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Grandiflodines A and B, two novel diterpenoid alkaloids from *Delphinium grandiflorum*†

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Two novel diterpenoid alkaloids, grandiflodines A and B (**1** and **2**), were isolated from *Delphinium grandiflorum*. Compound **1** represents a rare hetisine-type C₂₀-diterpenoid alkaloid in which the bond between the atoms of N and C-17 is broken. Compound **2** features an unusual lycotonine-type C₁₉-diterpenoid alkaloid skeleton with the cleavage of N–C₁₉ and C₇–C₁₇ bonds, and the construction of the N–C₇ bond. Structural elucidations of the isolates were performed by spectroscopic analysis, X-ray diffraction and comparison with the literature. These compounds were tested for their antiviral and anti-inflammatory activities.

Introduction

The genus *Delphinium* belongs to the family Ranunculaceae and consists of about 300 species distributed throughout the northern hemisphere.^{1,2} Among the 300 species, more than 113 ones are endemic to China and about 18 ones are used as folk medicines.^{1,2} As an important medicinal plant, *Delphinium* plants are used to treat traumatic injury, analgesia and rheumatism, *etc.*¹ Recent investigations showed that the diterpenoid alkaloids are the main components of *Delphinium* plants, and the alkaloids possess complex structure skeletons and exhibit a wide spectrum of pharmacological activities.^{3–7} Thus, the diterpenoid alkaloids have become an increasing, attractive target for medicinal chemists.⁸

Delphinium grandiflorum is a perennial herb mainly distributed in the Northwest of China and some regions of Siberia and

People's Republic of Mongolia.⁹ As a folk medicine, the *D. grandiflorum* is applied for the treatment of toothache, and used as native pesticide as well.⁹ As part of our ongoing research on the bioactive natural products from *Delphinium* plants,¹⁰ an extensive phytochemical investigation on *D. grandiflorum* was undertaken, leading to the isolation of two novel diterpenoid alkaloids, grandiflodines A and B (**1** and **2**). Compound **1** is a rare hetisine-type C₂₀-diterpenoid alkaloid with the cleavage of the bond between the atoms of N and C-17. Compound **2** features an unusual lycotonine-type C₁₉-diterpenoid alkaloid skeleton with the cleavage of N–C₁₉ and C₇–C₁₇ bonds, and construction of the N–C₇ bond. Herein, we report the isolation, structure elucidation and biological activities of **1** and **2** (Fig. 1).

Results and discussion

Grandiflodine A (**1**) was isolated as colorless block crystal. The molecular formula of **1** was established as C₂₂H₂₈N₂O₃ by its HR-ESI-MS (m/z 369.2175 [M + H]⁺, calcd for C₂₂H₂₉N₂O₃: 369.2173). The UV spectrum of **1** displayed the absorption maxima at 208 nm, and its IR spectrum showed the characteristic absorptions for hydroxyl groups (3479, 3423 cm⁻¹), cyanogroup (2228 cm⁻¹) and carbonyl group (1673 cm⁻¹). The ¹H NMR and HSQC spectroscopic data of **1** provided the resonances for two methyls [δ_{H} 1.18, 2.26 (each 3H, s); δ_{C} 25.6, 33.4], an olefinic methylene [δ_{H} 4.51, 4.67 (each 1H, d, J = 1.8 Hz); δ_{C} 103.4] and an oxygenated methine [δ_{H} 3.36 (1H, t, J = 5.8 Hz); δ_{C} 72.9]. The ¹³C and DEPT NMR data exhibited 22 signals of two methyls, seven methylenes, six methines and seven quaternary carbons, including a cyanogroup (δ_{C} 117.6), a pair of double bond (δ_{C} 103.4, 150.3) and a carbonyl group (δ_{C} 216.9). Detailed comparison of the ¹D NMR data of **1** (Table 1) with those of anhydroignavinol¹¹ showed that they were similar except for the presence of additional carbonyl and cyanogroup, and the

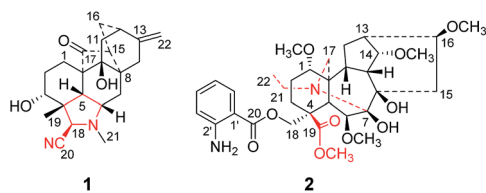


Fig. 1 Chemical structures of compounds **1** and **2**.

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Table 1 NMR spectroscopic data for grandiflodines A (**1**) and B (**2**) (δ in ppm)

Position	Grandiflodine A (1) ^a		Grandiflodine A (2) ^b	
	δ_C	δ_H (<i>J</i> in Hz)	δ_C	δ_H (<i>J</i> in Hz)
1	23.2	1.62 1.06 m	88.9	2.88 dd (10.8, 4.1)
2	26.6	2.60 m 1.62	21.7	1.98 m 1.75 m
3	72.9	3.36 t (5.8)	30.1	2.34 m 1.44 m
4	47.7	—	49.8	—
5	53.5	2.30	49.9	2.02 d (6.7)
6	58.6	3.07 m	90.5	3.71 m
7	31.2	2.30 1.75 dd (14.8, 4.5)	87.8	—
8	39.6	—	82.0	—
9	76.5	—	39.9	2.30 m
10	51.7	—	51.3	1.89 m
11	38.7	1.86 1.38 dd (14.1, 2.6)	42.7	—
12	35.9	2.16	29.6	1.83 m 1.35 m
13	150.3	—	45.3	2.43 m
14	32.7	1.62 1.56 dd (12.8, 4.3)	85.3	3.66 t (3.9)
15	49.2	2.30	33.2	2.37 m 1.57 dd (13.8, 8.1)
16	31.2	1.70 d (14.5) 2.16	84.2	3.12 m
17	216.9	—	42.3	2.99 d (11.0) 2.65 d (11.0)
18	55.3	3.81 s	70.5	4.66 d (10.9) 4.15 d (10.9)
19	25.6	1.18 s	174.9	—
20	117.6	—	167.7	—
21	33.4	2.26 s	43.7	3.15 m 2.75 m
22	103.4	4.51 4.67 d (1.8)	13.7	1.02 t (6.9)
3-OH		5.00 d (5.5)		
9-OH		4.86 s		
1-OCH ₃			56.8	3.24, s
6-OCH ₃			61.3	3.62, s
14-OCH ₃			57.9	3.38, s
16-OCH ₃			56.4	3.28, s
19-OCH ₃			52.1	3.72, s
1'			110.7	
2'			150.8	
3'			117.0	6.64, m
4'			134.4	7.24, m
5'			116.5	6.58, m
6'			131.0	7.71, dd (8.0, 1.4)

^a Measured at 500/125 MHz in DMSO-*d*₆. ^b Measured at 300/75 MHz in CDCl₃. Overlapped signals are reported without designating multiplicity.

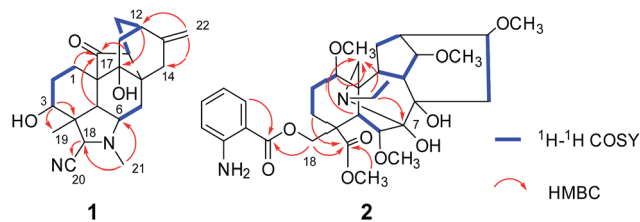


Fig. 2 Key ¹H–¹H COSY and HMBC correlations of **1** and **2**.

absence of two oxygenated methines in **1**. In the HMBC spectrum, the correlations (Fig. 2) between H-1/H-5/H-15 and the carbonyl group (δ_C 216.9) revealed that the carbonyl group was located at C-17. Moreover, the HMBC correlations between H-21 [δ_H 2.26, (3H, s)] and C-6/C-18 suggested that the methyl (δ_C 33.4, C-21) was connected to the nitrogen atom. The above information implied that the N–C₁₇ bond was broken to form a unique hetidines-type C₂₀-diterpenoid alkaloid skeleton as depicted. In addition, the cyanogroup (δ_C 117.6) was located at C-18 based upon the HMBC correlation between H-18 and C-20 (δ_C 117.6). And the HMBC correlations between H-3 (δ_H 3.36, 1H, t, *J* = 5.8 Hz) and C-4/C-18/C-19, between H-11/H-12/H-15/H-16 and C-9 (δ_C 76.5) indicated that the carbons at C-3 and C-9 were substituted by hydroxyls, respectively. In light of the evidences mentioned above, the planar structure of **1** was finally established.

The relative configuration of **1** could be elucidated by the NOESY experiment. The correlations (Fig. 3) between 3-OH and H-18, between H-19 and H-3/H-5/H-6, as well as between 9-OH and H-5 established the relative configuration of **1**. Finally, the structure and configuration were further elucidated by an X-ray diffraction analysis (Fig. 4). The final refinement of the Cu K α data resulted in a small flack parameter of -0.05 (6) allowing the assignment of the absolute configuration of **1** as 3*R*, 4*R*, 5*R*, 6*S*, 8*S*, 9*S*, 10*S*, 12*S*, 15*S*, 18*R*.

The molecular formula of **2** was deduced as C₃₃H₄₈N₂O₁₀ by HR-ESI-MS at *m/z* 633.3387 [M + H]⁺ (calcd for C₃₃H₄₉N₂O₁₀: 633.3382). The ¹H NMR spectrum of **2** displayed the signals of one *ortho*-substituted benzene ring at δ_H 7.71 (1H, dd, *J* = 8.0, 1.4 Hz), 7.24 (1H, m), 6.64 (1H, m), 6.58 (1H, m), and five methoxyls at δ_H 3.72, 3.62, 3.38, 3.28, 3.24 (each 3H, s). The ¹³C and DEPT NMR data displayed thirty-three carbon signals including six methyls, seven methylenes, thirteen methines and seven quaternary carbons. Detailed analysis of the ¹H and ¹³C NMR data (Table 1) of **2** showed a number of similarities to those of anthranoyllycoctonine.¹² The most notable differences were the existence of an additional carbonyl (δ_C 174.9) and an

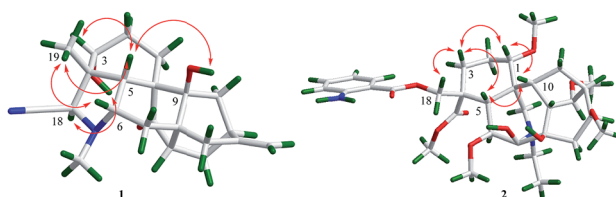


Fig. 3 NOESY correlations of **1** and **2**.



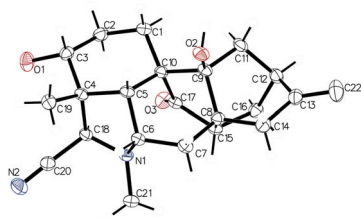


Fig. 4 Perspective drawing of the X-ray structure of 1.

additional methoxyl (δ_C 52.1) in 2. The HMBC correlations between H-3/H-18 and C-19 (δ_C 174.9), and between 19-OCH₃ (δ_C 52.1) and C-19 indicated that the N-C₁₉ bond was broken, and the carbon at C-19 was oxidized to be carbonyl. Furthermore, the HMBC correlations between H-1/H-5/H-21 and the methylene at C-17 (δ_C 42.3) revealed that the C₇-C₁₇ linkage was broken. In addition, the correlation from H-21 to C-7 suggested that a new bond was constructed between the nitrogen atom and C-7. Hence, the planar structure of 2 was established. The relative configuration of 2 was the same as that of anthranolylcoctonine by interpretation of the NOESY data (Fig. 3).¹²

Compounds 1 and 2 were tested for their antiviral effect against the respiratory syncytial virus (RSV), and anti-inflammatory activity on Nitric Oxide (NO) production. Both the two compounds showed no cell cytotoxicity towards the tested cells with the CC₅₀ values more than 100 μ M. Compound 2 displayed weak inhibitory effect on the growth of RSV and the production of NO in tested cells with the IC₅₀ values of 75.3 and 72.7 μ M, respectively, and 1 was virtually inactive with IC₅₀ values more than 100 μ M.

Conclusions

In summary, compounds 1 and 2, two novel diterpenoid alkaloids were isolated from *D. grandiflorum*. Compound 1 represents a rare hetisine-type C₂₀-diterpenoid alkaloid, and 2 features an unusual licoctonine-type C₁₉-diterpenoid alkaloid skeleton, revealing that the alkaloids in *Delphinium* plants possess complex structure skeletons and adding the diversity of alkaloid compositions isolated from *Delphinium* plants. Moreover, the assays of anti-RSV and anti-inflammatory activities showed that these two compounds had little cytotoxicity towards the tested cells, providing more potentiality for further pharmacologic study.

Experimental section

General

Melting point was obtained on an X-5 microscopic melting point apparatus. Optical rotations were recorded on a digital JASCO P-2000 polarimeter. UV spectra were obtained using a JASCO V-550 UV/VIS spectrophotometer. IR spectra were measured on a JASCO FT/IR-480 plus FT-IR spectrometer. NMR spectra were obtained by Bruker AV-500/300 spectrometers, with TMS as an internal standard. The chemical shifts (δ) were expressed in ppm and coupling constants (J) in Hz. HR-ESI-MS

data was recorded on an Agilent 6210 ESI/TOF mass spectrometer. Analytical HPLC was performed using a Dionex ultimate 3000 system with a Cosmosil C₁₈ analytical column (5 μ m, 4.6 \times 250 mm). Preparative HPLC was performed using an Agilent 1100 liquid chromatograph with a Cosmosil C₁₈ preparative column (5 μ m, 20 \times 250 mm). Column chromatographies were performed with silica gel (80–100, 200–300, 300–400 mesh; Qingdao Marine Chemical Group Co. Ltd, Qingdao, China), ODS (50 μ m, 120 \AA ; YMC) and Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden). Silica gel GF₂₅₄ plates (Yantai Chemical Industry Research Institute, Yantai, China) were used for thin-layer chromatography (TLC). Fractions were monitored by TLC, and spots were detected with modified Dragendorff's reagent.

Plant material

The dried rhizomes of *D. grandiflorum* were purchased in Guangzhou, Guangdong Province of China, in July, 2015. The plant was authenticated by Prof. Guang-Xiong Zhou (College of Pharmacy, Jinan University). A voucher specimen (no. 150713) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, P. R. China.

Extraction and isolation

The air-dried and powdered rhizome (10.0 kg) was extracted four times with 95% alcohol (4 \times 35 L) at room temperature. After evaporation of alcohol, the crude extract (492.2 g) was suspended in water (2 L) and acidified with HCl to pH = 4–5, then partitioned with CHCl₃ (3 \times 4 L) to give a water-soluble fraction. The water-soluble fraction was basified with NH₃·H₂O to pH = 9–10 and then partitioned with a H₂O/CHCl₃ mixture to give a CHCl₃-soluble fraction (63.0 g). The CHCl₃-soluble fraction was chromatographed on silica gel column (300–400 mesh, 1000 g) eluted with a solvent system of CHCl₃/CH₃OH (100 : 0 to 0 : 100, v/v), yielding six fractions (Fr.A-F). Fr.B (9.2 g) was further separated on an ODS column (200 g) eluted with MeOH/H₂O (30 : 70 to 100 : 0, v/v) to afford 9 sub-fractions (Fr.B1–B9). Fr.B5 (1.2 g) were purified by Sephadex LH-20 (MeOH/CHCl₃, 1 : 1, v/v) and compound 1 (15.0 mg) was crystallized from the eluent. Then 2 (12.3 mg) was obtained by the preparative HPLC with MeOH/H₂O (68 : 32, v/v) from Fr.B5.

Grandiflodine A (1). Colorless and block crystals (MeOH); mp 273–274 °C; $[\alpha]_D^{25} +6.8$ (c 0.6, DMSO); UV (MeOH) λ_{\max} (log ϵ) 208.6 (3.57) nm; IR (KBr) ν_{\max} 3480, 3423, 2933, 2876, 2228, 1674, 1462, 1057, 1054, 894 cm⁻¹; ¹H and ¹³C NMR data see Table 1; HRESIMS m/z 369.2175 (calcd for C₂₂H₂₉N₂O₃, 369.2173).

Grandiflodine B (2). White powder; $[\alpha]_D^{25} +10.7$ (c 0.96, MeOH); UV (MeOH) λ_{\max} (log ϵ) 219.5 (3.78), 250.2 (3.30), 340.5 (3.18) nm; IR (KBr) ν_{\max} 3455, 2931, 2874, 1677, 1453, 1360, 1189, 1055, 893 cm⁻¹; ¹H and ¹³C NMR data see Table 1; HRESIMS m/z 633.3382 (calcd for C₃₃H₄₉N₂O₁₀, 633.3387).

X-ray crystallographic analysis of 1. Colorless blocks, C₂₂H₂₈N₂O₃, $M_r = 368.46$; monoclinic, space group $P2_1$; $a = 9.7305$ (2) \AA , $b = 8.76406$ (16) \AA , $c = 11.0765$ (2) \AA , $\alpha = 90^\circ$, $\beta = 109.45$ (2)°, $\gamma = 90^\circ$; $V = 890.68$ (3) \AA^3 , $Z = 2$, $d_x = 1.374$ Mg m⁻³,



$F(000) = 396.0$, $\mu(\text{Cu K}\alpha) = 0.731 \text{ mm}^{-1}$. Data collection was performed on a Gemini S Ultra using graphite monochromated radiation ($\lambda = 1.54184 \text{ \AA}$); 2829 unique reflections were collected to $\theta_{\text{max}} = 125.536^\circ$, where 14 056 reflections were observed [$F_2 > 2\sigma(F_2)$]. The structure was solved by direct methods (SHELXS 97)¹³ and refined by full-matrix least-squares on F_2 . Final $R = 0.0297$, $R_w = 0.0840$, and $S = 1.111$. Crystallographic data for these structures have been deposited with the Cambridge Crystallographic Data Center as CCDC 1517870 for compound **1**.

Assay of anti-RSV activities on Hep-2 cells

The human larynx epidermoid carcinoma (HEp-2, ATCC CCL-23) cells and RSV A2 (ATCC VR-1540) strains were purchased from Medicinal Virology Institute, Wuhan University, China. HEp-2 cells were cultured in DMEM (Gibco) supplemented with 100 U mL^{-1} penicillin and streptomycin solution, and virus was propagated in HEp-2 cells and incubated in DMEM with 2 mM L-glutamine, 2% FBS, and 100 U mL^{-1} penicillin and streptomycin solution. All of the cells were cultured in a 95% humidified atmosphere supplied with 5% CO_2 at 37°C , and the ribavirin (Sigma, purity of 99%) was used as the positive control. The cytotoxicity of the compounds toward HEp-2 cells was detected by the MTT assay in 96-well plates (Corning) with the optical density (OD) values measured in an enzyme immunoassay reader (Thermo Labsystems Multiskan MK3) at 570 nm , and the 50% cytotoxic concentration (CC_{50}) was estimated by regression analysis. The antiviral activities of the isolates against the RSV-A2 strain were assessed by the CPE reduction assay as reported in previous paper.¹⁴ The concentration that reduces 50% of CPE with respect to the virus control was estimated from the plots of the data and was defined as the 50% inhibitory concentration (IC_{50}) of the tested compounds.

Assay of anti-inflammatory activities on NO production toward RAW 264.7 cells

RAW 264.7 cells were provided by the Medicinal Virology Institute of Wuhan University and maintained in DMEM (Gibco) containing 10% FBS (Gibco), and supplemented with 100 U mL^{-1} penicillin and streptomycin solution. Cells were cultured at 37°C in a 95% humidified atmosphere supplied with 5% CO_2 . The cytotoxicity of the compounds on RAW 264.7 cells was detected by the MTT assay in 96-well plates with the OD values measured at 570 nm , and the CC_{50} was estimated by regression analysis. The anti-inflammatory activities of the compounds were evaluated by the inhibitory effect on NO production. RAW 264.7 cells (4×10^4 cells per well) were incubated in a 96-well plate for 14 h and then pretreated with 100 ng mL^{-1} LPS and different concentrations of compounds (6.25 –

$100 \text{ }\mu\text{M}$) for 24 h. Then, the Griess reagent ($100 \text{ }\mu\text{L}$) was added and blended with the supernatant ($100 \text{ }\mu\text{L}$), and the absorbance was measured at 540 nm with an enzyme immunoassay reader. NO levels were determined *via* a calibration curve constructed with NaNO_2 concentrations of 3.12 – $100 \text{ }\mu\text{M}$. Inhibitory effects of compounds on NO production (IC_{50}) were calculated by regression analysis of the dose–response curve generated from the data.

Acknowledgements

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