HETEROCYCLES, Vol. 94, No. 10, 2017, pp. 1903 - 1908. © 2017 The Japan Institute of Heterocyclic Chemistry
Received, 20th June, 2017, Accepted, 24th July, 2017, Published online, 9th August, 2017
DOI: 10.3987/COM-17-13768

THREE NEW LACTONE-TYPE DITERPENOID ALKALOIDS FROM *ACONITUM ROTUNDIFOLIUM* KAR. & KIR.

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Abstract – Three new lactone-type C_{19}-diterpenoid alkaloids, rotundifosines A – C (**1** - **3**) along with two known diterpenoid alkaloids, heterophyllidine (**4**) and chellespontine (**5**) were isolated from the whole plant of *Aconitum rotundifolium* Kar. & Kir. Their structures were established on the basis of extensive spectroscopic analysis, including 1D and 2D NMR experiments. Compounds **1** - **3** were evaluated for cytotoxicity against MCF-7, MDA-MB-231, 786-0 and HepG2 human cancer cell lines.

Aconitum (Ranunculaceae) is a large genus comprising 400 species and distributed in the temperate regions of the Northern Hemisphere. About 76 Aconitum species in China are used as folk medicines, which are mainly used for the treatment of rheumatoid arthritis and various types of pains.[1] In our continuous phytochemical studies on the plants of the genera *Aconitum* and *Delphinium*, we obtained a series of structurally and chemotaxonomically diverse diterpenoid alkaloids.[2] *Aconitum rotundifolium* Kar. & Kir., a kind of herbaceous plants belongs to the Ranunculaceae family, is distributed in China (e.g., Xinjiang) and Russia and grows at altitudes 3100 m.[3] To the best of our knowledge, no phytochemical investigation of this plant has previously been undertaken. In the present paper, we report on the isolation and structural elucidation of three new lactone-type C_{19}-diterpenoid alkaloids, rotundifosines A – C (**1** - **3**) (Figure 1), by 1D, 2D NMR and HR-ESI-MS experiments. In addition, two known compounds (Figure 1) were isolated and identified as heterophyllidine (**4**)[4] and chellespontine (**5**).[5] The cytotoxic activity of those isolated compounds has not been studied yet, and here we have investigated the isolated compounds for their cytotoxicity *in vitro* against several human cancer cell lines.

The EtOH extract of *A. rotundifolium* Kar. & Kir. was treated in the usual procedures to give the crude alkaloid extract, and the crude alkaloid extract was sequentially subjected to column chromatography on silica gel to afford three new diterpenoid alkaloids and two known ones.

Rotundifosine A (**1**) was isolated as a white amorphous powder, gave a positive reaction with the Dragendorff reagent. The IR spectrum indicated the presence of hydroxy group (3354 cm^{-1}) and carbonyl group (1735 cm^{-1}). Its molecular formula was deduced to be $C_{21}H_{29}NO_5$ by HR-ESI-MS at *m/z* 376.2138 $[M + H]^+$ (calcd. for $C_{21}H_{30}NO_5$, 376.2124). Further support for this molecular formula came from the analysis of ^{13}C NMR and DEPT spectra (Table 1), which revealed the presence of 21 carbons, comprising resonances for two methyls, seven methylenes, seven methines, and five quaternary carbon atoms, consistent with the molecular formula of $C_{21}H_{29}NO_5$. The ^1H NMR and ^{13}C NMR data (Table 1) of **1** indicated the presence of the signals of an acetyl group [δ_H 2.01 (3H, s), δ_C 21.9 (q) and 171.1 (s)]. The remaining 19 carbons in the ^{13}C NMR spectrum were assigned based on 1D and 2D NMR data. The presence of the characteristic signal of a lactone group [δ_C 173.6 (s) and 76.2 (d)] in the skeleton indicated that compound **1** was a lactone-type diterpenoid alkaloid.[6] The existence of three oxygenated carbons [δ_C 72.3 (d), δ_C 76.2 (d) and δ_C 86.6 (s)] deduced from its ^{13}C NMR spectrum suggest that **1** has one hydroxy group, in addition to an ester group and a lactone group. The locations of the hydroxy group at C-1 and the lactone carbonyl carbon at C-14 were determined by the correlations in the HMBC experiment, respectively. The additional acetyl group was located at C-8 by the downfield chemical shift of carbon of C-8 (δ_C 86.6), with this observations supported by the HMBC correlations among H-6 (δ_H 2.02), H-7 (δ_H 3.61) and H-10 (δ_H 2.48) to C-8.[7] The complete planar structure of **1** was further verified by analysis of the HMBC and ^1H-^1H COSY spectra (Figure 2). A correlation between H-1 and H-10 in the NOESY experiment indicated that the OH group at C-1 was α-oriented (Figure 3).[4] Thus, the structure of **1** was determined as shown in Figure 1, named rotundifosine A.

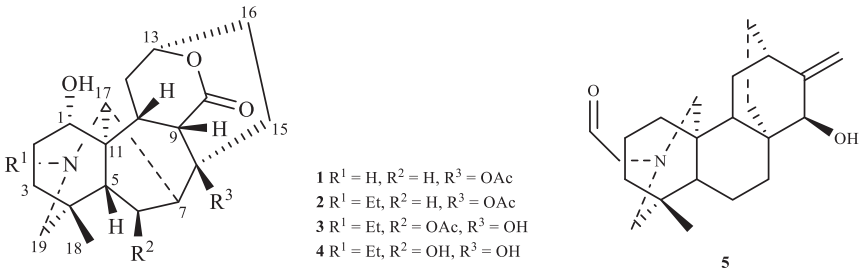

Figure 1. Structures of compounds **1-5**

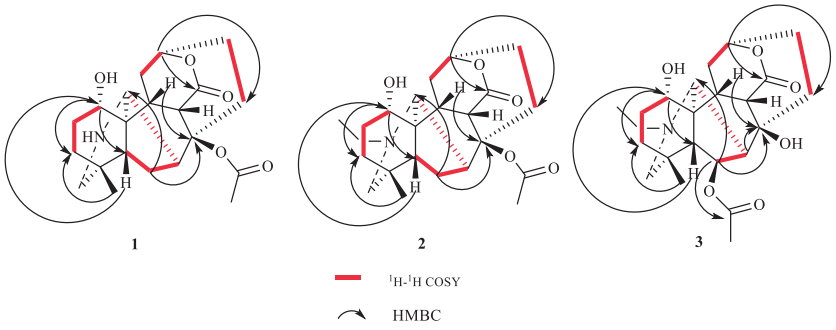

Figure 2. Key HMBC and ^1H-^1H COSY correlations of compounds **1 - 3**

Rotundifosine B (**2**) was isolated as a white amorphous powder and its molecular formula was deduced to be $C_{23}H_{33}NO_5$ by HR-ESI-MS at *m/z* 404.2445 $[M + H]^+$ (calcd. for $C_{23}H_{34}NO_5$, 404.2437). The spectroscopic data of compound **2** demonstrated that its structure was very similar to those of **1**, except for the additional signals for one *N*-ethyl group [δ_H 1.14 (3H, t, J = 7.2 Hz), 2.53 (1H, m), 2.62 (1H, m), δ_C 49.3 (t), 13.3 (q)] observed in the NMR spectra of **2**, and the signals of C-17 and C-19 in ^{13}C NMR spectrum were displaced downfield about δ_H 4.5 and 10.3 ppm, respectively. Therefore, the structure of **2** is elucidated as demonstrated.

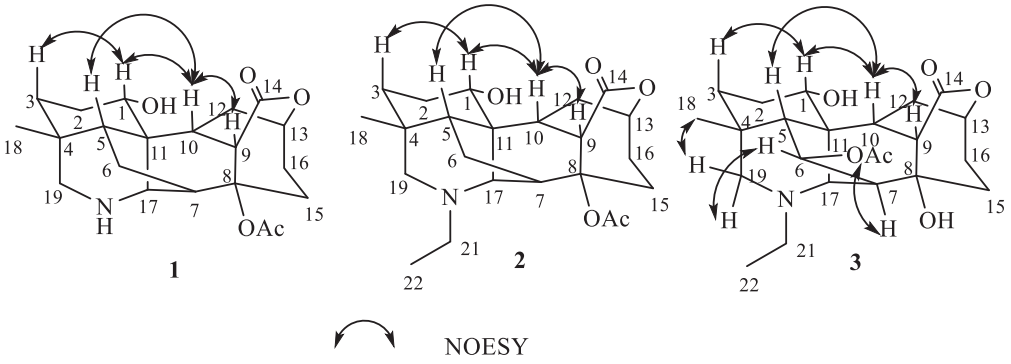

Figure 3. Key NOESY correlations of compounds **1** - **3**

Rotundifosine C (**3**) was obtained as a white amorphous powder, and its molecular formula was deduced to be $C_{23}H_{33}NO_6$ by HR-ESI-MS at *m/z* 420.2391 $[M + H]^+$ (calcd. for $C_{23}H_{34}NO_6$, 420.2386). Comprehensive analysis of the NMR spectra suggested that compound **3** was also a lactone-type diterpenoid alkaloid. The ^1H and ^{13}C NMR data (Table 1) evidenced the presence of an *N*-ethyl group [δ_H 1.12 (3H, t, J = 7.2 Hz), 2.04 (1H, m), 2.53 (1H, m), δ_C 48.6 (t), 13.2 (q)], an acetyl group [δ_H 2.09 (s); δ_C 21.9 (q) and 170.8 (s)], and four oxygen-bearing carbons [δ_C 72.6 (d), δ_C 73.2 (d), δ_C 75.9 (s) and δ_C 74.8 (d)]. The correlations in HMBC spectrum between H-6 (δ_H 6.47) and the carbonyl carbon of acetoxy group (δ_C 170.8) indicated that the acetoxy group was at the C-6 position. The existence of four oxygenated carbons suggests that **3** have two hydroxy groups, in addition to acetoxy group and the lactone group. The position of a hydroxy group could be tentatively assigned to C-8 because of the chemical shift for C-8 at δ 75.9 (s).[8] Further, the location of another hydroxy group at C-1 was established based on the HMBC correlations between H-3, H-5 and C-1 and COSY correlations of H-1 and H-2. In the NOESY spectrum of **3**, the cross-peak between H-1 and H-10 proved that OH at C-1 is the α-position. Analysis of the ^1H-^1H COSY spectrum (Figure 2), the absence of correlation between H-5 and H-6, indicating a dihedral angle of 90º between the two H-atoms, confirmed that 6-AcO was β-position.[4] All the available data led to the elucidation of the structure of **3** as depicted.

To evaluate the biological activities of these compounds isolated from the entire *A. rotundifolium* Kar. & Kir. for future applications, compounds **1** - **3** were evaluated for their cytotoxicity against human cancer

cell lines including MCF-7, MDA-MB-231, 786-0 and HepG2 by MTT method. However, none of those compounds had obvious inhibitory activity against those tumor cells used (IC_{50} > 50 μM, n = 3).

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **1** - **3** [a]

	1 [b]		**2** [b]		**3** [c]	
No.	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	4.07 m	72.3	3.81 m	74.1	3.83 m	72.6
2	1.59 m, 1.85 m	30.4	1.59 m, 1.80 m	30.2	1.23 m, 1.65 m	29.8
3	1.61m, 1.88 m	30.8	1.57 m, 1.78 m	32.1	2.31 m, 2.51 m	30.5
4	—	34.2	—	34.2	—	33.6
5	2.03 m	44.2	1.83 m	45.8	1.66 m	51.4
6	1.89 m, 2.02 m	26.2	1.69 m, 1.84 m	26.6	5.47 d (7.2)	73.2
7	3.61 d (7.8)	45.2	3.42 d (7.8)	41.9	2.61 d (7.2)	49.6
8	—	86.6	—	88.2	—	75.9
9	3.12 d (6.6)	48.7	3.07 d (7.2)	49.5	3.75 d (6.8)	48.9
10	2.48 m	40.2	2.29 m	40.5	2.40 m	40.2
11	—	50.4	—	51.1	—	49.8
12	1.62 m, 1.86 m	30.2	1.59 m, 1.68 m	30.6	1.27 m, 1.76 m	30.3
13	4.91 m	76.2	4.88 m	76.6	4.86 m	74.8
14	—	173.6	—	174.7	—	173.1
15	2.12 m, 2.49 m	30.5	2.63 m, 2.87 m	31.1	1.76 m, 2.00 m	36.0
16	1.63 m, 1.92 m	29.2	1.85 m, 2.50 m	30.9	1.67 m, 1.83 m	31.7
17	4.07 m	58.7	3.33 (overlapped)	63.2	3.48 m	64.1
18	1.04 s	27.4	0.93 s	27.9	1.03 s	27.7
19	2.83 ABq (13.2)	51.0	2.35 ABq (11.4)	61.3	2.17 m	61.8
	2.93 ABq (13.2)		2.18 ABq (11.4)		2.52 m	
21	—	—	2.53 m, 2.62 m	49.3	2.04 m, 2.53 m	48.6
22	—	—	1.14 t (7.2)	13.3	1.12 t (7.2)	13.2
Ac	—	171.1	—	171.3	—	170.8
	2.01 s	21.9	1.97 s	22.0	2.09 s	21.9

a. Data are based on DEPT, ^{1}H-^{1}H COSY, HMQC and HMBC experiments. Spectra were recorded in $CDCl_3$.
b. ^{1}H NMR (600 MHz, δ, J in Hz in parentheses), ^{13}C NMR (150 MHz, δ).
c. ^{1}H NMR (400 MHz, δ, J in Hz in parentheses), ^{13}C NMR (100 MHz, δ).

EXPERIMENTAL

General experimental procedure. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. 1D and 2D NMR spectra were recorded on a Bruker AV 600 or a Bruker AV 400 NMR spectrometers instrument. IR spectra were obtained with a ThermoFisher Nicolet 6700 spectrometer, KBr pellets in cm^{-1}. HR-ESI-MS were measured using a Q-TOF micro mass spectrometer (Waters, USA). All the stuffing used in column chromatography is silica gel (Qingdao Haiyang Chemical Co., Ltd., China). TLC plates were precoated with silica gel GF254 (Qingdao Haiyang Chemical Co., Ltd., China).

Plant Material. The plants of *A. rotundifolium* Kar. & Kir. were collected from Xinjiang, China, in October 2015, and were identified by Prof. Liang-Ke Song from School of Life Science and Engineering, Southwest Jiaotong University, Sichuan, China, where a voucher specimen (C. Ren & L. Wang 821) is deposited.

Extraction and Isolation. The whole plants of *A. rotundifolium* Kar. & Kir. (2 kg) were extracted with 95% EtOH at room temperature for three times (4 L × 3 days). After removal of the solvent, the extract was suspended in water (3 L) and adjusted to pH 2 with hydrochloric acid solution, and successively extracted with light petroleum (4 × 1 L) and EtOAc (4 × 1 L). Then, 28% aqueous ammonia soln. (2 L) was added to the acidic solution to adjust to pH 10. The solutions were extracted with CH_2Cl_2 (4 × 1 L), and the CH_2Cl_2 extracts were concentrated to produce the crude alkaloid extract (3.5 g). Column chromatography of the crude alkaloid extract over silica gel, using a CH_2Cl_2/MeOH (95:5 → 50:50, *v*/*v*) mixture with increasing polarity afforded fractions (I–VIII). Fractions I, II and V were further purified using an RP-18 silica gel column with MeOH: H_2O (5:100 → 95:5, *v*/*v*) to obtain compounds **1** (15 mg), **2** (3 mg) and **3** (12 mg), respectively. Fractions III and IV were subjected to silica gel column with petroleum ether: Me_2CO: Et_2NH (20:1:1 → 15:6:1, *v*/*v*/*v*) to yield compounds **4** (8 mg) and **5** (18 mg).

Rotundifosine A (**1**)

White amorphous powder; $[\alpha]_D^{20}$ +10.0 (c 0.75, MeOH); HR-ESI-MS *m/z* 376.2138 $[M + H]^+$ (calcd. for $C_{21}H_{30}NO_5$, 376.2124); ^1H and ^{13}C NMR data see Table 1. IR (KBr) ν_{max}: 3354, 2932, 2875, 1735, 1612, 1451, 1383, 1240, 1217, 1082, 1044, 580, 477 cm^{-1}.

Rotundifosine B (**2**)

White amorphous powder; $[\alpha]_D^{20}$ +7.5 (c 0.15, MeOH); HR-ESI-MS *m/z* 404.2445 $[M + H]^+$ (calcd. for $C_{23}H_{34}NO_5$, 404.2437); ^1H and ^{13}C NMR data see Table 1. IR (KBr) ν_{max}: 3441, 2930, 2871, 1743, 1453, 1369, 1240, 1217, 1041, 1008, 956, 895, 701, 609 cm^{-1}.

Rotundifosine C (**3**)

White amorphous powder; $[\alpha]_D^{20}$ +14.2 (c 0.60, $CHCl_3$); HR-ESI-MS *m/z* 420.2391 $[M + H]^+$ (calcd. for $C_{23}H_{34}NO_6$,420.2386); ^1H and ^{13}C NMR data see Table 1. IR (KBr) ν_{max}: 3446, 2926, 2852, 2818, 1716, 1659, 1470, 1456, 1248, 1217, 1044, 935, 871, 740, 688 cm^{-1}.

Cell culture and cytotoxicity assay

The *in-vitro* growth inhibitory activities of compounds **1 - 3** were assayed by the MTT method.[9] The MCF-7, MDA-MB-231 (human breast cancer cell line), 786-0 (human renal carcinoma cell line) and HCT-116 (human colon carcinoma cells) cell lines were obtained from ATCC. The cells were maintained in a growth medium containing DMEM high-sugar medium (containing 10% calf serum, 100 $KU \cdot L^{-1}$ penicillin, and 100 $mg \cdot L^{-1}$ streptomycin). All cells were cultured at 37 °C with 5% CO_2 (*v*/*v*). Cells treated with DMSO (0.1% *v*/*v*) were used as negative controls, while adriamycin (Sigma–Aldrich, Trading Co.,

Ltd., Shanghai, China) was used as the positive control. Cells were seeded in a 96-well plate at 1×10^4 cells per well. After 24 h, cells were treated with compounds **1** - **3** at different concentrations (20 μM, 40 μM, 60 μM, 80 μM, and 100 uM), incubated for 48 h and then compared to the untreated cells. Cell viability was determined with a MTT Cell Proliferating Assay Kit (Promega, China).

ACKNOWLEDGEMENTS

This work was financially supported by the Research Foundation for the Educational Commission of Sichuan Province (15TD0048) and the Fundamental Research Funds for the Central Universities (2682017QY04).

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