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CYTOTOXIC DITERPENOID ALKALOIDS FROM *Aconitum austroyunnanense*

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A phytochemical investigation on the roots of Aconitum austroyunnanense *led to the isolation of two previously undescribed diterpenoid alkaloids, austroyunnanines D and E (**1** and **2**). Their structures were determined by spectral methods such as 1D and 2D (^1H–^1H COSY, HMQC, NOESY, and HMBC) NMR spectroscopy, in addition to high-resolution mass spectrometry. The isolated compounds were tested* in vitro *for cytotoxic activity against three human gastric carcinoma cell lines. As a result, compound **1** exhibited some cytotoxicities against all the tested tumor cell lines with IC_{50} values less than 20.0 μM.*

Keywords: *Aconitum austroyunnanense*, Ranunculaceae, diterpenoid alkaloids, cytotoxic activity.

The genus *Aconitum* (Ranunculaceae), which comprises 400 species, is naturally distributed throughout Northern Asia and North America [1, 2]. A widely used Chinese and Japanese herbal medicine, the tubers and roots of some species of the genus are mainly employed for the treatment of collapse, syncope, rheumatic fever, painful joints, gastroenteritis, diarrhea, oedema, bronchial asthma, various tumors, and some endocrinal disorders like irregular menstruation [3, 4]. The *Aconitum* alkaloids were structurally classified as C_{18}-diterpenoid, C_{19}-diterpenoid, and C_{20}-diterpenoid alkaloids, which possess diverse bioactivities, including cytotoxic, antiplasmodial, anti-inammatory, antinociceptive, antioxidant, and tyrosinase inhibition activities [5–8]. We have reported the isolation of diterpenoid alkaloids from *Aconitum austroyunnanense* E. Pritz. ex Diels [9]. As an extension of a search for bioactive alkaloids, the present study was undertaken to systematically examine the roots of *A. austroyunnanense* affording two new diterpenoid alkaloids, austroyunnanines D and E (**1** and **2**). The structures of these compounds were elucidated mainly by NMR spectroscopic and mass spectroscopic methods; furthermore, the two alkaloids were evaluated *in vitvo* for their cytotoxic potential.

Compound **1** was obtained as a white amorphous powder, exhibiting a quasimolecular ion peak at *m/z* 482.2032 $[M + H]^+$ in the high-resolution mass spectrometry, which corresponded to the molecular formula $C_{23}H_{31}NO_{10}$ with 9 degrees of unsaturation. The IR spectrum showed the typical absorption assigned to OH (3444 cm^{-1}), ester CO (1780 cm^{-1}), and acetyl CO (1741 cm^{-1}) functions. The ^1H NMR spectrum of **1** exhibited proton signals for the presence of four methoxyl groups. Characteristic ^{13}C NMR (Table 1) and HSQC spectra determined 23 skeleton carbons in **1**, including five methylenes (one oxygenated), eight methines (three oxygenated) and six quaternary carbons (two carbonyl groups) and as well as four methoxys (δ 53.5, 55.0, 58.3, and 59.3). Inspection of its characteristic NMR data revealed that **1** possessed a rearranged-type skeleton in C_{18}-diterpenoid alkaloids similar to kusnezosine A [10]. The four methoxyl groups were attributed to C-1, C-6, C-15, and C-18 based on the HMBC correlations from 1-OCH_3 (δ 3.22) to C-1 (δ 80.6), 6-OCH_3 (δ 3.35) to C-6 (δ 82.0.), from 15-OCH_3 (δ 3.85) to C-15 (δ 173.6), and from 18-OCH_3 (δ 3.30) to C-18 (δ 80.3), respectively. The HMBC cross-peaks of H-17 (δ 3.56) to C-5 (δ 49.4), C-6, and C-8 (δ 74.2) indicated that C-17 was connected with C-7.

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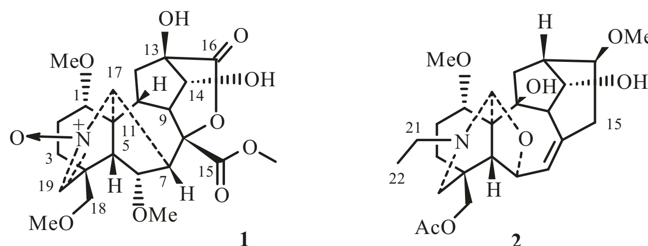
TABLE 1. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2** ($CDCl_3$, δ, ppm, J/Hz)

C atom	δ_H		C atom	δ_C	
	1	**2**		**1**	**2**
1α	3.37 (dd, J = 11.2, 6.2)	3.98 (dd, J = 11.0, 5.8)	1	80.6	78.7
2α	1.47 (m)	2.00 (m)	2	27.7	24.3
2β	2.58 (m)	2.55 (m)	3	30.7	31.8
3α	1.77 (m)	1.76 (m)	4	44.6	37.8
3β	1.84 (m)	1.53 (m)	5	49.4	43.2
5	2.38 (d, J = 6.2)	2.44 (d, J = 6.2)	6	82.0	75.3
6α	3.93 (dd, J = 6.6, 6.2)	4.40 (dd, J = 6.4, 6.2)	7	46.3	128
7α	1.98 (d, J = 6.6)	5.47 (d, J = 6.4)	8	74.2	136.4
9	2.97 (m)	2.83 (d, J = 6.6)	9	49.9	51.9
10	2.72 (m)	–	10	48.2	84.6
12α	2.91 (m)	2.42 (m)	11	51.1	55.8
12β	1.94 (m)	1.73 (m)	12	36.3	41.9
13β	–	2.64 (m)	13	81.0	37.7
14	8.10 (d, J = 6.6)	5.44 (dd, J = 6.6, 6.0)	14	86.7	76.9
15α	–	1.93 (m)	15	173.6	37.6
15β	–	1.98 (m)	16	177.6	84.7
16	–	3.69 (m)	17	71.2	93.4
17	3.56 (br.s)	4.23 (br.s)	18	80.3	68.7
18a	3.25 (d, J = 11.2)	3.48 (d, J = 10.8)	19	145.2	51.6
18b	3.66 (d, J = 11.2)	3.37 (d, J = 10.8)	21	-	49.1
19a	6.95 (s)	2.46 (d, J = 10.2)	22	-	13.1
19b	–	2.08 (d, J = 10.2)	MeO-1	55.0	56.9
21α	–	2.58 (m)	MeO-6	58.3	-
21β	–	2.38 (m)	MeO-15	53.5	-
22	–	0.95 (t, J = 7.0)	MeO-16	-	56.1
MeO-1	3.22 (s)	3.34 (s)	MeO-18	59.3	-
MeO-6	3.35 (s)	–	Ac-18	-	171.1
MeO-15	3.85 (s)	–		-	21.4
MeO-16	–	3.22 (s)			
MeO-18	3.30 (s)	–			
Ac-18	–	2.04 (s)			



A carbonyl groups were located at C-8 based on the HMBC cross-peaks from H-7 (δ 1.98, d, J = 6.6 Hz) and H-9 (δ 2.97, m) to C-8 (δ 74.2). A nitrone group was placed between C-17 and C-19, which was confirmed by the deshielding shifts of H-17, H-19 (δ 6.95), C-17 (δ 71.2), and C-19 (δ 145.2). The presence of a double bond between the *N*-atom and C-19 was verified by the HMBC correlations between the olefinic H-19 and C-4, C-5 and C-17. The long-range HMBC correlations between H-12 (δ 1.94 and 2.91, each, m) and H-14 (δ 8.10, d, J = 6.6 Hz) to C-16 (δ 177.6) suggested the ketone group of C-16. The stereochemistry of compound **1** was deduced from the NOESY experiment. The NOESY correlations between H-10 and H-1, H-5, H-7, H-9, H-14, and 13-OH elucidated that they were all *β*-oriented. Thus, the structure of compound **1** was structurally elucidated and named austroyunnanine D.

Compound **2** was isolated as a white amorphous powder with the molecular formula $C_{25}H_{37}NO_7$, as determined by positive-ion HR-ESI-MS, showing an $[M + H]^+$ ion at *m/z* 464.2643. The IR spectrum showed the typical absorption assigned to OH (3423 cm^{-1}) and acetyl CO (1738 cm^{-1}). In the ^{1}H NMR spectrum, the signals of an olefinic H atom at δ 5.47

(d, J = 6.4 Hz, H-7), two OMe groups at δ 3.22 and 3.34, and one *N*-ethyl [δ 2.38 and 2.58 (each, m, -NCH_2Me) and 0.95 (3H, t, J = 7.0 Hz, -NCH_2Me] were observed. The ^{13}C NMR and DEPT spectrum displayed 25 carbon signals, which were identified with the aid of the DEPT experiment as one acetyl group, three methyls (two oxygenated), seven methylenes (one olefinic and one oxygenated), nine methines (one olefinic and four oxygenated), and four quaternary carbons. The spectroscopic data revealed that compound **2** was an aconitine-type C_{19}-diterpenoid alkaloid possessing a structure similar to the known acotarine D [11]. The mere ^{13}C NMR difference of the downfield shift of signals to δ 49.2 to 84.6 for C-10 in **2**, relative to those of acotarine D, indicated that C-10 was linked with one hydroxyl group. The cross-peaks of H-6 and H-7 with C-8, of H-6 with C-7, and of H-7 with C-10 in the HMBC spectrum suggested that the C=C bond should be positioned between C-7 and C-8. Furthermore, the cross-peaks between H-6 and H-5/H-7, H-12 and H-13, H-16 and H-13/H-15 observed in the ^1H–^1H COSY spectrum supported the above conclusions. The NOESY correlations of H-1 with H-5, and H-9, of H-5 with H-18, and of H-13 with H-9 and MeO-16 indicated that they were all β-oriented. The relative configuration of compound **2** was established by the NOESY spectrum as being the same as acotarine D. Based on the above interpretation, the structure of compound **2** was substantiated and named austroyunnanine E.

The diterpenoid alkaloids obtained from genus *Aconitum* have been reported to show strong antioxidant activities [12, 13], which agrees well with our results. The isolated compounds were *in vitro* evaluated for their cytotoxic potential by using the revised MTT method. The results showed that compound **1** exhibited some cytotoxicity against three human gastric carcinoma cell lines (MGC-803, BGC-823, and SGC-7901) with IC_{50} values of 17.6, 14.3, 15.8 μM, respectively (IC_{50} values of doxorubicin: 2.3, 3.1, 3.7 μM), while no cytotoxicity was detected for compound **2** ($IC_{50} \geq 50$ μM).

EXPERIMENTAL

General Procedures. Optical rotations: Perkin-Elmer 341 polarimeter. UV Spectra: Hewlett-Packard-8452A diode-array spectrophotometer. IR Spectra: Nicolet Magna FT-IR 750 spectrophotometer in cm^{-1}. ^1H and ^{13}C NMR spectra: Bruker AM-600 spectrometer. HR-ESI-MS spectra: Micromass LC-MS/MS mass spectrometer. Column chromatography (CC): silica gel (200–300 mesh, 10–40 μm; Qingdao Marine Chemical Factory, Qingdao, China), Sephadex LH-20 (Amersham Pharmacia Biotech, Sweden). Thin-layer chromatography (TLC): silica gel GF_{254} (10–40 μm; Qingdao Marine Chemical Factory, Qingdao, China). All solvents were distilled before use. Preparative HPLC: Varian SD1 instrument with a 320 single-wave detector on C-18 columns (250 × 10 mm, 5 μm, Waters; 220 × 25 mm, 10 μm, Merck, respectively).

Plant Material. The roots of *Aconitum austroyunnanense* were collected at the Dali of Yunan Province of China in June of 2022. A specimen (AA20220601), identified by one of the authors (X. Mao), was deposited in the Herbarium of the College of Biological Resources and Food Engineering, Qujing Normal University, Qujing, Yunnan, China.

Extraction and Isolation. The dried roots of *A. austroyunnanense* (10.0 kg) were powdered and percolated with 0.05 M HCl (50 L) at room temperature. Wet resin (type: 001 × 7, dry weight 30 kg) was added to the percolate, followed by repeated washing on a suction filter with deionized H_2O. The air-dried resin was then alkalized with 10% aq. NH_4OH (3.0 L) and continuously extracted with Et_2O (5.0 L), and evaporated to yellowish amorphous powder. The yellowish amorphous powder was suspended in H_2O (l L) and then partitioned with $CHCl_3$ (8.0 L), and evaporated to render the total crude alkaloids as a yellowish amorphous powder. The crude alkaloids (98.0 g) were chromatographed over SiO_2 column (2 kg, 200–300 mesh) and eluted with petroleum ether (PE)–acetone–Et_2NH (from 10:1:1 to 5:1:1) to provide five fractions (F_1–F_5). Fraction F_3 (4.2 g) was chromatographed on a SiO_2 column eluting with $CHCl_3$–MeOH, 90:10 to afford three fractions F_{3a} (817 mg), F_{3b} (976 mg), and F_{3c} (948 mg). Fraction F_{3b} (842 mg) was chromatographed on preparative HPLC (MeOH–H_2O, from 60% to 70%, 240 nm, 220 × 25 mm, 10 μm, Merck), yielding **2** (66 mg, retention time: 14.6 min). Fraction F_4 (2.9 g) was chromatographed on a SiO_2 column eluting with $CHCl_3$–MeOH (85:15) to afford three fractions, F_{4a}–F_{4c}. Fraction F_{4c} (201 mg) was further separated by preparative HPLC (MeOH–H_2O, from 55% to 65%, 240 nm, 220 × 25 mm, 10 μm, Merck) to afford **1** (69 mg, retention time: 14.9 min).

Austroyunnanine D (1), white amorphous powder, $[\alpha]_D^{25}$–16.87° (*c* 0.10, $CHCl_3$). IR (KBr, ν_{max}, cm^{-1}): 3444, 3365, 2962, 2922, 2854, 1780, 1741, 1659, 1466, 1260, 1095, 803. UV (MeOH, λ_{max}, nm) (log ε): 195 (3.72). ^1H and ^{13}C NMR data, see Table 1. HR-ESI-MS *m/z* 482.2032 $[M + H]^+$ (calcd for $C_{23}H_{32}NO_{10}$, 482.2026).

Austroyunnanine E (2), white amorphous powder, $[\alpha]_D^{25}$–11.64° (*c* 0.10, $CHCl_3$). IR (KBr, ν_{max}, cm^{-1}): 3423, 2930, 2822, 1738, 1637, 1454, 1385, 1365, 1244, 1206, 1095, 1053. UV (MeOH, λ_{max}, nm) (log ε): 196 (3.92). ^1H and ^{13}C NMR data, see Table 1. HR-ESI-MS *m/z* 464.2643 $[M + H]^+$ (calcd for $C_{25}H_{38}NO_7$, 464.2648).

Cytotoxicity Assay *in vitro*. The isolated compounds (**1** and **2**) were subjected to cytotoxic evaluation against three human gastric carcinoma cell lines (MGC-803, BGC-823, and SGC-7901) by employing the revised MTT method [14]. Doxorubicin was used as the positive control. All tumor cell lines were cultured on RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in 25 cm^2 culture flasks at 37°C in a humidified atmosphere with 5% CO_2. For the cytotoxicity tests, cells in the exponential growth stage were harvested from culture by trypsin digestion and centrifuged at 180 × g for 3 min, then resuspended in fresh medium at a cell density of 5×10^4 cells per mL. The cell suspension was dispensed into a 96-well microplate at 100 μL per well, incubated in a humidified atmosphere with 5% CO_2 at 37°C for 24 h, and then treated with the compounds dissolved in DMSO at various concentrations (0, 1, 10, 100 μM). After 48 h of treatment, 50 μL of 1 mg/mL MTT solution was added to each well, and further incubated for 4 h. The cells in each well were then solubilized with DMSO (100 μL for each well) and the optical density (OD) was recorded at 570 nm. All cell lines were purchased from the Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences.

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REFERENCES

1. H. Niitsu, Y. Fujita, S. Fujita, R. Kumagai, M. Takamiya, Y. Aoki, and K. Dewa, *Forensic Sci. Int.*, **227**, 111 (2013).
2. J. Singhuber, M. Zhu, S. Prinz, and B. Kopp, *J. Ethnopharmacol*., **126**, 18 (2009).
3. A. Bello-Ramirez, J. Buendia-Orozco, and A. Nava-Ocampo, *Fund. Clin. Pharmacol.*, **17**, 575 (2003).
4. T. Yamamoto, K. Yuyama, K. Nakamura, T. Kato, and H. Yamamoto, *Eur. J. Pharmacol.*, **397**, 25 (2000).
5. J. Li, W. Ren, X. J. Huang, D. J. Zou, and X. Hu, *Brain Res. Bull.*, **97**, 81 (2013).
6. T. Kiss, P. Orvos, S. Bansaghi, P. Forgo, N. Jedlinszki, L. Talosi, J. Hohmann, and D. Csupor, *Fitoterapia*, **90**, 85 (2013).
7. M. Liang, S. C. Li, B. Shen, J. P. Cai, C. Li, Z. Y. Wang, X. G. Li, J. Gao, H. Y. Huang, X. Y. Zhang, and J. Y. Li, *Carbohyd. Polym.*, **88**, 973 (2012).
8. D. K. Zhao, H. L. Ai, S. H. Zi, L. M. Zhang, S. C. Yang, H. C. Guo, Y. Shen, Y. P. Chen, and J. J. Chen, *Fitoterapia*, **91**, 280 (2013).
9. J. Hu, Q. Wu, Q. Li, T. Lv, T. F. Peng, S. Yin, and H. Z. Jin, *J. Asian Nat. Prod. Res.,* **25**, 132 (2023).
10. Y. Z. Li, L. L. Qin, F. Gao, L. H. Shan, and X. L. Zhou, *Fitoterapia*, **144**, 104738 (2020).
11. Y. Si, X. Ding, T. A. Adelakuna, Y. Zhang, and X. J. Hao, *Fitoterapia*, **147**, 717 (2020).
12. Y. P. Fu, Y. F. Z, F. Y. Lei, and H. Wangensteen, *J. Ethnopharmacol.*, **291**, 115148 (2022).
13. H. L. Tao, X. F. g Liu, R. M. Tian, Y. Liu, Y. Zeng, X. L. Meng, and Y. Zhang, *J. Ethnopharmacol.,* **301**, 115726 (2023).
14. J. Hu, Y. Song, X. Mao, Z. Wang, and Q. J. Zhao, *J. Funct. Foods,* **20**, 1 (2016).