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Diterpenoid Constituents of the Roots of *Salvia digitaloides*

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Salvia digitaloides, belonging to the economically and medicinally important genus *Salvia*, is distributed in the northwest of Yunnan Province, People's Republic of China. The roots of this plant were soaked in alcohol by local Tibetans to make a special traditional "red wine", and many local people like to drink this traditional "red wine" to strengthen physical health. To investigate the bioactive diterpenoid constituents of the roots, a detailed phytochemical study was carried out, and 13 diterpenoids including two new norditerpenoids, dihydroneotanshinlactone (**1**) and 16,17-dinorpisferal A (**2**), were isolated. Their structures were established on the basis of detailed spectroscopic analysis. In addition, computational methods were applied to validate the stereochemistry of compound **1**. For the bioassay to inhibit the growth of five tumor cell lines, neotanshinlactone (**3**) exhibited selective cytotoxic activity toward the human breast cancer cell line SK-BR-3, while the other four tanshinones (**4**−**7**) showed significant toxicity effects against all of the tested five cell lines.

KEYWORDS: *Salvia digitaloides*; cytotoxic activity; diterpenoids; stereochemistry

INTRODUCTION

Salvia (including 700−1050 species) is the biggest genus in the economically and medicinally important family Labiatae and is distributed widely in the world (*1*). The diterpenoid constituents of this genus have attracted great interest due to their diverse structures and significant biological activities (*2*−*4*). Also, the polyphenolic constituents of this genus have also been extensively studied (*5*, *6*). *Salvia digitaloides* Diels is widely distributed in the northwest of Yunnan Province, People's Republic of China (*7*). The roots of this plant were used to soak in the liquor by local Tibetans to make a special traditional "red wine", and they like to drink this traditional "red wine" to strengthen their physical health. It is well-known that diterpenoids, especially tanshinones, are the major red pigments of the roots of *Salvia* plant (*2*−*4*). As far as we know, only two diterpenoids have been reported from the roots of this plant recently (*8*). To explore the diterpenoid constituents of the roots of *S. digitaloides* with novel structures and potent bioactivities, an intensive phytochemical investigation was carried out, which resulted to the isolation of two new norditerpenoids, dihydroneotanshinlactone (**1**) and 16,17-dinorpisferal A (**2**), together with 11 known diterpenoids, neotanshinlactone (**3**) (*4*), tanshinone IIA (**4**) (*9*), cryptotansinone (**5**) (*10*), dihydrotanshinone (**6**) (*10*), tanshinone I (**7**) (*9*), przewalskin (**8**) (*11*), 5,6-dehydrosugiol (**9**) (*12*), carnosol (**10**) (*13*), ferruginol (**11**) (*14*), sugiol (**12**) (*15*), and danshenspiroketallactone (**13**) (*16*). It was noteworthy that neotanshinlactone (**3**), featured with a novel seco-abietane norditerpenoid skeleton, has been reported to have significant and selective cytotoxic activity to human breast cancer cell lines (*4*). To the best of our knowledge, dihydroneotanshinlactone (**1**) can be seen as the first natural analogues of **3**. The stereochemistry of **1** was elucidated on the basis of computational study. Neotanshinlactone (**3**) exhibited selective cytotoxic activity toward the human breast cancer cell line SK-BR-3, and another four known tanshinones, tanshinone IIA (**4**), cryptotansinone (**5**), dihydrotanshinone (**6**), and tanshinone I (**7**), showed significant toxicity effects against the tested cancer cell lines. Reported herein are the isolation, structure elucidation, and cytotoxic activity evaluations of these compounds.

MATERIALS AND METHODS

Instrumentation. Optical rotations were measured with an Horiba SEPA-300 polarimeter (Horiba, Kyota). Ultraviolet absorption spectra were recorded by a UV-2401 PC spectrophotometer (Shimadzus, Kyoto). IR spectra were obtained from a Bio-Rad FtS-135 spectrometer (Bio-Rad, California). NMR spectra were measured on a Bruker AV-400 spectrometer with tetramethylsilane as the internal standard. Electron ionization−mass spectrometry (EI-MS) was determined on a Finnigan-4510 spectrometer (ThermoFinnigan, California). Electrospray ionization−mass spectrometry (ESI-MS) and high-resolution (HR) ESI-MS were recorded with an API QSTAR Pulsar 1 spectrometer (Advanced Biomics, Los Angeles, CA). Column chromatography was performed with silica gel 60 (200−300 mesh, Merck), Sephadex LH-20, and reversed-phase C_{18} silica gel (250 mesh, Merck). Precoated thin-layer chromatography (TLC) sheets of silica

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Table 1. ^{13}C and ^{1}H NMR Data of **1** and **2** in CD_3Cl_3[a]

	1		2	
	δ_C mult	δ_H (*J* in Hz)	δ_C mult	δ_H (*J* in Hz)
1α	121.1, d	8.43, d (8.1)	33.8, t	2.87, m
1β				1.18, m
2α	126.8, d	7.49, t (8.1)	19.6, t	1.63, m
2β				1.68, m
3α	129.6, d	7.45, t (8.1)	41.2, t	1.46, m
3β				1.30, m
4	134.5, s		33.8, s	
5	134.3, s		51.8, d	1.65, m
6	120.2, d	7.81, d (8.8)	18.3, t	2.04, m
7	117.9, d	7.62, d (8.8)	29.9, t	2.90, m
8	107.6, s		130.1, s	
9	153.0, s		133.5, s	
10	123.2, s		53.2, s	
11			113.4, d	6.62, s
12	160.6, s		152.7, s	
13	106.4, s		123.9, s	
14	167.7, s		131.7, d	6.87, s
15	34.9, d	3.70, dt (6.2, 6.8)	15.4, q	2.17, br s
16	81.5, t	4.99, t (9.2) 4.45, dd (6.2, 9.2)		
17	18.6, q	1.45, d (6.8)		
18	19.6, q	2.70, br s	32.7, q	0.99, br s
19			20.5, q	0.81, br s
20			201,3, d	9.88, s

[a] ^{1}H at 400 MHz and ^{13}C at 100 MHz.

gel 60 GF254 were used. An Agilent 1100 series instrument equipped with Alltima C_{18} column (4.6 mm × 250 mm) was used for high-performance liquid chromatography (HPLC) analysis, and a semipreparative Alltima C_{18} column (22 mm × 250 mm) was used in the sample preparation.

Plant Material. The roots of *S. digitaloides* were collected in Zhongdian, Yunnan Province, People's Republic of China, in August, 2006. The plant material was identified by Dr. Liu En-De, Kunming Institute of Botany, Chinese Academy of Sciences. A herbarium sample (200605) was deposited in Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and Isolation. The air-dried and powdered roots of *S. digitaloides* (3.0 kg) were extracted with acetone (3 × 10 L, each 2 days) at room temperature. The solution obtained was evaporated under reduced pressure to yield a dark-green residue (50.0 g), which was subjected to column chromatography over silica gel, eluting with a gradient petroleum ether/EtOAc (from 1:0 to 0:1, v/v). Six fractions (A−F) were obtained on the basis of TLC analysis. Compounds **7** (105.0 mg), **5** (210.0 mg), and **4** (168.0 mg) were crystallized from fractions B, C, and D, respectively. Fraction B was subjected to column chromatography over a silica gel column eluted with petroleum ether/$CHCl_3$/EtOAc (9/0.7/0.3) to afford **11** (8.2 mg). Fraction C was chromatographed over silica gel (cyclohexane/EtOAc, 85/15) to give **3** (5.1 mg), **10** (28.6 mg), and **12** (15.3 mg), along with several subfractions (C_1−C_4). Subfractions C_2 and C_3 were finally purified by semipreparative HPLC (MeOH/MeCN/H_2O, 80/5/15) to afford **1** (3.8 mg) and **2** (5.4 mg), respectively. Fraction D was repeatedly chromatographed over silica gel (CH_3Cl/EtOAc, 9/1) to afford five subfractions (D_1−D_5). Subfraction D_2 was further subjected to column chromatography over Lichroprep RP-18 (MeOH/H_2O from 7:3 to 1:0) to give **13** (4.7 mg). Subfraction D_3 was chromatographed on Sephadex LH-20 (CH_3Cl/MeOH, 1/1) to give **8** (23.8 mg) and **9** (6.2 mg). Compound **6** (5.1 mg) was purified employing silica gel (CH_3Cl/EtOAc, 4/1) and HPLC by MeOH/MeCN/H_2O (70/5/25) from the fraction E.

Physical Data for Dihydroneotanshinlactone (*1*). Red powder; $[\alpha]_D^{25}$ + 19.2 ($CHCl_3$, *c* 0.57). IR (KBr): υ_{max} 2965, 2925, 1708, 1639, 1621, 1601, 1571, 1488, 1407, 1385, 1325, 1299, 1174, 1152, 987 cm^{-1}. UV ($CHCl_3$): λ_{max} (log ε) 362.2 (1.42), 284.0 (2.11), 272.8 (2.01), 262.4 (1.86), 246.2 (1.78), 235.0 (1.77) nm. ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see **Table 1**. EI-MS *m*/*z* 267 (14), 266 (67), 251 (100), 223 (19), 207 (40), 195 (22), 128 (21), 97 (13). Positive HR-ESI-MS *m*/*z* 289.0832 [M + Na]$^+$, calcd for $C_{17}H_{15}O_3Na$, 289.0840.

Physical Data for 16,17-Dinorpisferal A (*2*). Yellow powder; $[\alpha]_D^{25}$ + 350.4 ($CHCl_3$, *c* 0.44). IR (KBr): υ_{max} 3424, 2927, 2849, 1699, 1619, 1509, 1459, 1439, 1413, 1367, 1342, 1269, 1243, 1200, 1131, 1037, 1009, 952, 889 cm^{-1}. UV ($CHCl_3$): λ_{max} (log ε) 291.2 (1.00), 218.0 (1.77), 202.2 (1.89) nm. ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see **Table 1**. EI-MS *m*/*z* 272 (19), 255 (11), 243 (100), 201 (17), 187 (25), 173 (46), 161 (43), 147 (34). Positive HR-ESI-MS *m*/*z* 295.1664 [M + Na]$^+$, calcd for $C_{18}H_{24}O_2Na$, 295.1673.

Bioassay. The cytotoxicity of compounds **1**−**7** against HL-60, SMMC-7721, A-549, SK-BR-3, and PANC-1 cell lines was assessed using the MTT method. Cells were plated in 96-well plates 12 h before treatment and continuously exposed to different concentrations of compounds. After 48 h, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (40 μM) was added to each well, which were incubated for a further 4 h. Then, 20% sodium dodecyl sulfate (100 μL) was added to each well. After 12 h at room temperature, the OD value of each well was recorded at 595 nm. The IC_{50} value of each compound was calculated by the Reed and Muench method (*17*).

Computational Section. All calculations were performed by the Gaussian03 program package (*18*). For circular dichroism, ground-state geometries were optimized at the B3LYP/6-31G* and B3LYP/6-31G** levels. Total energies were obtained, and vibrational analysis was done to confirm the minima. TDDFT at the levels of B3LYP/6-31G*, B3LYP/6-31G**, B3PW91/6-31G**, B3LYP/6-311++G**, and B3LYP/aug-cc-PVDZ was employed to calculate excitation energy (in nm) and rotatory strength *R* in the dipole velocity form (R_{vel}) and dipole length form (R_{len}). The calculated rotatory strengths were simulated into an ECD curve by using the Gaussian function. The self-consistent reaction field method (SCRF) with conductor-like continuum solvent model (COSMO) was further employed to perform ECD calculation in methanol solution at B3LYP-SCRF/6-31G*//B3LYP/6-31G* and B3LYP-SCRF/6-31G**//B3LYP/6-31G** levels (*19*−*21*).

For optical rotation, all compounds were obtained in the gas phase at the B3LYP/6-31G* level of theory. The optical rotation values were calculated using B3LYP/6-31G*, B3LYP/6-31+G*, B3LYP/6-311+G-(2d,p), B3LYP/6-311++G (2d,p), B3LYP/6-311++G(2d,2p), and B3LYP/aug-cc-pVDZ theories. The solvation effect was considered using chloroform in the calculations to resemble the experimental conditions. The polarized continuum model (PCM) of Tomasi et al. was used (*22*−*24*).

RESULTS AND DISCUSSION

The root of *S. digitaloides* was extracted with acetone and then chromatographed on silica gel eluted with a gradient petroleum ether/EtOAc. The detailed isolation of the pure compounds is shown in the Extraction and Isolation section. In total, 13 diterpenoids, including two new norditerpenoids, dihydroneotanshinlactone (**1**) and 16,17-dinorpisferal A (**2**) (**Figure 1**), and 11 previously described compounds, neotanshinlactone (**3**), tanshinone IIA (**4**), cryptotansinone (**5**), dihydrotanshinone (**6**), tanshinone I (**7**), przewalskin (**8**), 5,6-dehydrosugiol (**9**), carnosol (**10**), ferruginol (**11**), sugiol (**12**), and danshenspiroketallactone (**13**), were isolated. Their structures were established on the basis of detailed spectroscopic analysis conjugated by comparison with literature values, and computational methods were applied in the stereochemistry validation of compound **1**. The ability of the two new and five known diterpenoids to inhibit the growth of five tumor cell lines was evaluated. Neotanshinlactone (**3**) exhibited selective cytotoxic activity toward the human breast cancer cell line SK-BR-3, and other four known tanshinones, tanshinone IIA (**4**), cryptotansinone (**5**), dihydrotanshinone (**6**), and tanshinone I (**7**), showed significant toxicity effects against the tested cell lines, HL-60, SMMC-7721, A-549, SK-BR-3, and PANC-1.

Compound **1** was obtained as a red powder, and its molecular formula was determined to be $C_{17}H_{14}O_3$ by HR-ESI-MS and from the ^{13}C NMR spectrum (**Table 1**). The IR spectrum displayed bands at 1708 cm^{-1} for lactone carbonyl groups. The ^{1}H NMR spectrum ($CDCl_3$) of **1** displayed two methyl groups at δ_H 2.70 (s) and 1.45 (d, *J* = 6.8 Hz), a methylene at δ_H 4.99 and 4.45 (each 1 H),

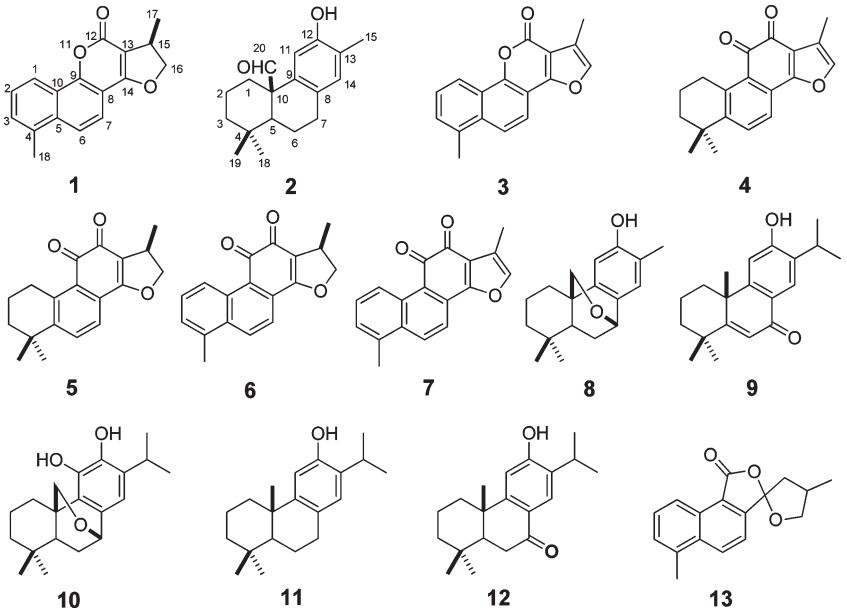

Figure 1. Structures of diterpenoids from *S. digitaloides*.

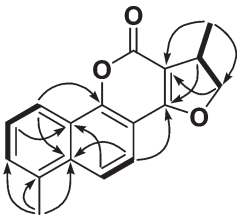

Figure 2. Key HMBC (→) and COSY (—) correlations of **1**.

a methine at δ_H 3.70 (dt, J = 6.2, 6.8), and an ABX pattern for 1,2,3-aromatic protons at δ_H 8.43 (d, J = 8.1 Hz), 7.49 (d, J = 8.1 Hz), and 7.45 (d, J = 8.1 Hz), and an AB pattern for *ortho*-aromatic protons at δ_H 7.81 (d, J = 8.8 Hz) and 7.62 (d, J = 8.8 Hz) for an typical abietane diterpenoid with aromatic A and B rings. The ^{13}C NMR spectrum of **1** exhibited the presence of a conjugated lactone carbonyl group at δ_C 160.6 (C-12), seven olefinic quaternary carbons at δ_C 134.5 (C-4), 134.3, (C-5), 107.6 (C-8), 153.0 (C-9), 123.2 (C-10), 106.4 (C-13), and 167.7 (C-14), one methane at δ_C 34.9 (C-15), one methylene at δ_C 81.5 (C-16), and two methyls at δ_C 18.6 (C-17) and 19.6 (C-18). Careful comparison of the NMR data of **1** with those of neotanshinlactone (**3**) indicated that they are similar to one another except for the signal for the double bond between C-15 and C-16 in **3** being replaced by the signals for a methine (C-15, δ_C 34.9; δ_H 3.70) and methylene (C-16, δ_C 81.5; δ_H 4.99 and 4.45, each 1 H) in **1**, respectively (*4*). This observation indicated that **1** is the 15,16-dihydro derivative of **3**, which was confirmed by the proton spin system (Me-15/H-16/H-17) observed from the ^{1}H−^{1}H correlation spectroscopy (COSY) spectrum and the heteronuclear multiple bond correlation (HMBC) correlations from Me-17 to C-13, C-15, and C-16 as well as the HMBC correlations of H-15/C-14, H-15/C-17, and H-16/C-13 (**Figure 2**).

The rotating frame Overhauser effect spectroscopy (ROESY) spectrum was inefficient to elucidate the stereochemistry of **1**, and a single crystal also cannot be obtained, so a computational chemistry study was carried out to determine the stereochemistry of **1**. The simulated and experimental ECD curves of **1** are shown in **Figure 3**. It is evident that the calculated ECD spectra of **1A** match with its experimental data in the region of 190−400 nm (**Figure 3**). However, for **1B**, the calculated ECD spectra are just the opposite. So, we can conclude that the configuration of **1** is structure **A** but not **B**. We also considered the solvation effect to calculate the ECD of **1A**. It seems that both can match better with the experimental ECD.

It should be emphasized that the absolute configuration of compound **1** determined by computational technique without single crystal may result in the wrong result (*25*). So, we also use additional geometry optimization at a higher level of theory to calculate the optical rotation and get the same conclusion. The computed optical rotation values for both enantiomers are summarized in **Table 2**. From the results, we can see that the B3LYP/aug-cc-pVDZ//B3LYP/6-31G(d) method in the chloroform solvent for prediction of the optical rotation in chiral compound **1** is closest to the experimental results. For **1A**, the calculated optical rotation is 55.76°, and for its enantiomer, **1B** is −56.47°. The former is positive and close to the experimental value of 19.24°, which further suggests that the configuration of **1** is structure **A** but not **B**.

The HR-ESI-MS indicated that **2** has a molecular formula of $C_{18}H_{24}O_2$, which was supported by the ^{13}C NMR spectrum (**Table 2**). In the ^{13}C and DEPT NMR spectra, the typical signals for an A/B rings system of an abietane type diterpenoid at δ_C 33.8 (t, C-1), 19.6 (t, C-2), 41.2 (t, C-3), 33.8 (s, C-4), 51.8 (d, C-5), 18.3 (t, C-6), 29.9 (t, C-7), and 53.2 (s, C-10) were observed. Careful comparison of the NMR data of **2** with those of pisiferal indicated that they are similar to one another except for the signal for isopropyl group (C-15−C-17) in pisiferal being replaced by methyl in **2** (*26*). This observation, in conjunction with the molecular formulas of both pisferal ($C_{20}H_{28}O_2$) and **2** ($C_{18}H_{24}O_2$) indicated that **2** is the 16,17-dinorderivative of pisferal, which was confirmed by the HMBC correlations from Me-15 to C-12, C-13, and C-14 as well as the HMBC correlations of H-14/C-12 and H-14/C-15. For biogenetic reasons, C-20 of **2** is expected to be β-oriented, and H-5 and Me-18 are α-oriented. This was confirmed by the NOE correlations of Me-18/H-3α, H-3α/H-5, Me-18/H-5, H-2β/Me-19, and H-20/Me-19 found in the ROESY spectrum. Accordingly, the structure of **2** (16,17-dinorpisiferal) was established as shown.

Limited by the quantity of the isolates, only seven compounds including the two new (**1** and **2**) and five known ones, neotanshinlactone (**3**), tanshinone IIA (**4**), cryptotanshinone (**5**), dihydrotanshinone (**6**), and tanshinone I (**7**), were tested for their

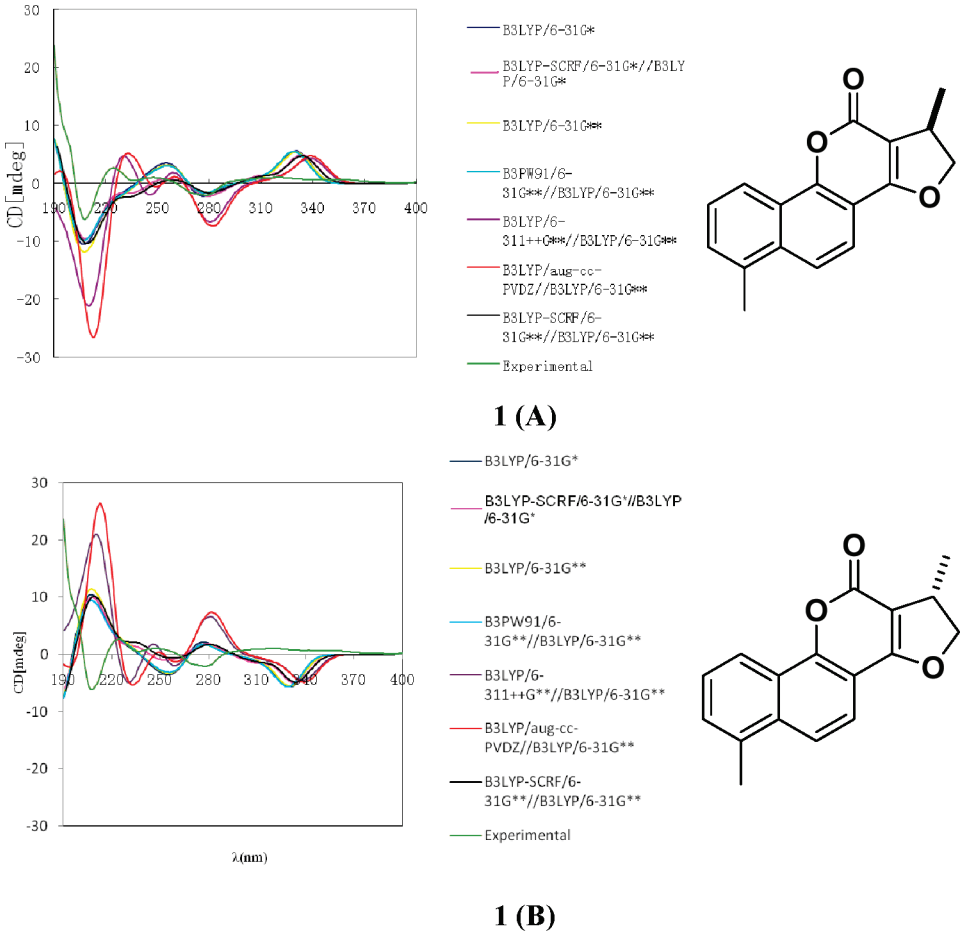

Figure 3. Calculated ECD spectra of **1** at different levels and experimental ECD spectra in the range of 190−400 nm.

Table 2. Computed Optical Rotation Considering the Solvation Effect of **1A** and **1B**

	6-31G*	6-31+G*	6-311+G(2d,p)	6-311++G(2d,p)	6-311++G(2d,2p)	aug-cc-pVDZ	experiment
1A	64.09	67.92	66.75	67.76	68.57	55.76	21.23
1B	−64.50	−68.46	−67.44	−68.37	−69.17	−56.47	

Table 3. Cytotoxicity of the Isolates on Five Cancer Cell Lines with IC_{50} (μM) Values[a]

compound	HL-60	SMMC-7721	A-549	SK-BR-3	PANC-1
1	>40	31.34	>40	>40	>40
2	>40	>40	>40	>40	>40
3	39.04	>40	>40	4.07	>40
4	8.61	14.00	6.39	4.18	>40
5	15.92	13.45	6.27	4.82	39.62
6	3.19	13.88	6.64	3.40	3.67
7	2.36	3.03	5.15	3.97	2.75
cisplatin[b]	1.60	13.59	11.83	19.88	14.36
taxol[b]	<0.008	0.20	0.04	0.008	0.04

[a] Cell lines: HL-60 acute leukemia, SMMC-7721 liver cancer, A-549 lung cancer, SK-BR-3 breast cancer, and PANC-1 pancreatic cancer. [b] Positive control.

cytotoxic effects on five human cancer cell lines HL-60, SMMC-7721, A-549, SK-BR-3, and PANC-1 using the MTT method descript previously (*17*). The result indicated that neotanshinone (**3**) can selectively reduce the viability of cell line SK-BR-3 (**Table 3**), which was consistent with the bioactive studies of **3** by Lee et al. (*4*, *27*−*29*). Meanwhile, the new isolated analogue, dihydroneotanshinlactone (**1**), did not show any cytotoxic effect on the same cell line SK-BR-3, which indicated that the furan ring should be one of the active center to the cytotoxic activity of **3** against breast cancer cell lines. It was also noteworthy that all four tanshinones tested, tanshinone IIA (**4**), cryptotansinone (**5**), dihydrotanshinone (**6**), and tanshinone I (**7**), showed significant toxicity against the five cell lines (**Table 3**), which indicated that the orthoquinone fragment should be one of the active centers for the cytotoxic activity of tanshinone type metabolites.

Previous studies indicated that diterpenoids, especially tanshinones, were the major red pigments of the roots of *Salvia* plant (*2*−*4*). In this study, 13 diterpenoids were isolated, most of which were red pigments. In the bioactive study, most of these isolates showed significant toxicity effects against the tested five cell lines. Our research findings, to some extent, might be responsible for the traditional usage of the roots by local Tibetans.

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