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Anti-inflammation C_{19}-diterpenoid Alkaloids from *Delphinium giraldii* Diels

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Abstract: Two new C_{19}-diterpenoid alkaloids named Delphinium alkaloid A (1) and Delphinium alkaloid B (**2**) were isolated from the *Delphinium* (Ranunculaceae). Their structures were elucidated by spectroscopic and mass-spectrometric analyses, including 1D-, 2D-NMR and HR-Q-TOF-MS. Compound **1** showed anti-inflammatory activity by regulating the inflammatory reaction induced by LPS in Caco2 cell.

Keywords: *Delphinium giraldii* Diels; chemical constituents; diterpenoid alkaloids; anti-inflammatory.

1. Introduction

Delphinium (Ranunculaceae) is a widely distributed plant grown in the north temperature zone. The genus includes more than 200 species, half of which are growing in China [1]. *Delphinium* were used to treat analgesia, sedation and rheumatism [2,3]. Phytochemical investigation revealed norditerpenoid and diterpenoid alkaloids were the characteristic bioactive ingredients of the plant [3,4].

Delphinium *giraldii* Diels (Ranunculaceae) is one of the common species of *Delphinium* which mainly distributed in Sichuan, Gansu, Ningxia, Shanxi, Hubei and Henan provinces of China. In previous reports, 14 diterpenoid alkaloids were isolated and identified from the plant [4]. As part of our search for new natural bioactive compounds, we carried out the phytochemical investigation on *Delphinium giraldii* Diels. Two new C_{19}-diterpenoid alkaloids were isolated and identified from *Delphinium giraldii* Diels, the anti-inflammatory effect of one of the compounds was assayed subsequently.

2. Materials and Methods

2.1. General Experimental Procedures

Optical rotations were measured using a Perkin-Elmer digital polarimeter (USA). HR-ESI-MS data were measured on an Agilent 1100 LC/MSD Trap-SL spectrometer (USA), 1D and 2D nuclear

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magnetic resonance (NMR) were recorded on a Bruck AVANCE 400 FT-NMR spectrometer with tetramethylsilane (TMS) as internal standard. Column chromatography was performed on silica gel (200–300 mesh, Marine Chemical Factory, Qingdao, China). All the reagents were of analytical grade and purchased from Tianjin Kermel Chemical Company (Tianjin, China).

2.2 Plant Material

Delphinium giraldii Diels was collected from Qinling, Shanxi Province of China in August, 2016. A voucher specimen was identified by Dr. Zhi Yang Liu and deposited in the Changchun Sci-Tech University (NO.20160811).

2.3 Extraction and Isolation

About 5 kg of dried *Delphinium giraldii* Diels were extracted with 95% EtOH for three times, and the extracted solution was combined and evaporated to dryness by a vacuum. The crude extract was then successively suspended in water and extracted with petroleum ether, dichloromethane, ethyl acetate and n-butanol, subsequently. The CH_2Cl_2 fraction (about 80 g) was then subjected to normal silica gel column chromatography and eluted with CH_2Cl_2-MeOH (100:0~1:1, v/v) to yield nine fractions (*Fr.1-9*), *Fr. 6* (13.2 g) was then subjected to normal silica gel column eluted with gradient solvent systems with PE-EtOAc (100:1~1:1) to afford eight major fractions (*Fr.6-1~6-8*). *Fr.6−4* was purified by silica gel column with PE-Acetone to give compound **1** (58mg) and **2** (9mg).

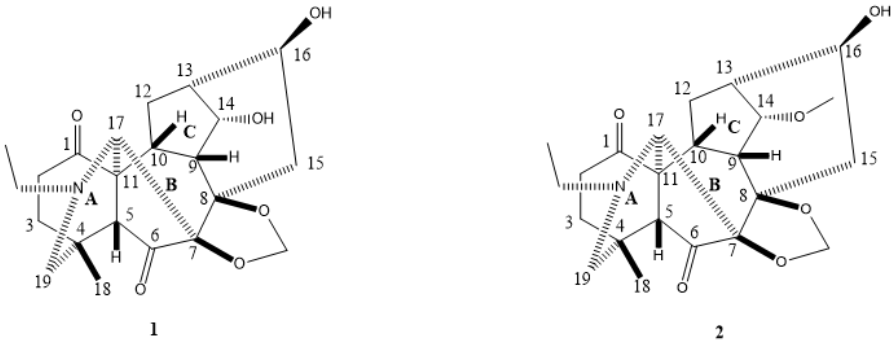

Figure 1. Structures for compounds **1** and **2**.

2.4 Cytotoxicity Assay

Cytotoxicity assay was performed as the method with minor modification [5]. Caco2 cells, obtained from American Type Culture Collection (ATCC), were seeded in 96-well plates at a density of 1×10^4/mL. The cells were cultured in DMEM cluture medium containing 10% fetal bovine serum (FBS, HyClone) for 48 h before use. 1, 10, 20, 40 μM of compound **1**, at the final concentration, were added into wells and incubated for 24h (n=6). Then, 20 μL (5 mg/mL) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT, Sigma) was added for another 4 h. Next, 150 μL of dimethyl sulfoxide (DMSO) was added to resolve the formazan. The OD values at 570nm of the wells were measured using a microplate reader.

2.5 Anti-inflammatory Assay

Caco2 cells were seeded in 6-well culture plates at a density of 3×10^5/mL. After washing in Hank's balanced salt solution for three times to remove the antibiotics, 1 and 10 μM of compound **1** were added into the medium for the first 24 h. Then, 3 mg/mL LPS was added for the next 24 h to trigger an inflammatory response, and five replicates were used per treatment. DMEM culture medium

was used as a blank control and BAY 11-7082 (1 μM) was used as positive drug. The relative levels of TNF-α, IL-6 and IL-8 were determined using RT-qPCR to estimate the anti-inflammatory activity of **1**.

2.6 RT-qPCR Analysis

Total RNA isolation and cDNA synthesis were conducted according to the manufacturer's instructions. All of the cDNA samples were stored at -20 °C until use. The RT-qPCR mixture system was set up as follows: 12.5 μL of SYBR Premix Ex Taq II (2×), 1 μL of a forward primer (10 μM), 1 μL of a reverse primer (10 μM), 1 μL of cDNA, and 9.5 μL of double-distilled water. The primers used were designed by RiboBio (Guangzhou, China). The reaction protocol was as follows: 95 °C for 5 min; 40 cycles at 95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s; and 72 °C for 5 min. All of the samples were run in triplicate, and the average cycle threshold values were calculated using the $2^{-\Delta\Delta Ct}$ method as previously described [6].

2.7 Statistical Analysis

All the data were presented as the means ± SE and were analyzed by one-way ANOVA using SPSS software, version 19.0. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was obtained as a colorless amorphous solid, $[\alpha]_D^{20}$-26.3. The HR-ESI-MS of **1** contained a $[M + H]^-$ ion at *m*/*z* 404.2065 corresponding to the formula $C_{22}H_{29}NO_6$ (calcd. 404.2073), suggesting the 9 degrees of unsaturation. The IR spectrum showed resonance for hydroxyl (3449 cm^{-1}) and carbonyl (1749 and 1685 cm^{-1}) groups. ^1H NMR spectrum indicated the presence of two methyl groups at δ_H 1.07 (3H, s, H-18) and 1.06 (3H, t, J = 7.5 Hz, N-$CH_2\underline{CH_3}$), and two oxygen-substituted methine groups at δ_H 4.13 (1H, m, H-14) and 3.17 (1H, m, H-16). Combined with the HSQC correlations for carbon signals of δ_C 40.3 (C-2), 39.2 (C-3), 28.1 (C-12), 35.1 (C-15), 58.4 (C-19), 50.5 (N-$\underline{CH_2}CH_3$), and 95.7 (-OCH_2O-), seven groups of methylene signals were determined as δ_H 2.40, 3.18 for C-2, δ_H 1.81, 1.87 for C-3, δ_H 1.31, 2.40 for C-12, δ_H 2.52, 1.75 for C-15, δ_H 2.75, 2.40 for C-19, δ_H 2.77, 2.72 for N-$\underline{CH_2}CH_3$, and δ_H 5.15, 5.61 for -OCH_2O-. The ^{13}C NMR spectrum gave 22 carbon signals, and in addition to those of the aforementioned groups, another two oxygen-substituted carbon signals at δ_C 81.0 (C-8) and 90.4 (C-7), along with two carbonyl carbon signals at δ_C 211.1 (C-1) and 213.9 (C-6) were observed in the ^{13}C NMR spectrum. Subsequently, all the proton and carbon signals were assigned as shown in table 1. By analysis of the skeleton of **1** via ^{13}C NMR data comparison, we found that all these ^1H and ^{13}C NMR data resembled those of the C_{19}-diterpenoid alkaloids [7,8]. In the HMBC spectrum, correlation from H-15 to C-8 and C-13; from -OCH_2O- (δ_H 5.61) to C-7 and C-8; from H-17 to C-7 and C-19 suggested the C-C linkages that bridged C-7 and C-8, C-8 and C-13, C-17 and C-7, C-17 and N-C-19 (Figure 2A), accounting for four of the nine degrees for unsaturation. As the two carbonyls (δ_C 211.1 and 213.9) contributed anther two of the nine degrees, the three degrees of unsaturation unassigned were attributed to ring A-C. Thus, the basic skeleton of **1** was considered to consist of seven rings, including ring A-C and another four ones bridging C-7 and C-8, C-8 and C-13, C-17 and C-7, C-17 and N-C-19. The detailed location of two hydroxyls, the ethyl group and two carbonyls were determined by the HMBC analysis as shown in Figure 2A. Therefore, the ultimate structure of **1** was determined as shown in Figure 1.

The relative structure of **1** was elucidated via analysis of NOESY spectrum, where NOE correlations between H-18/H-5, H-5/H-10, H-10/H-14 and H-17/H-15 were given (Figure 2B). Finally, the structure of **1** was established as shown in Figure 1 and named Delphinium alkaloid A.

Compound **2** was also obtained as a colorless amorphous solid, $[\propto]^{20}{}_D = -23.3$. The molecular formula of **2** was determined to be $C_{23}H_{31}NO_6$ as the HR-ESI-MS of **2** showed $[M + H]^-$ ion at *m*/*z*

418.2239 (calcd. 418.2238). By comparing the NMR data to those of **1**, we found that **1** and **2** possessed almost identical ^{1}H and ^{13}C NMR data, except for one methoxyl group at δ_H 3.42 (δ_C 58.0) that was only appeared in the ^{1}H and ^{13}C NMR spectrum of **2**, suggesting that **2** shared that same skeleton of **1**. As there were only two hydroxyls in the skeleton of **1** and **2**, we thus matching the ^{13}C NMR of **2** to those of **1** so as to find the potential location of this methoxyl. The results showed that all the ^{13}C NMR data of **2** matched those of **1** perfectly, except that the carbon signals at δ_C 82.1 of **2** showed significant difference to that at δ_C 73.9 (C-14) of **1**. We thus inferred that the methoxyl of **2** was linked to the hydroxyl of C-14 for **2**. HMBC correlation for the methoxyl (from δ_H 3.42 to δ_C 82.1) confirmed the inferred linkage between methoxyl and C-14. Therefore, the plain structure of **2** was determined as shown in Figure 1. Also, **2** showed the same NOE characteristics as **1**, including H-18/H-5, H-5/H-10, H-10/H-14 and H-17/H-15 correlations in the NOESY spectrum, which means **1** and **2** had the same stereo-configuration. Thus, the final structure of **2** was established as shown in Figure 1 and named Delphinium alkaloid B.

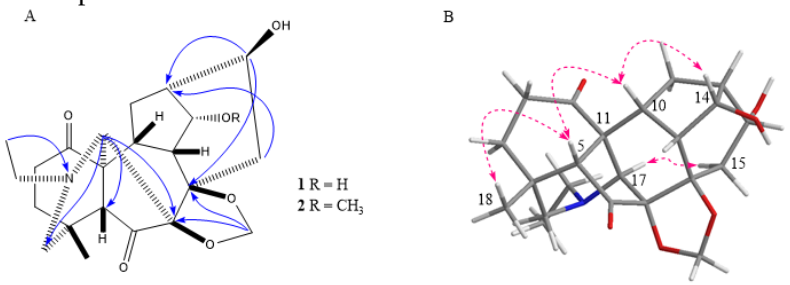

Figure 2. 2D NMR correlations for compounds 1 and 2. (A) HMBC (B) NOESY

Table 1. Chemical shifts of compound **1** and **2** in $CDCl_3$

	Compound **1**		Compound **2**	
NO.	δ_C	δ_H	δ_C	δ_H
1	211.1	–	211.1	–
2	40.3	3.18 *m*, 2.40 *m*	40.3	3.21 *m*, 2.38 *m*
3	39.2	1.81 *m*, 1.87 *m*	39.3	1.81 *m*, 1.85 *m*
4	35.9	–	35.8	–
5	61.5	2.25 *s*	61.6	2.25 *s*
6	213.9	–	213.7	–
7	90.4	–	90.3	–
8	81.0	–	81.1	–
9	44.7	2.17 *m*	41.2	2.46 *m*
10	41.1	2.22 *m*	42.1	2.23 *m*
11	56.6	–	56.3	–
12	28.1	2.40 *m*, 1.31 *m*	28.3	2.42 *m*, 1.34 *m*
13	36.2	2.55 *m*	39.0	2.52 *m*
14	73.9	4.13 *m*	82.1	3.73 *m*
15	35.1	2.52 *m*, 1.75 *m*	35.0	2.53 *m*, 1.70 *m*
16	71.4	3.71 *m*	71.1	3.73 *m*
17	63.2	3.69 *s*	63.2	3.70 *s*
18	24.0	1.07 *s*	24.1	1.06 *s*
19	58.4	2.75 *m*, 2.40 *m*	58.6	2.77 *m*, 2.42 *m*
N-$\underline{CH_2}CH_3$	50.5	2.77 *m*, 2.72 *m*	50.0	2.76 *m*, 2.68 *m*
N-$CH_2\underline{CH_3}$	13.5	1.06 *t* (7.5)	13.6	1.08 *t* (7.5)
14-OCH_3	–	–	58.0	3.42 (*s*)
OCH_2O	95.7	5.61 *s*, 5.15 *s*	95.8	5.62 *s*, 5.13 *s*

*3.2 Anti-inflammatory Activities of **1***

The MTT test indicated that the cytotoxicity of compound **1** has non-toxic with the concentration ranging from 1 to 20 μM (Figure 3A). Thus, 1 and 10 μM of **1** were adopted in the anti-inflammatory

assay subsequently. As shown in Figure 3B-3D, LPS stimulation increased the expression of pro-inflammatory factors TNF-α, IL-6 and IL-8 significantly ($P < 0.05$), while both 1 and 10 μM of **1** reversed the LPS-induced overexpression of these three pro-inflammatory factors, suggesting that **1** is a potential natural anti-inflammatory agent against intestinal inflammation. But the anti-inflammatory activities were less than the positive drug (BAY 11-7082).

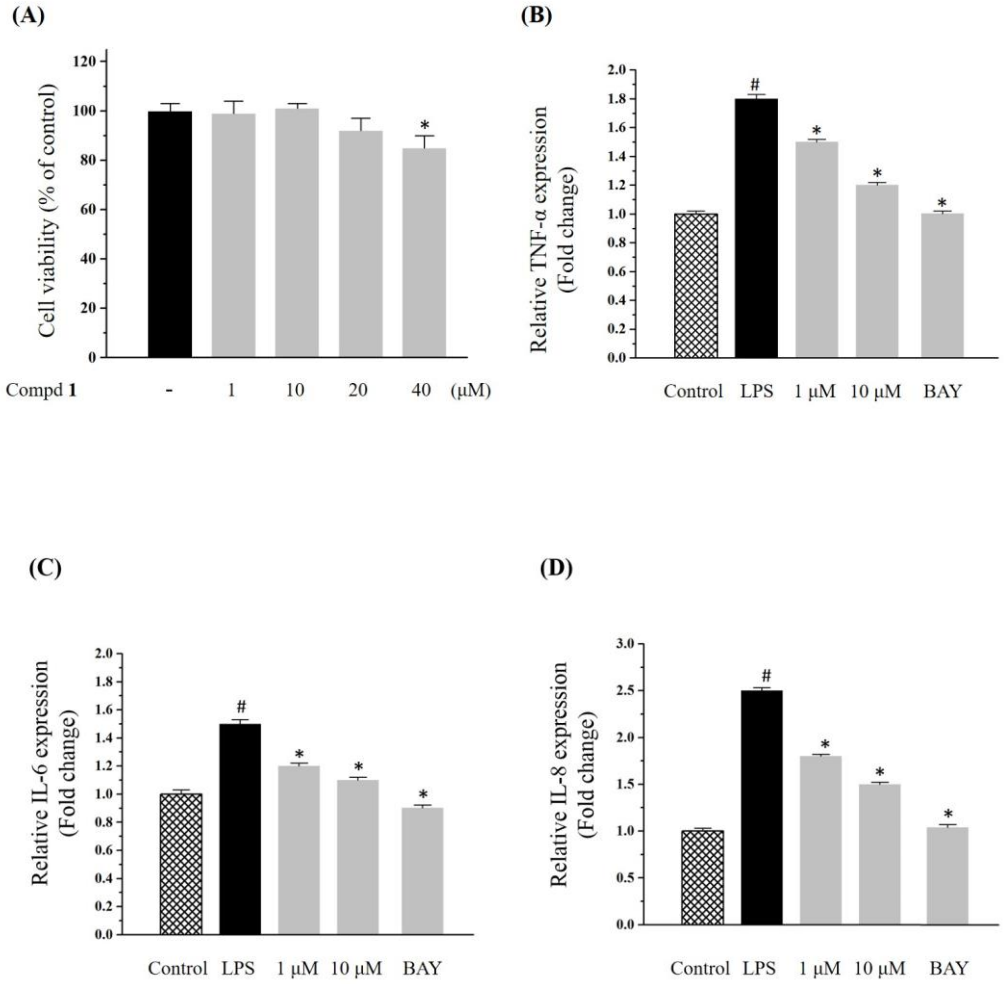

Figure 3. The anti-inflammatory effect of **1** on LPS-infected Caco2 cells. (A) **1** did not alter the survival rate of Caco2 cells with the concentration of 1-20 μM. (B) - (D) **1** abrogated the increased pro-inflammatory cytokines induced by lps (expressed with the $2^{-\Delta\Delta Ct}$ method; $^{\#}$ $P < 0.05$ referenced to the control group; * $P < 0.05$ referenced to the lps group).

In this study, two new C_{19} diterpenoid alkaloids were isolated from Delphinium *giraldii* Diels (Ranunculaceae). The anti-inflammatory activity of Delphinium alkaloid B (**2**) was not assayed due to its limit amount. But, we can also infer that **2** could be with the similar potential bioactivities of **1** because of the similar structure. This study could be a supplement for the research of Delphinium *giraldii* Diels (Ranunculaceae)

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