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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 C_{20}-diterpenoid alkaloid in which the bond between the atoms of N and C-17 is broken. Compound **2** features an unusual lycoctonine-type C_{19}-diterpenoid alkaloid skeleton with the cleavage of N–C_{19} and C_7–C_{17} bonds, and the construction of the N–C_7 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 C_{20}-diterpenoid alkaloid with the cleavage of the bond between the atoms of N and C-17. Compound **2** features an unusual lycoctonine-type C_{19}-diterpenoid alkaloid skeleton with the cleavage of N–C_{19} and C_7–C_{17} bonds, and construction of the N–C_7 bond. Herein, we report the isolation, structure elucidation and biological activities of **1** and **2** (Fig. 1).

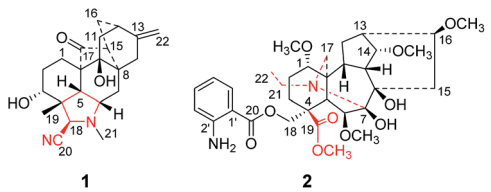

Fig. 1 Chemical structures of compounds **1** and **2**.

Results and discussion

Grandiflodine A (**1**) was isolated as colorless block crystal. The molecular formula of **1** was established as $C_{22}H_{28}N_2O_3$ by its HR-ESI-MS (*m*/*z* 369.2175 $[M + H]^+$, calcd for $C_{22}H_{29}N_2O_3$: 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 ^{13}C 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 anhydroignavinol[11] showed that they were similar except for the presence of additional carbonyl and cyanogroup, and the

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

	Grandiflodine A (**1**)[a]		Grandiflodine A (**2**)[b]	
Position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J 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 $CDCl_3$. Overlapped signals are reported without designating multiplicity.

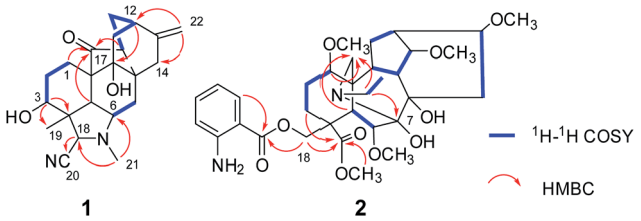

Fig. 2 Key ^{1}H–^{1}H COSY and HMBC correlations of **1** and **2**.

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–C_{17} bond was broken to form a unique hetidines-type C_{20}-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 $C_{33}H_{48}N_2O_{10}$ by HR-ESI-MS at m/z 633.3387 $[M + H]^+$ (calcd for $C_{33}H_{49}N_2O_{10}$: 633.3382). The ^{1}H 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 ^{13}C and DEPT NMR data displayed thirty-three carbon signals including six methyls, seven methylenes, thirteen methines and seven quaternary carbons. Detailed analysis of the ^{1}H and ^{13}C 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

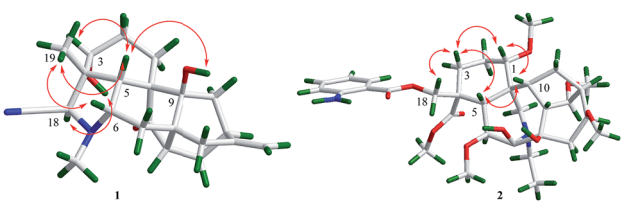

Fig. 3 NOESY correlations of **1** and **2**.

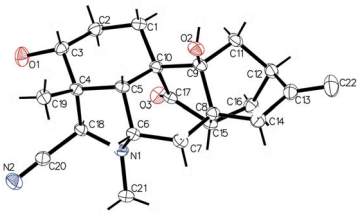

Fig. 4 Perspective drawing of the X-ray structure of **1**.

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-OCH_3 (δ_C 52.1) and C-19 indicated that the N–C_{19} 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 C_7–C_{17} 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 anthranoyllycoctonine 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 CC_{50} values more than 100 μM. Compound **2** displayed weak inhibitory effect on the growth of RSV and the production of NO in tested cells with the IC_{50} values of 75.3 and 72.7 μM, respectively, and **1** was virtually inactive with IC_{50} values more than 100 μM.

Conclusions

In summary, compounds **1** and **2**, two novel diterpenoid alkaloids were isolated from *D. grandiflorum*. Compound **1** represents a rare hetisine-type C_{20}-diterpenoid alkaloid, and **2** features an unusual lycoctonine-type C_{19}-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 C_{18} analytical column (5 μm, 4.6 × 250 mm). Preparative HPLC was performed using an Agilent 1100 liquid chromatograph with a Cosmosil C_{18} preparative column (5 μm, 20 × 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 μm, 120 Å; YMC) and Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden). Silica gel GF_{254} 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 × 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 $CHCl_3$ (3 × 4 L) to give a water-soluble fraction. The water-soluble fraction was basified with $NH_3 \cdot H_2O$ to pH = 9–10 and then partitioned with a H_2O/$CHCl_3$ mixture to give a $CHCl_3$-soluble fraction (63.0 g). The $CHCl_3$-soluble fraction was chromatographed on silica gel column (300–400 mesh, 1000 g) eluted with a solvent system of $CHCl_3$/CH_3OH (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/H_2O (30 : 70 to 100 : 0, v/v) to afford 9 subfractions (Fr.B1–B9). Fr.B5 (1.2 g) were purified by Sephadex LH-20 (MeOH/$CHCl_3$, 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/H_2O (68 : 32, v/v) from Fr.B5.

Grandiflodine A (1). Colorless and block crystals (MeOH); mp 273–274 °C; $[\alpha]_D^{19}$ +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 ^{13}C NMR data see Table 1; HRESIMS *m*/*z* 369.2175 (calcd for $C_{22}H_{29}N_2O_3$, 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 ^{13}C NMR data see Table 1; HRESIMS *m*/*z* 633.3382 (calcd for $C_{33}H_{49}N_2O_{10}$, 633.3387).

X-ray crystallographic analysis of 1. Colorless blocks, $C_{22}H_{28}N_2O_3$, M_r = 368.46; monoclinic, space group $P2_1$; a = 9.7305 (2) Å, b = 8.76406 (16) Å, c = 11.0765 (2) Å, α = 90°, β = 109.45 (2)°, γ = 90°; V = 890.68 (3) $Å^3$, Z = 2, d_x = 1.374 Mg m^{-3},

$F(000) = 396.0$, μ (Cu Kα) = 0.731 mm^{-1}. Data collection was performed on a Gemini S Ultra using graphitemonochromated radiation (λ = 1.541 84 Å); 2829 unique reflections were collected to θ_{max} = 125.536°, 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 μM) for 24 h. Then, the Griess reagent (100 μL) was added and blended with the supernatant (100 μL), 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 μM. 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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