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New *neo*-clerodane diterpenoids with neurotrophic activity from the aerial parts of *Salvia tiliifolia*

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ABSTRACT

Five new *neo*-clerodane diterpenoids, tiliifolins A–E (**1–5**), along with ten known ones, were isolated from the aerial part of *Salvia tiliifolia*. Their structures were proposed based on 1D and 2D NMR spectroscopic data analysis. All new compounds were evaluated for their neurotrophic activity on PC12 cells and cytotoxicity against five human cancer cell lines (HL-60, A-549, SMMC-7721, MCF-7, and SW480), and compound **5** showed statistically significant neuroprotective effect in vitro assay.

1. Introduction

Salvia genus is one of the largest genera of the Lamiaceae family with over 1000 species worldwide. Some members of the genus are used as folk medicine, culinary herb, spice, tea, in perfume and cosmetic industries [1,2]. *Salvia* genus is rich in diterpenoids, especially abietane and clerodane diterpenoids, which has attracted considerable interest within the chemical and biological research fields [3,4]. Among them, clerodane diterpenes possess various pharmacological activities beneficial to humans, including NGF-potentiating [5], antifeedant [6], cytotoxic [7], anti-inflammatory [8], antibacterial [9], and κ-opioid receptor agonistic activities [10].

Salvia tiliifolia, an alien species, which had been incorrectly identified as *S. dugesii* (a synonym of *S. melissodora*) in China, belonging to the Lamiaceae family, is mainly distributed in west of Guizhou, south-east of Tibet, west of Sichuan, and all municipalities/autonomous prefectures of Yunnan Province [11]. Our previous study on the title plant led to the isolation of seven new *neo*-clerodane diterpenoids, dugesins A-G (2) [12,13]. With the aim of searching for useful metabolites from this invasive plant and continuing our systematic studies of this genus [14–17], five new *neo*-clerodane diterpenoids were obtained, representing two 5,6-seco-clerodanes (**1** and **2**) with a furofuran moiety, one clerodane-17,12:18,19-diolides (**3**), and two rearranged clerodane diterpenoids (**4** and **5**) with salvigenane skeleton [18], along with ten known compounds, isopuberulin (**6**) [3], tilifodiolide (**7**), salvifolin (**8**) [4], salvifaricin (**9**) [19,20], salviandulin E (**10**) [21], 6,7-dehydrodugesin A (**11**) [22], puberulin (**12**) [3], dugesin B (**13**) [13], salyunnanins I (**14**) [23], dugesin F (**15**) [13].

The structures of these new metabolites were established by spectroscopic methods, especially, 2D NMR techniques and mass spectral data. Moreover, all new compounds were tested for their neurotrophic activity on PC12 cells and cytotoxicity against five human cancer cell lines (HL-60, SMMC-7712, A-549, MCF-7, and SW480). In this paper, we described the isolation, structural elucidation and biological activities of these new compounds.

2. Experimental

2.1. Gernal experimental procedures

IR spectra were run on a Bruker Tensor-27 spectrometer with KBr pellets. UV spectra were recorded using a Shimadzu UV2401PC instrument. Optical rotations were taken on a Horiba SEPA-300 polarimeter. CD spectra were measured on a Chirascan instrument. ESIMS and HRESIMS were collected on an Agilent 1290 UPLC/6540 Q-TOF. 1D NMR and 2D NMR were performed on a Bruker AV 600 or a AV 800 spectrometer. MPLC was performed on a Lisui EZ Purify III System (Shanghai Lisui Chemical Engineering Company, Shanghai, China). An Agilent 1200 series instrument equipped with an Agilent Zorbax SB-C_{18} column (5 μm, 10 mm × 250 mm) was used for high-performance liquid chromatography (HPLC) analysis. Semipreparative and preparative HPLC were performed on an Agilent 1260 chromatography with a Zorbax SB-C_{18} column (9.4 mm × 25 cm) and a Zorbax SB-C_{18} column (21.2 mm × 15 cm), respectively. Column chromatography

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(CC) was performed over MCI gel (CHP 20P, 75–150 μm; Mitsubishi Chemical Corporation, Japan), Sephadex LH-20 (Amersham Pharmacia Biotech, Sweden), silica gel (100–200 or 200–300 mesh; Qingdao Marine Chemical Co. Ltd., Qingdao, China), and compounds were visualized by spraying the dried plates with 8% aqueous H_2SO_4 followed by heating until dryness. Petroleum ether, chloroform, ethyl acetate, acetone, 2-propanol, and methanol were purchased from Tianjing Chemical Reagents Co. (Tianjing, People's Republic of China). PC12 cells, HL-60, A-549, HL-60, MCF-7, SMMC-7721 and SW480 cancer cells were purchased from ATCC (American type culture collection).

2.2. Plant material

The aerial parts of *S. tiliifolia* were collected from Kunming Botanical Garden, Yunnan Province, P. R. China in September 2015, and identified by Prof. Xiao Cheng of Kunming Institute of Botany, Chinese Academy of Sciences. A specimen of this plant was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences (voucher code 201509190P1).

2.3. Extraction and isolation

The aerial parts of *S. tiliifolia* (9 kg) were shade dried, powdered, and extracted three times with acetone (each 24 h) at room temperature. The resulting acetone extract was evaporated to dryness under reduced pressure, affording crude extract (230 g), which was purified by reverse-phase MPLC on an MCI column (7.0 × 46 cm) using a gradient solvent system EtOH-H_2O (50:50 → 100:0, 30 mL/min) to give six fractions (Fr.1-Fr.6). Of these, Fr.1 (4.6 g) was subjected to repeated Sephadex LH-20 CC (MeOH), and then purified by semipreparative HPLC-C_{18} column chromatography using MeCN-H_2O (21:49, 5 mL/min) to afford **1** (2.3 mg, t_R 15.1 min) and **5** (15.5 mg, t_R 19.4 min). Fr.2 (9.8 g) was chromatographed over Sephadex LH-20 CC (MeOH), and then chromatographed on silica gel CC (petroleum ether/$CHCl_3$/2-propanol, 10:80:2) to get three subfractions (Fr.2.1 to Fr.2.3). Fr.2.1 (3.0 g) was further purified by silica gel CC (petroleum ether/$CHCl_3$/2-propanol, 10:85:5), Sephadex LH-20 CC (MeOH), and semipreparative HPLC-C_{18} column chromatography using MeCN-H_2O (33:67, 5 mL/min) to yield **6** (9 mg, t_R 25.4 min) and **14** (7.3 mg, t_R 29.1 min). Then, Fr.2.2 (8.2 g) was successively subjected to silica gel columns (petroleum ether/$CHCl_3$/EtOAc, 5:4:2), and preparative HPLC-C_{18} column chromatography with solvent system MeCN-H_2O (36:64, 5 mL/min) to afford **12** (43.6 mg, t_R 35.5 min), semipreparative HPLC-C_{18} column chromatography with solvent system MeOH-H_2O (45:55, 5 mL/min) to yield **10** (12.4 mg, t_R 39.2 min) and **13** (7.8 mg, t_R 45.3 min). Fr.2.3 (2.6 g) was subjected to silica gel column ($CHCl_3$/acetone, 9:1), and further purified by semipreparative HPLC-C_{18} column chromatography using MeOH-H_2O (47:53) as mobile phase, and silica gel column (petroleum ether/$CHCl_3$/EtOAc, 2:2:1) to afford **11** (5.6 mg) and **15** (8.3 mg). Fr. 3 (22 g) was applied to column chromatography using Sephadex LH-20 (MeOH) to obtain **7** (1.2 g) and **8** (2.5 g). Fr.4 (10 g) was subjected to repeated Sephadex LH-20 CC (MeOH), and then purified by silica gel column (petroleum ether/$CHCl_3$/EtOAc, 3:1:1), and semipreparative HPLC-C_{18} column chromatography using MeCN-H_2O (50:50) as mobile phase to yield **2** (5.7 mg, t_R 35.8 min), **3** (3.2 mg, t_R 39.1 min), **4** (5.8 mg, t_R 45.8 min), and **9** (11.7 mg, t_R 49.3 min).

2.3.1. Tiliifolin A (1)

Colorless, amorphous powder; $[\alpha]_D^{22}$ – 10.4; (c 0.32, MeOH); UV (MeOH) λ_{max} (log ε) 207 (3.48), 254 (3.06), 297 (2.41) nm; IR (KBr) ν_{max} 3426, 2933, 1759, 1634, 1383, 1272, 1063, 1022, 754 and 600 cm^{-1}; ^1H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) *m/z* 395 $[M + Na]^+$; HRESIMS (positive) *m/z* 395.1111 $[M + Na]^+$ (calcd for $C_{20}H_{20}O_7Na$, 395.1101).

2.3.2. Tiliifolin B (2)

Colorless, amorphous powder; $[\alpha]_D^{20}$ – 57.7; (*c* 0.70, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.63), 222 (4.18), 281 (3.35) nm; IR (KBr) ν_{max} 2942, 1764, 1379, 1270, 1150, 1087, 1024, 877, 755 and 599 cm^{-1}; ^1H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) *m/z* 393 $[M + Na]^+$; HRESIMS (positive) *m/z* 393.1320 $[M + Na]^+$ (calcd for $C_{21}H_{22}O_6Na$, 393.1309).

2.3.3. Tiliifolin C (3)

Colorless, amorphous powder; $[\alpha]_D^{22}$ – 3.1; (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 213 (3.24), 374 (1.76) nm; IR (KBr) ν_{max} 3430, 2926, 2856, 1747, 1632, 1383, 1031 and 595 cm^{-1}; ^1H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) *m/z* 395 $[M + Na]^+$; HRESIMS (positive) *m/z* 395.1109 $[M + Na]^+$ (calcd for $C_{20}H_{20}O_7Na$, 395.1101).

2.3.4. Tiliifolin D (4)

Colorless, prism crystals (MeOH); mp 220–221 °C; $[\alpha]_D^{22}$ – 158.7; (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 209 (4.36) nm; IR (KBr) ν_{max} 3449, 2928, 1751, 1296, 1190, 1099, 1018, 974, 874, 760 and 602 cm^{-1}; ^1H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) *m/z* 379 $[M + Na]^+$; HRESIMS (positive) *m/z* 379.1162 $[M + Na]^+$ (calcd for $C_{20}H_{20}O_6Na$, 379.1152).

2.3.5. Tiliifolin E (5)

Colorless, amorphous powder; $[\alpha]_D^{22}$ – 78.1; (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 216 (4.56), 292 (3.78) nm; IR (KBr) ν_{max} 3451, 2957, 1755, 1631, 1506, 1316, 1214, 1021, 875, 742 and 603 cm^{-1}; ^1H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) *m/z* 375 $[M + Na]^+$; HRESIMS (positive) *m/z* 375.0849 $[M + Na]^+$ (calcd for $C_{20}H_{16}O_6Na$, 375.0839).

2.4. X-ray crystallographic analysis of 4

Crystal analysis was performed on a Bruker APEX DUO diffractometer equipped with an APEX II CCD, using Cu Kα radiation. Cell refinement and data reduction were performed with Bruker SAINT. The structure of **4** was solved by direct methods using SHELXS-2014. Refinements were performed with SHELXL-2014 using full-matrix least-squares, with anisotropic displacement parameters for all the non-hydrogen atoms. The H-atoms were placed in calculated positions and refined using a riding model. CCDC number 1561275 for compound **4** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB21EZ, UK; fax: + 44 1223 336,033; e-mail: deposit@ccdc.cam.ac.uk).

X-ray crystallography data of **4**: $C_{20}H_{20}O_6$, M = 356.36, a = 7.58640(10) Å, b = 10.5283(2) Å, c = 10.7982(2) Å, α = 90°, β = 100.15°, γ = 90°, V = 848.97(3) $Å^3$, T = 100(2) K, space group $P21$, Z = 2, μ (CuKα) = 0.856 mm^{-1}, 7787 reflections measured, 2917 independent reflections (R_{int} = 0.0289). The final R_1 values were 0.0282 ($I > 2\sigma(I)$). The final wR (F^2) values were 0.0727 ($I > 2\sigma(I)$). The final R_1 values were 0.0282 (all data). The final wR (F^2) values were 0.0727 (all data). The goodness of fit on F^2 was 1.071. Flack parameter = 0.19(4).

2.5. Neurite outgrowth-promoting activity assay

The neurotrophic activities of the test compounds were examined according to an assay using PC12 cells as reported [24]. Briefly, PC12 cells were maintained in 1640 medium supplemented with 10% horse serum (HS), and 5% fetal bovine serum (FBS), and incubated at 5% CO_2 and 37 °C. Test compounds were dissolved in DMSO. For the neurite outgrowth-promoting activity bioassay, PC12 cells were seeded at a density of 5 × 10^4 cells/mL in 48-well plate coated with poly-L-lysine.

Table 1
^{1}H NMR spectroscopic data of **1–5** (δ in ppm, J in Hz).

No.	1[a]	2[b]	3[c]	4[b]	5[b]
1a	7.74 (d, 7.7)	7.68 (d, 7.7)	2.06 (ddd, 14.3, 4.3, 3.5)	2.26 (ddd, 16.0, 5.9, 3.4)	3.08 (ddd, 16.2, 4.9, 2.5)
1b			1.61 (td, 14.3, 8.6)	1.34 (ddd, 16.0, 12.6, 3.4)	2.50 (ddd, 16.2, 12.7, 4.9)
2a	7.61 (dd, 7.7, 7.5)	7.59 (dd, 7.7, 7.5)	2.44 (dd, 8.6, 3.5)	2.37 (m)	2.71 (m)
2b				2.15 (m)	2.01 (m)
3	7.76 (d, 7.5)	7.74 (d, 7.5)	6.75 (t, 3.5)	6.83 (dd, 7.6, 1.9)	7.35 (dd, 7.6, 2.2)
6a	3.66 (dd, 11.6, 5.3)	1.48 (s)	1.57 (m)	1.99 (ddd, 14.2, 5.0, 3.6)	5.57 (d, 9.1)
6b	3.54 (dd, 11.6, 7.5)		1.43 (tdd, 14.3, 4.2, 1.7)	1.53 (td, 14.2, 4.5)	
7a			1.91 (m)	2.79 (m)	6.82 (d, 9.1)
7b			1.71 (tdd, 14.8, 6.2, 5.3)	2.68 (m)	
8	2.74 (q, 7.2)	2.46 (q, 7.2)	2.89 (d, 5.3)		
10				2.40 (dd, 12.6, 3.4)	
11a	2.91 (dd, 12.9, 5.4)	2.87 (dd, 13.0, 5.4)	2.82 (dd, 16.4, 7.5)		
11b	2.21 (dd, 12.9, 10.8)	2.20 (dd, 13.0, 10.7)	2.34 (dd, 16.4, 1.7)		
12	5.78 (dd, 10.8, 5.4)	5.42 (dd, 10.7, 5.4)	5.79 (dt, 7.5, 1.7)	6.16 (s)	6.71 (s)
13					
14	6.52 (br s)	6.50 (br s)	6.40 (dd, 1.7, 0.8)	6.44 (br s)	6.49 (br s)
15	7.54 (t, 1.6)	7.51 (t, 1.6)	7.66 (t, 1.7)	7.55 (t, 1.7)	7.59 (br s)
16	7.62 (br s)	7.62 (br s)	7.51 (dd, 1.7, 0.8)	7.72 (br s)	7.88 (br s)
17	1.14 (d, 7.2)	1.13 (d, 7.2)			
19a	5.75 (d, 15.8)	5.48 (s)	4.06 (dd, 8.2, 1.8)	4.51 (d, 8.5)	3.80 (d, 8.4)
19b	5.49 (d, 15.8)		3.92 (d, 8.2)	4.12 (dd, 8.5, 0.5)	3.27 (d, 8.4)
20a	6.09 (s)	6.10 (s)	9.91 (s)	0.99 (s)	4.61 (d, 12.9)
20b					4.41 (d, 12.9)
6-OH	4.21 (dd, 7.5, 5.3)				
7-OH/OCH3	5.19 (s)	3.33 (s)			
9-OH				4.57 (s)	
10-OH			5.69 (s)		

[a] Recorded at 800 MHz in acetone-d_6.
[b] Recorded at 600 MHz in acetone-d_6.
[c] Recorded at 800 MHz in DMSO-d_6.

Table 2
^{13}C NMR spectroscopic data of **1–5** (δ in ppm).

No.	1[a]	2[b]	3[c]	4[b]	5[b]
1	132.8	132.6	24.9	23.2	27.8
2	130.2	130.3	22.7	26.4	25.8
3	124.2	124.2	136.3	136.7	139.4
4	127.8	127.3	131.2	139.1	134.0
5	145.1	144.0	47.5	46.8	47.5
6	65.3	20.8	31.0	35.7	130.9
7	107.1	108.7	17.1	21.7	122.4
8	47.0	55.0	36.8	125.2	127.0
9	63.1	62.8	54.7	75.2	131.2
10	144.1	144.9	73.9	55.8	140.4
11	40.3	40.7	25.9	168.8	159.0
12	73.4	74.0	71.0	76.5	76.2
13	127.0	127.8	126.0	123.1	122.5
14	109.9	109.9	109.0	109.9	109.6
15	144.3	144.3	144.8	144.4	145.3
16	140.5	140.5	139.9	143.2	142.9
17	10.4	10.3	173.9	174.1	171.8
18	171.2	171.1	169.1	169.3	168.7
19	70.8	70.5	69.1	69.4	75.3
20	112.5	112.9	203.0	23.9	60.1
7-OCH_3		48.9			

[a] Recorded at 200 MHz in acetone-d_6.
[b] Recorded at 150 MHz in acetone-d_6.
[c] Recorded at 200 MHz in DMSO-d_6.

After 24 h, the medium was changed to that containing 10 μM of each test compounds plus 5 ng/mL NGF, or various concentrations of NGF (50 ng/mL for the positive control, 5 ng/mL for the negative control). The final concentration of DMSO was 0.05%, and the same concentration of DMSO was added into the negative control. After 72 h incubation, the neurite outgrowth was assessed under a phase-contrast microscope. Neurite processes with a length equal to or greater than the diameter of the neuron cell body were scored as neurite bearing cells. The ratio of the neurite-bearing cells to total cells (with at least 100 cells examined/view area; 5 viewing area/well) was determined and expressed as a percentage.

2.6. Cytotoxicity assay

The five cancer cell lines (A-549 lung cancer, HL-60 human myeloid leukemia, MCF-7 breast cancer, SMMC-7721 hepatocellular carcinoma, and SW480 colon cancer) were cultured in DMEM medium containing 10% fetal bovine serum and 100 U/mL penicillin/ streptomycin in a humidified incubator in a 5% CO_2 atmosphere at 37 °C. Cells (3×10^3/well) were plated in 96-well plates in 100 μL of medium, in which the test samples were added at various concentrations. After 48 h incubation, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution (5 mg/mL in phosphate-buffered saline) was added (20 μL/well). The incubation was continued for another 3 h to give a formazan product. The absorbance of the solution was measured at 492 nm using a MULTISKAN FC instrument. Compound concentrations inhibiting 50% of cell growth (IC_{50} values) were calculated by the Reed and Muench method [25].

3. Results and discussion

3.1. Chemistry

Compound **1** was isolated as colorless, amorphous powder, for which the molecular formula was established as $C_{20}H_{20}O_7$ by the HRESIMS peak at m/z 395.1111 $[M + Na]^+$ (calcd for $C_{20}H_{20}O_7Na$, 395.1101), implying 11 degrees of unsaturation. Its IR spectrum showed a strong absorption at 3426 cm^{-1} due to the presence of hydroxy group in the molecule. The ^{13}C NMR spectrum of **1** showed 20 signals (Table 2), which in combination with ^{1}H NMR (Table 1) and HSQC experiments indicated the presence of one trisubstituted aromatic ring (δ_C/δ_H 132.8/7.74, 130.2/7.61, 124.2/7.76, 127.8, 145.1, 144.1), one furan ring (δ_C/δ_H 127.0, 109.9/6.52, 144.3/7.54, 140.5/7.62), one ester carbonyl group (δ_C 171.2), three methylenes [two

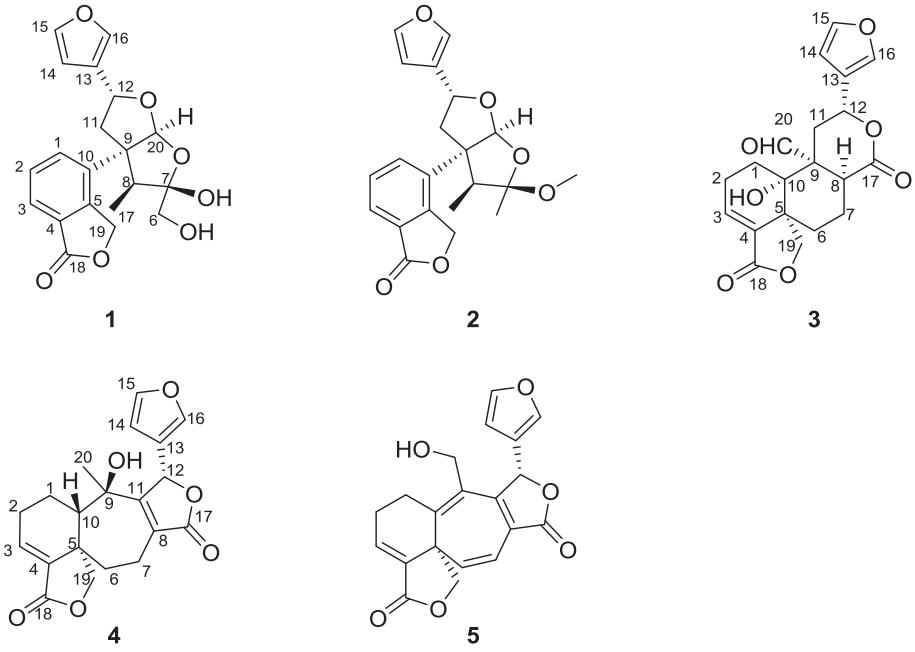


Fig. 1. Structures of the new compounds **1–5**.

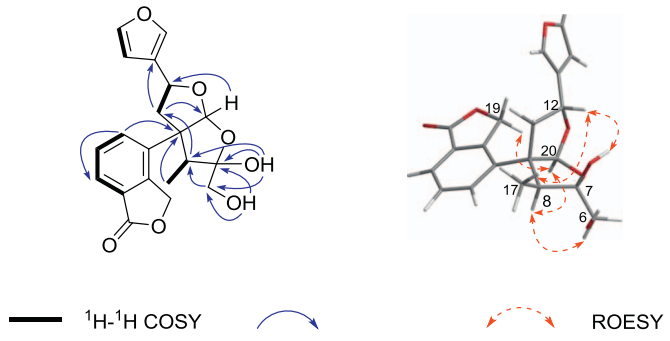


Fig. 2. The key 2D-NMR correlations of compound **1**.

oxygenated methylene (δ_C/δ_H 70.8/5.75, 5.49; 65.3/3.66, 3.54)], three methines [two oxygenated methines (δ_C/δ_H 73.4/5.78; 112.5/6.09)], and one methyl group. The structure of **1** seemed to be closely related to that of rhyacophiline [19] by comparison of their NMR spectroscopic data and mass spectra (Fig. 1). The molecular formula of **1** differs from that of rhyacophiline by two more oxygen atoms, suggesting that it is a dihydroxylated derivative of rhyacophiline, which was supported by the existence of one additional oxygenated methylene (δ_C 65.3) and one oxygenated quaternary carbon (δ_C 107.1) in **1** instead of one methyl group (δ_C 19.2) and one methine (δ_C 82.9) in the latter, respectively. The location of hydroxymethyl group at C-7 was confirmed by the HMBC correlations of OH-6 to C-6, C-7 and H-6 to C-7, C-8 (Fig. 2). The additional hydroxy group was also located at C-7 by the HMBC correlations of OH-7 to C-6, C-7 and C-8. To our knowledge, compound **1** was the fourth 5,6-secoclerodane skeleton diterpene with an aromatic A ring except the previously reported ones, rhyacophiline [26], 7-*epi*-rhyacophiline [27] and 7,8-didehydrorhyacophiline [28]. The ROSEY correlations of H-12 with OH-7 and CH_3-17 indicated that OH-7, H-12 and CH_3-17 were β-orientated, as well as the α-orientation of H-8; the correlation of H-20 with H-8 indicated that H-20 were α-orientated; the observed NOE between H_2-19 and H-20 revealed the *cis* connection of the furofuran moiety [28]. The above results are in accordance with structure **1** for the diterpene under study, which showed the 5,6-secoclerodane skeleton typical of *Salvia* species. Hence, the structure of **1** was elucidated as tiliifolin A.

Compound **2** was isolated as colorless, amorphous powder. The IR spectrum indicated that **2** had an ester carbonyl group (1764 cm^{-1}). Its HRESIMS peak at *m/z* 393.1320 $[M + Na]^+$ (calcd, 393.1309) established a molecular formula of $C_{21}H_{22}O_6$, which is 30 mass units more than that of rhyacophiline [26] (Fig. 1), suggesting the present of an additional methoxy group (δ_H/δ_C 3.33/48.9) (Tables 1 and 2) at C-7 in **2**, as supported by the HMBC correlations from OMe-7 (δ_H 3.33) to C-7 (δ_C 108.7), together with the correlations from H-6, H_3–17 and H-20 to C-7. As the fifth new diterpene with 5,6-secoclerodane skeleton, the planar structure of compound **2** was confirmed. The relative configurations at C-7, C-8, C-9, C-12 and C-20 of **2** were determined to be the same as those of rhyacophiline on the basis of ROESY correlations of CH_3–6/H-8, H-8/H-20, H-20/H_2-19; CH_3-17/H-12, H-12/OMe-7. Thus, the structure of **2** (tiliifolin B) was established as shown.

According to its HRESIMS data, compound **3** established a molecular formula of $C_{20}H_{20}O_7$, implying 11 degrees of unsaturation. Analysis of its 1D NMR spectra (Tables 1 and 2) showed the presence of six methylenes (one oxygenated at δ_C 69.1), seven methines (one oxygenated at δ_C 71.0, one aldehyde group at δ_C 203.0) and seven quaternary carbons (two ester carbonyl groups at δ_C 173.9 and 169.1). The ^{1}H and ^{13}C NMR spectroscopic data of **3** were in close correspondence to those of *ent*-(5*R*,9*R*)-15,16-epoxy-10*S*-hydroxycleroda-3,13(16),14-triene-17,12*S*;18,19-diolide [29], except for the presence of one aldehyde group (δ_H/δ_C 9.91/203.0) in **3** instead of the methyl group (δ_H/δ_C 1.27/27.3) in the latter. The HMBC experiment indicated the presence of the aldehyde group at C-9, which was supported by the correlations from H-20 (δ_H 9.91) to C-8 (δ_C 36.8) and C-9 (δ_C 54.7), H_2-11 (δ_H 2.82 and 2.34) to C-9 and C-20 (δ_C 203.0). The ROESY correlations of OH-10 with H-19a and H-20, H-20 with H-8 indicated that OH-10, Ha-19, H-20 and H-8 were α-orientated; the correlations of H-8 with H-14 and H-16 indicated that H-12 was β-orientated in Fig. 3. Therefore, the relative configuration of **3** was identical to those of *ent*-(5*R*,9*R*)-15,16-epoxy-10*S*-hydroxycleroda-3,13(16),14-triene-17,12*S*;18,19-diolide, the structure of **3** was established, and it was named tiliifolin C.

Compound **4** was obtained as colorless, prism crystals. Its HRESIMS showed a peak at *m/z* 379.1162 $[M + Na]^+$ (calcd for $C_{20}H_{20}O_6Na$, 379.1152), which in conjunction with the ^{13}C NMR data, corresponding to a molecular formula of $C_{20}H_{20}O_6$. The IR spectrum showed absorptions for a hydroxy and a carbonyl groups at 3449 and 1751 cm^{-1}, respectively. The 1D NMR spectra (Tables 1 and 2) was indicative of a

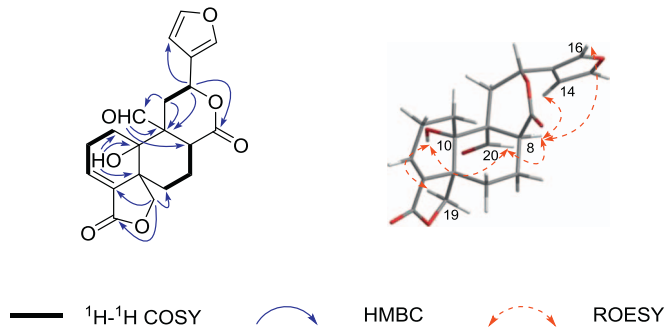


Fig. 3. The key 2D-NMR correlations of compound **3**.

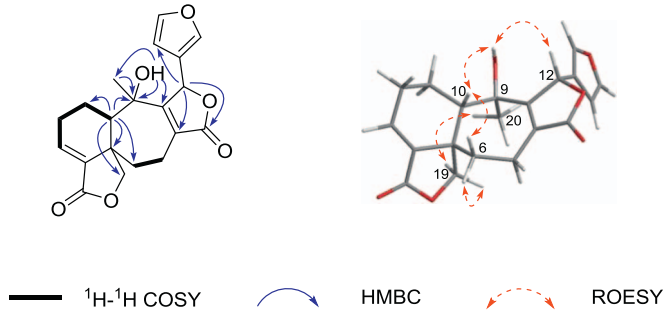


Fig. 4. The key 2D-NMR correlations of compound **4**.

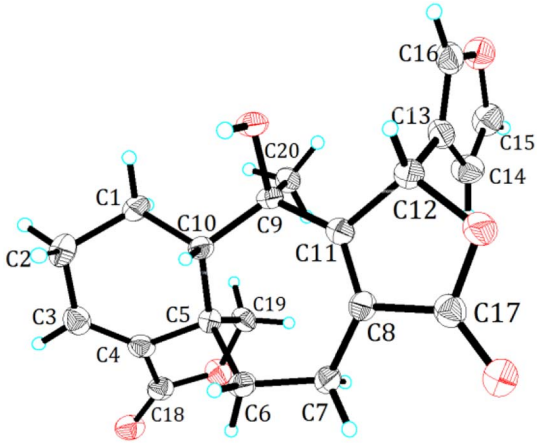


Fig. 5. X-ray crystallography structure of **4**.

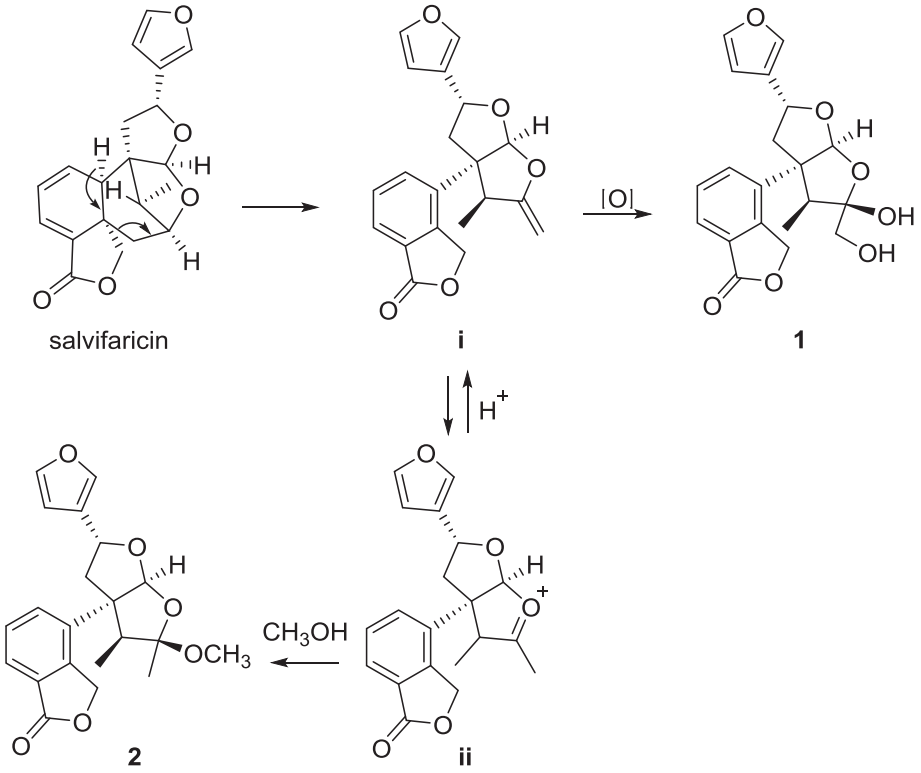
clerodane skeleton, as it showed 20 resonances that were attributed to one methyl, five methylenes (one oxygenated at δ_C 69.4), six methines (one oxygenated at δ_C 76.5), and eight quaternary carbons [30]. These data showed typical signals for a β-substituted furan ring [δ_H 6.44, (d, J = 0.9 Hz, H-14); δ_H 7.55, (t, J = 1.7 Hz, H-15; δ_H 7.72), (s, H-16)], two carbonyl groups at δ_C 174.1 and 169.3, and four olefinic carbons (δ_C 168.8, 139.1, 136.7, 125.2). Carefully analysis of the NMR spectral data indicated that the 1D NMR data of **4** was closely similar to those of dugesin D [13]. The main differences were the presence of an additional methylene (δ_C/δ_H 35.7/1.99, 1.53) and an additional quaternary carbon (δ_C 75.2) in **4** instead of two methines (δ_C/δ_H 67.4/4.45; 38.9/2.67) in the latter, indicating that the distinct substitution position of the hydroxy group was on the C-9 in **4** rather than C-6 in dugesin D, which was further confirmed by HMBC correlations from OH-9 to C-9, C-11 and C-20, H-10 and CH_3-20 to C-9. In the ROESY spectrum (Fig. 4), the observed correlations of OH-9/H-12, H_2-19/CH_3-20, H-19a/H-6a, and H-6b/H-10 demonstrated that H-10, OH-9, and H-12 were β-orientated. Finally, the structure of **4** was named as tiliifolin D. The single-crystal X-ray crystallographic (Fig. 5) analysis of **4** confirmed the above deduction. The value of the Flack parameter 0.19(4) allowed the assignment of the absolute configuration of **4** as 5*S*, 9*R*, 10*S*, 12*S*.

Compound **5** was isolated as a white amorphous powder, and its molecular formula was established as $C_{20}H_{16}O_6$ based on the quasi-molecular ion peak at m/z 375.0849 $[M + Na]^+$ (calcd for $C_{20}H_{16}O_6Na$, 375.0839) in the HRESIMS, requiring 13 degrees of unsaturation. The IR spectrum exhibited an absorption band at 1755 cm^{-1} due to a saturated γ-lactone, and the characteristic absorptions at 1506 and 875 cm^{-1} due to a furan ring. The ^{13}C NMR and DEPT spectra displayed 20 carbon signals, including four methylenes (two oxygenated at δ_C 76.2, 60.1), seven methines (one oxygenated at δ_C 76.2), and nine quaternary carbons (two ester carbonyl groups at δ_C 171.8 and 168.7) in Table 2. A comparison of the ^{13}C NMR data of **5** with those of 6,7-dehydrodugesin A [22] revealed similarities except that the methyl group was replaced by a hydroxymethyl group (δ_C/δ_H 60.1/4.61, 4.41) in the former, which was deduced from the HMBC cross-peaks of H_2-20 (δ_H 4.61, 4.41) with C-10 (δ_C 140.4), C-9 (δ_C 131.2) and C-11 (δ_C 159.0). The relative configuration of **5** was assigned by the ROESY spectrum. H-12 was assigned to be β-oriented based on the ROESY correlations of H-19a/H-14 and H-19a/H-16, which was identical to those of 6,7-dehydrodugesin A [22]. The absolute configuration of **5** was defined by comparing its ECD spectrum (Supplementary data, Fig. S46) with that of **10**. Since the 1D NMR data and optical rotation of **10** were identical to that of salviandulin E [21,22], which indicated that they were the same compound. Due to the lack of ECD spectrum of salviandulin E, whose absolute configuration has been unequivocally established by the X-ray crystallography as 5*S*,12*R* [22], the ECD spectrum (Supplementary data, Fig. S47) of salviandulin E (**10**) was carried out. The CD curve similarities between **5** and **10** with the negative Cotton effect at the corresponding UV absorption band around 245 nm suggested that they bear the same configurations, which was consistent with a biogenetic point of view. Thus, the absolute configuration of **5** was assigned as 5*S*,12*R*, and **5** was named tiliifolin E.

A plausible biogenetic pathway of tiliifolins A and B (**1** and **2**) was proposed as shown in Scheme 1. The biogenetic pathway of **1** and **2** could plausibly be traced back to salvifaricin [19,20], which is aromatized to afford intermediate **i**. Subsequently, dihydroxylation of **i** may lead to the formation of compound **1**. Otherwise, the intermediate **i** is protonized to give an oxonium ion **ii**, which was trapped by methanol to generate compound **2**.

3.2. Biological activity

The isolated new compounds **1–5** were evaluated for their ability to stimulate nerve growth factor (NGF)-mediated neurite outgrowth on PC12 cells. The differentiation rates of neurite-bearing cells were 4.29 and 18.43% following incubation with 5 ng/mL NGF (negative control) and 50 ng/mL NGF (positive control) after 72 h, respectively. Compound **5** was found to promote the differentiation of PC12 cells after 72 h at a concentration of 10 μM with the differentiation rate of 11.10%, which exhibited moderate activity. Compounds **1–5** were also evaluated for in vitro cytotoxicity against five human tumor cell lines (HL-60, A-549, SMMC-7721, MCF-7, and SW480), but showed no activity with IC_{50} values of more than 40 μM.



Scheme 1. Plausible biogenetic pathway of compounds **1** and **2**.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

The 1D NMR, 2D NMR, IR, UV, and HR-ESI-MS spectra for compounds **1–5**, are available in the Supplementary data for this article can be found online at https://doi.org/10.1016/j.fitote.2017.09.013.

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