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ARTICLE TYPE

Lanceolatins A-G, diterpenoids from *Cephalotaxus lanceolata* and their anti-inflammatory and anti-tumor activities†

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Seven new diterpenoids lanceolatins A-G (**1**-**7**), together with five known diterpenoids (**8**-**12**), were isolated from the branches and leaves of *Cephalotaxus lanceolata*. The structures of the new diterpenoids were elucidated based on spectroscopic analysis, including 1D, 2D NMR, and HR-ESI-MS. Single crystal X-ray diffraction (CuK_α radiation) was employed to confirm the structure of lanceolatin G (**7**), and finally established its absolute configuration. Compound **12** showed significant inhibition against human tumor cell lines HCT116 and HepG2 with IC_{50} values of 0.17 and 0.63 μg/mL, respectively, while compounds **3**-**5** can inhibit nitric oxide (NO) release in LPS-induced RAW264.7 macrophages with IC_{50} values of 8.72, 10.79, 12.73 μM.

Introduction

Cephalotaxus species of the Cephalotaxaceae (formerly being contained in Taxaceae), also called plum yews, are evergreen, dioecious coniferous trees or shrubs mainly distributed in southern and eastern Asia.[1] Since the first *Cephalotaxus* alkaloid cephalotaxine was isolated from *C. harringtonia* var. *drupacea* in 1963,[2] especially the discovery of several potent antileukaemia cephalotaxine esters,[3-6] such as harringtonine, homoharringtonine, and isoharringtonine, considerable attention has been paid to *Cephalotaxus* alkaloids and their antitumor activities, and leading to the isolation of a great number of alkaloids from *Cephalotaxus* species.[7] Among these reported alkaloids, harringtonine and homoharringtonine have been clinically used to treat acute leukemia in China,[8] while homoharringtonine has been submitted to marketing authorization application and new drug application for treatment of orphan leukaemia in European Medicines Agency and US Food and Drug Administration,[9] respectively. Unfortunately, great success of *Cephalotaxus* alkaloids led to the ignorance for diterpenoids, another type of important chemical constituents from *Cephaloaxus* species. Although Sun *et al*. described the isolation of hainanolide, a structurally novel diterpenoid with tropone core,[10] and reported its potent antitumor activity against lewis lung carcinoma, Walker carcinoma, Sarcoma-180, L-1210, L-615, and P-388 leukaemia cell,[11] so far only five this type of diterpenoids were reported from *Cephalotaxus* species.[12] Moreover, there are six abietane type diterpenoids isolated from the field-grown seeds of *C. harringtonia*.[13] Our group has been long focusing on the discovery of bioactive natural products from medicinally important herbs in China.[14-16] During our investigation about bioactive constituents from *Cephalotaxus lanceolata* native to Gongshan county of Yunnan province, China, besides alkaloids,[17] a special attention was also paid to diterpenoids of this plant, and leading to the isolation of 7 new diterpenoids lanceolatins A-G (**1**-**7**) and 5 known ones (**8**-**12**) (**Fig. 1**). Herein, we describe the isolation, structural elucidation, and *in vitro* anti-inflammatory and antitumor evaluation of these diterpenoids.

Results and discussion

The EtOAc-soluble fraction of the 95% EtOH extract of *C. lanceolata* was submitted to repeated column chromatography (CC) on silica gel, ODS, and Sephadex LH-20, and semi-preparative HPLC to afford seven new (**1**-**7**) and five known diterpenoids (**8**-**12**). Due to comparison of the NMR and MS data with previously reported data in literatures, five known compounds were characterized as imbricatolic acid (**8**),[18] 14-dien-18-oic acid (**9**),[19] dehydroabietic acid (**10**),[20] 15-hydroxydehydroabietic acid (**11**),[21] hainanolide (**12**).[22]

Lanceolatin A (**1**), white amorphous powder, was assigned a molecular formula $C_{20}H_{32}O_4$ with 5 degrees of unsaturation due to analysis of negative HRESIMS (*m/z* 335.2223 $[M-H]^-$, calcd. 335.2228). Its IR spectrum showed absorption bands at 3450 and 1704 cm^{-1}, indicative of hydroxyl and carboxyl groups.

Three olefinic protons at δ_H 5.62 (s), 4.97 (s) and 4.74 (s), two oxygenated protons at δ_H 3.13 (m) and 4.38 (s), and four singlet methyls at δ_H 2.12, 1.07, 1.14 and 1.00, were observed in the ^1H NMR spectrum of **1** (**Table 1**). The ^{13}C NMR spectrum showed 20 carbon resonances (**Table 2**), which was sorted into 4 methyls (δ_C 19.0, 28.6, 17.5, 17.4), 6 methylenes (including an exocyclic double bond at δ_C 109.9), 5 methines (including two oxymethines at δ_C 80.0 and 69.8, and one olefinic carbon at δ_C 118.0), and 5

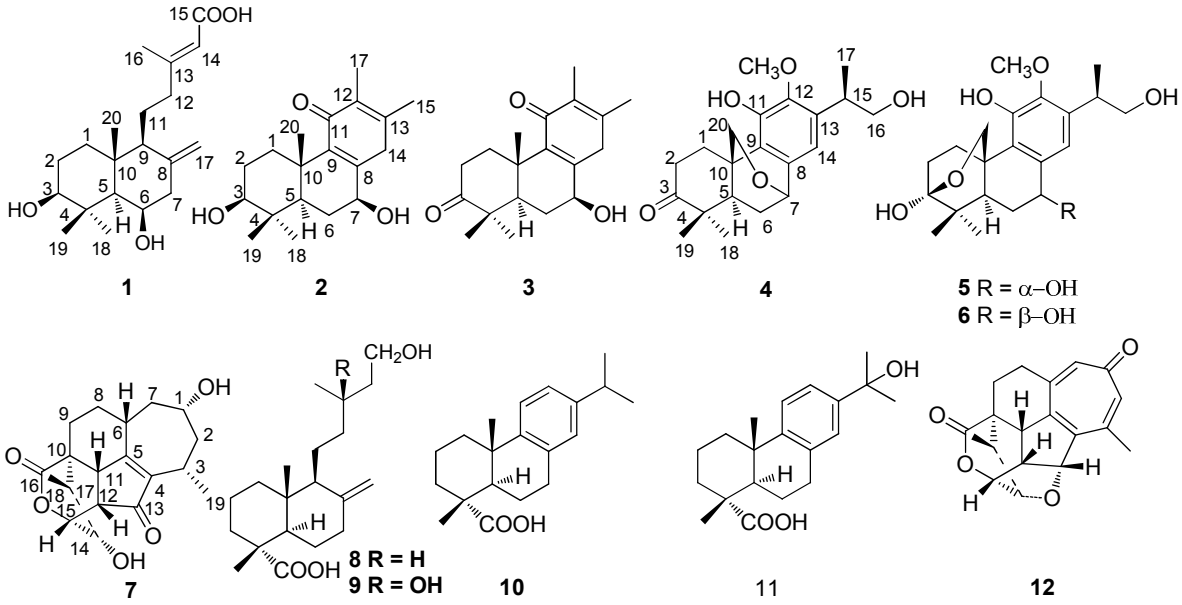

Fig. 1 Structures of compounds **1**-**12**.

quaternary carbons (including two olefinic carbons at δ_C 145.5 and 160.4, and one carboxyl carbon at δ_C 171.3). The above data, together with 5 degrees of unsaturation as indicated in molecular formula, implied that compound **1** should be a labdane-type diterpenoid with the functional groups of two double bonds, two hydroxyls, and one carboxyl. In the HMBC spectrum of **1** (**Fig. 2**), the key correlations between two methyls at δ_H 1.07 and 1.14 and the oxymethine at δ_C 80.0 indicated that a hydroxyl was substituted at C-3 (δ_C 80.0) position. Also, another hydroxyl was linked to C-6 position on the basis of the observation of the correlations between the oxygenated proton at δ_H 4.38 and C-10 (δ_C 41.5) and C-8 (δ_C 145.5). Two olefinic protons [δ_H 4.97 (s), 4.74 (s)], being assigned to a methylene at δ_C 109.9, exhibited key HMBC correlations (**Fig. 2**) with C-7 (δ_C 48.8) and C-9 (δ_C 57.8), indicative of the presence of one exocyclic double bond between C-8 and C-17. Moreover, the HMBC correlations of the methyl at δ_H 2.12 with C-12 (δ_C 40.6) and C-14 (δ_C 118.0), and of the olefinic proton at 5.62 (s) with C-12 and the carboxyl at δ_C 171.3, implied that one double bond was positioned between C-13 (δ_C 160.4) and C-14 (δ_C 118.0), and the carboxyl was assigned to C-15. Biogenetically, the CH_3-19 and CH_3-20 of labdane diterpenoid are β-oriented, while the CH_3-18, H-5 and H-9 are α-oriented. In the NOESY spectrum of **1**, the correlations (**Fig. 3**) from 19-CH_3 to 20-CH_3, and from H-5 to H-9 also confirmed the above relative configurations. Additionally, two hydroxyls at C-3 and C-6 positions were determined to be β-orientation due to key NOESY correlations (**Fig. 3**) of CH_3-18 with H-3 and H-6. Thus, the structure of **1** was identified as 3*β*,6*β*-dihydroxylabda-8(17),13*Z*-dien-15-oic acid, and named lanceolatin A.

Compound **2** was isolated as white amorphous powder with a specific optical rotation $[\alpha]_D^{20}$ = +34.7 (*c* 0.32, MeOH). Its molecular formula was inferred to be $C_{19}H_{28}O_3$ with 6 degrees of unsaturation as deduced by pseudomolecular ion peak $[M+H]^+$ at *m/z* 305.2100 (calcd. 305.2111) in positive HRESIMS spectrum. The IR spectrum exhibited the absorption bands for hydroxyl (3428 cm^{-1}), C=O (1644 cm^{-1}), double bond (1677 and 1619 cm^{-1}).

The ^{1}H NMR spectrum of **2** (**Table 1**) showed five singlet methyls (δ_H 2.24, 1.91, 0.84, 1.04, and 1.08) and two oxygenated protons at δ_H 3.23 (dd, *J* = 11.8, 5.2 Hz) and 4.35 (d, *J* = 4.2 Hz). The ^{13}C NMR spectrum showed 19 carbon resonances (**Table 2**), including five methyls (δ_C 20.0, 24.3, 16.4, 28.8, and 18.6), four methylenes (δ_C 28.3, 34.2, 29.5, and 33.6), three methines (δ_C 79.6, 47.1, and 66.7), and seven quaternary carbons (δ_C 39.7, 161.5, 152.6, 37.6, 197.8, 131.4, and 147.4). The above data were very close to those of abietane-type diterpenoid, but didn't exhibit typical features for isopropyl. Considering that only 19 carbon resonances were observed in the ^{13}C NMR spectrum, it was obvious that compound **2** may be an abietane-type diterpenoid derivative with methyl loss and migration in isopropyl. The structure of **2** was further determined by 2D-NMR experiments. The key HMBC correlations (**Fig. 2**) of the oxygenated proton at δ_H 3.23 with C-2 (δ_C 28.3), CH_3-18 (δ_C 28.8), and CH_3-19 (δ_C 16.4), of methyls at δ_H 1.04 and 0.84 with the oxygenated methine at δ_C 79.6, and of the oxygenated proton at δ_H 4.35 (d, *J* = 4.2 Hz) with C-5 (δ_C 47.1), C-9 (δ_C 152.6), and C-14 (δ_C 33.6), attached two hydroxyls to C-3 and C-7, respectively. Additionally, the singlet methyl at δ_H 1.91 exhibited the HMBC correlations (**Fig. 2**) with the ketone carbonyl (δ_C 197.8) and the olefinic carbon (δ_C 147.4), while another singlet methyl at δ_H 2.24 was observed the HMBC correlation with the methylene at δ_C 33.6. Two methylene protons at δ_H 3.25 and 2.89 exhibited key HMBC correlations with C-9 (δ_C 152.6) and C-12 (δ_C 131.4). The CH_3-20 at δ_H 1.08 showed the HMBC correlations with C-9 (δ_C 152.6) and C-5 (δ_C 47.1). The above information assigned two double bonds and ketone carbonyl to C-8, C-9, C-12, C-13, and C-11, and revealed that two methyls (δ_H 2.24, δ_C 20.0; δ_H 1.91, δ_C 24.3) were linked to C-13 and C-12, respectively. The relative configurations of **2** were determined by the NOESY correlations (**Fig. 3**) from H-5 to H-3 and H-7, from CH_3-18 to H-5, and from CH_3-19 to CH_3-20. Consequently, the structure of **2** was determined to be 3*β*,7*β*-dihydroxyl-16-nor-17-methyl (15→12)-

abeo-abieta-8,12-dien-11-one, and given name lanceolatin B.

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Table 1 ^{1}H NMR spectroscopic data for compounds **1-7** (*mult.*, *J* in Hz)

No.	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[c]	**6**[c]	**7**[c]
1	1.76 (m)	2.74 (dt, 13.5, 3.5)	2.83 (dt, 13.6, 6.2)	3.27 (ddd, 14.9, 10.2, 4.7)	3.46 (td, 12.4, 5.8)	3.43 (dt, 12.5, 5.7)	3.90 (m)
	1.19 (m)	1.21 (td, 13.5, 4.5)	1.68 (dt, 13.6, 8.7)	2.09 (ddd, 14.9, 8.1, 4.7)	1.49 (m)	1.36 (m)	
2	1.69 (overlap)	1.71 (m)	2.58 (2H, m)	2.74 (ddd, 15.2, 10.2, 4.7)	2.21 (overlap)	2.18 (m)	2.20 (m)
	1.64 (m)	1.69 (m)		2.56 (ddd, 15.2, 8.1, 4.7)	1.83 (m)	1.83 (td, 12.5, 3.3)	1.75 (m)
3	3.13 (m)	3.23 (dd, 11.8, 5.2)					2.78 (m)
4							
5	1.03 (s)	1.46 (dd, 12.8, 1.9)	2.15 (dd, 4.4, 10.8))	1.87 (m)	2.21 (overlap)	1.74 (dt, 13.5, 2.0)	
6	4.38 (s)	1.89 (m)	1.85 (m)	2.13 (ddd, 13.4, 6.9, 2.5)	1.86 (m)	2.02 (ddd, 11.9, 5.0, 2.0)	2.66 (m)
		1.81 (m)	1.30 (m)	1.62 (td, 13.4, 2.5)	1.77 (td, 13.7, 2.7)	1.61 (ddd, 11.9, 11.2, 2.0)	
7	2.36 (m)	4.35 (d, 4.2)	4.40 (dd, 2.4, 3.6)	4.78 (t, 2.5)	4.69 (t, 2.7)	4.53 (dd, 11.2, 5.0)	2.05 (m)
	2.26 (m)						1.85 (m)
8							1.98 (m), 1.43 (m)
9	1.62 (overlap)						1.90 (m), 1.80 (m)
10							
11	1.69 (overlap)						2.91 (overlap)
	1.62 (overlap)						
12	2.31 (m)						2.92 (overlap)
	2.01 (m)						
13							
14	5.62 (s)	3.25(d, 20.8)	3.31 (d, 20.9)	6.67 (s)	6.70 (s)	7.01 (s)	3.55 (m)
		2.89 (d, 20.8)	2.96 (d, 20.9)				
15		2.24 (s)	2.26 (s)	3.31 (m)	3.24 (m)	3.25 (m)	4.74 (m)
16	2.12 (s)			3.68 (dd, 10.7, 6.4)	3.65 (dd, 10.7, 6.3)	3.66 (dd, 10.7, 6.3)	
				3.56 (dd, 10.7, 7.6)	3.53 (dd, 10.7, 7.6)	3.54 (dd, 10.7, 7.6)	
17	4.97 (s)	1.91 (s)	1.93 (s)	1.22 (d, 7.0)	1.22 (d, 7.0)	1.22 (d, 7.0)	1.93 (m)
	4.74 (s)						
18	1.07 (s)	1.04 (s)	1.14 (s)	0.98 (s)	1.10 (s)	1.09 (s)	0.92 (d, 7.2)
19	1.14 (s)	0.84 (s)	1.09 (s)	1.32 (s)	1.03 (s)	1.06 (s)	1.27 (d, 7.3)
20	1.00 (s)	1.08 (s)	1.13 (s)	4.39 (d, 8.9)	4.72 (dd, 8.6, 1.6)	4.77 (dd, 8.7, 1.7)	
				3.22 (dd, 8.9, 1.7)	3.85 (dd, 8.6, 2.4)	3.94 (dd, 8.7, 2.5)	
CH_3O				3.73 (s)	3.69 (s)	3.69 (s)	

[a] Recorded at 400 MHz in CD_3OD. [b] Recorded at 500 MHz in CD_3OD. [c] Recorded at 600 MHz in CD_3OD

Table 2 ^{13}C NMR spectroscopic data for compounds **1**-**7**

No.	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[c]	**6**[c]	**7**[c]
1	40.5 (t)	34.2 (t)	34.2 (t)	28.4 (t)	30.7 (t)	31.3 (t)	70.2 (d)
2	28.8 (t)	28.3 (t)	35.1 (t)	36.8 (t)	29.9 (t)	29.9 (t)	44.0 (t)
3	80.0 (d)	79.6 (d)	220.0 (s)	219.1 (s)	99.9 (s)	100.0 (s)	28.2 (d)
4	41.1 (s)	39.7 (s)	48.1 (s)	48.9 (s)	41.5 (s)	41.8 (s)	146.9 (s)
5	57.6 (d)	47.1 (d)	46.6 (d)	46.2 (d)	42.8 (d)	48.9 (d)	170.8 (s)
6	69.8 (d)	29.5 (t)	30.5 (t)	30.0 (t)	29.4 (t)	31.7 (t)	38.0 (d)
7	48.8 (t)	66.7 (d)	65.9 (d)	72.6 (d)	69.5 (d)	71.1 (d)	43.2 (t)
8	145.5 (s)	161.5 (s)	161.5 (s)	137.8 (s)	136.7 (s)	139.7 (s)	30.9 (t)
9	57.8 (d)	152.6 (s)	150.9 (s)	129.4 (s)	124.8 (s)	124.1 (s)	26.7 (t)
10	41.5 (s)	37.6 (s)	36.9 (s)	40.4 (s)	37.9 (s)	38.6 (s)	47.9 (s)
11	23.1 (t)	197.8 (s)	197.6 (s)	146.0 (s)	150.3 (s)	149.8 (s)	48.6 (d)
12	40.6 (t)	131.4 (s)	131.2 (s)	147.7 (s)	147.4 (s)	146.6 (s)	46.1 (d)
13	160.4 (s)	147.4 (s)	148.0 (s)	137.0 (s)	137.2 (s)	136.8 (s)	206.6 (s)
14	118.0 (d)	33.6 (t)	33.8 (t)	114.2 (d)	121.3 (d)	116.2 (d)	76.0 (d)
15	171.3 (s)	20.0 (q)	20.0 (q)	36.3 (d)	36.1 (d)	36.2 (d)	80.4 (d)
16	19.0 (q)			68.6 (t)	68.5 (t)	68.6 (t)	177.6 (s)
17	109.9 (t)	24.3 (q)	24.3 (q)	18.7 (q)	18.5 (q)	18.5 (q)	35.2 (d)
18	28.6 (q)	28.8 (q)	27.5 (q)	25.2 (q)	27.1 (q)	27.4 (q)	16.4 (q)
19	17.5 (q)	16.4 (q)	21.4 (q)	21.3 (q)	18.9 (q)	18.9 (q)	22.1 (q)
20	17.4 (q)	18.6 (q)	18.1 (q)	69.5 (t)	67.2 (t)	67.9 (t)	
CH_3O				62.1 (q)	62.0 (q)	62.0 (q)	

[a] Recorded at 100 MHz in CD_3OD. [b] Recorded at 125 MHz in CD_3OD. [c] Recorded at 150 MHz in CD_3OD

Positive HRESIMS analysis of **3** gave a pseudomolecular ion peak $[M+H]^+$ at *m/z* 303.1969 (calcd. 303.1955), in agreement with the molecular formula $C_{19}H_{26}O_3$ with 7 degrees of unsaturation.

The ^{1}H- and ^{13}C NMR spectra of **3** were very similar to those of **2**, including 19 carbon resonances observed in ^{13}C NMR spectrum (**Table 2**), five typical singlet methyls (δ_H 2.26, 1.93, 1.14, 1.09, 1.13) in ^{1}H NMR spectrum (**Table 1**), two double bonds (δ_C 161.5, 150.9, 131.2, and 148.0) and a ketone carbonyl at δ_C 197.6 in ^{13}C NMR spectrum, disclosed that compound **3** shared the same 16-nor-17-methyl (15→12)-*abeo*-abietane skeleton as **2**. Comparison of the NMR data of **3** and **2** revealed that the structure of compound **3** had an additional ketone carbonyl (δ_C 220.0), instead of the signals for the oxygenated methine at δ_H 3.23 (H-3) and δ_C 79.6 (C-3) in the ^{1}H and ^{13}C NMR spectra of **2**. In the HMBC spectrum of **3** (**Fig. 2**), two angular methyls at δ_H 1.14 and 1.09 showed key correlations with the ketone carbonyl at δ_C 220.0 and C-5 (δ_C 46.6), suggesting the presence of C-3 carbonyl. Also, the HMBC correlations (**Fig. 2**) of the oxygenated proton at δ_H 4.40 (dd, *J* = 2.4, 3.6 Hz) with C-9 (δ_C 150.9), C-5 (δ_C 46.6), C-8 (δ_C 161.5), of H_2-14 (δ_H 3.31 and 2.96) with C-9 and C-12 (δ_C 131.2), of CH_3-20 (δ_H 1.13) with C-1 (δ_C 34.2) and C-9, of CH_3-15 (δ_H 2.26) with C-12 (δ_C 131.2), and of CH_3-17 (δ_H 1.93) with C-13 (δ_C 148.0), further confirmed the structure of **3**. The relative configurations of **3** were established to be identical with those of **2** due to analysis of the NOESY spectrum of **3**. Therefore, the structure of **3** was established to be 7*β*-hydroxyl-16-nor-17-methyl (15→12) -*abeo*-abieta-8,12-dien-3,11-dione, and named lanceolatin C.

Lanceolatin D (**4**), white amorphous powder with a specific optical rotation $[\alpha]_D^{20}$ = -19.3 (*c* 0.145, MeOH), had a molecular formula $C_{21}H_{28}O_5$ with 8 degrees of unsaturation as deduced by analysis of positive HRESIMS (*m/z* 361.2024 $[M+H]^+$, calcd. 361.2010). The absorption bands in IR spectrum suggested the presence of benzene ring (1650, 1579, 1452 cm^{-1}), carbonyl (1706 cm^{-1}), and hydroxyl (3426 cm^{-1}).

Analysis of the ^{1}H NMR spectrum of **4** (**Table 1**) indicated the presence of one aromatic proton at δ_H 6.67 (s), five oxygenated protons at δ_H 3.68 (dd, *J* = 10.7, 6.4 Hz), 3.56 (dd, *J* = 10.7, 7.6 Hz), 4.39 (d, *J* = 8.9 Hz), 3.22 (dd, *J* = 8.9, 1.7 Hz), and 4.78 (t, *J* = 2.5 Hz), together with two singlet methyl at δ_H 0.98 (s) and 1.32 (s), one doublet methyl at δ_H 1.22 (d, *J* = 7.0 Hz), and one methoxyl at δ_H 3.73 (s). The ^{13}C and DEPT NMR spectra (**Table 2**) displayed 21 carbon resonances, including three methyls (δ_C 18.7, 25.2, and 21.3), one methoxyl (δ_C 62.1), five methylenes (δ_C 36.8, 28.4, 30.0, 68.6, and 69.5), four methines (δ_C 46.2, 72.6, 114.2, and 36.3), eight quaternary carbons (δ_C 219.1, 48.9, 137.8, 129.4, 40.4, 146.0, 147.7, and 137.0). The above data showed typical features of the ring C aromatized abietane-type diterpenoid, and suggested the presence of the functionalities of one ketone carbonyl, two oxygenated methylenes and one oxymethine, one methoxyl, and one benzene ring. All protons and its related carbons were assigned by HMQC experiment. In the HMBC spectrum of **4** (**Fig. 2**), H_2-1 (δ_H 3.27 and 2.09), CH_3-18 (δ_H 0.98) and CH_3-19 (δ_H 1.32) was observed key correlations with the ketone carbonyl at δ_C 219.1, suggesting the presence of C-3 ketone carbonyl. The doublet methyl at δ_H 1.22 was correlated with C-13 (δ_C 137.0) and the hydroxymethyl at δ_C 68.6, along with the key HMBC correlations (**Fig. 2**) between hydroxymethyl protons (δ_H 3.68 and 3.56) and C-13, disclosed that the C-16 position was hydroxylated. The key HMBC correlations of the oxygenated proton at δ_H 4.78 with the oxymethylene at δ_C 69.5, C-5 (δ_C 46.2), C-9 (δ_C 129.4), and C-14 (δ_C 114.2), featuring the presence of the epoxyl ring between C-20 and C-7. Also, one hydroxyl and one methoxyl were substituted at C-11 and C-12 positions, respectively, on the basis

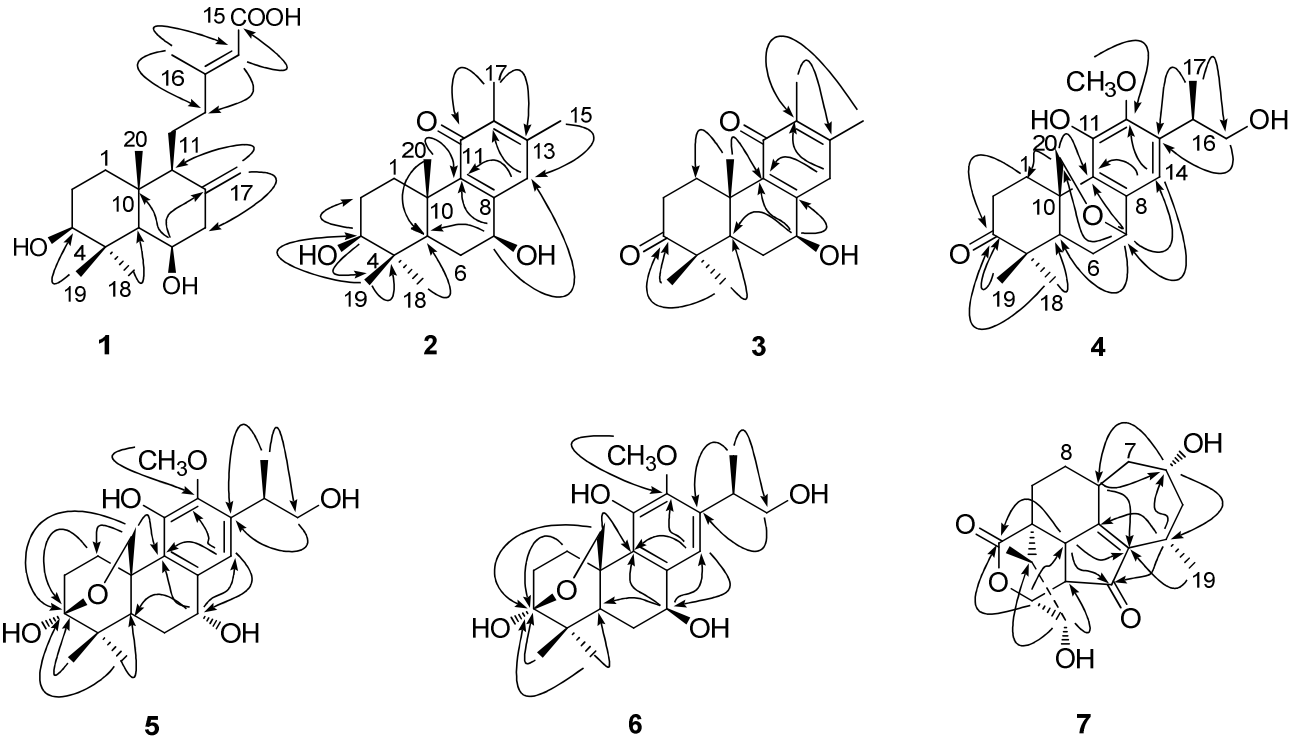

Fig. 2 Key HMBC correlations of compounds **1**-**7**

of the HMBC correlations (**Fig. 2**) of the methoxyl at δ_H 3.73 with C-12 (δ_C 147.7), of H-14 (δ_H 6.67) with C-9 (δ_C 129.4), C-12 (δ_C 147.7), and C-7 (δ_C 72.6). In the NOESY spectrum of **4** (**Fig. 3**), the key correlations between H-20 at δ_H 4.39 and CH_3-19 (δ_H 1.32) and between H-7 (δ_H 4.78) with H-5α (δ_H 1.87) revealed that the epoxyl ring was located above the molecule planar, namely β-orientation. According to *Luis* et al,[23] since abietane diterpenoid is biogenetically derived from isopimaradiene precursor, its CH_3-17 was a *pro* (*R*) methyl, while CH_3-16 was a *pro* (*S*) methyl. Therefore, due to biogenetic consideration, the absolute configuration of the C-15 position of C-16 hydroxylated abietane dieterpenoid should be assigned to be *R* configuration. On the basis of the above evidences, the structure of **4** was thus determined to be (15*R*)-7*β*,20-epoxy-11,16-dihydroxyl-12-methoxyl-abieta-3-one, and named lanceolatin D.

Compound **5** was isolated as white amorphous powder with a specific optical rotation $[\alpha]_D^{20}$ = +84.5 (*c* 0.355, MeOH), and its positive HRESIMS spectrum showed a pseudomolecular ion peak $[M+Na]^+$ at *m/z* 401.1930 (calcd. 401.1935), corresponding to the molecular formula $C_{21}H_{30}O_6$ with 7 degrees of unsaturaion. The IR spectrum displayed the absorption bands at 3396, 1616, 1421, 1313, 1039, 923, 869 cm^{-1}, indicative of the presence of the functionalities of hydroxyl and benzene ring.

The ^1H and ^{13}C NMR spectra (**Tables 1 and 2**) of **5** exhibited quite similar spectroscopic features, and possessed most functionalities as those of **4**, for example the phenyl [δ_H 6.70 (s); δ_C 136.7, 124.8, 150.3, 147.4, 137.2, 121.3], the methoxyl (δ_H 3.69; δ_C 62.0), two oxymethylenes [δ_H 3.65 (dd, *J* = 10.7, 6.3 Hz) and 3.53 (dd, *J* = 10.7, 7.6 Hz), δ_C 68.5; δ_H 4.72 (dd, *J* = 8.6, 1.6 Hz) and 3.85 (dd, *J* = 8.6, 2.4 Hz); δ_C 67.2], and the oxymethine [δ_H 4.69 (t, *J* = 2.7 Hz); δ_C 69.5], suggesting that compounds **5** and **4** shared the same C-16 hydroxylated abietane carbon skeleton with a aromatized C ring and similar substituent pattern. Comparing with **4,** the NMR spectra of **5** showed an additional hemiketal carbon at δ_C 99.9, rather than the ketone carbonyl at C-3 position of **4**. In the HMBC spectrum of **5** (**Fig. 2**), two oxygenated protons at δ_H 4.72 and 3.85, which were assigned to the methylene at δ_C 67.2 by HMQC experiment, were observed the cross peaks with the hemiketal carbon at δ_C 99.9, C-1 (δ_C 30.7), and C-9 (δ_C 124.8). Also, two angular methyls at δ_H 1.10 and 1.03 (CH_3-18 and CH_3-19) was exhibited the HMBC correlations (**Fig. 2**) with the hemiketal carbon. The 3,20-epoxyl ring was allowed to be established on the basis of the above information. In the NOESY spectrum of **5** (**Fig. 3**), two proton signals of H_2-6 at δ_H 1.77 and 1.86 were respectively observed the correlations with CH_3-19 (δ_H 1.03) and CH_3-18 (δ_H 1.10), revealed that the two protons were *β* and *α*-orientation, respectively. Moreover, the proton H-20 at δ_H 3.85 displayed key NOESY correlations (**Fig. 3**) with CH_3-19 and H-6 at δ_H 1.77, implying that the 3,20-epoxyl ring was positioned above the molecule planar. 7-OH was established to be α-oriented due to the NOESY correlation of H-7 (δ_H 4.69) with H-6 at δ_H 1.77 and H-14 (δ_H 6.70). Based on the same consideration as **4**, the absolute configuration of C-15 was assigned to be *R*. Consequently, the structure of **5** was determined to be (15*R*)-3*β*,20-epoxy-7α,11,16-trihydroxyl-12-methoxyl-abieta-3-one, given name lanceolatin E.

The molecular formula of **6** was assigned to be $C_{21}H_{30}O_6$ with 7 degrees of unsaturaion based on the pseudomolecular ion peak $[M+H]^+$ at *m/z* 379.2052 (calcd. 379.2047) in positive HRESIMS spectrum. The NMR spectral data (**Tables 1 and 2**) of **6** were very close to those of **5,** mainly differing in proton chemical shifts for H-5 [δ_H 1.74 (dt, *J* = 13.5, 2.0 Hz)], H-7 [δ_H 4.53 (dd, *J*

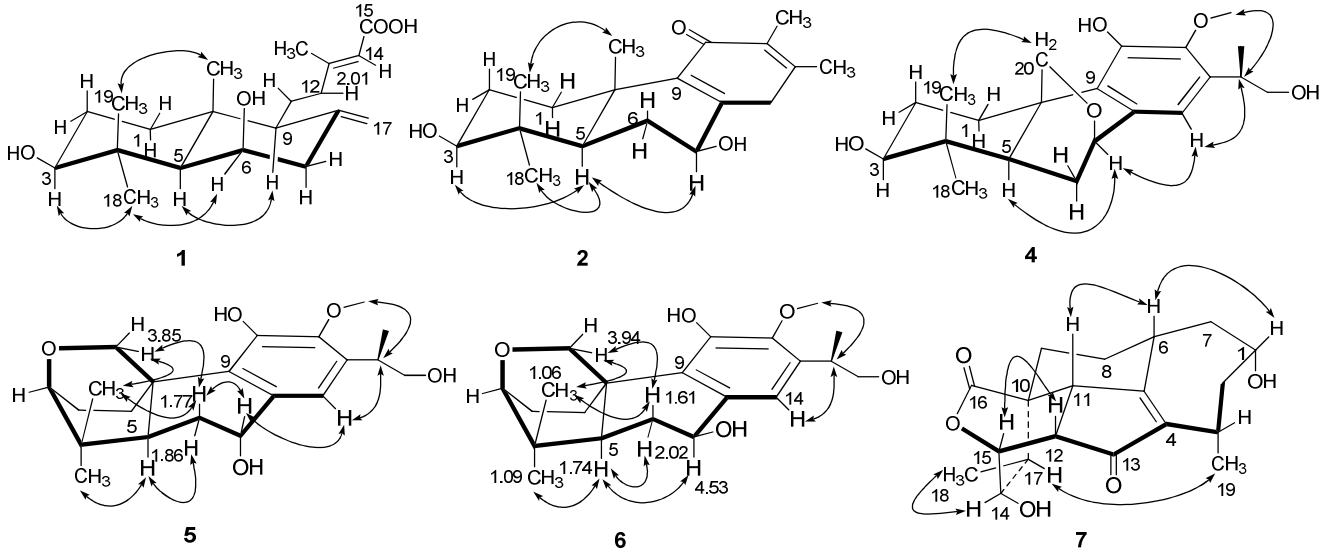

Fig. 3 Key NOESY correlations of compounds **1**-**2** and **4**-**7**.

= 11.2, 5.0 Hz)], and H-14 [δ_H 7.01 (s)], and carbon resonances for C-5 (δ_C 48.9), C-7 (δ_C 71.1), and C-14 (δ_C 116.2). These evidences revealed that compound **6** possessed the same planar structure as **5**, only difference in the configuration of 7-OH. In the HMBC spectrum of **6**, the observed correlations (**Fig. 2**) further supported the above inferences. In the NOESY spectrum of **6** (**Fig. 3**), H-7 (δ_H 4.53) was observed key correlation with H-5α (δ_H 1.74), indicative of the presence of 7β-OH. Additionally, the NOESY correlation between H-20 at δ_H 3.94 with 19-CH_3 (δ_H 1.06) and H-6 at δ_H 1.61 confirmed the presence of β-oriented 3,20-epoxyl ring. On the basis of the above evidences, the structure of **6** was identified as (15*R*)-3*β*,20-epoxy-7*β*,11,16-trihydroxyl-12-methoxyl-abieta-3-one, and named lanceolatin F.

Compound **7** was isolated as amorphous powder with a specific optical rotation $[\alpha]_D^{20}$ = -103.0 (*c* 0.30, MeOH), and grew block single crystal in MeOH. The positive HRESIMS gave a pseudomolecular ion peak $[M+H]^+$ at *m/z* 333.1690 (calcd. 333.1697), in agreement with the molecular formula $C_{19}H_{24}O_5$ with 8 degrees of unsaturaion. The IR spectrum of **7** showed typical absorption bands at 3363, 1735, 1629 cm^{-1}, indicative of the features of hydroxyl, carbonyl, and ester carbonyl.

The ^{1}H NMR spectrum of **7** (**Table 1**) gave two doublet methyls at δ_H 0.92 (d, *J* = 7.2 Hz) and 1.27 (d, *J* = 7.3 Hz), three oxygen-bearing protons at δ_H 3.90 (m), 3.55 (m), and 4.74 (m). The ^{13}C NMR spectrum (**Table 2**) exhibited 19 carbon resonances, including two methyls (δ_C 16.4 and 22.1), four methylenes (δ_C 44.0, 43.2, 30.9, and 26.7), eight methines (δ_C 70.2, 28.2, 38.0, 48.6, 46.1, 76.0, 80.4, and 35.2), and five quaternary carbons (δ_C 146.9, 170.8, 47.9, 206.6, and 177.6). These spectroscopic data showed typical characteristics of hainanolide type diterpenoid, a type of rare norditerpenoid only occurring in *Cephalotaxus* species so far. Comparing with the hainanolide (**12**),[22] the NMR spectra of **7** didn't show these signals for tropone moiety, but additionally gave one doublet methyl in ^{1}H NMR spectrum, three methines (including oxygenated one), two methylenes, and an additional ketone carbonyl. On the basis of the above information, it could be proposed that the tropone moiety of hainanolide was partially reduced and the 13,14-ether ring was open in compound **7**. Based on analysis of the HMBC spectrum of **7** (**Fig. 2**), the C-1 hydroxylation was verified by HMBC correlations of the oxygen-bearing proton at δ_H 3.90 with C-6 (δ_C 38.0) and C-3 (δ_C 28.2) and of H-6 (δ_H 2.66) and H-3 (δ_H 2.78) with the oxymethine at δ_C 70.2. An α,β-unsaturation ketone was assigned to C-4, C-5, and C-13 due to the HMBC correlations (**Fig. 3**) between H-3 and the olefinic carbon at δ_C 170.8 and the carbonyl at δ_C 206.6, between H-6 and the olefinic carbon at δ_C 146.9, and between H-11 and the olefinic carbon at δ_C 146.9 and the carbonyl at δ_C 206.6. Moreover, a hydroxyl was linked to C-14 position by the HMBC correlations from the oxygen-bearing proton at δ_H 3.55 to C-12 (δ_C 46.1) and C-17 (δ_C 35.2). The HMBC correlation of H-15 (δ_H 4.74) with the ester carbonyl (δ_C 177.6) also supported the presence of 15,16-lactone ring. The relative configurations of **7** were established by the NOESY correlations (**Fig. 3**) of H-6 with H-11 and H-1 (δ_H 3.90), of H-15 with H-12, of H-17 (δ_H 1.93) with CH_3-19, and of H-14 (δ_H 3.55) with CH_3-18 (δ_H 0.92). After repeated attempts, a suitable single crystal of **7** was obtained from methanol solution. The structure of **7** was further confirmed by the single crystal X-ray diffraction (CuK_α radiation) (**Fig. 4**), and its absolute configurations were unambiguously determined to be 1*S*, 3*S*, 6*R*, 10*S*, 11*S*, 12*S*, 14*R*, 15*R*, and 17*S*. The structure of **7** was thus named gongshanolide.

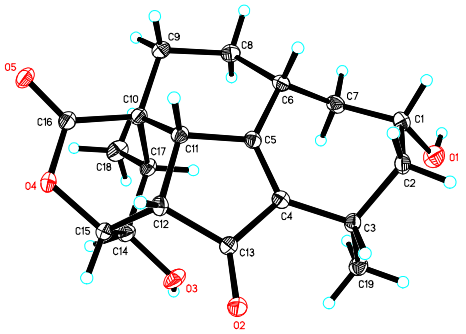

Fig. 4 Single crystal X-ray diffraction (CuK_α radiation) of **7**

Considering some diterpenoids from *Cephalotaxus* species, such as hainanolide,[11] have been reported significant anti-tumor activity, all isolates were evaluated for cytotoxicity against five human tumor cell lines A549, HCT116, SK-BR-3, HepG2, and HL-60. Only hainanolide (**12**) exhibited significant inhibition against two tumor cell lines HCT116 and HL-60 with IC_{50} values of 0.17 and 0.63 μg/mL, respectively. From these results, the tropone moiety and tetrahydrofuran ring could be two key pharmacophores, and their changes led to the loss of the antitumor activity of hainanolide.

In Chinese folk, *Cephalotaxus* species have been also used as traditional medicines for inflammatory treatment.[24] Nitric oxide (NO) is a key production of inflammation process, and plays central role in inflammation responses to diverse pathogens. New diterpenoids (**1**-**7**) were tested for anti-inflammation activity due to inhibition of NO release in LPS-induced RAW 264.7 macrophages. The results displayed that compounds **3**-**5** could obviously inhibit NO production in LPS-induced RAW 264.7 macrophages with IC_{50} values of 8.72, 10.79, and 12.73 μg/mL.

Conclusion

In the second metabolites of *Cephalotaxus* species, besides alkaloids, diterpenoids are also important chemical constituents due to the discovery of hainanolide type diterenoids,[11] which possess complex architecture featuring a fused tetracarbocyclic skeleton and strong cytotoxicity against Lewis lung carcinoma, Walker carcinoma, Sarcoma-180, L-1210, L-615 and P-388 leukaemia cells.[12] However, since the great success of *Cephalotaxus* alkaloids (such as harringtonine and homoharringtonine) in clinically use to treat acute leukemia in China and their potent inhibitory activities against different human tumor cell lines, especially against leukemia cell line, little attention was paid to diterpenoid metabolites from *Cephalotaxus* plants. So far, only six abietane-type and five hainanolide-type diterpenoids were isolated from the genus *Cephalotaxus*.[7] Our study led to the isolation of 12 diterpenes, among which compounds **2** and **3** are a type of rare 16-nor-17-methyl (15→12) *-abeo* abietane, and compound **7** is a hainanolide derivative with the ring opening of 13,14-epoxyl ring and partially reduced tropone moiety. We also isolated labdane type diterpenoids from *Cephalotaxus* species for the first time. These results revealed that *Cephalotaxus* species contains structurally diverse diterpneoids. Therefore, it will be interesting and valuable to pay more attention to exploring the diterpenoid constituents from *Cephalotaxus* species and their bioactivities.

Experimental section

General experimental procedures

Optical rotation was obtained on Perkin-Elmer 341 digital polarimeter at 589 nm. Melting point was measured on a X-4 Melting Point Apparatus with Microscope. A Shimadzu UV-2550 spectrometer was used to obtain UV spectra. IR spectra were recorded on a Bruker Vector-22 spectrometer with KBr pellets. Column chromatography (CC) was performed on Silica gel (200-300 mesh, Yantai Jiangyou Silica Gel Limited Company, Yantai, China), Sephadex LH-20 (Pharmacia Fine Chemicals) and ODS (Merck, Germany). TLC and prep.TLC: HSGF 254 silica gel plates (10-40 μm, Yantai Jiangyou Silica Gel Limited Company, Yantai, China). ^{1}H-, ^{13}C-, and 2D-NMR spectra were recorded on Bruker DRX-400 spectrometer (in CD_3OD; δ in ppm rel. to $SiMe_4$, J in Hz). MS were measured on Agilent-1100-LC/MSD-Trap (ESI-MS) and Agilent Micro-Q-Tof (HR-ESI-MS) spectrometer. Semi-preparative HPLC was performed on an Agilent 1100 liquid chromatography instrument with a Zorbax SB-C18 column (9.4 mm × 25 cm) and a UV detector at 210 nm.

Plant material

The branches and leaves of *C. lanceolata* were collected in August 2010, in Gongshan county, Yunnan province, China, and authenticated by Prof. Yuanchuan Zhou in Nujiang Institute of Medicinal Plants. A voucher specimen (No. 2010108070) is deposited in School of Pharmacy, Second Military Medical University.

Extraction and isolation

The branches and leaves of *C. lanceolata* (9.3 kg) was finely pulverized and extracted with 95% EtOH (50 L) for three times. The combined extracts were concentrated to a small volume under reduced pressure, and then dissolved in 2% HCl to adjust pH to 2-3 and then partitioned with $CHCl_3$. The aqueous layers were basified with sat. Na_2CO_3 aq. to adjust pH to 10 and then extracted with $CHCl_3$ to give a crude alkaloid fraction (9 g). The remaining aqueous layer was neutralized by 2% HCl to pH 7, and extracted with petroleum ether (PE) and EtOAc. The EtOAc extract (135 g) was subjected to silica gel column chromatography (CC) with gradient $CHCl_3$-MeOH (100：0→0：100) to afford several fractions (F_1- F_7). Fraction F_4 was divided into 3 subfractions (F_{4-1} – F_{4-3}) by using BUCHI RP-MPLC eluting with MeOH-H_2O (30-100%). F_{4-1} was chromatographed over Sephadex LH-20 with $CHCl_3$ – MeOH (1 : 1), followed purification using preparative TLC developed with $CHCl_3$ – MeOH (20 : 1) to give **1** (18 mg), **8** (25 mg) and **9** (12 mg), respectively. Similarly, F_{4-2} was subjected to preparative TLC using $CHCl_3$ - EtOAC (1 :1) and PE - Acetone (7 : 3) to give **2** (8 mg), **10** (10 mg), **11** (12 mg) and **12** (7 mg). F_{4-3} was isolated by reversed-phase semi-preparative HPLC (MeOH - H_2O, 65 : 35, flow rate of 2 ml/min) and CH_3CN – H_2O, 55 :45) to give **3** (11 mg, retention time = 14. 5 min) and **7** (32 mg, retention time = 11.7 min), respectively. By similar procedures, **4** (13 mg), **5** (17 mg), and **6** (11 mg) were isolated from F_5 by using $CHCl_3$ – MeOH (10 : 1) and/or EtOAC – MeOH (50 : 1) as eluent, respectively.

Lanceolatin A (**1**)： Amorphous powder; $C_{20}H_{32}O_4$; $[\alpha]_D^{20}$ = -10.3 (c = 0.39, MeOH); IR (KBr) ν_{max} 3450, 2931, 1704, 1228, 1153 cm^{-1}; for ^{1}H (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) spectroscopic data, see Tables 1 and 2; ESI-MS (positive) *m/z* 359 $[M+Na]^+$; HR-ESI-MS (positive) $[M-H]^-$ at *m/z* 335.2223 (calcd. for $C_{20}H_{33}O_4$, 335.2228).

Lanceolatin B (**2**): Amorphous powder; $C_{19}H_{28}O_3$; $[\alpha]_D^{20}$ = +34.7 (c = 0.32, MeOH); IR (KBr) ν_{max} 3428, 2931, 2867, 1677, 1619, 1371, 1286, 1041, 952 cm^{-1}; for ^{1}H (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) spectroscopic data, see **Tables 1-2**; ESI-MS (positive) *m/z* 305 $[M+H]^+$, 327 $[M+Na]^+$; HR-ESI-MS (positive) $[M+H]^+$ at *m/z* 305.2100 (calcd. $C_{19}H_{29}O_3$, 305.2111).

Lanceolatin C (**3**): Amorphous powder; $C_{19}H_{26}O_3$; $[\alpha]_D^{20}$ = +90.4 (*c* = 0.28, MeOH); IR (KBr) ν_{max} 3434, 2971, 2931, 1700, 1681, 1625, 1432, 1380, 1288, 1143, 1043 cm^{-1}; for ^1H (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) spectroscopic data, see Tables 1 and 2; ESI-MS (positive) *m/z* 325 $[M+Na]^+$, ESI-MS (negative) *m/z* 301 $[M-H]^-$; HR-ESI-MS (positive) $[M+H]^+$ *m/z* 303.1969 (calcd. $C_{19}H_{27}O_3$, 303.1955).

Lanceolatin D (**4**): Amorphous powder; $C_{21}H_{28}O_5$; $[\alpha]_D^{20}$ = -19.3 (*c* = 0.145, MeOH); IR (KBr) ν_{max} 3426, 2929, 2873, 1706, 1452, 1423, 1299, 1035, 858 cm^{-1}; ^1H (CD_3OD, 500 MHz) and ^{13}C-NMR (CD_3OD, 125 MHz) spectroscopic data, see Tables 1 and 2. ESI-MS (positive) *m/z* 383 $[M+Na]^+$, ESI-MS (negative) *m/z* 359 $[M-H]^-$; HR-ESI-MS (positive) *m/z* $[M+H]^+$ 361.2024 (calcd. $C_{21}H_{29}O_5$, 361.2010).

Lanceolatin E (**5**): Amorphous powder; $C_{21}H_{30}O_6$; $[\alpha]_D^{20}$ = +84.5 (*c* = 0.355, MeOH); IR (KBr) ν_{max} 3396, 2937, 1616, 1313, 1133, 1089, 1039, 923, 869 cm^{-1}; for ^1H (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz) spectroscopic data, see Tables 1 and 2; ESI-MS (positive) *m/z* 401 $[M+Na]^+$, ESI-MS (negative) *m/z* 377 $[M-H]^-$; HR-ESI-MS (positive) $[M+Na]^+$ *m/z* 401.1930 (calcd. $C_{21}H_{30}NaO_6$, 401.1935).

Lanceolatin E (**6**)**:** Amorphous powder; $C_{21}H_{30}O_6$; $[\alpha]_D^{20}$ = -84.5 (*c* = 0.355, MeOH); IR (KBr) ν_{max} 3407, 2929, 1637, 1421, 1309, 1051, 1029, 981, 910 cm^{-1}. For ^1H (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz) spectroscopic data, see Tables 1 and 2; ESI-MS (positive) *m/z* 401 $[M+Na]^+$, ESI-MS (negative) *m/z* 377 $[M-H]^-$; HR-ESI-MS (positive) $[M+Na]^+$ *m/z* 401.1962 (calcd. $C_{21}H_{30}NaO_6$, 401.1935).

Gongshanolide (**7**)**:** Colorless block; $C_{19}H_{24}O_5$; M.p. 180-182 °C; $[\alpha]_D^{20}$ = -103.0 (*c* = 0.30, MeOH); IR (KBr) ν_{max} 3363, 2925, 1735, 1683, 1629, 1361, 1043 cm^{-1}. for ^1H (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz) spectroscopic data, see Tables 1 and 2; ESI-MS (positive) *m/z* 355 $[M+Na]^+$, ESI-MS (negative) *m/z* 331 $[M-H]^-$; HR-ESI-MS (positive) $[M+H]^+$ *m/z* 333.1690 (calcd. $C_{19}H_{25}O_5$, 333.1697).

Single crystal X-ray diffraction crystallographic data of gongshanolide (7)

$C_{19}H_{24}O_5 \cdot H_2O$, M = 350, colorless block, λ = 1.54178 Å (Cu*Ka* radiation), *T* = 133 (2) K, orthorhombic, space group P2(1)2(1)2(1), a =9.6069 (10) Å, α = 90 °; b = 10.2192 (10) Å, β = 90 °; c = 17.6653 (2) Å, γ = 90 °; V = 1734.29 (3) $Å^3$, Z = 4, D_{calcd} = 1.342 mg/m^3, crystal size 0.25 × 0.18 × 0.11 mm^3, *F*(000) = 752, final R values were R = 0.0323, and R_w = 0.0862 [I > 2δ(I)]. The data were collected using Bruker APEX-II CCD diffractometer, and the structure was solved by direct methods using SHELXL-97. The crystal structure has been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 875594.

Assay for cytotoxicity against five human tumor cell lines

Assay was performed in 96-well plates. Cell cultures were diluted with fresh medium consisting of RPMI1640 with 15% newborn bovine serum (NBS), 100 IU/mL penicillin, and 100 IU/mL phytomycin to 5×10^4 cells/mL and plated in 96-well microplates at 100 μL/well. After 24 h incubation at 37° in a 5% CO_2 atmosphere, the tested compounds at six different concentrations (10^{-2}-10^2 μg/mL) were added to the microplates in 10-μL amounts. The five tumor cell lines, A549, HCT116, SK-BR-3, HepG2, and HL-60 were exposed to the drugs for another 72 h. Then, 20 μL of MTT soln. (5 mg/mL) were added to each well, and the plate was incubated for 4 h at 37° with 5% CO_2. The OD of each well was measured on a plate reader (*Wellscan MK-2, Labsystems*, Finland) at 570 nm. Adriamycin (purchased from *Nanjing Tianzun Zezhong Chemical Co. Ltd.*, P. R. China) was used as positive reference substance with concentrations of 10^{-3}-10^2 μg/mL. The cell lines were all preserved in Shanghai Institute for Pharmaceutical Industry, P. R. China.[25]

Assay for inhibition against NO release in LPS-induced RAW 264.7 macrophages

The tested compounds were dissolved in DMSO, and diluted with deionized water to a final volume of DMSO ≤ 0.5% prior to experiment. The macrophages were seeded in 48-well plates (2×10^5 cells/well). The cells were co-incubated with drugs and LPS (1 μg/ml) for 18 h, with aminoguanidine as positive control. The amount of NO was assessed by determining the nitrite concentration in the supernatants with Griess reagent. Aliquots of supernatants (100 μL) were incubated, in sequence, with 50 μL 1% sulphanilamide and 50 μL 0.1% naphthylethylenediamine in 2.5% phosphoric acid solution. The absorbances at 570 nm were read using a microtiter plate reader.[26]

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Notes and references

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† Electronic Supplementary Information (ESI) available: 1D, 2D NMR spectra of compounds **1**-**7**. For ESI See DOI: 10.1039/b000000x/

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Lanceolatins A-G, diterpenoids from *Cephalotaxus lanceolata* and their anti-inflammatory and anti-tumor activities

Yi-Ren He, Yun-Heng Shen, Lei Shan, Xi Yang, Bo Wen, Ji Ye, Xing Yuan, Hui-Liang Li, Xi-Ke Xu, and Wei-Dong Zhang

Seven new diterpenoids lanceolatins A-G (**1**-**7**), including two rare 16-nor-17-methyl (15→12) -*abeo* abietanes and one structurally novel hainanolide derivative with the ring opening of 13,14-epoxyl ring and partially reduced tropone moiety, were isolated from the branches and leaves of *Cephalotaxus lanceolata*.