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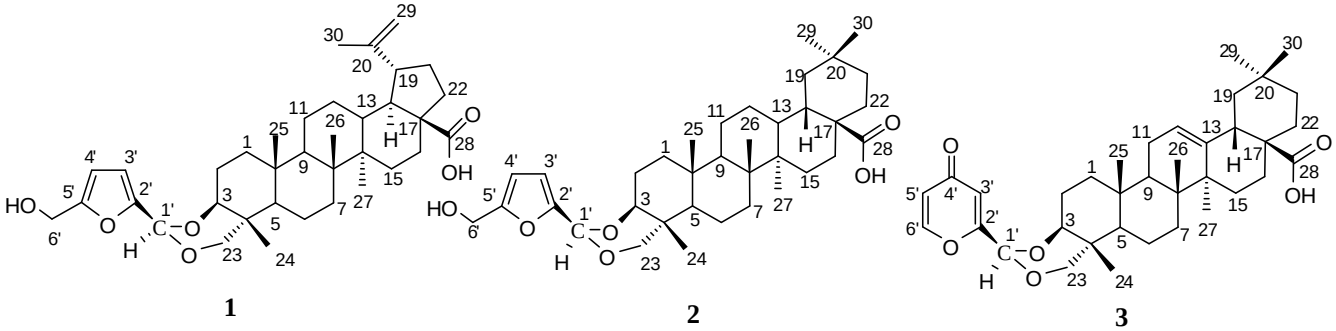
THREE NEW TRITERPENOIDS FROM *PULSATILLA CHINENSIS* (BUNGE) REGEL AND THEIR CYTOTOXIC ACTIVITIES

Zhan Shu, Zhong Chen, Xiu-juan Ding, Bing-qian Lu, Chen-jiao Ji, Qiong-ming Xu,* Xiao-ran Li, and Shi-lin Yang

School of Pharmacy, Soochow University, Suzhou, 215123, P. R. China. Phone: +86-512-69561421; Fax: +86-512-65882089; E-mail: xuqiongming@suda.edu.cn

Abstract - Three new triterpenoids, pulsatilla triterpenic acids A - C (**1-3**) were isolated from the roots of *Pulsatilla chinensis* (Bunge) Regel, and their structures were established on the basis of their spectral data. The three new compounds showed some cytotoxic activities by MTT assay.

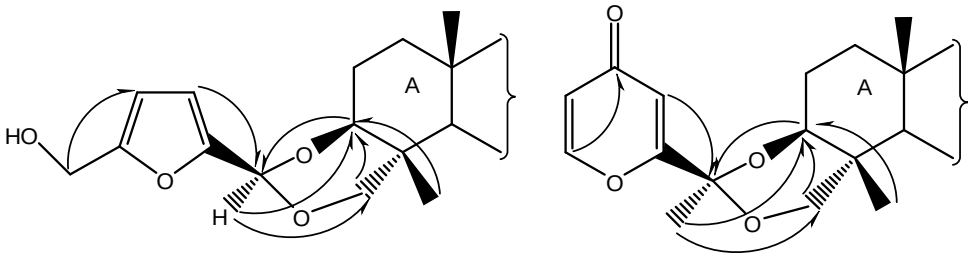
The roots of *Pulsatilla chinensis* (Bunge) Regel (Ranunculaceae) have been used in Traditional Chinese Mdicine for treatment of intestinal amebiasis, malaria, vaginal trichomoniasis, and bacterial infections.[1] Some studies suggested that a triterpene named pulsatillic acid from *P. Chinensis* showed cytotoxic activities against P-388 murine leukemia, Lewis lung carcinoma, and human large-cell lung carcinoma cells.[2] During the course of our investigation for cytotoxic agents from the chloroform-soluble fraction of the EtOH extract of roots of *Pulsatilla chinensis* (Bunge) Regel, three new triterpenoids, pulsatilla triterpenic acids A - C (**1-3**), together with six known triterpenoids, betulinic acid (**4**),[3] oleanolic acid (**5**),[4] ursolic acid (**6**),[5] 23-hydroxybetulinic acid (**7**),[2] hederagenin (**8**),[6] and hederagonic acid (**9**),[7] were obtained. In this paper we describe the isolation and structural elucidation of the three new compounds, as well as the evaluation of their cytotoxic activities against human HeLa, SMMC-7721, and HL-60 tumor cell lines.



1 **2** **3**

Figure 1

Pulsatilla triterpenic acid A (**1**) was isolated as white powder. The molecular formula was determined to be $C_{36}H_{52}O_6$ by high resolution (HR) - electrospray ionization (ESI) - mass spectrometry. Its ^{13}C NMR spectrum accounted for thirty six carbons (Table 1) in its molecular formula and presented six signals at δ 156.7 (s), 152.0 (s), 151.5 (s), 110.1 (t), 108.6 (d), and 107.8 (d) in the olefinic carbon region, indicating the presence of three double bonds, and a signal at δ 97.3 (d) that was assigned to acetal carbon. The ^1H NMR spectrum (Table 1) presented five signals at δ 1.91 (3H, s), 1.29 (3H, s), 1.14 (3H, s), 1.05 (3H, s), and 0.85 (3H, s), which were assigned to five methyl groups. The olefinic protons at δ 5.03 (1H, brs) and 4.86 (1H, brs) located at C-29 suggested that compound **1** belong to the lupane-type pentacyclic triterpene, which was confirmed by the four methine proton signals at δ 0.89 (1H, dd, J = 10.5, 1.5 Hz), 1.47 (1H, dd, J = 12.5, 2.0 Hz), 2.78 (1H, dt, J = 9.0, 1.5 Hz) and 1.82 (1H, m) corresponding to H-5, H-9, H-13 and H-18, respectively. The acetal proton at δ 5.91 (1H, s) and a hydroxylmethylene proton at δ 4.91 (2H, s) suggested the existence of furan in substituent groups, which was supported by the correlation between C-2' at δ 152.0 (s) and H-4' at δ 6.50 (1H, d, J = 3.0 Hz), as well as the correlation between H-3' at δ 6.74 (1H, d, J = 3.0 Hz) and C-5' at δ 156.7 (s) in the heteronuclear multiple bond connectivity (HMBC) spectrum (Figure 1). The 5'-hydroxymethylfurylmethylene group was established at C-3 and C-23 on the basis of the down field shifts of C-3 at δ 86.1 and C-23 at δ 78.8, which was supported by the correlation between H-1' at δ 5.91 (1H, s) and C-3, as well as the correlation between H-1' and C-23 in the HMBC spectrum (Figure 2).



1, 2 **3**

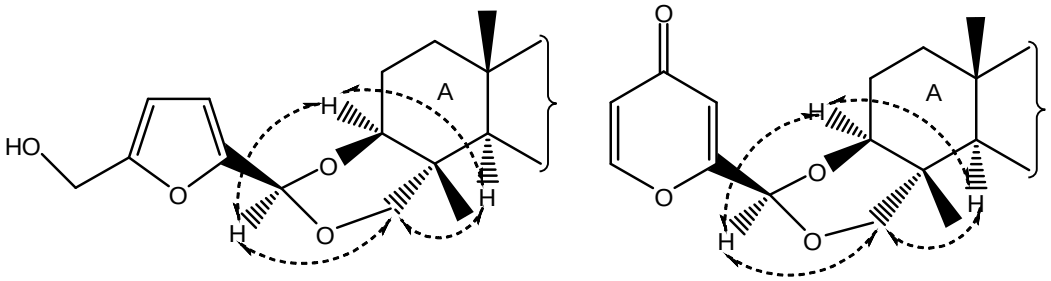
Figure 2. Key HMBC correlations of compounds **1**, **2** and **3**

Compound **1** was derived from the acetalation of compound **7** with 5-hydroxymethylfurfural,[8] which was comfirmed by comparison of ^1H and ^{13}C NMR spectra between them.

The relative stereochemistry of **1** was further established from its nuclear Overhauser effect spectroscopy (NOESY) spectrum (Figure 3). Interactions between H-1' (δ 5.91) and H-3 (δ 3.53) indicated that they were on the same side of the hexatomic ring, so the configuration of H-1' was α.

Pulsatilla triterpenic acid B (**2**) was isolated as white powder. The molecular formula was determined to

be $C_{36}H_{52}O_6$ by HR-ESI-mass spectrometry. The ^{1}H NMR spectrum of **2** showed singlet resonances of six tertiary methyl groups at δ 0.93 (Me-25), 1.05 (Me-26), 1.05 (Me-29), 1.10 (Me-30), 1.32 (Me-24), and 1.36 (Me-27) and three methine proton signals at δ 0.95 (1H, dd, J = 10.5, 1.5 Hz), 1.79 (1H, t, J = 9.0 Hz), and 3.61 (1H, m) corresponding to H-5, H-9 and H-18, respectively. The presence of olefinic proton at δ 5.57 (1H, brs) corresponding to H-12, compatible with the absence of two secondary methyl signals at C-19 and C-20 position, which suggested that compound **2** belong to the Δ^{12} oleanane-type pentacyclic triterpene. In the ^{13}C NMR (Table 1) and distortionless enhancement by polarization transfer (DEPT) spectra of **2**, most signals were quite similar to hederagenin (**8**). However, six carbon signals at δ 156.7, 151.9, 108.5, 107.7, 97.3, and 57.1 due to 5-hydroxymethylfurylmethylene group emerged in **2**, and did not exist in compound **8**. The 5-hydroxymethylfurylmethylene group was established at C-3 and C-23 on the basis of the down field shifts of C-3 at δ 86.0 and C-23 at δ 78.6, which was supported by the correlation between H-1' at δ 5.91 (1H, s) and C-3, as well as the correlation between H-1' and C-23 in the HMBC spectrum (Figure 2).



1, 2 **3**

Figure 3. Key NOESY correlations of compounds **1**, **2** and **3**

The relative stereochemistry of compound **2** was further established from its NOESY spectrum (Figure 3). Interactions between H-1' (δ 5.91) and H-3 (δ 3.55) indicated that they were on the same side of the hexatomic ring, so the configuration of H-1' was α.

Pulsatilla triterpenic acid C (**3**) was isolated as white powder. The molecular formula was determined to be $C_{36}H_{50}O_6$ by HR-ESI- mass spectrometry. The ^{1}H NMR spectrum of compound **3** showed singlet resonances of six tertiary methyl groups at δ 0.92 (Me-25), 1.04 (Me-29), 1.05 (Me-26), 1.10 (Me-30), 1.30 (Me-24), and 1.35 (Me-27) and three methine proton signals at δ 0.95 (1H, dd, J = 10.5, 1.5 Hz), 1.79 (1H, t, J = 9.0 Hz), and 3.60 (1H, m) corresponding to H-5, H-9 and H-18, respectively. The presence of olefinic proton at δ 5.56 (1H, brs) corresponding to H-12, compatible with the absence of two secondary methyl signals at C-19 and C-20 position, which suggested that compound **3** belong to the Δ^{12}

oleanane-type pentacyclic triterpenes. In the ^{13}C NMR (Table 1) and DEPT spectra of **3**, most signals were quite similar to hederagenin (**8**). However, six carbon signals at δ 195.8, 150.0, 148.9, 136.0, 127.6, and 103.7 due to 4-oxo-4*H*-pyranylmethylene group[9] emerged in **3**, and did not exist in compound **8**. The 4-oxo-4*H*-pyranylmethylene group was established at C-3 and C-23 on the basis of the down field shifts of C-3 at δ 85.6 and C-23 at δ 78.5, which was supported by the correlation between H-1' at δ 5.22 (1H, s) and C-3, as well as the correlation between H-1' and C-23 in the HMBC spectrum (Figure 2).

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectral data of compounds **1**, **2, 3, 5** and 7 (C_5D_5N, δ ppm).

	1		**7**		**2**	**5**		**3**
	δ_C	δ_H	δ_C	δ_C	δ_H	δ_C	δ_C	δ_H
1	39.4	1.72 (m), 1.12 (m)	39.2	39.4	1.60 (m), 1.10 (m)	38.4	39.4	1.58 (m), 1.10 (m)
2	24.1	1.73 (m), 1.84 (m)	28.0	23.9	1.15 (m), 1.20 (m)	27.7	23.9	1.15 (m), 1.20 (m)
3	86.1	3.53 (dd, 10.5, 5.5*)	73.5	86.0	3.55 (dd, 10.5, 5.5)	79.0	85.6	3.54 (dd, 10.5, 5.5)
4	37.2		43.1	39.9		38.7	39.9	
5	51.8	0.89 (dd, 10.5, 1.5)	47.9	51.6	0.95 (dd, 10.5, 1.5)	55.2	51.4	0.95 (dd, 10.5, 1.5)
6	18.0	1.40 (m), 1.12 (m)	18.6	18.1	1.40 (m), 1.21 (m)	18.3	18.0	1.40 (m), 1.21 (m)
7	34.3	1.40 (m)	34.6	32.7	1.54 (m), 1.31 (m)	32.6	32.7	1.54 (m), 1.31 (m)
8	41.4		41.2	37.7		39.3	37.5	
9	50.9	1.47 (dd, 12.5, 2.0)	51.1	48.1	1.79 (t, 9.0)	47.6	48.0	1.79 (t, 9.0)
10	37.7		37.4	37.2		37.0	37.1	
11	21.1	1.42 (m), 1.24 (m)	21.3	23.8	1.84 (m), 1.71 (m)	22.9	23.8	1.84 (m), 1.71 (m)
12	26.1	2.00 (m), 1.25 (m)	26.2	122.3	5.57 (brs)	122.6	122.3	5.56 (brs)
13	38.7	2.78 (dt, 9.0, 1.5)	38.7	144.9		143.6	144.9	
14	43.0		42.9	42.3		41.6	42.2	
15	30.4	1.93 (m), 1.33 (m)	30.4	28.4	2.23 (m), 1.15 (m)	27.2	28.4	2.22 (m), 1.17 (m)
16	33.0	2.70 (m), 1.60 (m)	33.0	23.7	2.22 (m), 2.20 (m)	23.4	23.7	2.22 (m), 2.20 (m)
17	56.7		56.7	46.7		46.5	46.7	
18	47.9	1.82 (m)	48.8	42.2	3.61 (m)	41.0	42.1	3.60 (m)
19	50.0	3.61 (m)	49.8	46.6	1.91 (m), 1.41 (m)	45.9	46.6	1.90 (m), 1.42 (m)
20	151.5		151.4	31.1		30.6	31.1	
21	31.4	2.33 (m), 1.60 (m)	31.3	34.3	1.55 (m), 1.30 (m)	33.8	34.3	1.56 (m), 1.32 (m)
22	37.7	2.33 (m), 1.63 (m)	37.7	33.3	2.06 (m), 1.90 (m)	32.4	33.3	2.05 (m), 1.90 (m)
23	78.8	4.05 (d, 10.5), 3.53 (d, 10.5)	67.9	78.6	4.08 (d, 10.5), 3.55 (d, 10.5)	28.1	78.5	4.06 (d, 10.5), 3.53 (d, 10.5)
24	13.6	1.29 (s)	13.0	13.8	1.32 (s)	15.5	13.8	1.30 (s)
25	17.5	0.85 (s)	16.9	16.6	0.93 (s)	16.3	16.5	0.92 (s)
26	16.4	1.05 (s)	16.6	17.4	1.05 (s)	17.1	17.4	1.05 (s)
27	15.0	1.14 (s)	15.0	26.3	1.36 (s)	25.9	26.3	1.35 (s)

28	179.0		180.0	179.0		182.4	180.3	
29	110.1	5.03(brs), 4.86 (brs)	110.0	33.4	1.05 (s)	33.0	33.4	1.04 (s)
30	19.6	1.91 (s)	19.5	23.9	1.10 (s)	23.5	23.9	1.10 (s)
1′	97.3	5.91(s)		97.3	5.91 (s)		103..7	5.22 (s)
2′	152.0			151.9			150.0	
3′	108.6	6.74 (d, 3.0)		108.5	6.74 (d, 3.0)		136.0	7.66 (d, 3.0)
4′	107.8	6.50 (d, 3.0)		107.7	6.50 (d, 3.0)		195.8	
5′	156.7			156.7			127.6	6.47 (dd, 10.5, 3.0)
6′	57.2	4.91(s)		57.1	4.92(s)		148.9	7.71 (d, 10.5)

* Data in parentheses are *J* values (in Hz).

The relative stereochemistry of compound **3** was further established from its NOESY spectrum (Figure 3). Interactions between H-1' (δ 5.22) and H-3 (δ 3.54) indicated that they were on the same side of the hexatomic ring, so the configuration of H-1' was α.

Cytotoxic activities of compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9** were determined by MTT assay and expressed as IC_{50} values. The IC_{50} values of compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9** and norcantharidin for HeLa uterocervical carcinoma cell were 27.35, 31.14, 26.72, 21.72, 29.16, 17.85, 19.53, 15.24, 11.88 and 0.70 μg/mL respectively, for SMMC-7721 hepato carcinoma cell those were 29.18, 29.25, 23.96, 20.47, 30.06, 21.32, 22.39, 12.95, 13.02 and 0.88 μg/mL, and for HL-60 leukocythemia carcinoma cell those were 26.32, 27.34, 24.66, 22.62, 31.22, 19.97, 21.57, 16.38, 14.35 and 0.58 μg/mL. Our results suggested all these compounds showed moderate to weak cytotoxic activities, and triterpenoids might be, at least in part, responsible for the proposed therapeutic effect of roots of *P. chinensis.*

EXPERIMENTAL

General

Melting points were determined by the XT5 micro-melting-point apparatus (XT5, Beijing families instrument light instrument plant, China) and are uncorrected. Optical rotations were obtained on a Perkin-Elmer model 241 polarimeter. IR spectra were taken on a Perkin-Elmer 983 G spectrometer. ^{1}H-, ^{13}C-NMR and 2D NMR spectra were recorded on a Varian Inova 500 spectrometer in C_5D_5N using tetramethylsilane (TMS) as internal standard. EI-MS spectra were determined on a Micromass Zabspec spectrometer. HR-ESI-MS spectra were determined on a Micromass Q-TOF2 spectrometer. Semi-preparative HPLC was carried out on a column of ODS (250 mm × 9.4 mm i.d., Agilent Zorbax SB-C_{18}, Palo Alto, USA) with a Waters 2996 detector, the flow rate was 2 mL/min and the wavelength for detection was 254 nm. Medium pressure liquid chromatography (MPLC) was carried out on a column

of silica gel H (460 mm × 26 mm i.d., Büchi Borosilikat 4.6, Flawil, Swiss). Silica gel (200-300 mesh) for column chromatography was obtained from Qingdao Marine Chemical Factory, Qingdao, China. Precoated plates of silica gel used for TLC were obtained from Qingdao Marine Chemical Factory, Qingdao, China. Compounds on the TLC were colored by 10% sulfuric acid alcohol solution. HeLa uterocervical carcinoma cell, SMMC-7721 hepato carcinoma cell and HL-60 leukocythemia carcinoma cell were purchased from Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Plant material

Dried roots of *Pulsatilla chinensis* (Bunge) Regel were purchased from a Chinese herbal store in Suzhou City of Jiangsu provinces in November 2008, and were authenticated by Professor Chun-yu Liu in Soochow University. A voucher specimen (No. 01-03-24-10) has been deposited in the herbarium of the School of College of Pharmacy, Soochow University.

Extraction and isolation

The dried plant material (20 kg) was extracted three times with 70% EtOH (200 L) under reflux. The solvent was subsequently dried under reduced pressure to give the residue (2.40 kg), which was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ - soluble fraction (125.5 g) further partitioned between petroleum ether and 90% MeOH (V/V). The 90% MeOH fraction (62.5 g) was chromatographed over silica gel column (60.0 cm × 5.0 cm i.d.), which was eluted with petroleum ether/EtOAc gradiens (0-100%), to afford 10 fractions. Subfraction 3 (2.16 g) was isolated by MPLC eluted with petroleum ether/EtOAc (90 : 10) to give betulinic acid (**4**, 120 mg, yield 0.00060%), oleanolic acid (**5,** 246 mg, yield 0.00123%), ursolic acid (**6**, 152 mg, yield 0.00076%). Fraction 5 (4.55 g) was further purified by MPLC on silica gel (500-600 mesh), with a petroleum ether/EtOAc gradient eluents (the ratios of petroleum ether/EtOAc were from 9:1 to 7:3), yielded pure 23-hydroxybetulinic acid (**7**, 62 mg, yield 0.00031%), hederagenin (**8**, 1.26 g, yield 0.00610%), hederagonic acid (**9**, 32 mg, yield 0.00016%). Fraction 9 (0.22 g) was subjected to semi-preparative RP-HPLC with 90% acetonitrile to give pulsatilla triterpenic acid A (**1**, 6.8 mg, yield 0.000034%), pulsatilla triterpenic acid B (**2**, 8.2 mg, yield 0.000041%) and pulsatilla triterpenic acid C (**3**, 4.6 mg, yield 0.000023%).

Pulsatilla triterpenic acid A (**1**): white powder, mp 366−367 °C; $[\alpha]_D^{24}$ -3.5 (*c* 0.06, MeOH); UV ($CHCl_3$) λ_{max} nm: 205, 261; IR (KBr) cm^{-1}: 3300, 3012, 2953, 2925, 2860, 2502, 1610, 1424, 1304, 1285, 1180, 893, 710 cm^{-1}; ^1H and ^{13}C NMR spectral data see Table 1; EI-MS *m/z*: 580 (5), 562 (2), 547 (9), 534 (5), 331 (16), 313 (45), 298 (21), 248 (100), 234 (16), 220 (28), 203 (35), 110 (69). HR-ESI-MS *m/z*: 581.3840 $[M+H]^+$ (Calcd for $C_{36}H_{53}O_6$ 581.3842).

Pulsatilla triterpenic acid B (**2**): white powder, mp 342−344 °C; $[\alpha]_D^{24}$ -12.2 (*c* 0.04, MeOH); UV ($CHCl_3$) λ_{max} nm: 203, 258; IR (KBr) cm^{-1}: 3324, 3010, 2956, 2923, 2861, 2500, 1605, 1426, 1304, 1292, 1220,

996, 715 cm^{-1}; ^1H and ^{13}C NMR spectral data see Table 1; EI-MS *m/z*: 580 (1), 552 (2), 493 (8), 331 (46), 313 (25), 298 (11), 248 (100), 133 (35), 110 (69). HR-ESI-MS *m/z*: 581.3836 $[M+H]^+$ (Calcd for $C_{36}H_{53}O_6$ 581.3842).

Pulsatilla triterpenic acid C (**3**): white powder, mp 322−325 °C; $[\alpha]_D^{24}$ -4.8 (*c* 0.02, MeOH). UV ($CHCl_3$) λ_{max} nm: 203, 262; IR (KBr) cm^{-1}: 3012, 2951, 2500, 1665, 1603, 1446, 1296, 1093, 925, 727 cm^{-1}; ^1H and ^{13}C NMR spectral data see Table 1; EI-MS *m/z*: 578 (1), 560 (1), 545 (7), 532 (2), 329 (16), 311 (45), 296 (21), 248 (100), 234 (30), 218 (25), 203 (31), 108 (55); HR-ESI-MS *m/z*: 579.3681 $[M+H]^+$ (Calcd for $C_{36}H_{51}O_6$ 579.3686).

Cytotoxic activity

To evaluate the cytotoxic activities of triterpenoids from the roots of *Pulsatilla chinensis* (Bunge) Regel. against human HeLa, SMMC-7721, and HL-60 tumor cell lines, the MTT colorimetric assay had been performed. The amount of formazan was determined by photometer at 570 nm. Cells were plated into 96-well flat-bottomed cultured plates at a concentration of 5×10^4 cells per well in complete RPMI 1640 culture medium. Twenty-four hours after plating, the medium containing foetal calf serum was removed and test solutions were given to cells in various final concentrations such as 2.5, 5, 10, 20, 50, 100 μg/mL. After incubation with drugs for 24 h, MTT solution was added to the wells and plates were incubated at 37 °C for 4 h. Results were expressed as percentage of the absorbance in control cells compared to that in the drug-treated cells.

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