New *neo*-Clerodane Diterpenoid Alkaloids from *Scutellaria barbata* with Cytotoxic Activities

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Four new *neo*-clerodane diterpenoid alkaloids, named scutebarbatines I—L (1—4), were isolated from the whole plant of *Scutellaria barbata* D. Don. Their structures were established on the basis of detailed spectral analyses. *In vitro*, the four new compounds showed significant cytotoxic activities against three human cancer lines (HONE-1 nasopharyngeal, KB oral epidermoid carcinoma, and HT29 colorectal carcinoma cells), and gave IC_{50} values in the range 3.2—8.3 μM.

Key words *Scutellaria barbata*; Labiatae; *neo*-clerodane diterpenoid alkaloid; scutebarbatine; cytotoxic activity

In our previous phytochemical studies on *Scutellaria barbata* D. Don, we reported the isolation of ten *neo*-clerodane diterpenoid alkaloids, which showed significant cytotoxic activities.[1—3] As a continuous search for more novel *neo*-clerodane diterpenoids, we have further investigated the aerial parts of this species and isolated four new *neo*-clerodane diterpenoid alkaloids, named scutebarbatines I—L (**1**—**4**). By means of detailed spectroscopic methods, the structures of four new compounds, **1**—**4**, were elucidated. In addition, the four new compounds were screened for cytotoxity 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 3.2—8.3 μM. Herein we report on the isolation, structure elucidation, as well as evaluation of the cytotoxic effects of these four new compounds.

Results and Discussion

Compound **1** was isolated as white needles, and showed a positive response to many alkaloid reagents. The molecular formula was established as $C_{30}H_{41}NO_8$ by HR-FAB mass spectrum, which gave a *quasi*-molecular ion at *m*/*z* 544.2919 $[M+H]^+$. The IR spectrum displayed absorption bands at 1726, 1710, 1590, 1478, 1440, 1251, 888 and 730 cm^{-1}, which were indicative of carbonyl and nicotinic acid ester moieties.[1—3] The ^{1}H- and ^{13}C-NMR spectra of **1** exhibited the presence of the following groups: a tertiary methyl (δ_H 1.02, 3H, s, H-20; δ_C 14.3 q, C-20), a secondary methyl (δ_H 0.89, 3H, d, J=6.1 Hz, H-17; δ_C 16.6 q, C-17), an acetoxyl group (δ_H 1.64, s, 3H; δ_C 170.8 s, 21.5 q), an ethoxyl group (δ_H 3.41 1H, m; 3.72, 1H, m; 1.16, 3H, t, J=7.1 Hz; δ_C 63.1 t, 15.4 q), a nicotinic acid ester moiety (δ_H 9.40, 1H, br s, H-3′; 8.87, 1H, br d, J=4.6 Hz, H-5′; 7.43, 1H, dd, J=4.6, 7.8 Hz, H-6′; 8.54, 1H, br d, J=7.8 Hz, H-7′; δ_C 165.3 s, C-1′; 127.1 s, C-2′; 151.6 d, C-3′; 153.7 d, C-5′; 123.5 d, C-6′; 138.2 d, C-7′), a C_4–C_{18} epoxide function (δ_H 2.60, 1H, d, J=4.2 Hz, H_a-18; 2.79, 1H, d, J=4.2 Hz, H_b-18; δ_C 65.2 s, C-4; 48.3 t, C-18), as well as a hexahydrofurofuran moiety (δ_H 4.03, 1H, dd, J=4.3, 12.0 Hz, H-11; 1.56, 1H, m, H_a-12; 1.78, 1H, m, H_b-12; 2.88, 1H, m, H-13; 1.63, 1H, m, H_a-14; 2.24, 1H, m, H_b-14; 5.24, 1H, d, J=4.2 Hz, H-15; 5.72, 1H, d, J=5.4 Hz, H-16; δ_C 83.6 d, C-11; 33.0 t, C-12; 40.4 d, C-13; 38.4 t, C-14; 103.8 d, C-15; 107.4 d, C-16), which were like other *neo*-clerodane derivatives previously isolated from *Scutellaria* plants.[4,5] Based on the above data and comprehensive 2D NMR experiments (^{1}H–^{1}H COSY, HMQC, HMBC), the structure of **1** was established as shown in Fig. 1. The relative stereochemistry of the chiral centers in **1** was resolved by 2D ROESY data. In the ROESY experiment (Fig. 1), the cross peaks were observed from H-10 to H_2-18 and H-6, and from H_3-20 to H-11, H_3-17 and H_2-19. Thus, H_3-17, H_2-19, H_3-20 and H-11 were on the same molecular plane (α-configuration) while H-6, H-10 and H_2-18 were on the opposite side of the molecular plane (β-configuration).

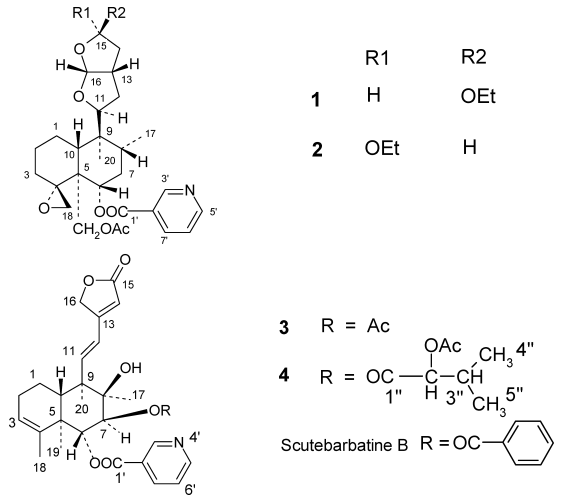

Chart 1. The Structures of Scutebarbatine B and New Compounds Isolated from *Scutellaria barbata*

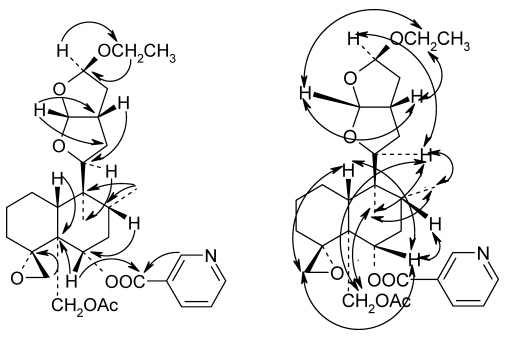

Fig. 1. Key HMBC and ROESY Correlations of **1**

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Table 1. ^{1}H-NMR Data of Compounds **1**—**4** (400 MHz, in $CDCl_3$)[a,b]

Position	1	2	3	4
1	1.41 (m, H_a-1)	1.43 (m, H_a-1)	1.32 (m, H_a-1)	1.33 (m, H_a-1)
	1.90 (m, H_b-1)	1.95 (m, H_b-1)	1.61 (m, H_b-1)	1.62 (m, H_b-1)
2	2.20 (m, 2H)	2.23 (m, 2H)	2.04 (m, 2H)	2.04 (m, 2H)
3	1.14 (m, Ha-3)	1.13 (m, Ha-3)	5.24 (br s)	5.23 (br s)
	1.72 (m, Hb-3)	1.71 (m, Hb-3)		
6	4.75 (dd, 4.4, 11.3)	4.47 (dd, 4.3, 11.2)	5.71 (d, 9.9)	5.78 (d, 9.9)
7	1.48 (m, Ha-7)	1.48 (m, Ha-7)	5.36 (d, 9.9)	5.43 (d, 9.9)
	1.67 (m, Hb-7)	1.69 (m, Hb-7)		
8	1.51 (m)	1.50 (m)		
10	1.74 (dd, 3.2, 11.3)	1.73 (dd, 3.0, 11.8)	2.32 (dd, 1.9, 12.1)	2.28 (dd, 1.8, 12.0)
11	4.03 (dd, 4.3, 12.0)	4.47 (dd, 5.6, 11.2)	6.45 (d, 16.8)	6.41 (d, 16.8)
12	1.56 (m, Ha-12)	1.53 (m, Ha-12)	6.36 (d, 16.8)	6.36 (d, 16.8)
	1.78 (m, Hb-12)	1.77 (m, Hb-12)		
13	2.88 (m)	2.87 (m)		
14	1.63 (m, Ha-14)	1.61 (m, Ha-14)	5.94 (br s)	5.95 (br s)
	2.24 (m, Hb-14)	2.20 (m, Hb-14)		
15	5.24 (d, 4.2)	5.08 (d, 5.6)		
16	5.72 (d, 5.4)	5.79 (d, 5.5)	4.99 (br d, 16.4, H_a-16)	4.98 (br d, 16.3, H_a-16)
			5.02 (br d, 16.4, H_b-16)	5.01 (br d, 16.3, H_b-16)
17	0.89 (d, 6.1)	0.91 (d, 6.1)	1.02 (s, 3H)	1.03 (s, 3H)
18	2.60 (d, 4.2, Ha-18)	2.57 (d, 4.2, Ha-18)	1.55 (s, 3H)	1.54 (s, 3H)
	2.79 (d, 4.2, Hb-18)	2.81 (d, 4.2, Hb-18)		
19	4.58 (d, 11.8, Ha-19)	4.57 (d, 12.0, Ha-19)	1.40 (s, 3H)	1.41 (s, 3H)
	5.27 (d, 11.8, Hb-19)	5.29 (d, 12.0, Hb-19)		
20	1.02 (s, 3H)	0.99 (s, 3H)	1.22 (s, 3H)	1.21 (s, 3H)
3′	9.40 (br s)	9.40 (br s)	9.23 (br s)	9.22 (br s)
5′	8.87 (br d, 4.6)	8.86 (br d, 4.5)	8.81 (br d, 4.5)	8.78 (br d, 4.6)
6′	7.43 (dd, 4.6, 7.8)	7.43 (dd, 4.5, 7.7)	7.43 (dd, 4.5, 7.8)	7.40 (dd, 4.6, 7.7)
7′	8.54 (br d, 7.8)	8.51 (br d, 7.7)	8.28 (br d, 7.8)	8.29 (br d, 7.7)
2″				4.90 (d, 3.3)
3″				1.27 (m)
4″				0.48 (3H, d, 6.8)
5″				0.79 (3H, d, 6.8)
OAc	1.64 (s, 3H)	1.64 (s, 3H)	1.84 (3H, s)	2.16 (3H, s)
OEt	3.41 (1H, m)	3.46 (1H, m)		
	3.72 (1H, m)	3.78 (1H, m)		
	1.16 (3H, t, 7.1)	1.18 (3H, t, 7.1)		

a) Chemical shift values are in ppm and *J* values (in Hz) are presented in parentheses. *b*) The assignments were based on HMQC, HMBC, and ^{1}H–^{1}H COSY experiments.

Detailed examination of the ROESY spectrum indicated important information about the stereochemistry of the hexahydrofurofuran moiety. H-16 showed NOE cross peaks with H-(OEt) and H-13. Moreover H-11 displayed NOEs with H-15, H_3-17, H_2-19 and H_3-20. These data clearly established the stereochemistry of the hexahydrofurofuran side-chain in **1**, and confirmed the β-configuration of the ethoxyl group. This proposal was reinforced by the absence of significant NOEs between H-15 and any other protons such as H-13 and H-16 which were assigned as β-configuration.

Compound **2** was homogenous on TLC and its ^{1}H- and ^{13}C-NMR showed essentially the same signals as those present in the spectra of **1** (Tables 1, 2). In fact the observed differences between these spectra were in the chemical shifts of H-11 ($\Delta\delta$ −0.44 ppm), H-13 ($\Delta\delta$ +0.01 ppm) and H-15 ($\Delta\delta$ +0.16 ppm). The observed differences between the NMR data of **2** and **1** were in agreement with the former being of the epimer of **1**. In the ROESY spectrum of **2**, correlations of H-11 with the OEt protons at C-15 reinforced the α-configuration of the ethoxyl group.

Compound **3** was obtained as white needles and assigned a molecular formula of $C_{28}H_{33}NO_7$ from HR-FAB-MS. Comparison of the NMR spectra of **3** (Tables 1, 2) and scutebarbatine B[1] showed similarities except for the substitution of a benzoyloxy group in scutebarbatine B with an acetoxyl group in **3**. Compound **4** was isolated as white needles and a molecular formula of $C_{33}H_{41}NO_9$ based on its HR-FAB-MS were established. Comparison of its ^{1}H- and ^{13}C-NMR data (Tables 1, 2) with those of **3** showed that **4** had many spectral features in common with **3**. The differences in their NMR spectra could be accounted for by the change of attachment of the acetoxyl group. Instead, a 2-acetoxy-3-methylbutanoyloxy group was attached to C-7 in **4**. The stereochemical assignments of the chiral centers in **3** and **4** were accomplished in a similar manner as that described for scutebarbatine B,[1] with H_3-17, H_3-19, H_3-20 and H-7 being an α-configuration while H-6 and H-10 were a β-configuration.

The four isolated compounds (**1**—**4**) were evaluated for their cytotoxic activities against HONE-1, KB, and HT29 cancer cell lines by using the methylene blue dye assay and the anti-cancer drugs etoposide and cisplatin[6,7] as positive controls. These new *neo*-clerodane diterpenoids exhibited significant cytotoxicity as shown in Table 3.

Experimental

General Experimental Procedures Melting points were measured on an XT-4 micro-melting point apparatus and are uncorrected. Optical rota-

Table 2. ^{13}C-NMR Data of Compounds **1**—**4** (100 MHz, in $CDCl_3$)[a]

Carbon	1	2	3	4
1	25.2 t	25.3 t	19.2 t	19.2 t
2	22.3 t	22.4 t	26.1 t	26.1 t
3	32.4 t	32.9 t	123.4 d	123.5 d
4	65.2 s	65.3 s	140.6 s	140.6 s
5	46.0 s	46.1 s	43.3 s	43.3 s
6	72.1 d	72.2 d	75.4 d	75.2 d
7	33.5 t	33.6 t	76.4 d	77.1 d
8	36.6 d	36.3 d	77.2 s	77.3 s
9	40.3 s	40.2 s	48.1 s	48.1 s
10	48.5 d	48.7 d	42.7 d	42.6 d
11	83.6 d	83.7 d	146.7 d	146.6 d
12	33.0 t	33.1 t	121.9 d	121.8 d
13	40.4 d	40.7 d	162.0 s	161.9 s
14	38.4 t	39.8 t	115.1 d	115.0 d
15	103.8 d	104.1 d	173.9 s	173.8 s
16	107.4 d	109.3 d	70.7 t	70.5 t
17	16.6 q	16.6 q	22.7 q	22.7 q
18	48.3 t	48.4 t	20.1 q	20.1 q
19	62.5 t	62.6 t	17.4 q	17.3 q
20	14.3 q	14.4 q	15.3 q	15.5 q
1′	165.3 s	165.2 s	164.6 s	164.3 s
2′	127.1 s	126.7 s	126.2 s	126.3 s
3′	151.6 d	151.6 d	150.9 d	151.0 d
5′	153.7 d	153.5 d	153.7 d	153.4 d
6′	123.5 d	123.6 d	123.6 d	123.2 d
7′	138.2 d	137.9 d	137.1 d	137.1 d
1″				168.7 s
2″				75.9 d
3″				29.1 d
4″				16.1 q
5″				18.7 q
OAc	170.8 s	170.1 s	170.0 s	171.8 s
	21.5 q	21.3 q	20.5 q	20.6 q
OEt	63.1 t	63.3 t		
	15.4 q	15.3 q		

a) The assignments were based on HMQC, HMBC, and ^{1}H–^{1}H COSY experiments.

Table 3. Cytotoxicity of Compounds **1**—**4** against Cultured HONE-1, KB and HT29 Cancer Cell Lines

Compound	Growth inhibition constant (IC_{50})[a] [μM]		
	HONE-1	KB	HT29
Etoposide[b]	1.1±0.5	1.3±0.7	2.3±0.9
Cisplatin[b]	2.7±0.8	3.1±1.3	3.6±1.4
1	4.2±2.2	4.7±2.7	7.5±2.6
2	4.4±1.9	5.1±1.8	8.3±1.1
3	3.9±2.2	5.5±2.0	5.9±2.7
4	3.2±2.3	5.6±1.3	6.0±1.5

a) IC_{50} is defined as the concentration that resulted in a 50% decrease in cell number and the results are the means±standard deviation of 3 independent replicates. An IC_{50} greater than 10 μM was considered to indicate no cytotoxicity. *b*) Positive control substance.

tions 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 (^{1}H, ^{13}C) spectra were recorded on a Varian Unity BRUKER 400. HPLC separation was performed on a CONSTA METRIC 3200 and a UV detector at 254 nm.

Plant Material *Scutellaria barbata* D. Don was collected in Linyi district, Shandong Province, People's Republic of China, in September 2006, and identified by Professor Yan-yan Zhao of the 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 the School of Pharmaceutical Science, Yantai University.

Extraction and Isolation The air-dried whole plant (30.0 kg) of *Scutellaria barbata* was finely cut and extracted three times with refluxing EtOH. Evaporation of the solvent under reduced pressure provided the ethanolic extract. The extract 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 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, 50 : 50) to give eight fractions. Fraction 5 (3.1 g) was separated by reversed-phase silica gel (150 g, 40—50 μ) CC [eluted by MeOH–H_2O, 55 : 45, v/v], giving **3** (26 mg), **4** (12 mg) and a mixture (56 mg). The mixture was further separated by semipreparative HPLC (Alltech C-18, 250×10 mm, eluted by MeOH–CH_3CN–H_2O, 20 : 20 : 60) to give **1** (19 mg), and **2** (14 mg) in 23 min and 29 min.

Scutebarbatine I (**1**): White needles, mp 150—151 °C, $[\alpha]_D^{29}$ −13.9° (c=0.12, $CHCl_3$). UV ($CHCl_3$) λ_{max}: 221, 255 nm. IR (KBr) ν_{max}: 1726, 1710, 1590, 1478, 1440, 1251, 888, and 730 cm^{-1}. FAB-MS *m/z*: 544.3 $[M+H]^+$. HR-FAB-MS *m/z*: 544.2919 $[M+H]^+$ (Calcd for $C_{30}H_{41}NO_8$, 544.2910). ^{1}H- and ^{13}C-NMR data, see Tables 1, 2.

Scutebarbatine J (**2**): White needles, mp 149—150 °C, $[\alpha]_D^{29}$ −7.7° (c=0.13, $CHCl_3$). UV ($CHCl_3$) λ_{max}: 220, 255 nm. IR (KBr) ν_{max}: 1725, 1710, 1477, 1439, 1248, 890, and 729 cm^{-1}. FAB-MS *m/z*: 544.2 $[M+H]^+$. HR-FAB-MS *m/z*: 544.2923 $[M+H]^+$ (Calcd for $C_{30}H_{41}NO_8$, 544.2910). ^{1}H- and ^{13}C-NMR data, see Tables 1, 2.

Scutebarbatine K (**3**): White needles, mp 155—156 °C, $[\alpha]_D^{29}$ −110.8° (c=0.14, MeOH). UV ($CHCl_3$) λ_{max}: 220, 257 nm. IR (KBr) ν_{max}: 3341, 1776, 1727, 1633, 1592, 1500, 1458, and 1409 cm^{-1}. FAB-MS *m/z*: 496.4 $[M+H]^+$. HR-FAB-MS *m/z*: 496.2341 $[M+H]^+$ (Calcd for $C_{28}H_{33}NO_7$, 496.2335). ^{1}H- and ^{13}C-NMR data, see Tables 1, 2.

Scutebarbatine L (**4**): White needles, mp 153—155 °C, $[\alpha]_D^{29}$ −103.7° (c=0.13, MeOH). UV ($CHCl_3$) λ_{max}: 217, 221 and 256 nm. IR (KBr) ν_{max}: 3443, 1769, 1731, 1628, 1603, 1511, 1450 and 1412 cm^{-1}. FAB-MS *m/z*: 596.3 $[M+H]^+$. HR-FAB-MS *m/z*: 596.2853 $[M+H]^+$ (Calcd for $C_{33}H_{41}NO_9$: 596.2860). ^{1}H- and ^{13}C-NMR data, see Tables 1, 2.

Antitumoral Cytotoxic Bioassays Cytotoxic activities against HONE-1, KB, and HT29 cancer cell lines of the four new compounds were evaluated by methods reported previously.[1—3]

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