Cytotoxic Cycloartane Triterpene Saponins from *Actaea asiatica*

Jingchun Gao,[†,‡] Feng Huang,[†] Jinchao Zhang,[§,⊥] Guoyuan Zhu,[‡] Mengsu Yang,*[,§] and Peigen Xiao*[,†]

Institute of Medicinal Plant Development, Chinese Academy of Medical Science, Peking Union Medical College, Beijing 100094, People's Republic of China, Department of Biology and Chemistry, City University of Hong Kong, Hong Kong, People's Republic of China, Shenzhen Research Institute of Hongkong City University, Shenzhen 518057, People's Republic of China, and Department of Chemistry, College of Chemistry & Environmental Science, Hebei University, Baoding 071002, People's Republic of China

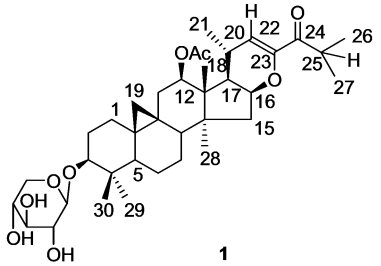
Received March 15, 2006

Three new 9,19-cycloartane triterpene glycosides, asiaticoside A (**1**), asiaticoside B (**2**), and 25-*O*-ethylcimigenol-3-*O*-*β*-D-xylopyranoside (**3**), together with cimiacemoside I (**4**), 25-*O*-acetylcimigenol-3-*β*-*O*-D-xyloside (**5**), and 25-anhydrocimigenol-*β*-*O*-D-xyloside (**6**) were isolated from the roots/rhizomes extract of *Actaea asiatica*, and their structures were established by spectroscopic methods (IR, HRESIMS, and NMR). Compounds **1**−**3**, **5**, and **6** had notable cytotoxicity against HepG2 and MCF-7 cancer cell lines.

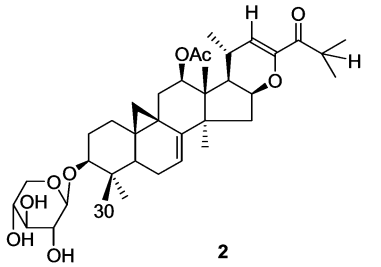
Actaea asiatica Hara (Ranunculaceae, tribe Cimicifuga), commonly known as baneberry, is an herb distributed mainly in the north temperate zone. Its rhizomes have been used as an anti-inflammatory, analgesic, and antipyretic remedy in traditional Chinese medicine.[1] Chemical and pharmacological studies on the genera *Cimicifuga* (now *Actaea*) have reported that 9,19-cycloartane triterpene glycosides are major chemical constituents;[2,3] however, little work has been done on the chemical constituents of *A. asiatica* Hara. Previously, our group reported that three 9,19-cycloartane triterpene glycosides from *C. dahurica* showed cytotoxicity against several cancer cell lines.[4]

In this study, three new 9,19-cycloartane triterpene glycosides (**1**−**3**), together with three known compounds, cimiacemoside I (**4**), 25-*O*-acetylcimigenol-3-*β*-*O*-D-xyloside (**5**), and 25-anhydrocimigenol-*β*-*O*-D-xyloside (**6**), were isolated from the roots/rhizomes of *A. asiatica* L. Their cytotoxicity was screened against HepG2 and MCF-7 cancer cell lines using the MTT assay.

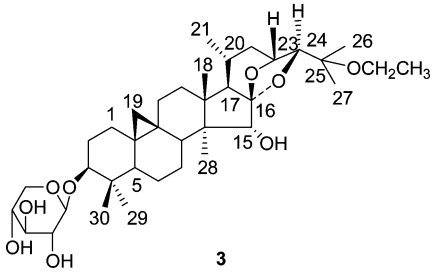
Compound **1** was obtained as a white powder. The HRESIMS of **1** showed a significant quasi-molecular ion peak at *m*/*z* 667.3815, corresponding to the molecular formula $C_{37}H_{56}O_9$ (calcd 667.3822 for $[M + Na]^+$). The IR spectrum showed hydroxyl and carbonyl absorptions at 3395 and 1732 cm^{-1}, respectively. The UV showed a significant absorption maximum at 265 nm and the usual 210 nm absorption typical of triterpene compounds. The ^1H NMR spectrum (Table 1) showed signals due to a cyclopropane methylene at δ 0.19 and 0.61 (each 1H, d, $J = 4.0$ Hz); four *tert*-methyl singlet signals at δ 0.90, 1.01, 1.22, and 1.32, three overlapped methyl doublet signals at δ 1.14, 1.15, and 1.16, an acetyl methyl group at δ 2.15, an olefinic proton at δ 6.21 (d, $J = 4.0$ Hz), and an anomeric proton at δ 4.82 (d, $J = 7.6$ Hz). The ^{13}C and DEPT NMR spectra (Table 1) showed five oxygenated carbons assignable to a xylose moiety at δ 107.9, 79.2, 76.0, 71.6, and 67.5, two oxygenated carbon signals at δ 77.2 and 75.9, and two unsaturated carbon signals at δ 116.5 and 150.0. The above evidence suggested that **1** was a 9,19-cycloartane triterpene monoglycoside with an unsaturated double bond. In the ^1H NMR spectrum of compound **1** there were two additional secondary methyl signals at δ 1.14 and 1.15 overlapped with a secondary methyl group signal for 21-CH_3; therefore we assumed a partial structure of $-CH(CH_3)_2$ at C-25. A multiplet proton signal at δ 3.32 supported the existence of an isopropyl group in the structure. In the ^{13}C NMR, two carboxyl signals appeared at δ 171.1 and 200.8; the former was assigned to an acetyl group and the latter was assigned to a keto group at C-24, which was supported by the significant correlation between the methyl signal at δ 1.14 and 1.15 (each 3H, d, $J = 6.8$ Hz, H-26, 27) and the keto signal at δ 200.8 (HMBC). In compound **1**, an olefinic proton was observed at δ 6.21 (d, $J = 4.0$ Hz), which showed strong correlation with a methine carbon at δ 25.9 (C-20), a secondary methyl at δ 24.8 (C-21), and a keto carbon at δ 200.8 (C-24) in the HMBC, consistent with an unsaturated double bond between C-22 and C-23.



1



2



3

The HMBC spectrum showed significant correlations between Hα-11 (δ 2.64) and an oxygenated methine signal at δ 77.2 (C-12); between H_α-15 (δ 2.06) and signals of two quaternary carbons at δ 49.3 (C-13) and 48.5 (C-14) and an oxygenated methine signal at δ 75.9 (C-16); between H-16 (δ 4.27) and a quaternary carbon at δ 48.5 (C-14); between H-17 (2.12) and a alkene carbon signal

* Corresponding author. Tel: +86-10-63011294. Fax: +86-10-63038753. E-mail: xiaopg@public.bta.net.cn; bhmyang@cityu.edu.hk.
† Peking Union Medical College.
‡ Shenzhen Research Institute of Hongkong City University.
§ City University of Hong Kong.
⊥ Hebei University.

10.1021/np060113h CCC: $33.50
Published on Web 09/12/2006

Table 1. ^{1}H NMR and ^{13}C NMR Data for Compounds **1**−**3** in Pyridine-d_5[a] (400 MHz for ^{1}H and 100 MHz for ^{13}C)

	1		**2**		**3**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	32.4	1.09 m, 1.51 m	30.9	1.13 m, 1.59 m	32.9	1.24 m, 1.31 m
2	30.2	1.85 m, 2.28 m	30.0	1.85 m, 2.28 m	30.6	1.96 m, 2.36 m
3	88.4	3.45 dd (4.0,11.6)	88.4	3.43 dd (4.0,11.6)	89.0	3.51 (4.0,11.6)
4	41.6		41.0		41.8	
5	47.4	1.28 m	42.9	1.18 m	47.7	1.35 m
6	20.5	0.75 m, 1.51 m	22.4	1.57 m, 1.85 m	21.5	0.71 m, 1.52 m
7	26.1	1.02 m, 1.32 m	114.7	5.16 d (6.8)	26.8	1.06 m, 1.20 m
8	46.9	1.68 dd (11.5, 4.4)	148.5		49.1	1.69 dd (11.5, 4.6)
9	21.2		21.1		20.6	
10	27.6		27.7		27.1	
11	36.8	1.15 overlapped	36.8	1.21 overlapped	26.9	1.13 overlapped
		2.64 dd (16,9.2)		2.85 dd (16,9.2)		2.08 m
12	77.2	5.29 dd (4.0,9.2)	77.3	5.38 brd (8.4)	34.5	1.55, 1.66
13	49.3		51.1		42.3	
14	48.5		48.6		48.0	
15	46.0	2.06, 1.93	44.9	2.25, 2.63	80.6	4.22 s
16	75.9	4.27 m	76.2	4.34 m	112.4	
17	52.2	2.12	53.2	2.07	59.9	1.45
18	12.9	1.22 s	14.7	1.24	20.0	1.13
19	30.5	0.19 d (4.0)	30.0	0.47 d (3.6)	31.4	0.27 d (3.6)
		0.61 d (4.0)		0.86 d (3.6)		0.52 d (3.6)
20	25.9	2.49 m	26.1	2.50 m	24.5	1.62
21	24.8	1.16 d (6.4)	24.8	1.13 d (6.4)	20.1	0.83 d (6.4)
22	116.5	6.21 d (4.0)	117.1	6.22 d (3.6)	38.6	0.94, 2.22
23	150.0		150.5		72.0	4.64 d (9.2)
24	200.8		200.9		88.7	3.66 s
25	35.4	3.32 m	35.6	3.29 m	76.5	
26	18.7	1.15 d (6.8)	19.1	1.11 d (6.4)	20.5	1.28 s
27	18.7	1.14 d (6.8)	19.1	1.12 d (6.4)	23.4	1.28 s
28	21.6	0.90 s	28.4	1.07 s	12.3	1.17 s
29	26.2	1.32 s	26.3	1.32 s	26.2	1.31 s
30	15.5	1.01 s	14.8	1.01 s	15.9	1.05 s
1′	107.9	4.82 d (7.6)	108.1	4.72 d (7.6)	108.1	4.86 d (7.6)
2′	76.0	4.00 t (8.0)	76.4	4.02 t (8.0)	76.0	4.02 t (8.0)
3′	79.2	4.13 t (8.4)	79.2	4.14 t (8.4)	79.1	4.15 t (8.4)
4′	71.6	4.21 m	71.8	4.21 m	71.7	4.20 m
5′	67.5	3.71 t (10.4)	67.7	3.71 t (10.4)	67.6	3.73 t (10.4)
		4.33 dd (11.2,5.2)		4.33 dd (11.2,5.2)		4.35 dd (11.2,5.2)
CH_3CO	171.1		171.3			
CH_3CO	20.9	2.15 s	21.8	2.18		
$-OCH_2-$					57.3	3.41 m
$-CH_3$					16.9	1.11t(6.8)

[a] Chemical shifts (δ) in ppm, coupling constants (Hz) in parentheses.

at δ 116.5 (C-22) and an oxygenated methine signal at δ 77.2 (C-12); and between H-20 (δ 2.49) and an alkene methine signal at δ 116.5 (C-22) and an oxygenated quaternary alkene carbon signal at δ 150.0 (C-23). Therefore, the aglycone structure of **1** was elucidated as 16β:23-expoxy-12β-acetoxy-22,23-didehydro-24-one-25-hydro-9,19-cyclolanostane.

The coupling constant (J = 7.6 Hz) of the anomeric proton in the ^{1}H NMR spectrum of **1** indicated the D-xylose was in the β-configuration. The β-D-xylopyranoside unit was shown to be attached at C-3 by observation of a long-range cross-peak between H-1′ and C-3 (δ 88.4) in the HMBC spectrum. The H-3 α signal was assigned from its chemical shift and coupling patterns (Table 1). The relative stereochemistry of **1** was determined on the basis of the coupling constants from the ^{1}H and NOESY experiments and comparison with those of known 9,19-cycloartane triterpene glycosides.[5] Thus, compound **1** was established as 16β:23-expoxy-12β-acetoxy-22,23-didehydro-24-one-25-hydro-9,19-cyclolanostane-3-*O*-β-D-xylopyranoside and was named asiaticoside A.

Compound **2** gave the molecular formula $C_{37}H_{54}O_9$, as determined by HRESIMS (found *m*/*z* 665.3686, calcd 665.3666 for $[M + Na]^+$). The IR spectrum showed hydroxyl and carbonyl absorptions at 3445 and 1736 cm^{-1}, respectively. The UV showed a significant absorption at 265 nm and other absorptions typical of triterpene compounds. The ^{13}C NMR signals of **2** were assigned with the help of 2D NMR spectroscopy and were similar to those of **1** (Table 1), except for the signals of a C-7, C-8 double bond and their α- and β-carbons (C-5, C-6, C-28, C-15). By comparison of ^{13}C NMR data of **2** and **1** and consideration of substituent effects, the double bond was assigned to C-7, C-8. Thus, compound **2** was determined to be 16β:23-expoxy-12β-acetoxy-22,23-didehydro-24-one-25-hydro-9,19-cyclolanostane-7-ene-3-*O*-β-D-xylopyranoside and was named asiaticoside B.

Compound **3** was obtained as a white powder. The molecular formula of $C_{37}H_{60}O_9$ was established for compound **3** (HRESIMS). The IR spectrum showed a hydroxyl absorption at 3421 cm^{-1}. The ^{1}H NMR spectra showed signals due to a cyclopropane methylene at δ 0.27 and 0.52 (each 1H, d, J = 3.6 Hz); six *tert*-methyl groups at δ 1.05, 1.13, 1.17, 1.28, 1.28, and 1.31; a secondary methyl group at δ 0.83 (d, J = 6.4 Hz); and an anomeric proton at δ 4.86 (d, J = 7.6 Hz). The ^{13}C NMR spectrum showed data consistent with a xyloside moiety. The evidence suggested that **3** was a highly oxygenated 9,19-cycloartane triterpene monoglycoside. Comparing the ^{1}H NMR and other data with those of known 9,19-cycloartane triterpene glycosides,[2] the aglycone of **3** was very similar to that of cimicifugoside (cimigenol-3-O-β-D-xylopyranoside) except that there was an additional proton multiplet at δ 3.41 (2H) and a triplet methyl signal at δ 1.11 (t, J = 6.8 Hz), which corresponded to the carbon signals at δ 57.3 and 16.9, respectively. In the ^{13}C NMR, signals for C-26 and C-27 shifted downfield. In the HMBC, significant correlation was observed between the ^{1}H (3.41) and the ^{13}C signal at δ 76.5 (C-25) and the triplet methyl carbon at δ 16.9, consistent with an −OEt group at C-25. The ^{1}H and ^{13}C signals

for compound **3** were fully assigned using a combination of HMQC, HMBC, ^{1}H−^{1}H COSY, and NOESY experiments (Table 1).

The known compounds cimiacemoside I, 25-*O*-acetylcimigenol-3-*β*-*O*-D-xyloside, and 25-anhydrocimigenol-3-*β*-*O*-D-xyloside were confirmed by comparison of their physical and spectroscopic data with published data.[5−7]

As noted in the introduction, several 9,19-cycloartane triterpene glycosides isolated from the genus *Cimicifuga* significantly inhibit proliferation of some cancer cell lines.[4] One report also indicated that triterpene glycosides from *Cimicifuga racemosa* inhibit proliferation of the MCF-7 estrogen-responsive breast cancer cell line.[8] The compounds isolated in the present study (**1**−**3**) were strongly cytotoxic, with IC_{50} values of 9.90, 9.74, and 11.79 μM against HepG2 cancer cells and 9.78, 8.32, and 11.99 μM against MCF-7 cancer cells, respectively. The IC_{50} of compounds **5** and **6** against HepG2 cells was consistent with the previous reports,[4,9] and they also exerted significant cytotoxicity against MCF-7 cells with IC_{50} values of 9.83 and 19.82 μM, respectively. Compound **4** was inactive.

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Optical rotations were performed on a Perkin-Elmer digital polarimeter. ^{1}H and ^{13}C NMR spectra were measured on a Brucker Avance 400 MHz instrument. Chemical shifts (δ) were expressed in ppm with reference to TMS. HR-ESI data were recorded on a Accu(TOFCS)NC-MS. IR spectra were run on a Shimadzu FTIR 8400 infrared spectrometer (KBr). TLC was performed on Si gel GF_{254} sheets (0.20−0.25 mm) (Qingdao Haiyang Chemical Group Co., Qingdao, Shandong Province, P. R. China) or $RP\text{-}18F_{254}$ (Merck) reversed-phase plates with compounds visualized by spraying the dried plates with 5% H_2SO_4 in EtOH, followed by heating. Semipreparative HPLC was carried out on a Waters 2695 separation system with a Waters 2696 photodiode array detector and Millennium^{32} Chromatography Manager (Waters Co., MA) on a YMC-pack ODS-A semipreparative column (5 μm, 250 × 10 mm) with a flow rate of 2.5 mL/min. GC was conducted on a 6890N gas chromatograph (Agilent) with a flame ionization detector (FID). Medium-pressure reversed-phase column chromatography was carried out on a Büchi BOROSILIKAT 3.3 column filled with Merck RP_{18} with a fluid pump (Büchi B-688); low-pressure reversed-phase column chromatography was carried out on a glass colum (i.d. 200 × 20 mm) with an electronic air pump. Silica gel (100−300 mesh, Qingdao Haiyang Chemical Group Co., Qingdao, Shandong Province, P. R. China) was used for column chromatography.

Cytotoxicity Bioassay. The assay for cytotoxicity against HepG2 and MCF-7 cancer cell lines was performed as previously described.[4]

Plant Material. *Actaea asiatica* Hara roots/rhizomes were collected in LuoPing Village, SuiJiang County, ShaoTong Region, YunNan Province (July 2004), and identified by Dr. Bao-lin Guo, Peking Union Medical College, Beijing, China. A voucher specimen (AA-04-0717) has been deposited in the Herbarium of Institute of Medicinal Plant Development, Chinese Academy of Medical Science and Peking Union Medical College.

Extraction and Isolation. The air-dried, milled roots/rhizomes of *A. asiatica* (4.0 kg) were exhaustively extracted with 70% EtOH (20 L, 3 times) under reflux, and the extracts were evaporated to yield a syrup residue (1 kg). A sample (950 g) of the residue was suspended in H_2O (1500 mL) and fractionated by successive partition with petroleum ether (2 L × 2), EtOAc (2 L × 5), and *n*-BuOH (2 L × 3) to give a petroleum ether-soluble portion (10 g), an EtOAc-soluble portion (300 g), and *n*-BuOH solubles (200 g). A portion (270 g) of the EtOAc extract was subjected to low-pressure column chromatography (LPLC) on silica gel. Gradient elution with petroleum ether−Me_2CO−MeOH gave five fractions: I (102 g), II (103 g), III (25 g), IV (9 g), V (16 g). Fraction I (100 g) was subjected to LPLC on silica gel eluted with a gradient of $CHCl_3$−MeOH to give fractions A−I. Fraction C (8.0 g) was subjected to medium-pressure reversed-phase chromatography eluted with MeOH−H_2O (60:40, 2 L, 70:30, 1 L, 80:20, 1 L) to give four subfractions (CI−CIII). Subfraction CIII (2.0 g) was subjected to reversed-phase chromatography eluted with MeOH−H_2O (60:40, 1 L, 70:30, 500 mL, 80:20, 500 mL) to give three subfractions (CIII1−CIII3). CIII3 (1.0 g) was subjected to semipreparitive HPLC (MeOH−H_2O, 93:7, flow rate 3 mL/min) to yield compound **3** (10 mg). Fraction G (7.5 g) was fractionated by medium-pressure reversed-phase chromatography eluted with MeOH−H_2O (40:60, 2 L, 50:50, 1 L, 60:40, 1 L, 70:30, 1 L, 80:20, 1 L, 90:10, 1 L) to give four subfractions (GI−GIV). Subfraction GIV (2.5 g) was subjected to reversed-phase chromatography eluted with MeOH−H_2O (40:60, 1 L, 50:50, 1 L, 60:40, 1 L) to give three subfractions (GIV1−GIV3). GIV3 (1.2 g) was subjected to semipreparative HPLC (MeOH−H_2O, 80:20, flow rate 2.5 mL/min) to yield compounds **1** (40 mg), **2** (10 mg), **4** (6 mg), **5** (500 mg), and **6** (8 mg).

Determination of the Absolute Configuration of Sugars. Compounds (1−3 mg) were dissolved in 1 N HCl (1 mL) and then heated to 80 °C for 4 h. The solvent was removed under N_2. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2. The residue was dissolved in 0.1 mL of dry pyridine, and then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL) was added to the solution. The reaction mixture was heated at 60 °C for 2 h. After drying by N_2, 0.1 mL of *N*-(trimethylsilyl)imidazole was added, followed by heating at 60 °C for 1 h. The product was partitioned between cyclohexane and H_2O (0.1 mL, each), and the organic layer was analyzed by gas chromatography (GC). Peaks of the hydrolyzate were detected at 25.85 min (D-xylose). Treated in the same way, standard D-xylose (Sigma) gave a peak at t_R (min) 25.91 and L-xylose (Sigma) gave a peak at t_R (min) 26.42.

Asiaticoside A (1): white powder, mp 142−145 °C, $[\alpha]^{20}_D$ −67.0 (*c* 1.52, MeOH); ^{1}H, ^{13}C NMR, Table 1; IR (KBr) ν_{max} (cm^{-1}) 3395, 2943, 2869, 1732, 1678, 1632, 1458, 1385, 1242, 1041, 980; HRESIMS *m*/*z* 667.3815 (calcd 667.3822 for $C_{37}H_{56}O_9Na$).

Asiaticoside B (2): white powder, mp 143−147 °C, $[\alpha]^{20}_D$ −105.0 (*c* 0.52, MeOH); ^{1}H, ^{13}C NMR, Table 1; IR (KBr) ν_{max} (cm^{-1}) 3445, 2966, 2874, 1736, 1686, 1458, 1381, 1242, 1038, 988; HRESIMS *m*/*z* 665.3686 (calcd 665.3666 for $C_{37}H_{54}O_9Na$).

25-*O*-Ethylcimigenol-3-*O*-*β*-D-xylopyranoside (3): white powder, mp 237−329 °C, $[\alpha]^{20}_D$ 16.6 (*c* 0.30, MeOH); ^{1}H, ^{13}C NMR, Table 1; IR (KBr) ν_{max} (cm^{-1}) 3422, 2931, 2870, 1072, 1037, 972; HRESIMS *m*/*z* 671.4123 (calcd 671.4135 for $C_{37}H_{60}O_9Na$).

Acknowledgment. The authors are grateful to B. Qiu, Yunnan Institute of Materia Medica, Kunming, Yunnan Province, China, for the collection of plant material. This work was supported by State Hi-Tech Development Program of China (863 Program) (No. 2003-AA2Z2052) and National Nature Science Foundation of China (No. 30530860). We are grateful to Dr. Y. Dai at Jinan University for the GC technical support.

Supporting Information Available: ^{1}H NMR and ^{13}C NMR spectra of asiaticoside A are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (1) Wang, W. T. *Flora Reipublicae Popularis Sinicae*; Science Press: Beijing, 1979.
(2) Li, C. J.; Li, Y. H.; Chen, S. F.; Xiao, P. G. *Acta Pharm. Sin.* **1994**, *29*, 449−453.
(3) Li, J. X.; Kadota, S.; Li, H. Y.; Miyahara, T.; Namba, T. *Yakugaku Zasshi.* **1996**, *13*, 50−58.
(4) Tian, Z.; Yang, M. S.; Huang F.; Li, K. G.; Si, J. Y.; Shi, L.; Chen, S. B.; Xiao, P. G. *Cancer Lett.* **2005**, *226*, 65−75.
(5) Chen, S. N.; Fabricant, D. S.; Lu, Z. Z.; Fong, H. H. S.; Farnsworth, N. R. *J. Nat. Prod.* **2002**, *65*, 1391−1397.
(6) Li, C. J.; Chen, D. H.; Xiao, P.G. *Acta Pharm. Sin*. **1993**, *28*, 777−781.
(7) Kadota, S.; Li, J. X.; Tanaka, K.; Namba, T. *Tetrahedron* **1995**, *51*, 1143−1166.
(8) Hostanska, K.; Nisslein, T.; Freudenstein, J.; Reichling, J.; Saller, R. *Biol. Pharm. Bull.* **2004**, *27*, 1970−1975.
(9) Tian, Z.; Pan, R. L.; Si, J. Y.; Xiao, P. G. *Fitoterapia* **2006**, *77*, 39−42.

NP060113H