China Tobacco Yunnan Industrial Co., Ltd, Kunming 650231, P.R. China. E-mail: jszxtg_2015@163.com

Abstract – Three new isoflavones, 4′-hydroxy-6-methoxy-7methylisoflavone (**1**), 4′,6-dihydroxy-7-methylisoflavone (**2**), and 4′-hydroxy-7-hydroxymethyl-6-methoxyisoflavone (**3**), together with two known isoflavones (**4** and **5**), were isolated from the root of *Pueraria lobata*. Their structures were elucidated by spectroscopic methods, including extensive ^{1}D- and ^{2}D NMR techniques. Compounds **1-5** were evaluated for their anti-tobacco mosaic virus (anti-TMV) activities. The results showed that compounds **1** and **2** exhibited comparable anti-TMV activities with inhibition rates of 34.2 and 33.5%. The other compounds also showed potential anti-TMV activities with inhibition rates in the range of 21.8-25.6%, respectively. The cytotoxicities of compounds **1-5** against five human tumor cell lines (NB4, A549, SHSY5Y, PC3, and MCF7) were also tested. The results revealed that compounds **1-5** showed weak inhibitory activities against some tested human tumor cell lines with IC_{50} values in the range of 3.9-9.2 μM.

The dried root of *Pueraria lobata* (Wild.) Ohwi (Yege) belonging to the family of Fabaceae or Leguminosae, which is a twining perennial herb with woody base native to South East Asia regions, such as China, Korea and Japan.[1-3] Since 2005, this herbal medicine is also called Gegen in the Chinese Pharmacopeia.[4] Gegen has traditionally been used in TCM for improving the body function, such as promoting circulation and increasing the blood flow.[1,4] Puerarin, the first isoflavone isolated from the root of *Pueraria lotaba* in the late 1950s bearing a specific carbon-glycoside bond,[5] has proven to be responsible for the pharmacological actions on the cardiovascular systems of this herbal medicine.[1,4]

Actually its high medicinal value and nutritional value have rightfully earned Gegen herb a reputation as the "Asian ginseng" and "longevity powder" in Japan, where it is honored as the "Royal Special food".[6] Previous phytochemical studies of root of *Pueraria lobata* have shown the presence of isoflavones,[7-9] triterpenes,[2,10,11] coumarins,[12,13] steroids,[14] and the homologous. Today's research also discovered that the concentration and activity of isoflavones contained in the root of *Pueraria lobata* are far more than that found in soybeans, and it is enjoying great popularity in the west and Japan for a variety of purposes, e.g. weight loss, breast enlargement, hair loss treatment, alcoholism prevention, liver tonic, and so on.[15,16] In continuing efforts to the phytochemistry research on the root of *Pueraria lobata* led to the isolation of three new (**1-3**) and two known (**4** and **5**) isoflavones. This paper deals with the isolation, structural elucidation, and bioactivities of these compounds, as well as their anti-tobacco mosaic virus (anti-TMV) activities and cytotoxicities.

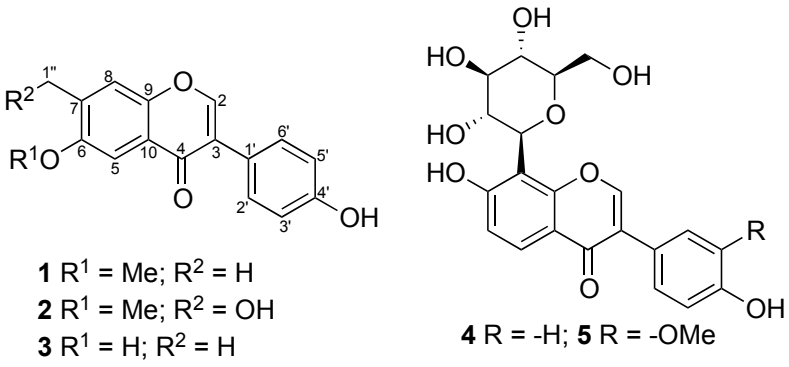

Figure 1. Isoflavones from the root of *Pueraria lobata*

A 90% aq. MeOH extract prepared from the root of *Pueraria lobata* was subjected repeatedly to column chromatography and preparative HPLC to afford three new isoflavones, 4′-hydroxy-6-methoxy-7-methylisoflavone (**1**), 4′,6-dihydroxy-7-methylisoflavone (**2**), and 4′-hydroxy-7-hydroxymethyl-6-methoxyisoflavone (**3**), and two known isoflavones (**4** and **5**). The structures of the compounds **1-5** were as shown in **Figure 1**, and the ^{1}H and ^{13}C NMR data of **1-3** were listed in **Table 1**. The known compounds, compared with literature, were identified as puerarin (**4**),[17] and 3′-methoxypuerarin (**5**).[18v]

Compound **1** was obtained as an orange-yellow gum. The molecular formula of $C_{17}H_{14}O_4$ was determined from the HRESIMS spectra showing the sodiated molecular ion at *m/z* 305.0798 $[M+Na]^+$ (calcd 305.0790). The ^{1}H and ^{13}C NMR spectrum of **1** (**Table 1**) along with analysis of the DEPT spectra displayed 17 carbon signals and 14 proton signals, respectively, corresponding to 4′,6,7-trisubstituted isoflavones nucleus (C-2 ~ C-10 and C-1′ ~ C-6′; H-5, H-8, H-2′,6′, and H-2′,6′),[19] one methyl group (δ_C 16.6 q; δ_H 2.73 s), one methoxy groups (δ_C 55.9 q; δ_H 3.86 s), and one phenolic

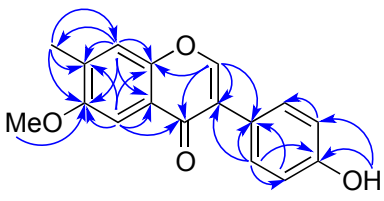

Figure 2. key HMBC (↷) correlations of **1**

hydroxy group (δ_H 11.20 s). The 4′,6,7-trisubstituted isoflavones nucleus was also supported by the HMBC correlations (**Figure 2**) of H-5 with C-4, C-6, C-7, C-9, C-10, of H-8 with C-6, C-7, C-9, C-10, of H-2 with C-1′, C-3, C-4, C-9, and of H-2′,6′ with C-3. The location of methyl group was assigned to C-7 position on the basis of HMBC correlations of H_3-1″ (δ_H 2.37) with C-6 (δ_C 154.1), C-7 (δ_C 132.0), C-8 (δ_C 117.8), and of H-8 (δ_H 6.69) with C-1″ (δ_C 16.6). The HMBC correlations from the methoxy proton (δ_H 3.86) to C-6 (δ_C 154.1) concluded the linkage of the methoxy group at C-6. Finally, The phenolic hydroxy group located at C-4′ was supported by the HMBC correlations of phenolic hydroxy proton (δ_C 157.5) with C-4′ and C-3′,5′ (δ_C 116.0). In addition, the typical proton signals of H-5 (δ_H 7.13 s), H-8 (δ_H 6.69 s), H-2′,6′ (δ_H 7.73, d, J = 8.8), and H-3′,5′ (δ_H 6.78, d, J = 8.8) also supported the above substituents pattern. Thus, the structure of **1** was established as 4′-hydroxy-6-methoxy-7-methylisoflavone.

Table 1. ^{1}H and ^{13}C NMR data for compounds **1-3** ($CDCl_3$, 125 and 500 MHz)

No.	Compound (**1**)		Compound (**2**)		Compound (**3**)	
	δ_C (mult.)	δ_H (mult, *J*, Hz)	δ_C (mult.)	δ_H (mult, *J*, Hz)	δ_C (mult.)	δ_H (mult, *J*, Hz)
2	152.6 d	7.85 s	152.3 d	7.85 s	152.8 d	7.87 s
3	123.6 s		123.9 s		123.1 s	
4	176.2 s		176.5 s		176.1 s	
5	114.9 d	7.13 s	116.3 d	7.07 s	115.5 d	7.18 s
6	154.1 s		151.2 s		153.3 s	
7	132.0 s		133.3 s		134.9 s	
8	117.8 d	6.69 s	118.2 d	6.65 s	116.9 d	6.71 s
9	149.0 s		149.2 s		149.6 s	
10	121.4 s		121.8 s		122.7 s	
1′	124.9 s		125.0 s		124.4 s	
2′,6′	130.5 d	7.73 (d) 8.8	130.2 d	7.74 (d) 8.8	130.2 d	7.79 (d) 8.8
3′,5′	116.0 d	6.78 (d) 8.8	115.9 d	6.80 (d) 8.8	115.9 d	6.81 (d) 8.8
4′	157.5 s		157.4 s		157.1 s	
1″	16.6 q	2.37 s	16.2 q	2.34 s	63.2 t	4.61 s
OMe-6	55.9 q	3.86 s			56.3 q	3.85 s
Ar-OH-6				11.21 s		
Ar-OH-4′		11.20 s		10.91 s		10.84 s

4′,6-Dihydroxy-7-methylisoflavone (**2**) was isolated as orange gum and it gave an $[M+Na]^+$ peak at *m/z* 291.0628, consistent with a molecular formula of $C_{16}H_{12}O_4$. The data of ^{1}H NMR were assigned to ^{13}C NMR with the help of HSQC spetrum (**Table 1**). Its ^{1}H and ^{13}C NMR spectroscopic data were similar to those of **1**, which suggested that compound **2** was structurally related to **1**. The marked differences between them were due to the inexistence of a methoxy group, and appearance of a phenolic hydroxy group (δ_H 10.91 s) in compound **2**. These changes indicated that a methoxy group in **1** was replaced by a phenolic hydroxy group in compound **2**. In addition, the obvious chemical shift differences of the upfield shift of C-6 from δ 154.1 ppm to δ 151.2 ppm suggested the substituent groups should be varied at C-6.

This was also supported by the HMBC correlations of the phenolic hydroxy proton signal (δ_H 10.91) with C-5 (δ_C 116.3), C-6 (δ_C 151.2), and C-7 (δ_C 133.3). According to above informations, the structure of compound **2** was assigned.

Compound **3** was assigned a molecular formula of $C_{17}H_{14}O_5$ as supported by the HRESIMS (*m/z* 321.0744 $[M+Na]^+$), corresponding to 11 degrees of unsaturation. Its ^1H and ^{13}C NMR spectroscopic data were also similar to those of compound **1**, except for the presence of a hydroxymethyl group signal (δ_H 4.61 s), and the absence of a methyl proton signal. These changes indicated that the methyl group in **1** was substituted by a hydroxymethyl group in compound **3**. The HMBC correlations from the methoxy protons (δ_H 3.85) to C-6 (δ_C 153.3) suggested that the methoxy groups located at C-6. The phenolic hydroxy group located at C-4′ was supported by the HMBC correlations of the phenolic proton signal (δ_H 10.84) with C-3′,5′ (δ_C 115.9) and C-4′ (δ_C 157.1). Finally, the location of hydroxymethyl group located at C-7 was supported by the HMBC correlations of H_2-1″ (δ_H 4.61) with C-6 (δ_C 153.3), C-7 (δ_C 134.9), and C-8 (δ_C 116.9), and of H-8 (δ_H 6.71) with C-1″ (δ_C 63.2). Accordingly, the structure of 4′-hydroxy-7-hydroxymethyl-6-methoxyisoflavone (**3**) was determined as shown.

Since certain of the isoflavones exhibit potential anti-TMV activity,[20-22] compounds **1-5** were tested for their anti-TMV activity. The inhibitory activity of compounds **1-5** against TMV replication were tested using the half-leaf method.[23] Ningnanmycin (with inhibition rate of 31.5%), a commercial product for plant disease in China, was used as a positive control. The antiviral inhibition rates of compounds **1-5** at the concentration of 20 μM were listed in **Table 2**. The results showed that compounds **2** and **3** exhibited comparable anti-TMV activities with inhibition rates of 34.2% and 33.5%. The inhibition rates are higher than that of positive control. The other compounds also showed potential anti-TMV activities with inhibition rates in the range of 21.8-25.6%, respectively.

Table 2. The TMV Inhibition rates compounds **1-5**

Compounds	Inhibition rates (%)	Compounds	Inhibition rates (%)
1	25.6 ± 2.8	**4**	23.4 ± 2.9
2	34.2 ± 3.2	**5**	21.8 ± 2.6
3	33.5 ± 3.3	ningnanmycin	31.5 ± 3.2

All results are expressed as mean ± SD; n = 3 for all groups.

Since certain of the isoflavones exhibit potential cytotoxic activity,[24-26] the cytotoxicities of compounds **1-5** were also tested using a previously reported

Table 3. Cytotoxic activity of compounds **1-5**

Compounds	Cell lines and IC_{50} (μM)				
	NB4	A549	SHSY5Y	PC3	MCF7
1	>10	>10	9.2 ± 0.8	>10	>10
2	>10	7.8 ± 0.5	8.5 ± 0.5	>10	>10
3	6.4 ± 0.6	4.8 ± 0.4	3.9 ± 0.4	5.5 ± 0.4	7.3 ± 0.7
4	8.8 ± 0.5	>10	>10	7.6 ± 0.6	9.2 ± 0.8
5	>10	7.9 ± 0.6	6.8 ± 0.7	8.2 ± 0.5	7.1 ± 0.5
Taxol	0.03	0.02	0.05	0.05	0.05

NB4, human leukemia cell; A549, carcinomic human alveolar basal epithelial cell; SHSY5Y, human neuroblastoma cell; PC3, human prostate cancer cell; MCF7, human breast adenocarcinoma cell.
All results are expressed as mean ± SD; n = 3 for all groups.

procedure.[27] the cytotoxic abilities against five human tumor cell lines (NB4, A549, SHSY5Y, PC3, AND MCF7) by MTT-assay were summarized in **Table 3**. the results revealed that compounds **1-5** showed weak inhibitory activities against some tested human tumor cell lines with IC_{50} values in the range of 3.9-9.2 μM.

EXPERIMENTAL

General. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. 1D and 2D NMR spectra were recorded on DRX-500 spectrometers with TMS as internal standard, and the chemical shifts (δ) were expressed in ppm. HRESIMS was performed on an API QSTAR time-of-flight spectrometer and a VG Autospec-3000 spectrometer, respectively. Preparative HPLC was performed on a Shimadzu LC-8A preparative liquid chromatograph with a ZORBAX PrepHT GF (21.2 mm × 25 cm, 7 μm) column or a Venusil MP C_{18} (20 mm × 25 cm, 5 μm) column. Column chromatography was performed with silica gel (200-300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China). The fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 5% H_2SO_4 in EtOH.

Plant Material. The root of *Pueraria lobata* was collected fromYuxi Prefecture, Yunnan province, China, in September 2016 and identified by Prof. Ning Yuan. A voucher specimen (YNNI-16-09-28) has been deposited in the Key Laboratory of Chemistry in Ethnic Medicinal Resources, Yunnan University.

Extraction and Isolation. The air-dried and powdered root of *Pueraria lobata* (6.8 kg) were extracted four times with 90% aqueous MeOH (3 × 5 L) at room temperature and filtered. The solvent was evaporated in vacuo, and the crude extract was dissolved in H_2O and partitioned with EtOAc. The EtOAc partition (182 g) was applied to silica gel (200-300 mesh) column chromatography, eluting with a $CHCl_3$-MeOH gradient system (9:1, 8:2, 7:3, 6:4, 5:5, 4:6), to give six fractions A-F. Further separation of fraction B (8:2, 12.5 g) by silica gel column chromatography, eluted with $CHCl_3$-Me_2CO (9:1-1:2), yielded mixtures B1–B7. Fraction B3 (7:3, 1.15 g) was subjected to silica gel column chromatography using petroleum ether-acetone and semi-preparative HPLC (50% MeOH-H_2O, flow rate 20 mL/min) to give **1** (12.2 mg) and **3** (10.8 mg). Fraction B4 (6:4, 1.86 g) was subjected to silica gel column chromatography using petroleum ether-acetone and semi-preparative HPLC (44% MeOH-H_2O, flow rate 20 mL/min) to give **2** (12.6 mg). Fraction B5 (5:5, 1.86 g) was subjected to silica gel column chromatography using petroleum ether-acetone and semi-preparative HPLC (38% MeOH-H_2O, flow rate 20 mL/min) to give **4** (33.6 mg) and **5** (42.1 mg).

Anti-TMV Assays. The anti-TMV activities were tested using the half-leaf method,[23] and Ningnanmycin (2% water solution), a commercial product for plant disease in China, was used as a positive control.

Cytotoxicity Assay. The cytotoxicity tests for the isolates were performed by against NB4, A549,

SHSY5Y, PC3, and MCF7 tumor cell lines by MTT-assay (with taxol as the positive control).[27]

4′-Hydroxy-6-methoxy-7-methylisoflavone (**1**), $C_{17}H_{14}O_4$, obtained as orange-yellow gum; UV (MeOH), λ_{max} (log ε) 328 (3.46), 262 (3.85), 210 (4.32) nm; IR (KBr) ν_{max} 3310, 3068, 2935, 1642, 1610, 1558, 1506, 1440, 1365, 1257, 1148, 1059, 962, 847 cm^{-1}; ^1H NMR and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), **Table 1**; ESIMS *m/z* 305; HRESIMS (positive ion mode) *m/z* 305.0798 $[M+Na]^+$ (calcd 305.0790 for $C_{17}H_{14}NaO_4$).

4′,6-Dihydroxy-7-methylisoflavone (**2**), $C_{16}H_{12}O_4$, obtained as orange-yellow gum; UV (MeOH), λ_{max} (log ε) 325 (3.52), 262 (3.76), 210 (4.41) nm; IR (KBr) ν_{max} 3318, 3065, 2938, 1640, 1610, 1554, 1502, 1436, 1362, 1254, 1143, 1050, 938, 862 cm^{-1}; ^1H NMR and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), **Table 1**; ESIMS *m/z* 291; HRESIMS (positive ion mode) *m/z* 291.0628 $[M+Na]^+$ (calcd 291.0633 for $C_{16}H_{12}NaO_4$).

4′-Hodraxy-7-hydroxymethyl-6-methoxyisoflavone (**3**), $C_{17}H_{14}O_5$, obtained as orange-yellow gum; UV (MeOH), λ_{max} (log ε) 332 (3.48), 265 (3.80), 210 (4.36) nm; IR (KBr) ν_{max} 3356, 3060, 2934, 1715, 1645, 1612, 1561, 1507, 1432, 1369, 1248, 1146, 1059, 972, 844 cm^{-1}; ^1H NMR and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz, respectively), **Table 1**; ESIMS *m/z* 321; HRESIMS (positive ion mode) *m/z* 321.0744 $[M+Na]^+$ (calcd 321.0739 for $C_{17}H_{14}NaO_5$).

ACKNOWLEDGEMENTS

This research was supported by the Research Foundation of China Tobacco Yunnan Industrial Co., Ltd (No. 2016JC03), the Research Foundation of China Tobacco Company (No. 110201502006), the National Natural Science Foundation of China (Nos. 21562049, 31360081 and 31400303), and the Applied Fundamental Foundation of Yunnan Province (Nos. 2014FB097, 2014FB163, and 2015FB162).

REFERENCES

1. Jiangsu New Medicine College. Dictionary of Chinese Medicinal Materials, Shanghai: Shanghai Scientific and Technological Publisher; 1997.
2. W. Song, Y. J. Li, X. Qiao, Y. Qian, and M. Ye, *J. Chin. Pharm. Sci*., 2014, **23**, 347.
3. Z. Zhang, T. N. Lam, and Z. Zuo, *J. Clin. Pharmacol*., 2013, **53**, 787.
4. *The Pharmacopoeia Commission of PRC*. Pharmacopoeia of the People's Republic of China Version 2010, Vol. 1, Beijing: Chemical Industry Press, 2010.
5. S. Shibata, T. Murakami, Y. Nishikawa, and M. Harada, *Chem. Pharm. Bull*., 1959, **7**, 134.
6. Y. Q. Yang, A. Chin, L. K. Zhang, J. J. Lu, and R. W. K. Wong, *Phytother. Res*., 2014, **28**, 1.
7. P. Li, Y. Lu, S. Du, J. Bai, H. Liu, Q. Guo, and Y. Guo, *Trop. J. Pharm. Res*., 2013, **12**, 919.

8. H. J. Rong, J. F. Stevens, M. L. Deinzer, L. De Cooman, and D. De Keukeleire, *Planta Med.*, 1998, **64**, 620.
9. W. Xiao, Q. Zhang, C. Chen, Q. H. Zhang, Y. J. Hu, Z. N. Xia, and F. Q. Yang, *J. Chromatogr. Sci.*, 2016, **54**, 1678.
10. T. Arao, J. Kinjo, T. Nohara, and R. Isobe, *Chem. Pharm. Bull.*, 1995, **43**, 1176.
11. M. Ceng and Y. H. Yi, *Acta Pharmacol. Sin.*, 2000, **35**, 438.
12. T. Nohara, J. Kinjo, J. Furusawa, Y. Sakai, M. Inoue, Y. Shirataki, Y. Ishibashi, I. Yokoe, and M. Komatsu, *Phytochemistry*, **33**, 1993, 1207.
13. Y. Dong, B. Xu, L. Lin, and Q. Za, *Food & Macinery*, 2005, **21**, 85.
14. G. H. Li, Q. W. Zhang, and Y. T. Wang, *China J. Chin. Mater. Med.*, 2010, **35**, 3156.
15. K. H. Lu, C. T. Liu, R. Raghu, and L. Y. Sheen, *J. Tradit. Chin. Complem. Med.*, 2012, **2**, 115.
16. W. Xiao, F. Q. Wang, C. H. Li, Q. Zhang, Z. N. Xia, and F. Q. Yang, *Anal. Methods-UK*, 2015, **7**, 1098.
17. R. Zhao, T. W. Tan, and C. Sandstrom, *J. Biol. Phys.*, 2011, **37**, 387.
18. J. F. Chi, G. G. Zhang, P. Li, and L. P. Kou, *Chin. J. Med. Chem.*, 2007, **17**, 47.
19. Z. Y. Chen, J. L. Tan, G. Y. Yang, M. M. Miao, Y. K. Chen, and T. F. Li, *Phytochem. Lett.*, 2012, **5**, 233.
20. X. M. Gao, H. X. Mu, X. S. Li, G. Y. Yang, G. P. Li, and Q. F. Hu, *J. Chin. Chem. Soc.*, 2012, **59**, 540.
21. Q. F. Hu, D. Y. Niu, B. Zhou, Y. Q. Ye, G. Du, C. Y. Meng, and X. M. Gao, *Bull. Korean Chem. Soc.*, 2013, **34**, 3013.
22. L. Li, Q. P. Shen, C. B. Liu, Y. Wang, J. J. Yao, T. Zhang, F. M. Zhang, P. He, X. X. Shi, Z. H. Liu, M. M. Miao, and G. Y. Yang, *Phytochem. Lett.*, 2015, **13**, 156.
23. M. Zhou, M. M. Miao, G. Du, S. Z. Shang, W. Zhao, Z.-H. Liu, G.-Y. Yang, C. T. Che, Q.-F. Hu, and X.-M. Gao, *Org. Lett.*, 2014, **16**, 5016.
24. X. Z. Huang, X. S. Bai, H. Liang, C. Wang, W. J. Li, J. M. Guo, and Z. Y. Jiang, *Bull. Korean Chem. Soc.*, 2013, **34**, 1421.
25. Y. P. Li, Y. K. Li, G. Du, H. Y. Yang, X. M. Gao, and Q. F. Hu, *J. Asian Nat. Prod. Res.*, 2014, **16**, 735.
26. H. Wang, J. X. Yang, G. Y. Liu, J. Lou, L. M. Li, Q. F. Hu, and X. M. Gao, *Asian J. Chem.*, 2015, **27**, 2019.
27. Q. F. Hu, B. Zhou, J. M. Huang, Z. Y. Jiang, X. Z. Huang, L. Y. Yang, X. M. Gao, G. Y. Yang, and C. T. Che, *J. Nat. Prod.*, 2013, **76**, 1866.