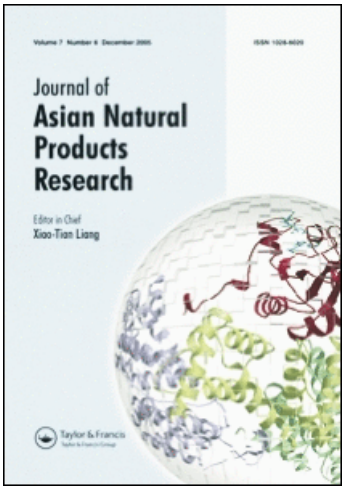

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Two new *neo*-clerodane diterpenoid alkaloids from *Scutellaria barbata* with cytotoxic activities

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Two new *neo*-clerodane diterpenoid alkaloids, scutebarbatine O (**1**) and 6-*O*-nicotinoylscutebarbatine G (**2**), were isolated from the whole plant of *Scutellaria barbata*. Their structures were elucidated by spectroscopic methods including extensive 1D and 2D NMR analyses. *In vitro*, compounds **1** and **2** showed cytotoxic activities against three human tumor cell lines, namely, HONE-1 nasopharyngeal, KB oral epidermoid carcinoma, and HT29 colorectal carcinoma cells, and with IC_{50} values in the range of 2.1–5.7 μM.

Keywords: *Scutellaria barbata* D. Don; *neo*-clerodane diterpenoid alkaloid; scutebarbatine O; 6-*O*-nicotinoylscutebarbatine G; cytotoxic activity

1. Introduction

The traditional Chinese medicinal herb 'Banzhilian', derived from the dry whole plant of *Scutellaria barbata* D. Don (Labiatae), is commonly used for the treatment of tumors, hepatitis, cirrhosis, and other diseases [1]. In our previous phytochemical studies on *S. barbata*, we reported the isolation of *neo*-clerodane diterpenoid alkaloids [2–6], which showed significant cytotoxic activities. As a continuous search for more novel *neo*-clerodane diterpenoid alkaloid, we have further investigated the aerial parts of this species and isolated two new *neo*-clerodane diterpenoid alkaloids, scutebarbatine O (**1**) and 6-*O*-nicotinoylscutebarbatine G (**2**). By means of detailed spectroscopic methods, the structures of two new compounds **1** and **2** were elucidated. In addition, the two new compounds were screened for cytotoxicity against three tumor cell lines (HONE-1 nasopharyngeal, KB oral epidermoid carcinoma, and HT29 colorectal carcinoma cells), with IC_{50} values being in the range of 2.1–5.7 μM. Herein we report on the isolation, structure elucidation, as well as the evaluation of cytotoxic effects of these two new *neo*-clerodane diterpenoid alkaloids.

2. Results and discussion

Compound **1** was obtained as white needles, and showed positive response to alkaloid reagents. The molecular formula was established as $C_{32}H_{34}N_2O_8$ by HR-FAB mass spectrum, which displayed a quasi-molecular ion at *m/z* 575.2398 $[M + H]^+$. The IR spectrum showed absorption bands at 3347, 1777, 1740, 1717, 1633, 1604, 1482, and 1388 cm^{-1}, which were assignable to hydroxyl, conjugated carbonyl,

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α,β-unsaturated γ-lactone, and aromatic ring groups. The ^{1}H NMR spectrum of **1** revealed the presence of the following fragments: three tertiary methyl groups at δ 1.09 (3H, s, H-17), 1.28 (3H, s, H-20), and 1.54 (3H, s, H-19); an α,β-unsaturated γ-lactone moiety at δ 5.96 (1H, br s, H-14) and 5.00 (2H, br s, H-16); a double bond with *E*-configuration at δ 6.34 (1H, d, $J = 16.8$ Hz, H-11) and 6.45 (1H, d, $J = 16.8$ Hz, H-12); two nicotinic acid ester moieties at δ 9.08 (1H, br s, H-3′), 8.98 (1H, br s, H-3″), 8.65 (1H, br d, $J = 4.6$ Hz, H-5′), 8.73 (1H, br d, $J = 4.6$ Hz, H-5″), 7.23 (1H, dd, $J = 4.6$, 7.8 Hz, H-6′), 7.31 (1H, dd, $J = 4.6$, 7.7 Hz, H-6″), 8.14 (1H, br d, $J = 7.8$ Hz, H-7′), and 8.03 (1H, br d, $J = 7.7$ Hz, H-7″); a terminal double bond group at δ 4.96 (1H, br s, H_a-18) and 5.03 (1H, br s, H_b-18); and an oxygenated methenyl group at δ 4.58 (1H, dd, $J = 4.8$, 11.7 Hz, H-3). In addition, the ^{1}H–^{1}H COSY experiment revealed two spin systems. The first spin system included the signals of a methine (δ 2.11, 1H, dd, $J = 2.3$, 12.0 Hz, H-10), two methylenes (δ 1.28, 1H, m, H_a-1; 2.15, 1H, m, H_b-1; 1.52, 1H, m, H_a-2; 1.79, 1H, m, H_b-2), and an oxygenated methine (δ 4.58, dd, $J = 4.8$, 11.7 Hz, H-3). Thus, H_a-1 and H_b-1 coupled with the signals of H-10, H_a-2, and H_b-2 which in turn was vicinally coupled with H-3. The latter, together with the crucial long-range correlations observed in the HMBC spectrum of **1** (Figure 1), indicated the presence of the hydroxyl group at C-3. The second spin system was traced from two aliphatic protons on oxygenated carbons at δ 6.29 (1H, d, $J = 9.8$ Hz, H-6) and 5.81 (1H, d, $J = 9.8$ Hz, H-7). Observation of the cross-peaks in the HMBC spectrum from H-6 to C-1′ and from H-7 to C-1″ proved that two nicotinic acid ester moieties were connected to C-6 and C-7, respectively. Based on the above data and comprehensive 2D NMR experiments (^{1}H–^{1}H COSY, HMQC, and HMBC), the structure of **1** was established as shown in Figure 1. The relative stereochemistry of the chiral centers in **1** was resolved by 2D ROESY data. In the ROESY spectrum (Figure 1), the cross-peaks were observed from H-6 to H-10, from H_3-20 to H-7, H_3-17, and H_3-19, as well as from H-7 to H_3-17, H_3-19, and H_3-20. Therefore, H_3-17, H_3-19, H_3-20, and H-7 were on the same molecular plane (α-configuration), while H-6 and H-10 were on the opposite side of the molecular plane (β-configuration). Furthermore, the resonance of H-3 was not enhanced by irradiation of H_3-19, indicating β-configuration of H-3. Thus, based on the above analysis, the structure of compound **1** was confirmed as shown in Figure 2.

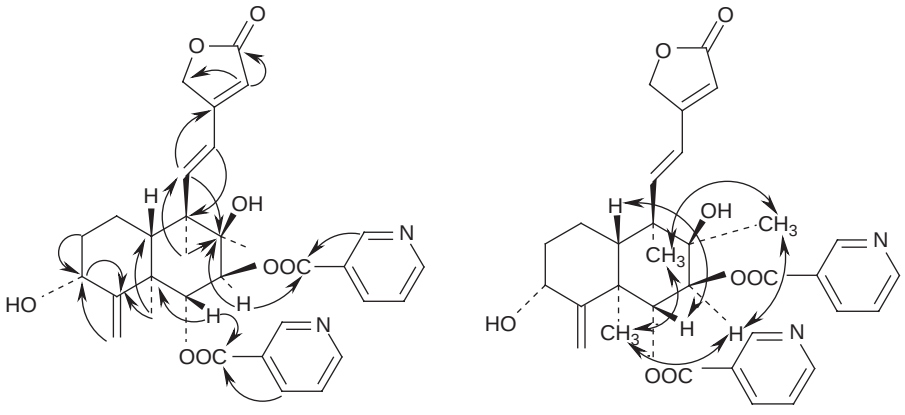

Figure 1. Key HMBC and ROESY correlations of **1**.

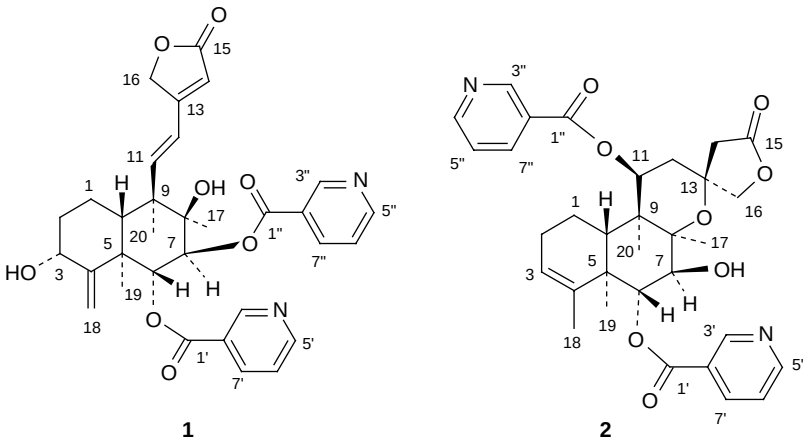

Figure 2. Structures of compounds **1** and **2**.

Compound **2** was isolated and purified as white needles. In the HR-FAB mass spectrum, **2** gave a quasi-molecular ion peak at *m/z* 577.2553 $[M + H]^+$, corresponding to a molecular formula $C_{32}H_{36}N_2O_8$. The IR spectrum showed absorption bands at 3344, 1780, 1735, 1629, 1596, 1500, 1472, and 1388 cm^{-1}, which were assignable to hydroxyl, conjugated carbonyl, aromatic ring, and γ-lactone groups. Comparison of its ^1H and ^{13}C NMR data (Table 1) with those of scutebarbatine G showed that **2** had many spectral features in common with scutebarbatine G [3]. The differences in their NMR spectra could be accounted for the change of attachment of the hydroxyl group. Instead, a nicotinic acid ester moiety (δ_H 9.27, 1H, br s; 8.80, 1H, br d, $J = 4.5$ Hz; 7.43, 1H, dd, $J = 4.5$, 7.6 Hz; 8.35, 1H, br d, $J = 7.6$ Hz; and δ_C 165.6, 126.0, 150.8, 153.7, 123.6, 137.1) was attached to C-6 in **2**. The stereochemical assignments of the chiral centers in **2** were accomplished in the similar manner as described for scutebarbatine G, with H_3-17, H_3-19, H_3-20, H-7, H-11, and H_b-16 being on the α-configuration, while H-6 and H-10 being on the β-configuration (Figure 3).

The two isolated compounds (**1** and **2**) were evaluated for their cytotoxic activities against HONE-1, KB, and HT29 tumor cell lines using methylene blue dye assay and anti-cancer drugs, etoposide and cisplatin [7,8] as positive controls. These compounds exhibited cytotoxicity as shown in Table 2.

3. Experimental

3.1 General experimental procedures

Melting points were measured on an XT-4 micro-melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. UV spectra were obtained on a Shimadzu UV-160 spectrophotometer. IR spectra were recorded on a Perkin-Elmer 683 infrared spectrometer with KBr disks. FAB-MS and HR-FAB-MS were recorded on an Autospec-Ultima ETOF MS spectrometer. NMR spectra were recorded on a Bruker Avance 400 spectrometer at 400 MHz (^1H) and 100 MHz (^{13}C), with TMS as the internal standard. Silica gel (200–300 mesh) for column chromatography and silica gel GF254 for preparative TLC were obtained from Qingdao Marine Chemical Factory, Qingdao, China.

3.2 Plant material

S. barbata D. Don was collected in Linyi District, Shandong Province, China, in

Table 1. ^{1}H and ^{13}C NMR spectral data of compounds **1** and **2** (in $CDCl_3$)[a,b].

	1		**2**	
No.	δ_H	δ_C	δ_H	δ_C
1	1.28 (m, H_a-1), 2.15 (m, H_b-1)	32.1 CH_2	1.65 (m, H_a-1), 2.01 (m, H_b-1)	28.5 CH_2
2	1.52 (m, H_a-2), 1.79 (m, H_b-2)	21.5 CH_2	2.76 (m, 2H)	32.7 CH_2
3	4.58 (dd, 4.8, 11.7)	82.4 CH	5.36 (br s)	120.1 CH
4		151.6 C		143.7 C
5		46.4 C		44.2 C
6	6.29 (d, 9.8)	74.4 CH	5.44 (d, 10.0)	77.1 CH
7	5.81 (d, 9.8)	76.0 CH	3.71 (d, 10.0)	74.6 CH
8		77.2 C		82.1 C
9		48.5 C		38.4 C
10	2.11 (dd, 2.3, 12.0)	46.0 CH	2.74 (dd, 2.4, 12.2)	43.6 CH
11	6.34 (d, 16.8)	145.5 CH	5.79 (dd, 3.9, 12.0)	72.1 CH
12	6.45 (d, 16.8)	122.2 CH	1.68 (m, H_a-12), 2.03 (m, H_b-12)	29.1 CH_2
13		161.6 C		76.3 C
14	5.96 (br s)	115.6 CH	2.58 (d, 17.0, H_a-14), 2.79 (d, 17.0, H_b-14)	42.4 CH_2
15		173.8 C		174.3 C
16	5.00 (br s, 2H)	70.6 CH_2	4.25 (d, 8.7, H_a-16), 4.44 (d, 8.7, H_b-16)	79.5 CH_2
17	1.09 (s, 3H)	22.5 CH_3	1.12 (s, 3H)	21.8 CH_3
18	4.96 (br s, H_a-18), 5.03 (br s, H_b-18)	102.2 CH_2	1.69 (s, 3H)	20.4 CH_3
19	1.54 (s, 3H)	17.3 CH_3	1.46 (s, 3H)	16.3 CH_3
20	1.28 (s, 3H)	15.2 CH_3	1.40 (s, 3H)	20.3 CH_3
1′		164.8 C		165.6 C
2′		125.5 C		126.0 C
3′	9.08 (br s)	150.1 CH	9.27 (br s)	150.8 CH
5′	8.65 (br d, 4.6)	153.9 CH	8.80 (br d, 4.5)	153.7 CH
6′	7.23 (dd, 4.6, 7.8)	123.2 CH	7.43 (dd, 4.5, 7.6)	123.6 CH
7′	8.14 (br d, 7.8)	137.1 CH	8.35 (br d, 7.6)	137.1 CH
1″		164.6 C		164.4 C
2″		125.1 C		126.5 C
3″	8.98 (br s)	150.7 CH	9.17 (br s)	150.6 CH
5″	8.73 (br d, 4.6)	153.6 CH	8.83 (br d, 4.5)	153.5 CH
6″	7.31 (dd, 4.6, 7.7)	123.3 CH	7.46 (dd, 4.5, 7.7)	123.5 CH
7″	8.03 (br d, 7.7)	136.9 CH	8.27 (br d, 7.7)	137.4 CH

[a] Coupling constants (in Hz) were presented in parentheses.
[b] The assignments were based on DEPT, HMQC, HMBC, and $^{1}H-^{1}H$ COSY experiments.

September 2006, and identified by Prof. Yan-Yan Zhao, School of Pharmaceutical Science, Yantai University. The whole plants of *S. barbata* were harvested and air-dried at room temperature in the dark. A voucher specimen (YP03063) has been deposited at the Herbarium of School of Pharmaceutical Science, Yantai University.

3.3 Extraction and isolation

The air-dried whole plant (30 kg) of *S. barbata* was finely cut and extracted

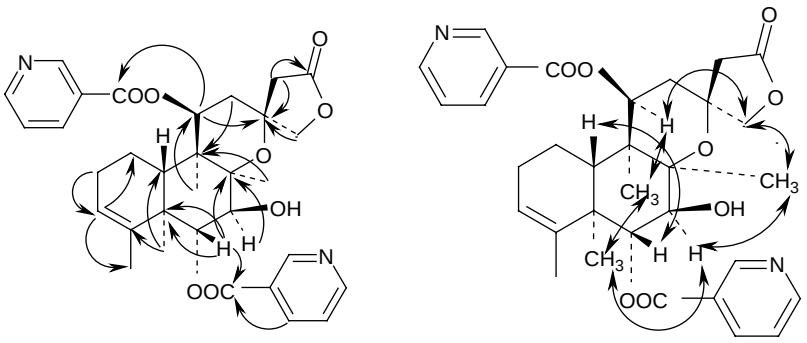

Figure 3. Key HMBC and ROESY correlations of **2**.

Table 2. Cytotoxicity of compounds **1** and **2** against cultured HONE-1, KB, and HT29 cancer cell lines.

	Growth inhibition constant (IC_{50})[a] (μM)		
Compounds	HONE-1	KB	HT29
Etoposide[b]	1.1 ± 0.7	1.3 ± 0.2	1.6 ± 0.6
Cisplatin[b]	2.3 ± 1.5	2.8 ± 0.9	3.9 ± 1.2
1	2.9 ± 1.5	2.2 ± 1.8	4.3 ± 2.3
2	3.1 ± 1.4	2.1 ± 3.0	5.7 ± 1.8

[a] IC_{50} is defined as the concentration that resulted in a 50% decrease in cell number and the results are means ± SD of the three independent replicates. The IC_{50} greater than 10 μM was considered to be no cytotoxicity.
[b] Positive control substance.

three times with refluxing EtOH. Evaporation of the solvent under reduced pressure provided the ethanolic extract. The extract (1.2 kg) was dissolved and suspended in H_2O, and partitioned with $CHCl_3$ and EtOAc. The $CHCl_3$ fraction (179.8 g) was subjected to extraction with 3% HCl. Following this, the aqueous solution was adjusted with NH_4OH to pH 10 and extracted with $CHCl_3$. The organic fractions were combined, and the solvent was evaporated under vacuum to yield the $CHCl_3$ alkaloidal fraction (48.3 g). The alkaloidal fraction (47.1 g) was initially subjected to column chromatography on silica gel, eluted with cyclohexane–acetone (95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 60:40, and 50:50) to give eight fractions. Fraction 7 (2.4 g) was separated by reversed-phase silica gel (150 g, 40–50 μm) column chromatography (eluted by MeOH–H_2O, 55:45, v/v) and purified on Sephadex LH-20 (100 g, eluting with $CHCl_3$–CH_3OH, 10:40, v/v) to afford **1** (33 mg) and **2** (28 mg).

3.3.1 *Scutebarbatine O (**1**)*

White needles; mp 150–151°C, $[\alpha]_D^{29}$ −107.3 ($c = 0.13$, $CHCl_3$). UV ($CHCl_3$) λ_{max} 220 and 260 nm. IR (KBr) ν_{max} 3347, 1777, 1740, 1717, 1633, 1604, 1482, and 1388 cm^{-1}. FAB-MS *m/z* 575.3 $[M + H]^+$. HR-FAB-MS *m/z* 575.2398 $[M + H]^+$ (calcd for $C_{32}H_{35}N_2O_8$, 575.2393). For ^1H and ^{13}C NMR spectral data, see Table 1.

3.3.2 *6-O-nicotinoylscutebarbatine G (**2**)*

White needles; mp 151–153°C, $[\alpha]_D^{29}$ −87.6 ($c = 0.14$, $CHCl_3$). UV ($CHCl_3$) λ_{max} 221 and 259 nm. IR (KBr) ν_{max} 3344, 1780, 1735, 1629, 1596, 1500, 1472, and

1388 cm^{-1}. FAB-MS *m/z* 577.4 $[M + H]^+$. HR-FAB-MS *m/z* 577.2553 $[M + H]^+$ (calcd for $C_{32}H_{37}N_2O_8$, 577.2550). For ^1H and ^{13}C NMR spectral data, see Table 1.

3.4 Antitumoral cytotoxic bioassays

Human nasopharyngeal carcinoma HONE-1, oral epidermoid carcinoma KB, and colorectal carcinoma HT29 cells were maintained in RPMI-1640 medium supplied with 5% fetal bovine serum. Cells in logarithmic phase were cultured at a density of 5000 cells/ml per well in a 24-well plate. The cells were exposed to various concentrations of the tested drugs for 72 h. The methylene blue dye assay was used to evaluate the effects of the tested drugs on cell growth, as described previously [9]. The IC_{50} value resulted from 50% inhibition of cell growth was calculated graphically as a comparison with the control.

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