Pergamon S0031-9422(96)00043-X

Phytochemistry, Vol. 42, No. 3, pp. 799–802, 1996

0031-9422/96 $15.00 + 0.00

TRITERPENOIDS FROM *PULSATILLA CHINENSIS*

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(*Received* 9 *November* 1995)

Key Word Index—*Pulsatilla chinensis*; Ranunculaceae; triterpenic acid; triterpenoid glycosides; 23-hydroxybetulinic acid; pulsatillic acid; pulsatilloside A and B.

Abstract—A new lupane type triterpenic acid, pulsatillic acid, and two new lupane type triterpenoid glycosides, pulsatilloside A and B, along with the known 23-hydroxybetulinic acid were isolated from the roots of *Pulsatilla chinensis*. Their structures were characterized as 3-oxo-23-hydroxy-lup-20(29)-en-28-oic acid, 3β, 23-dihydroxy-lup-20(29)-en-28-oic acid 3-*O*-α-L-arabinopyranoside and 3β, 23-dihydroxy-lup-20(29)-en-28-oic acid 28-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside on the basis of hydrolysis and spectral evidence including two-dimensional relay HOHAHA, one-dimensional multiple relay COSY and ROESY NMR techniques. Pulsatillic acid exhibited cytotoxic activities against P-388, Lewis lung carcinoma and human large-cell lung carcinoma.

INTRODUCTION

Pulsatilla chinensis (Bunge) Regel is one of the most important crude drugs in traditional chinese medicine and has been used for the treatment of amoebic dysentery and malaria [1]. Previously, we reported the isolation and structure elucidation of a new lupane-type triterpenoid glycoside; its structure was shown to be 3β, 23-dihydroxy-lup-20(29)-en-28-oic acid 3-*O*-α-L-rhamnoopyranosyl-(1 → 2)-L-arabinopyranoside [2]. In the present communication the isolation and structure eludication of a new lupane, pulsatillic acid (**2**), and two new lupane-type triterpenoid glycosides pulsatilloside A(**3**) and B(**4**) as well as a known 23-hydrosybetulinic acid (**1**) are reported from the roots of *Pulsatilla chinensis*.

RESULTS AND DISCUSSION

The chloroform-soluble part of the methanol extract of the dried roots of *P. chinensis* was fractionated by silica gel and sephadex LH-20 to yield 23-hydroxybetulinic acid (**1**) [3] and pulsatillic acid (**2**). Column chromatography of the *n*-butanol soluble fraction resulted in the isolation of two new lupane-type triterpenoid glycosides, pulsatilloside A (**3**) and B (**4**).

Pulsatillic acid (**2**), mp 214–217°C, was positive for the Liebermann–Burchard reaction and showed distinctive absorptions in it IR spectrum owing to carboxyl (1710, 1695 cm^{-1}) and exo-methylene (1640, 880 cm^{-1}) groups. The molecular formula $C_{30}H_{46}O_4$ was determined by high-resolution mass spectroscopy (*m/z* 470. 3437 $[M]^+$). The ^1H NMR spectrum of compound **2** showed the presence of five tertiary methyl groups, two hydroxymethyl protons (δ 3.64 and 3.41, *d*, J = 11.0 Hz, each 1H), two exo-methylene protons (4.62 and 4.47, *br s*, each 1H). Similarly, the ^{13}C NMR spectrum revealed signals for the corresponding one hydroxymethyl carbon (δ 68.0), two olefinic carbons (δ 151.1 and 109.6), one carboxylic carbon (δ 178.6) and one ketonic carbon (δ 217.2). In comparing the ^1H and ^{13}C NMR spectra of **2** with those of **1**, the signal due to C3-αH was not observed and one additional carbonyl signal (δ 217.2) was seen (Table 1). Furthermore, compound **1** could be converted to compound **2** by Oppenauer oxidation. Thus, the structure of pulsatillic acid was confirmed as 3-oxo-23-hydroxy-20(29)-en-28-oic acid (**2**). Pulsatillic acid (**2**) exhibited cytotoxic activities against P-388 (IC_{50} 4.8 μg ml^{-1}), Lewis lung carcinoma (IC_{50} 5.9 μg ml^{-1}) and human large-cell lung carcinoma (IC_{50} 1.9 μg ml^{-1}).

Pulsatilloside A (**3**) exhibited the $[M]^+$ at *m/z* 604 in the field desorption (FD)-mass spectrum. The IR spectrum of **3** showed distinctive absorptions due to hydroxyl (3400 cm^{-1}), carboxyl (1700 cm^{-1}) and exo-methylene (1640, 880 cm^{-1}) groups. The ^1H NMR spectrum of **3** exhibited signals due to five tertiary methyl groups (δ 0.87, 0.90, 1.01, 1.06 and 1.77), two olefinic protons (δ 4.75 and 4.93), one hydroxyl methine proton (δ 3.55) and one anomeric proton (δ 4.97, *d*, J = 7.12 Hz). Similarly, the ^{13}C NMR spectrum of **3** revealed signals for the corresponding

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Table 1. ^{13}C NMR spectral data of compounds **1**, **2**, **3** and **4** (δ, pyridine-d_5)

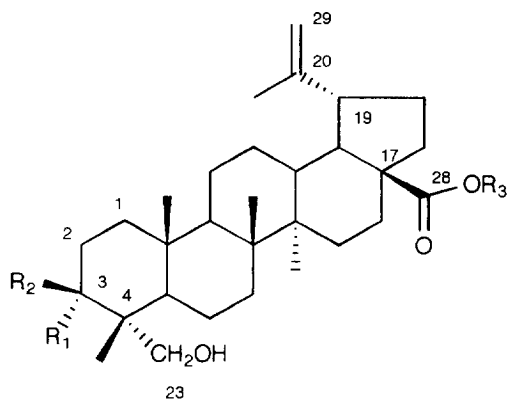
C	1	2	3	4
1	39.2	38.4	39.3	39.1
2	27.9	36.2	26.4	27.8
3	73.5	217.2	82.3	73.2
4	42.9	47.0	42.3	43.0
5	48.8	49.6	48.4	48.6
6	18.6	19.9	18.4	18.5
7	34.5	33.5	34.7	34.3
8	41.1	40.8	41.4	41.1
9	51.0	52.4	51.2	50.9
10	37.4	36.6	37.4	36.8
11	21.3	21.7	21.6	21.1
12	26.1	26.0	26.4	26.0
13	38.6	38.6	38.9	38.3
14	42.9	42.7	42.7	42.7
15	31.2	31.1	31.5	30.8
16	32.9	32.6	33.1	32.2
17	56.6	56.4	56.8	56.9
18	47.7	47.5	48.0	47.4
19	49.7	49.6	50.1	49.7
20	151.2	151.1	151.5	150.9
21	30.3	30.0	30.5	30.1
22	37.6	37.3	37.8	37.3
23	68.0	68.0	66.7	67.6
24	12.9	17.2	13.5	12.9
25	16.8	16.0	17.1	16.8
26	16.5	15.9	16.7	16.4
27	14.9	14.6	15.1	14.8
28	178.9	178.6	180.0	175.0
29	109.9	109.6	110.0	110.0
30	19.5	19.3	19.7	19.4
C-3 Sugar				
ara 1			106.4	
2			73.2	
3			74.7	
4			69.6	
5			64.8	
C-3 Sugar				
glc 1′				95.2
2′				74.0
3′				78.4
4′				70.9
5′				78.0
6′				69.4
1″				105.3
2″				75.1
3″				78.6
4″				71.5
5″				78.3
6″				62.6

ara: α-L-arabinopyranosyl.
glc: β-D-glucopyranosyl.

anomeric carbon (δ 106.4), two olefinic carbons (δ 110.0 and 151.5) and one carboxylic carbon (δ 180.0). The detailed ^{13}C NMR spectral data for **3** are given in Table 1.

Acid hydrolysis of **3** with 10% HCl yielded 23-hydroxybetulinic acid **1** and L-arabinose. The hydroxyl groups at C-3 and C-23 and the carboxyl groups at C-17 in 23-hydroxybetulinic acid, are all available for glycosidic linkage with the sugar chains. In the ^{13}C NMR spectrum of **3**, glycoslylation shifts were observed only at C-2 (−1.5 ppm), C-4 (−0.6 ppm) and C-3 (+8.87 ppm), by comparison with the spectrum of authentic 23-hydroxybetulinic acid (**1**) [3]. This led to the conclusion that the arabinose is united to the hydroxyl at C-3 of 23-hydroxybetulinic acid (**1**). Pulsatilloside A is thus a new kupane-type triterpenoid glycoside and has been elucidated as 3β, 23-dihydroxy-lup-20(29)-en-28-oic acid 3-*O*-α-L-arabinopyranoside (**3**).

The field desorption mass spectrum of pulsatilloside B (**4**) showed the molecular ion $[M + Na]^+$ at m/z 819. On acid hydrolysis, compound **4** yielded 23-hydroxybetulnic acid (**1**) and D-glucose. The IR spectrum of pulsatilloside B (**4**) displayed bands for a hydroxyl (3400, 1070 cm^{-1}), an ester (1730 cm^{-1}) and exomethylene (1640, 880 cm^{-1}) groups. The ^1H NMR spectrum of **4** exhibited five tertiary methyl singlets, two olefinic protons signals and two anomeric protons signals (δ 6.32, *d*, $J = 8.24$ Hz and δ 4.98, *d*, $J = 7.69$ Hz), which indicated that the sugar chain consisted of two glucose units and the configurations at the anomeric centres of both glucose units was β. This conclusion was supported by the ^{13}C NMR chemical shifts of the anomeric carbons (Table 1).



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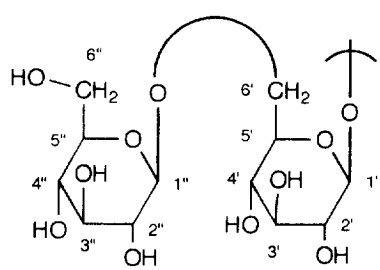
1 $R_1 = R_3 = H$, $R_2 = OH$
2 $R_1 + R_2 = O$, $R_3 = H$
3 $R_1 = R_3 = H$, R_2 = O-α-L-arabinopyranosyl
4 $R_1 = H$, $R_2 = OH$, R_3 =



Basic hydrolysis of **4** yielded 23-hydroxybetulinic acid. This indicated that the sugar carbohydrate as an ester unit was attached to the C-28 position of the aglycone, compound **4** is a monodesmosiic saponin. Further evidence for this conclusion was the observations that one of the anomeric carbon signals (δ 95.21) and the C-28 carboxylic carbon signal (δ 174.97) resonated at relatively high field in the ^{13}C NMR spectrum of compound **4**.

It is difficult to give a complete assignment of the sugar residue signals through the one-dimensional ^1H NMR spectrum alone. Overlapping signals from two glucose units in the ^1H NMR spectrum of compound **4** could be separated and identified by using one-dimensional multiple relay COSY and two-dimensional relay HOHAHA techniques. Upon selective excitation of the terminal glucose anomeric proton signal (δ 4.99, H-1″), the signal at δ 3.98 (H-2″) was correlated to that in the one-dimensional ^1H–^1H COSY spectrum of compound **4**, and one relay transfer signal (δ 4.18, H-1″) of magnetization in addition to the H-2″ signal was exhibited by the one-dimensional relay COSY spectrum. Furthermore, one more higher relay transfer signal (δ 4.16, H-4″) of magnetization was shown by the one-dimensional two-relay COSY spectrum. Therefore, all of the proton signals of the terminal glucose unit have been identified by using the one-dimensional four-relay COSY spectrum of **4**. Similarly, upon selective excitation of the inner glucose anomeric proton (δ 6.34, H-1′), the inner glucose protons signals could be also identified by the same method (Table 2).

In the one-dimensional ROESY spectrum with selective excitation of the terminal glucose anomeric proton (H-1″) of **4**, gave not only the H-3″ (δ 4.18) and H-5″ (δ 3.86) signals but also the H-6′a (δ 4.70) and H-6′b (δ 4.31) could be shown. This revealed that the C-6′ hydroxyl of the inner glucose must be linked to the terminal glucose. Consequently, pulsatilloside B was formulated as 3β, 23-dihydroxy-lup-20(29)-en-28-oic acid 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside(**4**). This is very interesting as there are no lupane triterpenoids in the genus *Pulsatilla* [4–5] except for *P. chinensis*.

EXPERIMENTAL

General. Mps uncorr. IR spectra were recorded as KBr discs. The ^1H NMR spectra were run at 400 MHz or 90 MHz, and the ^{13}C NMR spectra at 100 MHz or 22.5 MHz with TMS as int. standard. The EI- and FD-MS were measured on NIC FTMS-2000 and MAT-711 instruments. CC was carried out using silica gel GF_{254} vanillin–H_2SO_4 (saponins, sapogenin) and Sephadex LH-20. TLC was carried out using silica GF_{254}. Vanillin–H_2SO_4 (Saponin, Sapogenin) and thymol–H_2SO_4 (Sugars) were used as staining reagents.

Plant material. The roots of *P. chinensis* (Bunge) Regel were collected at Nanjing, China. A voucher specimen has been deposited in the Herbarium of China Pharmaceutical University.

Extraction and isolation. The defatted powdered plant (1.0 kg) was extracted with MeOH. The combined extract was evapd under red. pres. at 60°. The resulting residue (85 g) was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was evapd to dryness. The dark brown residue (5.0 g) was subjected to CC over silica gel using $CHCl_3$–CH_3OH (49:1, 19:1) as eluant to yield **1** (220 mg) and **2** (530 mg). The H_2O-soluble part was extracted with *n*-BuOH and on evapn provided a gummy mix. (35 g). The mixt. was subjected to CC over silica gel using a $CHCl_3$–CH_3OH solvent system of gradually increasing polarity (9:1, 4:1) and two frs (A, B) were collected. Frs A (410 mg) and B (280 mg) were purified by Sephadex LH-20 to yield **3** (160 mg) and **4** (250 mg), respectively.

23-hydroxybetulinic acid, **1**. Needles, mp 300–302° (lit. mp 300–302° [3]), IR ν_{max}^{KBr} cm^{-1}: 3420, 1690, 1640, 880. ^1H NMR (pyridine-d_5): δ 0.86 (6H, *s*, $2 \times CH_3$), 0.94 (3H, *s*, CH_3), 0.98 (3H, *s*, CH_3), 1.70

Table 2. ^1H NMR chemical shifts and coupling constants for sugar moieties of **4** (pyridine-d_5, TMS)

	Proton	δ (ppm)	Coupling constants (J, Hz)
glc	1′	6.34	$J(1',2') = 8.2$
(inner)	2′	4.08	$J(2',1') = 8.2$, $J(2',3') = 9.2$
	3′	4.21	$J(3',2') = 9.2$, $J(3',4') = 8.2$
	4′	4.31	$J(4',3') = 8.2$, $J(4',5') = 8.2$
	5′	4.09	$J(5',4') = 8.2$, $J(5',6'b) = 4.1$
	6′a	4.70	$J(6'a,6'b) = 11.5$
	6′b	4.31	$J(6'b,6'a) = 11.5$, $J(6'b,5') = 4.1$
glc	1″	4.99	$J(1'',2'') = 7.9$
(terminal)	2″	3.98	$J(2'',1'') = 7.9$, $J(2'',3'') = 9.0$
	3″	4.18*	
	4″	4.16*	
	5″	3.86*	
	6″a	4.46	$J(6''a,6''b) = 13.2$
	6″b	4.32	$J(6''b,6''a) = 13.2$, $J(6''b,5'') = 5.1$

* Obscured by other signals; therefore, coupling constants could not be determined accurately.

(3H, *s*, CH_3), 3.02 (1H, *m*, C_{19}-H), 3.46 (1H, like *t*, CH_3-αH), 3.44 (1H, $J = 10.0$ Hz, 23-H), 3.74 (1H, $J = 10.0$ Hz, 23-H), 4.63 and 4.76 (1H each, *brs*, 29-H). ^{13}C NMR data is given in Table 1. EI-MS *m/z*: 472 $[M]^+$, 454, 426, 248, 236, 203, 189, 187.

Oppenauer oxidation of **1**. A mixture of compound **1** (120 mg), aluminum *t*-butoxide (0.5 g), Me_2CO (5.0 ml) and benzene (10.0 ml) were stirred with a magnetic stirring bar for 1 hr at room temp. The reaction mixt. was diluted with H_2O (30.0 ml) and Et_2O (30.0 ml). The Et_2O layer was sepd, dried, and evapd to leave a powder. Crystallization from EtOAc provided colourless needles (50 mg) which were identified as pulsatillic acid (**2**) from its IR spectrum and mp.

Pulsatillic acid (**2**). Needles, mp 214–17°, IR ν_{max}^{KBr} cm^{-1}: 3450, 2950, 1710, 1690, 1640, 1460, 880. ^{1}H NMR (pyridine-d_5): δ 0.99 (6H, *s*, $2 \times CH_3$), 1.01 (3H, *s*, CH_3), 1.04 (3H, *s*, CH_3), 1.69 (3H, *s*, CH_3), 3.04 (1H, *m*, 19-H), 3.41 (1H, $J = 11.0$ Hz, 23-H), 3.64 (1H, $J = 11.0$ Hz, 23-H), 4.62 and 4.74 (1H each, *brs*, 29-H). ^{13}C NMR: Table 1. HR-MS *m/z*: 470.3437 $[M]^+$ (theor. 470.3394).

Pulsatilloide A (**3**). Amorphous powder, mp 160–165°, IR ν_{max}^{KBr} cm^{-1}: 3400, 2940, 1695, 1640, 1455, 1040, 1080, 880. ^{1}H NMR (pyridine-d_5) δ: 0.87 (3H, *s*, CH_3), 0.90 (3H, *s*, CH_3), 1.01 (3H, *s*, CH_3), 1.06 (3H, *s*, CH_3), 1.77 (3H, *s*, CH_3) 3.30 (1H, *m*, 19-H), 3.55 (1H like *t*, 3α-H), 4.75 and 4.93 (1H each, *brs*, 29-H) 4.97 (1H, *d*, $J = 7.12$ Hz, arabinose anomeric H). ^{13}C NMR: Table 1. FD–MS *m/z*: 627 $[M + Na]^+$.

Acid hydrolysis of **3**. Compound **3** (100 mg) was heated with 10% HCl (40 ml) under reflux for 6 hr. The reaction mixt. was diluted with H_2O and extracted with EtOAc. The EtOAc soln was washed with H_2O, dried and concd to give a residue, which was chromatographed on silica gel using hexane-EtOAc (1:1) as eluant to yield **1** (25 mg), mp 300–302°. Compound **1** was identical to authentic 23-hydroxybetulinic acid (mmp, IR and NMR), which was obtained from the $CHCl_3$ part of the MeOH extract of this material. The aq. layer of the hydrolysate was neutralized with Ag_2CO_3. The neutral H_2O layer revealed the presence of L-arabinose by PC and TLC with its authentic sample.

Pulsatilloside B (**4**). Amorphous power, mp 200–202°, IR ν_{max}^{KBr} cm^{-1}: 3400, 1730, 1640, 1100, 1070, 880. ^{1}H NMR (pyridine-d_5) δ 0.87 (3H, *s*, CH_3), 0.93 (3H, *s*, CH_3), 0.99 (3H, *s*, CH_3), 1.13 (3H, *s*, CH_3), 1.68 (3H, *s*, CH_3) 3.37 (1H, *m*, 19-H), 3.66 (1H, *d*, $J = 10.44$, 3α-H), 4.68 and 4.82 (1H each, *brs*, 29-H), 4.99 (1H, *d*, $J = 7.89$ Hz, terminal glucose 1″-H), 6.34 (1H, *d*, J = 8.24 Hz, inner glucose 1′-H). The signals of sugar residue protons are shown in Table 2. ^{13}C NMR data are given in Table 1. FD-MS *m/z*: 819 $[M + Na]^+$.

Acid hydrolysis of **4**. Compound **4** (100 mg) in MeOH was hydrolysed with 2 N HCl (20 ml) at 80° for 6 hr. Water was added to the reaction mixt. and the aglycone was extracted with EtOAc, and crystallized from MeOH. The aglycone was found to be the same as obtained from pulsatilloside A (**3**) on the basis of mmp, TLC and IR. The aq. phase was neutalized with Ag_2CO_3. The neutral hydrolysate revealed the presence of D-glucose by PC and TLC with its authentic sample.

Alkaline hydrolysis of **4**. Compound **4** (50 mg) was refluxed with 5 M NH_4OH in 50% EtOH (20 ml) for 8 hr. On cooling, the reaction mixt. was neutralized with HCl and extracted with *n*-BuOH. The *n*-BuOH layer was evapd to dryness to give a residue, which was chromatographed on silica gel ($CHCl_3$-CH_3OH) to yield a compound which was identified as 23-hydroxybetulinic acid (**1**) (there are no prosapogenins) by TLC, IR and NMR. The extract from the H_2O layer was hydrolysed with 2 M HCl and neutalised with Ag_2CO_3. The neutral hydrolysate revealed the presence of D-glucose by PC and TLC with its authentic sample.

Acknowledgements—This study was supported by a grant from the national Science Foundation of China, to which the authors' thanks are due. We thank Professor Zhen-Chun MIAO and Dr Rui Feng (Academy of Military Medical Science, Beijing) for high-field one-dimensional multiple-relay COSY, two-dimensional relay HOHAHA and one-dimensional ROESY difference measurements. We are thankful to Professor Katsuya Tokura, Shionogi Research Laboratories, Shionogi Co. Ltd, Japan for bioassay.

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