Two New Diterpenoids from Cell Cultures of *Salvia miltiorrhiza*

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A new spiroketallactone, *epi*-danshenspiroketallactone A (1**) and a new C18-norditerpenoid, normiltioane (**2**) along with 21 known compounds, were isolated from cell cultures of *Salvia miltiorrhiza*. Their structures were elucidated on the basis of extensive spectroscopic analyses. In the *in vitro* assays, the compounds **9**–**11**, **21**–**23** exhibited the significant antitumor activity with the IC₅₀ ranges of 1.0–8.3 μ M.**

Key words diterpenoid; cell culture; *Salvia miltiorrhiza*

Salvia miltiorrhiza BUNGE (Labiatae), which is well-known as the traditional Chinese medicine “Danshen,” has been used for the treatment of menstrual disorders, menorrhagia, menostasis, insomnia and cardiovascular diseases, particularly angina pectoris and myocardial infarction.^{1–3} Numerous diterpenoid tanshinones have been isolated from *S. miltiorrhiza*, and some of which have been shown to possess various biological and pharmacological properties, including antitumor, antioxidant and antiplatelet aggregation activities.^{4–9} Large-scale exploration of plants causes damage to and destruction of natural resources, whereas *in vitro* plant cell culture may represent sustainable and renewable source of valuable medicinal compounds, including new compounds that have not been isolated from whole plants.^{10–13}

In this context and as part of our search for bioactive compounds, the secondary metabolites produced by *S. miltiorrhiza* cell cultures were systematically investigated, which led to the isolation of two new diterpenoids (Fig. 1), *epi*-danshenspiroketallactone A (**1**) and normiltioane (**2**), together with twenty-one known compounds (Fig. 2). The new structures were elucidated by a detailed analysis of spectroscopic data. Herein, we reported the isolation and structural elucidation of the new compounds, as well as the cytotoxic activity of isolated compounds against five human cancer cell lines.

Results and Discussion

Compound **1** was obtained as white powder, and gave an high resolution-electrospray ionization (HR-ESI)-MS ion peak at m/z 341.1365 [M+H]⁺, corresponding to a molecular formula of C₂₀H₂₀O₅ with eleven degrees of unsaturation. The IR absorption at 1758 and 1739 cm^{−1} suggested the presence of carbonyl group. The ¹H-NMR spectrum showed the

presence of AMX spin system for three consecutive protons at δ 8.87 (1H, d, J =8.4 Hz, H-1), 7.61 (1H, dd, J =8.4, 7.6 Hz, H-2) and 7.46 (1H, d, J =7.6 Hz, H-3), a pair of *ortho*-coupled aromatic doublets at δ 8.29 (1H, d, J =8.4 Hz, H-6) and 7.48 (1H, d, J =8.4 Hz, H-7). In high magnetic field, ¹H-NMR displayed three methyl units at δ 2.74 (3H, s, H₃-18), 1.27 (3H, d, J =6.4 Hz, H₃-17), and 0.60 (3H, t, J =6.8 Hz, H₃-21), two oxygen-bearing methylenes at δ 4.45 (1H, t, J =8.0 Hz, H-14a), 3.94 (1H, t, J =8.8 Hz, H-14b), 3.78 (1H, m, H-20a), 3.66 (1H, m, H-20b), and two methines at δ 3.40 (1H, d, J =9.6 Hz, H-16), and 3.13 (1H, m, H-15). The carbon signals at δ 169.8 and 168.0 in ¹³C-NMR spectrum suggested the presence of ester carbonyls. Comparison of the ¹H- and ¹³C-NMR spectra of **1** with those of *epi*-danshenspiroketallactone (**17**)¹⁴ (reported from *Salvia miltiorrhiza*) indicated that the methylene (δ_{H} 2.67, 2.19, δ_{C} 44.5) at C-16 in **17** were substituted by an ester carbonyl (δ_{C} 169.8) and one ethoxy group (δ_{H} 3.78 and 3.65, δ_{C} 60.9; δ_{H} 0.60, δ_{C} 13.4) in **1**. In the heteronuclear multiple bond connectivity (HMBC) spectrum (Fig. 3), the correlations between C-19 with H-15 and H-16 supported that the ester carbonyl was attached to C-16. The location of ethoxy group was confirmed by HMBC correlation from H-20 to C-19.

The relative configuration of **1** was determined based on nuclear Overhauser effect (NOE) experiment. In the NOE difference spectrum experiment, irradiation of H₃-17 enhanced H-16, indicating that H-16 and H₃-17 were *syn*-oriented. Furthermore, no enhancement of H-7 when H-16 irradiated suggested the *opp*-orientation of the H-16 and benzene ring (Fig. 4). The absolute configuration of **1** was established by circular dichroism (CD) spectrum. In a previous paper,¹⁵ it was reported that the *S* configuration of chiral center (C-13) in five-membered α,β -unsaturated lactone ring exhibits positive

Fig. 1. The Structures of Compounds **1**, **2**, and **2a**

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Fig. 2. The Structures of Compounds 3–23

Cotton effect at 230–260 nm. Since the negative Cotton effect at 257 nm in the CD spectrum (see Fig. S7) of **1** indicated the 13*R* configuration. Thus, the absolute configuration of **1** was 13*R*, 15*R*, 16*R*. Hence, the structure of **1** was established as

shown in Fig. 1 and named to be *epi*-danshenspiroketalactone **A**. It might be an artificial product resulting from the process of the extraction/purification.

Compound **2** was isolated as white powder, and its

Fig. 3. Key HMBC Correlations of Compounds **1** and **2**

Fig. 4. Key NOEs of Compound **1**

molecular formula was established as $C_{18}H_{26}O_2$ by HR-ESI-MS at m/z 275.1994 $[M+H]^+$ (calcd for $C_{18}H_{27}O_2$, 275.2006). The IR spectrum showed the absorption bands for hydroxyl (3400 cm^{-1}), carbonyl (1713 cm^{-1}) groups and double bonds (1622 cm^{-1}). The ^{13}C -NMR and the distortionless enhancement by polarization transfer (DEPT) spectra indicated 18 carbon resonances, including four methyls, five methylenes, three methines (two olefinic) and six quaternary (one carbonyl, two olefinic, one oxygen-bearing) carbons. The ^1H - and ^{13}C -NMR spectra of **2** were similar to those of 8β -hydroxy-9(11),13-abietadien-12-one (**2a**).¹⁶ The isopropyl group in **2a** was absent, instead of methyl in **2**. Assignments of the proton and carbon signals were based on the detailed analysis of ^1H -NMR, ^{13}C -NMR, DEPT, correlation spectroscopy (gCOSY), heteronuclear multiple quantum coherence (HMQC) and HMBC spectroscopic data. The 8β configuration of **2** was elucidated by CD spectrum, in which the negative Cotton effect at 257 nm was observed (see Fig. S13).¹⁷ The levorotatory optical activity of **2** further supported the above deduction.^{16–18} Therefore, **2** was identified as shown in Fig. 1, and named normiltioane.

By comparing their physical and spectroscopic data with those reported in the literature or analyzing their 2D-NMR spectral data, the known compounds (Fig. 2) were identified as ferruginol (**3**),¹⁹ 7-dehydroabietanone (**4**),²⁰ sugiol (**5**),²¹ 6α -hydroxysugiol (**6**),²⁰ 6,12-dihydroxyabieta-5,8,11,13-tetraen-7-one (**7**),²⁰ 5,6-dehydrosugiol (**8**),²² dihydrotanshinone I (**9**),²³ cryptotanshinone (**10**),²⁴ dihydroisotanshinone I (**11**),²⁵ 2-isopropyl-8-methylphenanthrene-3,4-dione (**12**),²⁵ dehydromiltirone (**13**),²⁶ cryptoacetalide (**14**),²⁷ epicryptoacetalide (**15**),²⁷ danshenspiroketalactone (**16**),¹⁴ *epi*-danshenspiroketalactone (**17**),¹⁴ 12-hydroxy-6,7-secoabieta-8,11,13-triene-6,7-dial (**18**),²⁸ tanshinketolactone (**19**),²⁹ dihydroneotanshinolactone

(**20**),³⁰ danshenol A (**21**),³¹ danshenol B (**22**),³¹ and danshenol C (**23**).³²

Compounds **1–23** were evaluated for their cytotoxic activities against five human tumor cell lines (HCT-8, Bel-7402, BGC-823, A549 and A2780) with paclitaxel as a positive control. In addition, the cytotoxic activity of tanshinone IIA and tanshinone I, which were major and typical bioactive constituents in *S. miltiorrhiza*, were also tested. Compounds **9–11**, **21–23** showed selective cytotoxicity against HCT-8, BGC-823, A549, and A2780 cell lines (Table 2), which were comparable with those of tanshinone IIA and tanshinone I. The other compounds displayed low anti-tumor activity with $\text{IC}_{50} > 10\text{ }\mu\text{M}$. These results indicated that the carbonyl group might play an important role in their anti-tumor activities.

Experimental

General Experimental Procedures Optical rotations were measured on a Perkin-Elmer Model-343 digital polarimeter. The CD spectra were recorded on a JASCO J-815 spectropolarimeter. IR spectra were acquired on a Nicolet 5700 FT-IR microscope spectrometer (FTIR Microscope Transmission). Also, 1D and 2D NMR spectra were obtained at 300, 400, and 600 MHz for ^1H -NMR and 100 and 150 MHz for ^{13}C -NMR on Mercury-300, Mercury-Plus-400 and Bruker ARX-600 spectrometers. Chemical shifts (δ) are given in ppm, and coupling constants (J) are given in hertz (Hz). ESI-MS data and HR-ESI-MS data were measured using an Agilent Technologies 6520 Accurate Mass Q-TOF LC/MS spectrometer. Column chromatography (CC) was carried out with silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, P. R. China). Semi-preparative HPLC was performed on a Shimadzu HPLC instrument equipped with a Shimadzu RID-10A detector and a Grace Allsphere silica column (250 mm $\times$ 10 mm, i.d., 5 μm) by eluting with mixtures of *n*-hexane and EtOAc or a Grace Adsorbosphere C_{18} column (250 mm $\times$ 10 mm, i.d., 5 μm) by eluting with mixtures of CH_3OH and H_2O . Analytical TLC was carried out on pre-coated silica gel GF₂₅₄ plates (Qingdao Marine Chemical Inc.), and spots were visualized under UV light or by spraying with 10% H_2SO_4 in 90% EtOH followed by heating at 120°C.

Tissue and Cell Culture of *S. miltiorrhiza* Seedlings of *S. miltiorrhiza* (identified by Professor Weihua Zhu, Institute of Materia Medica, Chinese Academy of Medical Sciences) were collected from Hebei Province, People's Republic of China. Young leaves and stems were used as explants to initiate calli. The explants were disinfected by immersion in 70% ethanol for 30 s followed by saturated solution of sodium hypochlorite for 10 min and were washed five times with sterilized water. Next, the explants were aseptically transferred to Murashige and Skoog's (MS) medium supplemented with 0.5 mg/L of 6-benzylaminopurine (6-BA), 0.5 mg/L of α -naphthaleneacetic acid (NAA) and 0.2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D). The pH value of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. The initiated callus cultures were cultivated in 250 mL Erlenmeyer flasks with 100 mL of medium at $(23 \pm 2)^\circ\text{C}$ in the dark and sub-cultured every 2 weeks. A total of 1.2 kg of 2-week-old cell cultures were collected and dried at 60°C for extraction and isolation.

Extraction and Isolation The dried cell cultures of *S. miltiorrhiza* (1.2 kg) were refluxed with 80% EtOH (3 h $\times$ 3),

Table 1. NMR Data of Compounds **1**, **2**, and **17** in CDCl₃^{a)}

No.	1		17	2	
	$\delta_{\text{H}}^{\text{b)}$	$\delta_{\text{C}}^{\text{c)}$	$\delta_{\text{C}}^{\text{b)}$	$\delta_{\text{H}}^{\text{c)}$	$\delta_{\text{C}}^{\text{c)}$
1	8.87 (d, 8.4)	122.1	118.0	1.70 (m) 1.40 (m, overlapped)	37.8
2	7.61 (dd, 8.4, 7.6)	129.0	131.9	1.74 (m) 1.57 (m)	18.6
3	7.46 (d, 7.6)	128.7	128.8	1.42 (m) 1.15 (td, 13.2–4.2)	41.8
4		135.1	135.0		34.2
5		122.0	122.0	0.95 (m)	54.8
6	8.29 (d, 8.4)	131.6	128.4	1.88 (dd, 12.6, 3.0) 1.66 (m)	17.6
7	7.48 (d, 8.4)	119.0	122.0	1.91 (dt, 12.6, 3.0) 1.40 (m, overlapped)	39.6
8		145.7	146.9		69.4
9		128.9	129.2		169.7
10		133.5	133.3		41.4
11		168.0	168.2	5.97 (s)	121.4
12					188.1
13		113.0	113.1		132.4
14	4.45 (t, 8.0) 3.94 (t, 8.8)	75.8	77.5	6.42 (s)	148.4
15	3.13 (m)	37.8	37.8	1.84 (s)	15.0
16	3.40 (d, 9.6)	61.0	44.5		
17	1.27 (d, 6.4)	15.2	18.4		
18	2.74 (s)	19.8	20.2	0.89 (s)	33.5
19		169.8		0.94 (s)	21.8
20	3.78 (m) 3.66 (m)	60.9		1.35 (s)	20.3
21	0.60 (t, 6.8)	13.4			

a) Chemical shift values were in ppm and *J* values (in Hz) were presented in parentheses. b) NMR data in 400 MHz for ¹H and 100 MHz for ¹³C. c) NMR data in 600 MHz for ¹H and 150 MHz for ¹³C.

Table 2. The Cytotoxicity of the Isolated Compounds

Compound ^{a)}	IC ₅₀ (μM)			
	HCT-8	BGC-823	A549	A2780
9	>10	2.2	2.4	>10
10	3.9	8.3	2.6	>10
11	>10	>10	2.7	>10
21	3.8	1.0	2.1	>10
22	3.9	3.5	1.0	4.6
23	3.7	3.7	1.4	5.2
Tanshinone IIA	2.3	2.1	1.4	2.8
Tanshinone I	>10	3.8	1.7	>10
Paclitaxel ^{b)}	3.6	0.04	1.0×10 ⁻³	0.9

a) Compounds **1–8**, **12–20** were inactive against all cell lines tested (IC₅₀>10 μM). b) Positive control.

and the crude extract (370 g) was successively fractionated with petroleum ether (60–90°C), EtOAc, and *n*-BuOH. The petroleum ether fraction (41 g) was initially subjected to silica gel column (400 g, 200–300 mesh, 6×60 cm) chromatography eluting with a gradient of petroleum ether in acetone to provide 8 fractions. Fraction 1 (7.1 g) was submitted to vacuum-pressure silica gel column (210 g, 200–300 mesh, 4.5×40 cm) chromatography and eluted with petroleum ether–EtOAc (100:0–80:20, v/v) to obtain six fractions (1.1–1.6). Fraction 1.2 (2.5 g) was subjected to vacuum-pressure silica gel column chromatography eluting with petroleum ether–EtOAc (100:0–85:15, v/v) and further purified by semi-preparative reversed phase HPLC to yield **3** (325 mg), **4** (7 mg), **6** (8 mg) and **20** (2 mg). Fraction 1.3 (1.0) was separated by semi-preparative RP-HPLC to obtain **1** (1.6 mg), **11** (5 mg), **12** (22 mg). Fraction 1.5 (1.3 g) was purified by using semi-preparative RP-HPLC and CC over Sephadex LH-20 to yield **7** (3 mg), **13** (6 mg), **14** (14 mg), **15** (7 mg), **16** (15 mg), **17** (5 mg), **18** (12 mg).

Fraction 3 (3.0 g) was achieved by using semi-preparative normal phase HPLC to yield **5** (120 mg). Fraction 5 (5.8 g) was subjected to silica gel column chromatography and eluted with a gradient of petroleum ether in acetone to give seven fractions (5.1–5.7). Fraction 5.2 (1.2 g) was further purified by semi-preparative RP-HPLC to afford **2** (0.8 mg), **10** (290 mg), **19** (4 mg), **22** (2 mg). Fraction 5.4 (1.5 g) was isolated by semi-preparative RP-HPLC to yield **8** (4 mg), **9** (80 mg), **21** (110 mg), **23** (48 mg).

epi-Danshenspiroketallactone A (1) White powder; [α]_D²⁵ –55.0 (*c* 0.08, CHCl₃); CD (CHCl₃) λ_{max} ($\Delta\epsilon$): 257 (–3.84), 309 (–1.17) nm; IR ν_{max} : 2971, 2969, 2876, 1758, 1739, 1583, 1469, 1207, 1137, 1030, 933 cm⁻¹; ¹H- and ¹³C-NMR data, see Table 1; ESI-MS *m/z*: 339.1 [M–H][–]; HR-ESI-MS *m/z*: 341.1365 [M+H]⁺ (calcd for C₂₀H₂₁O₅, 341.1384).

Normiltioane (2) White powder; [α]_D²⁵ –133.5 (*c* 0.04, CHCl₃); CD (CHCl₃) λ_{max} ($\Delta\epsilon$): 257 (–4.36) nm; IR ν_{max} : 3400, 2962, 2925, 2851, 1713, 1668, 1622, 1457, 1371, 1265,

1013 cm⁻¹; ¹H- and ¹³C-NMR data, see Table 1; ESI-MS *m/z*: 275.1 [M+H]⁺, 297.1 [M+Na]⁺, 273.2 [M-H]⁻; HR-ESI-MS *m/z*: 275.1994 [M+H]⁺ (calcd for C₁₈H₂₇O₂, 275.2006).

Cytotoxic Bioassays HCT-8 (human colon cancer cell line), Bel-7402 (human hepatoma cancer cell line), BGC-823 (human gastric cancer cell line), A549 (human lung cancer cell line), and A2780 (human ovarian cancer cell line) were obtained from ATCC. All six tumor cell lines were maintained in the RPMI 1640 medium containing 10% fetal bovine serum supplemented with L-glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Cultures were incubated at 37°C in 5% CO₂ in air. HCT-8, Bel-7402, BGC-823, A549 and A2780 cells (1.5×10³) were seeded in 96-well tissue culture plates, and 100 μL of cell suspension was placed in each well. After 24 h, 100 μL of dimethyl sulfoxide (DMSO) solution containing the test compounds was added to give final concentrations of 0.01–10 μmol/mL; 100 μL of DMSO was added into control wells. The cells were treated with various concentrations of the test compounds for 96 h, and then cell growth was evaluated by an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay procedure. A 100 μL aliquot of 0.5 mg/mL MTT in RPMI 1640 was added to every well, and the plate was reincubated in 5% CO₂ in air for 4 h at 37°C. The plate was then centrifuged to precipitate cells and formazan. An aliquot of 150 μL of DMSO was added to dissolve the formazan crystals. The plate was mixed on a microshaker for 10 min and then read on a microplate reader at 570 nm. All compounds were tested at six concentrations, and each concentration of the compounds was tested in three parallel wells. A dose–response curve was plotted for each compound, and the IC₅₀ value was calculated as the concentration of the test compound resulting in 50% reduction of optical density compared with the positive control (paclitaxel).

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Supplementary Data Selected NMR, MS, IR and CD spectra of new compounds **1** and **2**.

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