TWO NEW C₁⁹-DITERPENOID ALKALOIDS FROM ACONITUM FRANCHETII VAR. VILLOSULUM

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Abstract – Two new C₁⁹-diterpenoid alkaloids, named villosudine A (1) and villosudine B (2), along with seven known diterpenoid alkaloids, were isolated from the root of Aconitum franchetii var. villosulum. Their structures were elucidated by extensive spectroscopic analyses including 1D, 2D NMR, and HR-ESI-MS. Compounds (1-7) were evaluated for their cytotoxicity against the MCF-7 and HepG2 human cancer cell lines.

Aconitum franchetii var. villosulum belongs to the family Ranunculaceae and has a wide distribution in Sichuan province of China. Our previous phytochemical studies on this herb have led to the isolation of eleven diterpenoid alkaloids.¹ Diterpenoid alkaloids are typical chemical components of the genus Aconitum, and display various bioactivities such as anti-inflammatory, anti-arrhythmia, antifungal, and cytotoxic properties, as well as antiviral, insecticidal and antifeedant activities.² To find more biologically active secondary metabolites, two new C₁⁹-diterpenoid alkaloid, named villosudine A (1) and villosudine B (2), along with seven known diterpenoid alkaloids, were isolated from this plant. Meanwhile, compounds (1-7) were tested for their cytotoxicity against the MCF-7 and HepG2 human cancer cell lines. Herein, isolation, structural elucidation, as well as cytotoxicity of these diterpenoid alkaloids are reported. The hydrochloric acid extract of A. franchetii was alkalinized with 28% NH₄OH soln. and then extracted with CH₂Cl₂ to give the total alkaloid. The alkaloid fraction was chromatographed over silica gel to afford two new C₁⁹-diterpenoid alkaloids, villosudine A (1) and villosudine B (2), together with seven known analogues (3-9). The structures of the isolated compounds were as shown in Figure 1, and the ¹H and ¹³C NMR data of 1 and 2 were listed in Table. The known compounds, 6-epichasmanine (3),¹ ezochasmaconitine (4),³ ezochasmanine (5),³ pseudoaconitine (6),⁵ ludaconitine (7),⁵ leucanthsine A
(8), and vilmorisine (9) were established by comparison of their spectroscopic data with those reported in the literature.

Figure 1. Structures of compounds 1-9

Compound 1 was isolated as a white amorphous powder and gave a positive reaction to Dragendorff’s reagent. Its molecular formula, C_{35}H_{49}NO_{9}, was deduced from the HR-ESI-MS m/z: 628.3488 [M+H]^+, calcd. for 628.3486 and \(^{13}\)C NMR spectroscopic data. The IR (KBr) spectrum showed absorption bands for hydroxy group (3502 cm\(^{-1}\)) and ester carbonyl group (1713 cm\(^{-1}\)). The NMR data (Table) showed signals characteristic for an \(\text{N-ethyl group}: \delta_H 1.10 (3H, t, J = 7.2 \text{ Hz}), 2.50 (2H, overlapped); \delta_C 48.7 \text{ t}, 13.4 \text{ q}\), five methoxyl groups \([\delta_H 3.12, 3.23, 3.28, 3.29, \text{ and } 3.52 (each 3H, s); \delta_C 48.8 \text{ q}; 56.0 \text{ q}, 58.7 \text{ q}, 58.9 \text{ q}, \text{ and } 59.2 \text{ q}\]. The quaternary carbon signals \((\delta_C 43.1 \text{ and } 50.8)\) characteristic for C-4 and C-11, as well as an O-bearing methylene \((\delta_C 77.4, \text{ t})\) for C-18 suggesting compound 1 might be a typical C\(_{19}\)-diterpenoid alkaloid. In addition, resonances of a trans-double bond \([\delta_H 6.42 (1H, d, J = 16.0 \text{ Hz}) \text{ and } 7.68 (1H, d, J = 16.0 \text{ Hz}); \delta_C 118.5 \text{ d and } 144.9 \text{ d}\), an aromatic ring \([\delta_H 7.36-7.53 (5H, m); \delta_C 128.2 \times 2, 129.0 \times 2, 130.3 \text{ and } 134.7\], and a carboxyl group \((\delta_C 166.9)\) in the NMR spectra, along with the key HMBC correlations (Figure 2), H-9' \((\delta_H 7.68)\) to C-2' \((\delta_C 128.2)\) and C-7' \((\delta_C 166.9)\), suggested the presence of a trans-cinnamyl moiety.

The trans-cinnamyl moiety was located at C-14 by the long-range correlation in the HMBC experiment from H-14 \([\delta_H 4.76 (1H, d, J = 5.2 \text{ Hz})]\) to C-7' \((\delta_C 166.9)\). The existence of eight oxygenated carbons deduced from its HR-ESI-MS and \(^{13}\)C-NMR spectrum suggested 1 possess two hydroxy groups. A hydroxy group could be placed at C-13 based on the multiplicity of H-14 and the \(\delta\) value of 16-OCH\(_3\) \((\delta_H 3.52, \text{ s})\). Another hydroxy group was positioned at C-3, due to the HMBC correlations from H-3 \((\delta_H 3.07, \text{ s})\).
3.78, m) to C-1 (δC 82.6), C-5 (δC 48.0), and C-18 (δC 77.4). The NMR data of compound 1 were very similar to those of villosutine, a known alkaloid isolated from this plant, except for lacking a signal for acetyl group. The chemical shift of C-8 at δC 85.8 in villosutine was shifted upfield to δC 78.7 in compound 1 suggesting that 8-OAc was substituted by a methoxyl group, which was confirmed by the difference of 28 mass units between those two compounds. The remaining methoxyl groups were assigned to at C-1, C-6, C-16, and C-18, due to the long-range correlations between 1-OCH₃ (δH 3.23)/C-1 (δC 82.6), 6-OCH₃ (δH 3.28)/C-6 (δC 83.1), 16-OCH₃ (δH 3.52)/C-16 (δC 83.7), and 18-OCH₃ (δH 3.29)/C-18 (δC 77.4) in HMBC spectrum (Figure 2). Accordingly, the substitution pattern and assigned planar structure of 1 was confirmed by complete ¹H-¹H COSY, HMQC, and HMBC spectroscopic analyses.

The relative configuration of compound 1 was deduced from the vicinal coupling constants (Table) and NOESY experiment (Figure 2). The coupling constant between H-5 and H-6 (J = 6.4 Hz) confirmed the β-position of H-6, which was further supported by the cross-peaks between H-6/H-9β in the NOESY spectrum. The β-methoxyl group at C-16 was demonstrated by the correlations between the H-16 and H-17α in the NOESY spectrum. Furthermore, the NOESY cross-peak between H-1/H-3, H-1/H-10β and H-10β/H-14 showed that H-1, H-3 and H-14 were β-oriented. Thus, the structure and absolute configuration of villosudine A (1) was determined as shown in Figure 1, and the full assignment of its spectroscopic data was achieved based on 1D and 2D NMR analyses.

Figure 2. Key ¹H-¹H COSY, HMBC (a) and NOESY (b) correlations of 1

Compound 2 exhibited a pseudo-molecular-ion peak at m/z 672.3384 [M+H]⁺ in the HR-ESI-MS, corresponding to the molecular formula C₃₆H₄₉NO₁₁. Compound 2 exhibited characteristic NMR (Table) spectral features of an aconitine-type C₁₉-diterpenoid alkaloid bearing an N-ethyl group [δH 1.08 (3H, t, J = 7.2 Hz); δC 49.0 t, 12.6 q], four methoxyl groups [δH 3.26 and 3.50 (each 3H, s); 3.28 (6H, s); δC 55.4 q; 58.8 q, 58.9 q, and 59.1 q], and an acetyl group [δH 1.78 (3H, s); δC 22.3 q, 169.8 s]. The acetyl signal at higher field (δH 1.78) and the chemical shift of C-8 at δC 85.9 suggested that the acetyl group was
presented on C-8.\textsuperscript{2} Comparison of the NMR data of 2 with those of the known compound atropurpurisine\textsuperscript{15} showed that the only difference between 2 and atropurpurisine was a substitute group at C-14. The \textsuperscript{1}H-NMR spectrum of 2 showed the presence of one CH=CH moiety at $\delta_H$ 6.58 (1H, d, $J = 16$ Hz) and 7.74 (1H, d, $J = 16$ Hz), together with those of five aromatic protons at $\delta_H$ 7.43-7.46 (3H, m) and 7.67-7.69 (2H, m), which were assigned to a trans-cinnamoyl group.\textsuperscript{2,10} The doublet signal at $\delta_H$ 4.77 ($J = 5.2$ Hz) could be assigned to H$_\beta$-14, suggesting the presence of an ester function at C-14.\textsuperscript{14} The long-rang correlations between H-14 ($\delta_H$ 4.77) and H-9' ($\delta_H$ 7.74, d, $J = 16.0$ Hz) with the carbonyl carbon signal at $\delta_C$ 166.9 (s) in HMBC confirmed the presence of the trans-cinnamoyl group at C-14 (Figure 3). In addition, compound 2 possessed a similar configuration to that of 1, according to the deduction from its NOESY experiment (Figure 3). Accordingly, the structure of villosidine B (2) was confirmed by extensive analysis of its NMR spectra.

Table. \textsuperscript{1}H (400 MHz) and \textsuperscript{13}C NMR (100 MHz) Data for Compounds 1 and 2 ($\delta$ in ppm, $J$ in Hz)

<table>
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<th>1 in CDCl$_3$</th>
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<td>33.3 t</td>
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<td>66.0 d</td>
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<td>3.78 m</td>
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<td>21 2.50$^a$</td>
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<tr>
<td>4</td>
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<td>-</td>
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$^a$ Overlapped signals.
To evaluate the biological activities of these compounds isolated from the roots of *A. franchetii* for future applications, alkaloids 1-7 were tested for their *in vitro* cytotoxicity against the MCF-7 and HepG2 human cancer cell lines. Unfortunately, all of the compounds were inactive (IC\(_{50} > 50 \mu M, n = 3\)).

**EXPERIMENTAL**

**General experimental procedure.** Optical rotations were measured in CHCl\(_3\) using a PerkinElmer polarimeter with a sodium lamp operating at 598 nm and 20 °C. The IR spectra were obtained using a Thermo Fisher Nicolet 6700 spectrometer. The HR-ESI-MS data were measured using a Q-TOF micro mass spectrometer (Waters). The NMR spectra were recorded on Bruker AV 400 spectrometer. The TLC plates were precoated with silica gel GF\(_{254}\) (Qingdao Haiyang Chemical Co., Ltd., China), and it was visualized under a UV lamp at 254 nm or by spraying with Dragendorff’s reagent or iodine.

**Plant materials.** *Aconitum franchetii* var. *villosulum* were collected in Baoxing, Sichuan province of China, in August 2014 and identified by Prof. Liang-ke Song of the Institute of Life Science and Engineering, Southwest Jiaotong University. A voucher specimen (No. SC20140805) was deposited in the School of Life Science and Engineering, Southwest Jiaotong University, Sichuan, China.

**Extraction and isolation.** Dried and powdered roots of *A. franchetii* var. *villosulum* (9.2 kg) were extracted with 0.1 mol/L hydrochloric acid seven times at room temperature, for two days each soaking. The combined solvent was adjusted to pH 9-10 with 28% NH\(_4\)OH soln., and then extracted with CH\(_2\)Cl\(_2\). The CH\(_2\)Cl\(_2\) extracts were concentrated to produce the crude alkaloid extract (100 g). Column chromatography of the crude alkaloid extract over silica gel, using a CH\(_2\)Cl\(_2\): MeOH (100:1, \(v/v\)) mixture with increasing polarity afforded fractions A–F based on TLC analysis. Fraction C was separated by silica gel CC (petroleum ether: Me\(_2\)CO 20:1, \(v/v\)) to obtain 2 (13 mg) and 9 (1 mg). CC (silica gel, petroleum ether: Me\(_2\)CO: Et\(_2\)NH 15:1, \(v/v\)) of fraction D afforded 7 (12 mg) and 8 (1 mg). Fraction E was chromatographed on silica gel column and eluted with petroleum ether: Me\(_2\)CO: Et\(_2\)N (10:1:0.1-0:1:0.1, \(v/v/v\)) to afford 1 (9 mg), 5 (5 mg) and 3 (6 mg). Fraction F was subjected to CC on
silica gel and eluted with petroleum ether: CH₂Cl₂ (1:1-0:1, v/v) to give 6 (6 mg) and 4 (8 mg).

**Villosudine A (1)**
White amorphous powder; [α]_D^20 +10.38 (c 0.55, CHCl₃); IR (KBr) v_max 3502, 3060, 2963, 2931, 2889, 2821, 1713, 1638, 1578, 1496, 1450, 1385, 1335, 1309, 1281, 1176, 1089, 1043, 983, 921, 766, 709, 684, 491; ¹H NMR (400 MHz, CDCl₃) data and ¹³C NMR (100 MHz, CDCl₃) data, see Table; HR-ESI-MS (m/z): 628.3488 [M + H]^+, calcd. for C₃₅H₅₀N₂O₉, 628.3486.

**Villosudine B (2)**
White amorphous powder; [α]_D^20 +11.59 (c 0.55, CHCl₃); IR (KBr) v_max 3503, 3060, 2971, 2927, 2890, 2820, 1716, 1637, 1577, 1495, 1450, 1369, 1277, 1228, 1175, 1128, 1098, 985, 964, 921, 880, 841, 769, 709, 685, 620, 498; ¹H NMR (400 MHz, (CD₃)₂CO) data and ¹³C NMR (100 MHz, (CD₃)₂CO) data, see Table; HR-ESI-MS (m/z): 672.3384 [M + H]^+, calcd. for C₃₆H₅₀N₁₁O₁₁, 672.3384.

**Cell lines and cell culture and cytotoxicity assay**
The in-vitro growth inhibitory activities of compounds 1-7 were assayed by the MTT method.¹⁶ The HepG2 (human hepatic carcinoma) and MCF-7 (human breast cancer) cell lines were obtained from ATCC. Cells were plated in 96-well plates 24 h before treatment and continuously exposed to different concentrations of compounds for 72 h. DMSO (0.1%, v/v) was used as the negative controls and adriamycin (≥ 98%; Sigma Chemical Co., Ltd., Shanghai, China) was used as the positive control.

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**REFERENCES**


