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Clerodane diterpenoids with potential anti-inflammatory activity from the leaves and twigs of *Callicarpa cathayana*

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[ABSTRACT] Phytochemical investigation of the leaves and twigs of *Callicarpa cathayana* led to the isolation of six new clerodane diterpenoids, cathayanalactones A−F (**1**−**6**), together with seven analogues (**7**−**13**). Their structures were established by extensive NMR analyses together with experimental and calculated ECD spectra analyses. Compounds **1**, **2**, **3**, **7** and **11** showed inhibitory activities on lipopolysaccharide-induced nitric oxide production in RAW264.7 cells.

[KEY WORDS] *Callicarpa cathayana*; Clerodane diterpenoids; Nitric oxide; Anti-inflammatory activity

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Introduction

The genus *Callicarpa* (Verbenaceae) is composed of approximately 190 species and mainly distributed in Asia and Oceanica, 46 species of them exist in China, throughout the tropics and sub-tropics [1]. Some *Callicarpa* species, such as *C. macrophylla*, *C. nudiflora*, and *C. dichotoma*, have been used as folk medicines for the treatment of various medical indications since ancient times, such as internal hemorrhage, rheumatism, furuncle and carbuncle [2]. Phytochemical researches indicated that many components exist in this genus, including terpenoids (especially diterpenoids and triterpenoids) [3-10], phenylethanoids [11], lignans [12], and flavonoids [13]. In our previous studies, 15 abietane diterpenoids were isolated from a close species, *C. bodinieri* [14]. As a continue research on bioactive secondary metabolites in this genus, the ethyl acetate extract of *C. cathayana* was subsequently investigated and thirteen clerodane diterpenoids (**1**−**13**), including six new compounds, cathayanalactones A−F (**1**−**6**), were obtained. The structures of compounds **1**−**13** were elucidated based on extensive 1D and 2D NMR data analysis, and the absolute configuration of new compounds was established by ECD analyses. Herein, we report the details of isolation, structure elucidation of six new compounds, as well as their inhibition on LPS-induced NO production.

Results and Discussion

Cathayanalactone A (**1**) was obtained as colorless oil, and its molecular formula was determined as $C_{23}H_{34}O_6$ by HR-ESI-MS at *m/z* 429.2244 $[M + Na]^+$, accounting for seven degrees of unsaturation. ^{1}H NMR spectra of **1** exhibited signals for four methyl groups [δ_H 0.70 (3H, s, H_3-20), 0.80 (3H, d, J = 6.3 Hz, H_3-17), 1.03 (3H, s, H_3-19), 1.91 (3H, s, H-2′)], one methoxy group [δ_H 3.54 (3H, s, 16-OMe)]. Two terminal ethylenic protons [δ_H 5.55 (2H, s, H-18)], and two oxymethine protons [δ_H 6.16 (1H, s, H-16), 5.67 (1H, d, J = 9.6 Hz, H-12)] (Table 1). The ^{13}C NMR spectra of **1** showed 23 carbon reso-

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Dedicated to Professor SUN Han-Dong on the Occasion of His 80th Birthday

nances, including two carbonyl groups [δ_C 170.4 (C-15), and 170.6 (C-1′)], four olefinic carbons [δ_C 167.7 (C-13), 119.7 (C-14), 164.2 (C-4), and 100.4 (C-18)], two oxygenated carbons [δ_C 104.7 (C-16), and 69.2 (C-3)], and one methoxy [δ_C 58.1 (16-OMe)] (Table 1). An *α*, *β*-unsaturated lactone part existed in **1** based on two downfield shifted protons [δ_H 6.43 (1H, s, H-14), 6.16 (1H, s, H-16)], two methine groups [δ_C 119.7 (C-14), 104.7 (C-16)], as well as two quaternary carbons [δ_C 170.6 (C-1′), 167.7 (C-13)]. Characteristic proton signals [δ_H 5.55 (1H, s), 4.86 (1H, s), H_2-18], and olefinic carbon signals [δ_C 164.2 (C-4), 100.4 (C-18)] indicated the presence of terminal double bond of carbon. Its NMR data indicated a clerodane diterpenoid similar to 16-methoxy-cleroda-4(18), 13-dien-15, 16-olide [24], except that two methylene groups were oxidized, and the presence of acetyl group. Based on the long-range correlations of H-14 to C-12, H-11 to C-1′, H-2′ to C-1′, and H-3 to C-4 in HMBC spectra (Fig. 2), planar structure of **1** was assigned. Chemical shift of C-19 (δ_C 21.8) indicated that the A/B ring junction was *trans*, because this carbon appears downfield by approx. 10 ppm in *cis* clerodanes [16]. Moreover, correlations of H-3/H_3-19, H_3-17/H_3-19, H-11/H-8, and H-11/H-10 in ROESY (Fig. 3) spectra revealed the orientation of H-3, H_3-17, H_3-19, H_3-20, and H-10 as *α*, *α*, *α*, *β*, and *β*, respectively.

Cathayanalactone B (**2**) was also obtained as colorless oil, and its molecular formula was determined as $C_{23}H_{34}O_6$ by HR-ESI-MS at *m/z* 429.2247 $[M + Na]^+$. The NMR spectra of **1** and **2** were really similar, except the chemical shift in C-16 (δ_C 104.7 for **1** and 103.4 for **2**) (Table 1). The presence of an *α*, *β*-unsaturated carboxylic acid next to the stereo center at C-12 and C-16 suggested that the absolute configuration of **1** and **2** could possibly be solved by ECD [16-17]. As to the stereochemistry of C-12 and C-16, four stereoisomers were possible (12*R*, 16*R*; 12*R*, 16*S*; 12*S*, 16*R* and 12*S*, 16*S*). The experimental ECD spectra of **1** showed one weak positive Cotton effect (CE) at about 245 nm. The calculated spectra of the (12*S*, 16*S*) stereoisomer showed excellent fit with the experimental data of **1** (Fig. 4). The experimental ECD spectra of **2** showed a positive and a negative CE at 220 and 245 nm, respectively, which was similar to the calculated spectra of the (12*S*, 16*R*) stereoisomer. Hence, the absolute configuration of **1** and **2** was confirmed as 3*S*, 5*R*, 8*R*, 9*S*, 10*R*, 12*S*, 16*S*, and 3*S*, 5*R*, 8*R*, 9*S*, 10*R*, 12*S*, 16*R*, respectively (Fig. 5).

Table 1 ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for compounds 1 and 2 in C_5H_5N

Position	1		2	
	δ_H[a] (*J* in Hz)	δ_C	δ_H[a] (*J* in Hz)	δ_C
1	1.51, m	21.9, CH_2	1.53, m; 1.89, m	21.9, CH_2
2	2.47, m	38.9, CH_2	2.49, m; 1.47, m	39.0, CH_2
3	4.57, m	69.2, CH	4.59, d (5.8)	69.2, CH
4		164.2, C		164.2, C
5		40.9, C		40.9, C
6	1.48, m	37.9, CH_2	2.40, m	37.9, CH_2
7	1.40, m; 1.46, m	27.9, CH_2	1.40, m; 1.54, m	27.8, CH_2
8	1.54, m	37.8, CH	1.55, m	37.9, CH
9		40.9, C		40.9, C
10	1.23, m	50.3, CH	1.21, d (11.9)	50.2, CH
11	2.01, dd (16.1, 9.7) 1.76, d (16.1)	41.1, CH_2	2.05, dd (16.3, 9.4) 1.66, d (16.3)	40.7, CH_2
12	5.67, d (9.7)	67.0, CH	6.00, d (9.2)	67.2, CH
13		167.7, C		167.9, C
14	6.43, s	119.7, CH	6.37, s	119.0, CH
15		170.4, C		170.1, C
16	6.16, s	104.7, CH	6.20, s	103.4, CH
17	0.80, d (6.3)	16.2, CH_3	0.84, d (6.6)	16.4, CH_3
18	5.55, s 4.86, s	100.4, CH_2	5.57, s 4.86, s	100.5, CH_2
19	1.03, s	21.8, CH_3	1.02, s	21.8, CH_3
20	0.70, s	17.9, CH_3	0.72, s	17.9, CH_3
12-OAc	1.91, s	170.6, C; 21.2, CH_3	2.00, s	170.2, s; 21.2, CH_3
16-OMe	3.54, s	58.1, CH_3	3.55, s	56.6, CH_3

[a] "m" means signals were in overlapped regions or multiplicities

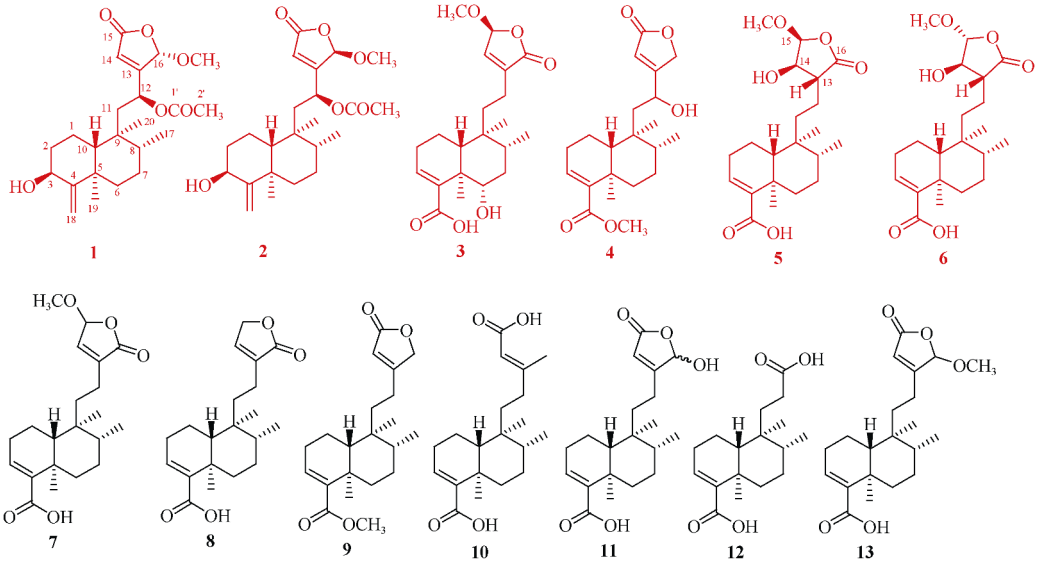

Fig. 1 Structures of compounds 1−13 (red for new compounds)

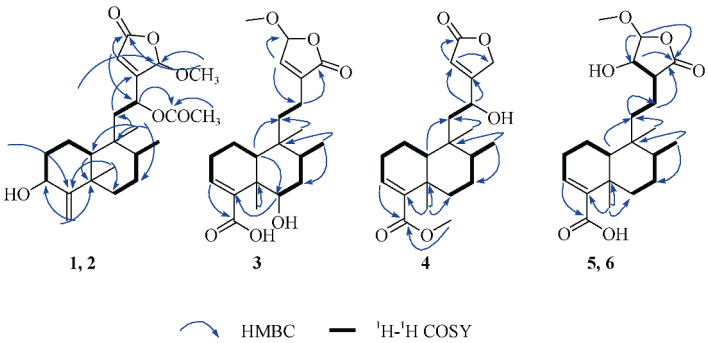

Fig. 2 Key ^{1}H-^{1}H COSY and HMBC correlations of compounds 1−6

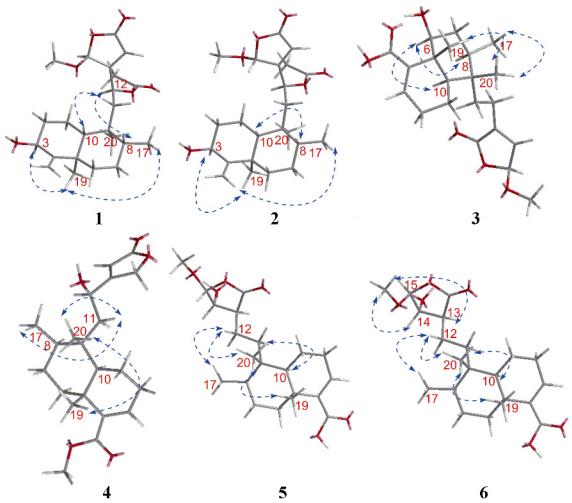

Fig. 3 Key ROESY/NOESY correlations of compounds 1−6

Cathayanalactone C (**3**) was obtained as white solid, and its molecular formula was determined as $C_{21}H_{30}O_6$ by HR-ESI-MS at *m/z* 401.1935 $[M + Na]^+$, accounting for seven degrees of unsaturation. The ^{1}H NMR and HMQC spectra of **3** exhibited signals for three aliphatic methyl groups [δ_H 0.77 (3H, s, H_3-20), 0.88 (3H, d, J = 6.8 Hz, H_3-17), 1.23 (3H, s, H_3-19)], one methoxy group [δ_H 3.63 (3H, s)], two olefinic protons [δ_H 6.98 (1H, m, H-3), 5.83 (1H, br s, H-13)], and two oxymethine protons [δ_H 6.82 (1H, m, H-15), 3.63 (1H, dd,

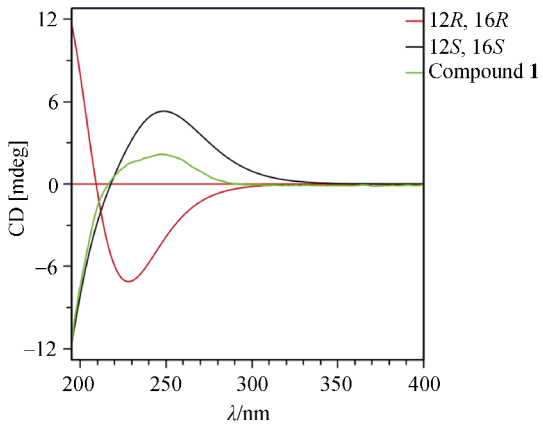

Fig. 4 Experimental and calculated ECD spectra for compound 1

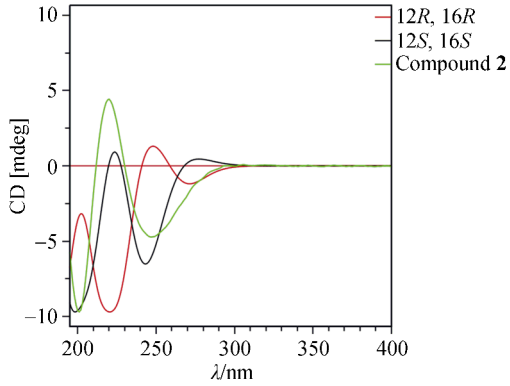

Fig. 5 Experimental and calculated ECD spectra for compound 2

J = 11.1, 4.8 Hz, H-6)] (Table 2). The ^{13}C NMR spectra of **3** showed 21 carbon resonances, which consisted of two carbonyl groups [δ_C 174.1 (C-16), and 173.4 (C-18)], four olefinic carbons [δ_C 144.3 (C-14), 143.5 (C-4), 140.6 (C-3), and 139.5 (C-13)], two oxygenated carbons [δ_C 104.4 (C-15), and 75.9 (C-6)], and one methoxy group (δ_C 57.1) (Table 2). Its NMR data indicated a clerodane diterpenoid which was similar to 15-methoxypatagonic acid [15], except that a methylene group was oxidized. The HMBC spectra showed the long-range correlations of H-6 to C-19 and C-4, H-10 to C-6, verifying that hydroxy group was linked to C-6 (Fig. 2). The long-range correlation of methoxy protons (δ_H 3.57) to C-15 suggested the methoxy group was linked to C-15. Moreover, in the ROESY spectra, correlations of H_3-20/H_3-19, H_3-20/H_3-17, H-6/H-8, and H-6/H-10 revealed the relative configuration of H_3-17, H_3-19, H_3-20, H-6, and H-10 as *α*, *α*, *α*, *β*, and *β*, respectively (Fig. 3). The absolute configuration of C-15 in **3** could be solved by ECD method. The calculated ECD spectra for the 15*S* stereoisomer excellently fitted to the experimental-curve, with one negative CE at 220 and one positive CE at 250 nm. The calculated spectra of the 15*R* had a negative CE at 250 nm, which was different from the experimental data (Fig. 6). Herein, the absolute configuration of **3** was assigned as 5*R*, 6*S*, 8*R*, 9*S*, 10*R*, and 15*S*.

Table 2 ^{1}H and ^{13}C NMR data for compounds 3 and 4

Position	**3**[a]		**4**[b]	
	δ_H[c] (*J* in Hz)	δ_C	δ_H[c] (*J* in Hz)	δ_C
1	1.68, m; 2.31, m	18.2, CH_2	1.97, dd (8.2, 4.7) 1.47, dd (11.1, 4.6)	19.1, CH_2
2	2.28, m	28.1, CH_2	2.22, m	27.6, CH_2
3	5.83, m	140.6, CH	6.56, m	138.2, CH
4		143.4, C		142.9, C
5		45.9, C		38.5, C
6	3.63, dd (11.1, 4.8)	75.9, CH	2.30, dt (12.8, 3.5) 1.10, td (13.1, 3.8)	36.5, CH_2
7	1.52, m	37.1, CH_2	1.52, m; 1.43, m	28.2, CH_2
8	1.70, m	35.3, CH	1.68, m	37.6, CH
9		39.8, C		40.5, C
10	1.52, m	47.0, CH	1.73, m	47.8, CH
11	2.18, m	37.2, CH_2	1.94, d (8.7) 1.62, d (15.7)	44.9, CH_2
12	2.04, td (13.9, 13.3, 4.9)	19.6, CH_2	4.85, m	65.4, CH
13		139.4, C		177.1, C
14	6.98, s	144.3, CH	5.93, s	114.0, CH
15	6.82, s	104.4, CH		173.9, C
16		173.4, C	4.94, s	71.6, CH_2
17	0.88, d, (6.7)	15.9, CH_3	0.83, d (6.8)	16.4, CH_3
18		174.0, C		167.9, C
19	1.23, s	16.9, CH_3	1.27, s	21.3, CH_3
20	0.77, s	18.0, CH_3	0.78, s	18.1, CH_3
15-OMe	3.53, s	57.1, CH_3		
18-OMe			3.63, s	51.2, CH_3
12-OH			4.56, d (5.6)	

[a] measured at 400 MHz in CD_3OD; [b] measured at 600 MHz in CD_3COCD_3; [c] "m" means signals were in overlapped regions or multiplicities

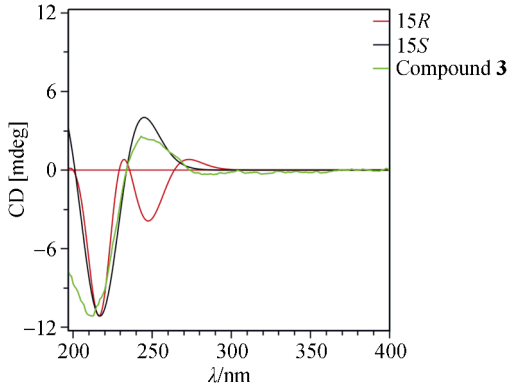

Fig. 6 Experimental and calculated ECD spectra for compound 3

Cathayanalactone D (**4**) was isolated as white powder, with amolecular formula of $C_{21}H_{30}O_5$, evidenced by HR-EIS-MS at *m/z* 385.1981 $[M + Na]^+$ (Calcd. 385.1985) and NMR data, with seven degrees of unsaturation. The ^{1}H NMR and HMQC spectra showed the presence of two olefinic protons at δ_H 6.56 (1H, m, H-3), and 5.93 (1H, s, H-14); one oxygenated methylene group at δ_H 4.94 (2H, s, H-16); one oxygenated methane group at δ_H 4.85 (1H, m, H-12); one methoxy group at 3.63 (3H, s, 18-OMe); three methyl groups at δ_H 1.27 (3H, s, H_3-19), 0.83 (3H, d, J = 6.8 Hz, H_3-17), and 0.78 (3H, s, H_3-20) (Table 2). The ^{13}C NMR and HMBC spectra (Table 2) revealed 21 carbon signals, comprising two ester carbonyl groups at δ_C 173.9 (C-15) and 167.9 (C-18); two double bonds of carbon at δ_C 177.1 (C-13), 142.9 (C-4), 138.2 (C-3), and 114.0 (C-14); two oxygenated carbons at δ_C 71.6 (C-16), and 65.4 (C-12); one methoxyl group at δ_C 51.2 (18-OMe); and three methyl groups at δ_C 21.3 (C-19), 18.1 (C-20), and 16.4 (C-17) (Table 2). The NMR spectra of **4** was very similar to 1-naphthalenecarboxylic acid [18], except that a methylene group was oxidized. The HMBC correlations of H_3-20 to C-11, H-9 to C-11, H-10 to C-11, and H-12 to C-13, C-14, demonstrated that hydroxy group was linked to C-12 (Fig. 2). As a result, the planar structure of **4** was assigned. Chemical shift of C-19 (δ_C 21.3), and correlations of H_3-19 to H_3-20, H-11a to H_3-17/H_3-20 in the ROESY spectra, indicated the orientation of H_3-17, H_3-19, H_3-20, and H-10 as α, α, α, and β, respectively (Fig. 3). However, the absolute configuration of C-12 couldn't be solved by ECD, because two stereoisomers had similar CEs (Fig. 7).

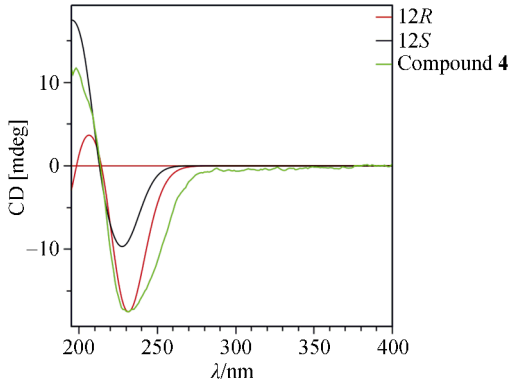

Fig. 7 Experimental and calculated ECD spectra for compound 4

Cathayanalactones E and F (**5** and **6**) were isolated as white solid assigned the same molecular formula of $C_{21}H_{32}O_6$ based on the HR-ESI-MS at *m/z* 379.2130 $[M - H]^-$ for **5**, and 379.2121 $[M - H]^-$ for **6** (Calcd. 379.2126), respectively, with six degrees of unsaturation. The NMR spectra of **5** and **6** were very similar to those of 15-methoxypatagonic acid [15], except that one double bond of carbon was oxidized (Table 3). The long-range HMBC correlations of H_3-19 to C-4 and H-3 to C-4 indicated that double bond of C-3 and C-4 existed in **5** and **6**. The HMBC spectra also showed the long-range correlations of H-12 to C-16, H-14 to C-16, and H_3-OMe to C-15 suggested that double bond of C-13 and C-14 was oxidized, and methoxy group was linked to C-15, respectively (Fig. 2). Chemical shift of C-19 (δ_C 21.0 for **5**, 20.9 for **6**), and correlations of H_3-19 to H_3-20, H-12a to H_3-17/H_3-20 in **5** and **6** in the NOESY spectra, revealed the orientation of H_3-17, H_3-19, H_3-20, and H-10 as α, α, α, and β, respectively (Fig. 3).

Table 3 ^{1}H and ^{13}C NMR data for compounds 5 and 6 in CD_3COCD_3

Position	**5**[a]		**6**[b]	
	δ_H[c] (J in Hz)	δ_C	δ_H[c] (J in Hz)	δ_C
1	1.83, m	18.1, CH_2	1.83, m	18.1, CH_2
2	2.27, m	27.9, CH_2	2.20, m	27.8, CH_2
3	6.71, m	137.9, CH	6.67	138.0, CH
4		143.0, C		143.1, C
5		38.4, C		38.4, C
6	1.64, m	36.8, CH_2	1.54, m	36.8, CH_2
7	1.56, m; 1.36, m	28.1, CH_2	1.56, m; 1.42, m	28.1, CH_2
8	1.52, m	37.2, CH	1.51, m	37.1, CH
9		39.5, C		39.5, C
10	1.39, m	47.7, CH	1.34, m	47.8, CH

Continued

No.	**5**[a]		**6**[b]	
	δ_H[c] (*J* in Hz)	δ_C	δ_H[c] (*J* in Hz)	δ_C
11	2.43, m; 1.11, m	36.8, CH_2	2.43, m; 1.13, m	36.8, CH_2
12	1.48, m	17.6, CH_2	1.55, m	17.6, CH_2
13	2.57, m	44.0, CH	2.57, m	44.1, CH
14	4.27, d (5.1)	72.7, CH	4.28, d (4.9)	72.6, CH
15	5.17, s	109.0, CH	5.18, s	109.0, CH
16		178.1, C		178.1, C
17	0.82, d (6.2)	16.3, CH_3	0.86, d (6.6)	16.3, CH_3
18		168.3, C		168.6, C
19	1.26, s	21.0, CH_3	1.25, s	20.9, CH_3
20	0.76, s	18.7, CH_3	0.76, s	18.8, CH_3
15-OMe	3.45, s	56.6, CH_3	3.44, s	56.6, CH_3

[a] measured at 400 MHz; [b] measured at 600 MHz; [c] "m" means signals were in overlapped regions or multiplicities

The difference between compound **5** and **6** was the configuration of C-13, C-14, and C-15. NOESY correlations from H-13 to H-15, and H-14 to H_3-OMe were in **6**, but no such correlations in NOESY spectra of **5**, indicated the relative configuration was 13*S**, 14*S**, 15*R** in **5**, and 13*S**, 14*S**, 15*S** in **6**. As for **5**, two stereoisomers were possible (13*S*, 14*S*, 15*R* and 13*R*, 14*R*, 15*S*). The experimental ECD spectra of **5** showed strong negative CE at about 240 nm, which was in the line with calculated spectra of (13*R*, 14*R*, 15*S*) stereoisomer. Therefore, the absolute configuration of **5** was confirmed as 5*R*, 8*R*, 9*S*, 10*R*, 13*R*, 14*R*, and 15*S* (Fig. 8). The experimental ECD spectra of **6** was similar to calculated spectra of (13*R*, 14*R*, 15*S*), so the absolute configuration of **6** was confirmed as 5*R*, 8*R*, 9*S*, 10*R*, 13*R*, 14*R*, and 15*R* (Fig. 9).

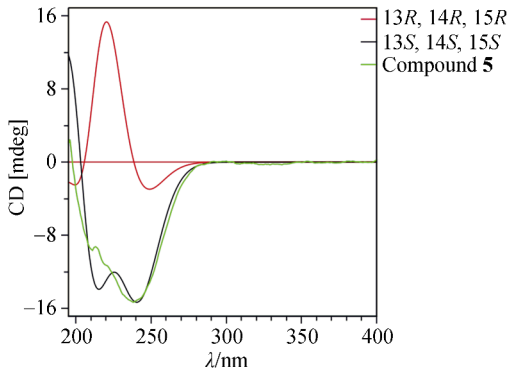

Fig. 8 Experimental and calculated ECD spectra for compound 5

Based on NMR data and reported literature, the remainning known compounds were elucidated as 15-methoxypatagonic acid (**7**) [15], patagonic acid (**8**) [19], 1-naphthalenecarboxylic acid (**9**) [18], kolavic acid (**10**) [20], 16-hydroxycleroda-3, 13-dien-16, 15-olide-18-oic acid (**11**) [21], norhardwickiic acid (**12**) [22], and dodovislactone B (**13**) [23].

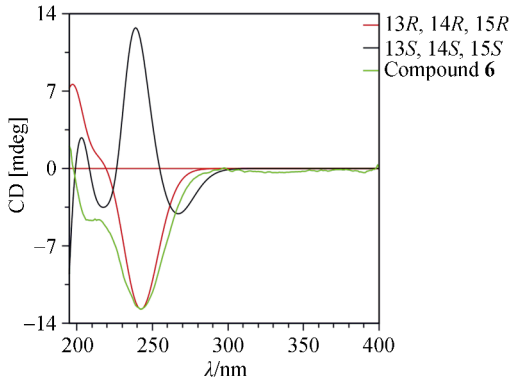

Fig. 9 Experimental and calculated ECD spectra for compound 6

The anti-inflammatory activity of these compounds was first screened by evaluating nitric oxide (NO) levels in LPS-stimulated RAW264.7 cells (Fig. 10a). As a result, compounds **1**, **2**, **3**, **7** and **11** showed significant NO inhibitory activity with IC_{50} values ranging from 13.25 to 82.82 μmol·L^{-1} (Figs. 10b−10f) at noncytotoxic concentrations (Figs. 10g−10k). We next explored the pro-inflammatory cytokines secretion by treatment with large amount compounds. As shown in Fig. 11, compounds **3**, **7** and **11** inhibited IL-1*β*, IL-6 and TNF-*α* levels in LPS-stimulated RAW264.7 cells in a dose-responsive manner.

Conclusions

In summary, our phytochemical research on *C. cathayana* has led to the isolation and structure elucidation of six new (**1**−**6**) and seven known clerodane diterpenoids (**7**−**13**) with five-membered-lactone ring. Furthermore, the absolute configurations of compounds **1**−**3**, **5**, and **6** were determined based on NMR data analysis, and experimental and calculated ECD spectra. Compounds **1**, **2**, **3**, **7** and **11** showed inhibitory activities on LPS-induced NO production in RAW264.7 cells.

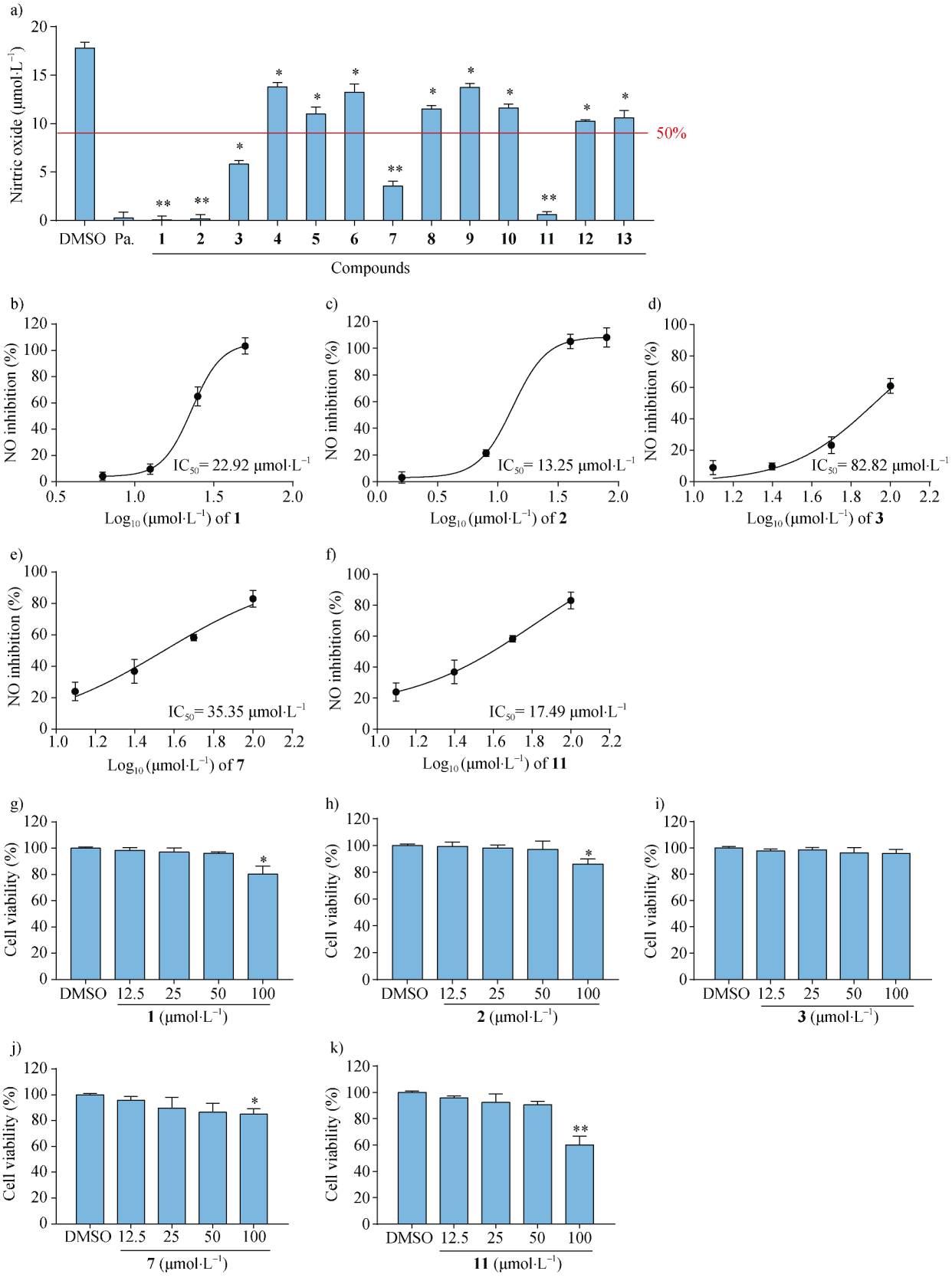

Fig. 10 The effects of compounds on NO production and cytotoxicity. a) Primary screen of all compounds against NO production in LPS treated RAW264.7 cells. b−f) NO inhibition of compounds 1, 2, 3, 7 and 11 in RAW264.7 cells. g−k) Cell viability of compounds 1, 2, 3, 7 and 11 in RAW264.7 cells. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* DMSO group (Pa.: Parthenolide, as a positive control)

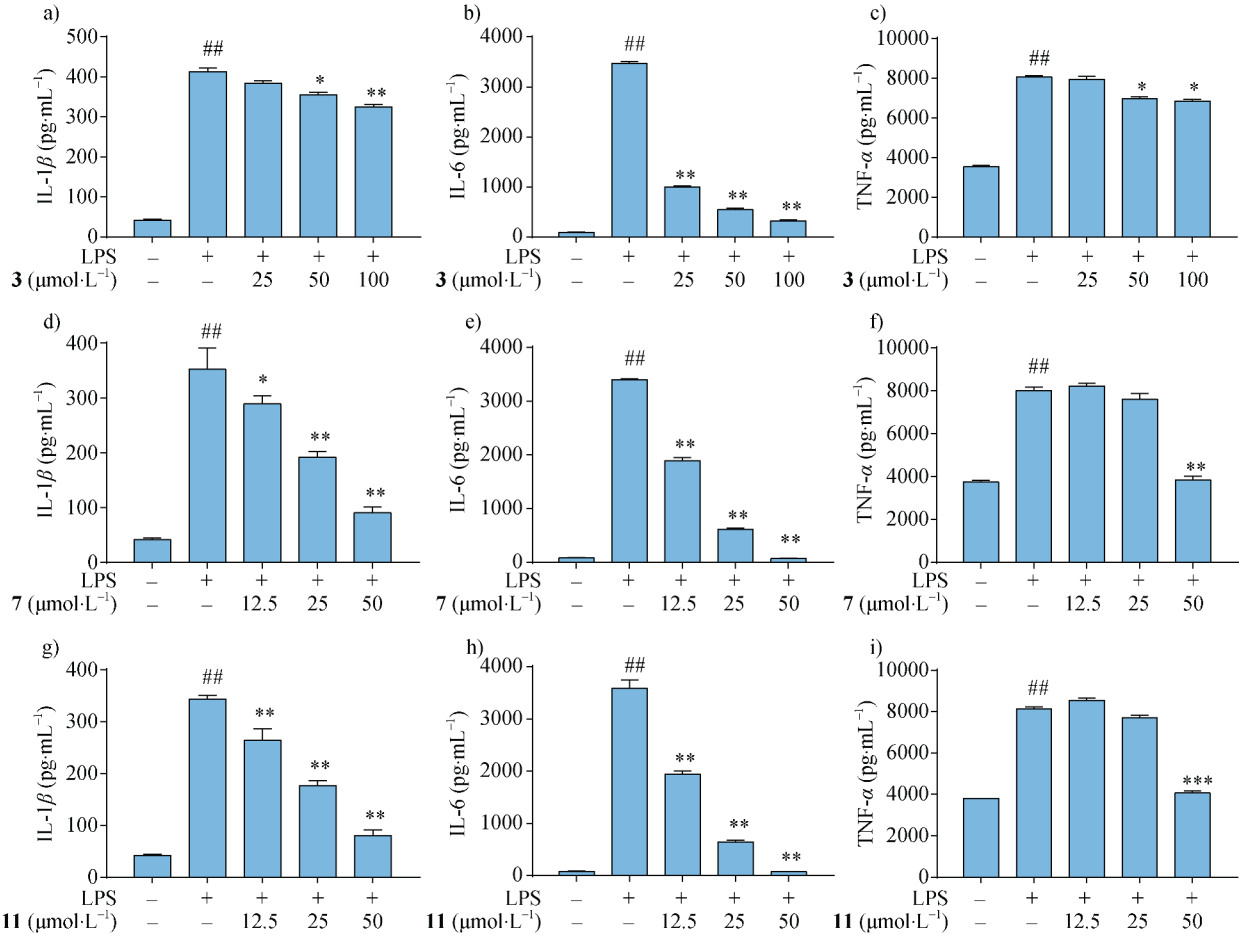

Fig. 11 The effects of compounds 3, 7 and 11 on LPS (1 μg·mL^{-1}) induced secretion of pro-inflammatory cytokines. a−c) IL-1*β*, IL-6 and TNF-*α* levels with the treatment of compound 1. d−f) IL-1*β*, IL-6 and TNF-*α* levels with the treatment of compound 7. g−i) IL-1*β*, IL-6 and TNF-*α* levels with the treatment of compound 11. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* LPS-treated group; $^{\#\#}P < 0.01$ *vs* untreated group

Experimental

General experimental procedures

NMR data were collected on a Bruker AV-400 and AV-III-600 HD spectrometers in CD_3COCD_3, CD_3OD or C_5D_5N (Bruker, Fällanden, Switzerland). HRESI-MS were recorded in methanol on an Agilent Technologies 6520 Accurate Mass Q-TOF LC/MS spectrometer (Agilent Technologies, Santa Clara, USA). IR spectra were confirmed using a Bruker Tensor-27 FT-IR spectrometer (Bruker Optics, Ettlingen, Germany). UV spectra were recorded in MeOH on a Shimadzu UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan). Optical rotations were confirmed using a JASCO P-1020 polarimeter (JASCO, Tokyo, Japan). CD spectra were taken in MeOH on a JASCO J-815 spectrometer (JASCO, Tokyo, Japan). Semipreparative HPLC was performed on an Agilent 1260 liquid chromatograph system (Agilent Technologies Inc., Waldbronn, Germany) equipped a diode array detector (DAD) with a column named Zorbax SB-C_{18} (9.4 mm × 250 mm, 5 μm, Agilent Technologies Inc., Santa Clara, USA). Column chromatography (CC) was performed on Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden), MCI gel CHP 20P/P120 (Mitsubishi Chemical Corporation, Tokyo, Japan), together with silica gel (80−100, 100−200 and 200−300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), and reversed-phase C_{18} (150−200 mesh, Merck, Germany). Fractions were monitored by using TLC (GF_{254} plates, Qingdao Marine Chemical Factory, Qingdao, China). Chromogenic agent consisted of 8%−10% H_2SO_4 in ethanol, followed by heating.

Plant material

The leaves and twigs of C. cathayana were collected in September 2017 from Honghe, Yunnan Province, China. The botanical identification was made by Prof. CHEN Yu, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (YNU 20170045) was deposited at the Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education, Yunnan University.

Extraction and isolation

The dried and powdered leaves together with twigs of *C. cathayana* (7.3 kg) were extracted three times (20 L, each) with 85% MeOH−H2O at room temperature to obtain crude

extract (450 g). Then suspended the crude extract in water, and extracted with ethyl acetate (EtOAc) to collect EtOAc solution (107 g). EtOAc soluble part was subjected to a silica gel column chromatography, using a gradient mixture of chloroform−acetone (50 : 1 to 0 : 1, *V/V*) to afford and combine into 5 fractions (F1−F6) based on TLC analysis. F3 (17 g) was subjected to MCI gel column eluted with MeOH−H_2O mixtures (1 : 1 to 1 : 0) to yield 5 fractions (F3.1−F3.3). F3.2 was purified in Sephadex LH-20 and eluted with $CHCl_3$−MeOH (1 : 1, *V/V*) to get 2 fractions (F3.2a, F3.2b). F3.2a was fractionated by RP-C_{18}, eluted with MeOH−H_2O mixtures (1 : 1 to 1 : 0) to give F3.2a1−F3.2a5. F3.2a2 was separated by silica gel CC, eluted with petroleum ether (PE) − acetone (3 : 1, *V/V*) and then purified by semi-preparative HPLC (3.0 mL·min^{-1}, 48% MeCN−H_2O) to afford **1** (17.5 mg, t_R = 15 min), **2** (7.1 mg, t_R = 17 min). Then F4 (25 g) was subjected to separate over MCI gel column, eluted with MeOH−H_2O mixtures (1 : 1 to 1 : 0) to yield 3 fractions (F4.1−F4.3). F4.3 was applied to a Sephadex LH-20 column and eluted with MeOH to give 3 fractions (F4.3a−F4.3d). F4.3b was separated by silica gel CC, eluted with PE−acetone (4 : 1 to 0 : 1, *V/V*) to obtain 2 fractions (F4.3b1 and F4.3b2), and then F4.3b1 was purified by semi-preparative HPLC (3.0 mL·min^{-1}, 60% MeCN−H_2O) to afford **12** (1.4 mg, t_R = 10 min), **8** (15.2 mg, t_R = 12.5 min), and **9** (7.1 mg, t_R = 13 min). F4.3b2 was purified by semi-preparative HPLC (3.0 mL·min^{-1}, 50% MeCN−H_2O) to obtain **5** (6.3 mg, t_R = 11.5 min) and **6** (2.3 mg, t_R = 12.5 min). F4.3c was separated using the same method as F4.3b to collect 2 fractions (F4.3c1 and F4.3c2). F4.3c1 was further purified by semi-preparative HPLC (3.0 mL·min^{-1}, 50% MeCN−H_2O, t_R = 10.3, 12.2 and 13.2 min, respectively) to collect **11** (17.4 mg), **7** (12.3 mg), and **13** (11.9 mg). F4.3c2 was further purified by semi-preparative HPLC (3.0 mL·min^{-1}, 47% MeCN−H_2O, t_R = 10.3, 13.1 and 14.0 min, respectively) to collect **10** (17.4 mg), **3** (7.8 mg), and **4** (11.4 mg).

Cathayanalactone A (**1**), $C_{23}H_{34}O_6$; colorless oil; $[\alpha]_D^{22}$ +10.6 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 196 (4.31) nm; IR (KBr) ν_{max} 3435, 2969, 2863, 1796, 1767, 1745, 1641, 1448, 1121, 1056 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESI-MS (positive) *m/z* 429.2244 $[M + Na]^+$ (Calcd. for $C_{23}H_{34}O_6Na$, 429.2248).

Cathayanalactone B (**2**), $C_{23}H_{34}O_6$; colorless oil; $[\alpha]_D^{22}$ –61.2 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 197(4.08) nm; IR (KBr) ν_{max} 3435, 2963, 2928, 2862, 1797, 1768, 1749, 1641, 1448, 1120, 1057 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HRESI-MS (positive) *m/z* 429.2247 $[M + Na]^+$ (Calcd. for $C_{23}H_{34}O_6Na$, 429.2248).

Cathayanalactone C (**3**), $C_{21}H_{30}O_6$; white powder; $[\alpha]_D^{22}$ –97.16 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 200 (3.93) nm, IR (KBr) ν_{max} 3429, 2961, 2930, 2876, 1768, 1668, 1384, 1024 cm^{-1}, ^{1}H and ^{13}C NMR data see Table 2, HRESI-MS (positive) *m/z* 401.1937 $[M + Na]^+$ (Calcd. for $C_{21}H_{30}O_6Na$, 401.1935).

Cathayanalactone D (**4**), $C_{21}H_{30}O_5$; white powder; $[\alpha]_D^{22}$ –70.44 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 212 (3.75) nm, IR (KBr) ν_{max} 3432, 2957, 2925, 2876, 1720, 1631, 1136, 1028 cm^{-1}, ^{1}H and ^{13}C NMR data see Table 2, HR-ESI-MS (positive) *m/z* 385.1981 $[M + Na]^+$ (Calcd. for $C_{21}H_{30}O_5Na$, 385.1985).

Cathayanalactone E (**5**), $C_{21}H_{32}O_6$; white powder; $[\alpha]_D^{24}$ –27.72 (*c* 0.16, MeOH), UV (MeOH) λ_{max} (log ε) 208 (4.33) nm, IR (KBr) ν_{max} 3427, 2959, 2919, 2872, 1739, 1679, 1420, 1263 cm^{-1}, ^{1}H and ^{13}C NMR data see Table 3, HR-ESI-MS (negative) *m/z* 379.2130 $[M - H]^-$ (Calcd. for $C_{21}H_{31}O_6$, 379.2126).

Cathayanalactone F (**6**), $C_{21}H_{32}O_6$; white powder; $[\alpha]_D^{24}$ –101.52 (*c* 0.16, MeOH), UV (MeOH) λ_{max} (log ε) 208 (4.27) nm, IR (KBr) ν_{max} 3413, 2959, 2919, 2873, 1740, 1680, 1262, 1128 cm^{-1}, ^{1}H and ^{13}C NMR data see Table 3, HR-ESI-MS (negative) *m/z* 379.2121 $[M - H]^-$ (Calcd. for $C_{21}H_{31}O_6$, 379.2126).

ECD computational methods

Conformation analysis of compounds **1**−**6** were performed with ROESY/NOESY spectra and Chem3D modeling, conformation searches were carried out firstly using the program BALLOON [25] and then conformers within a 2 kcal/mol energy were selected were selected for geometry optimizations. Geometry optimizations on the B3LYP/6-311 + G (d, p) level with a CPCM solvent model in methanol were finished by the Gaussian 09 package. Time-dependent density function theory (TD-DFT) ECD calculations in methanol for the optimized conformers were performed at the B3LYP/6-311 + G (d, p) level, and the calculated ECD spectra of different conformers were simulated with a half bandwidth of 0.3 eV. The ECD curves were extracted by using SpecDis 1.64 software. The spectra of all compounds were summed from individual conformers' spectra, which based on their contribution to Boltzmann-weighting.

Cytotoxicity assay

RAW264.7 is a mouse macrophage cell line and maintained in DMEM media with 10% heat inactive fetal bovine serum. Cytotoxicity of compounds in RAW264.7 were evaluated by MTT colorimetry as previously described [28].

NO release assay

NO release level was determined by Griess reagent as previously described [29]. Briefly, RAW264.7 cells were seeded into 96-well plates at a density of 2 × 10^5 cells per well overnight. After being pretreated with compounds (12.5, 25, 50 or 100 μmol·L^{-1}) or parthenolide (0.625, 1.25, 2.5 or 5 μmol·L^{-1}, as a positive) for 2 h, LPS (1 μg·mL^{-1}) was then added to the compounds containing medium and cultured for 24 h. Supernatant (100 μL) of the sample was added to an equal volume of Griess reagent in a 96-well plate, incubated at RT for 10 min. Then the absorbance was measured at 540 nm using a microplate reader.

ELISA for TNF-α, IL-6 and IL-1β

After being pretreated with compounds (12.5, 25, 50 or 100 μmol·L^{-1}) or parthenolide (0.625, 1.25, 2.5 or 5 μmol·L^{-1}, as a positive) for 2 h, LPS (1 μg·mL^{-1}) was then added to the compounds containing medium and cultured for 24 h. The

medium was collected, and the secretion levels of IL-1β, IL-6 and TNF-α were determined using ELISA kits from R&D Inc. according to the manufacturer's instructions.

Supporting Information

Detailed ^{1}D and ^{2}D NMR, HRESI-MS, IR, UV spectra and quantum chemical calculations of compounds **1**−**6** are in supporting information file online, and can be requested by sending E-mails to the corresponding author.

References

[1] Flora of China Editorial Committee of Chinese Academy of Sciences. *Flora of China* [M]. Beijing: Science Press, 2002: 25.

[2] Tu Y, Sun L, Guo M, *et al*. The medicinal uses of *Callicarpa* L. in traditional Chinese medicine: An ethnopharmacological, phytochemical and pharmacological review [J]. *J Ethnopharmacol*, 2013, **146**(2): 465-481.

[3] Luo YH, Fu HZ, Huang B, *et al*. Hepatoprotective iridoid glucosides from *Callicarpa nudiflora* [J]. *J Asian Nat Prod Res*, 2015, **18**(3): 1-6.

[4] Cheng HH, Cheng YB, Hwang TL, *et al*. Anti-inflammatory diterpenoids from *Callicarpa randaiensis* [J]. *Planta Med*, 2016, **81**(S01): S1-S381.

[5] Zhou GP, Yan Y, Yuan MM, *et al*. Four new triterpenoids from *Callicarpa kwangtungensis* [J]. *Molecules*, 2015, **20**(5): 9071-9083.

[6] Xu J, Sun Y, Wang M, *et al*. Bioactive diterpenoids from the leaves of *Callicarpa macrophylla* [J]. *J Nat Prod*, 2015, **78**(7): 1563-1569.

[7] Yuan MM, Zhong RJ, Chen G, *et al*. Two new triterpenoids from *Callicarpa kwangtungensis* [J]. *J Asian Nat Prod Res*, 2015, **17**(2): 138-142.

[8] Dong L, Zhang L, Zhang X, *et al*. Two new 3, 4-seco-labdane diterpenoids from *Callicarpa nudiflora* and their inhibitory activities against nitric oxide production [J]. *Phytochem Lett*, 2014, **10**: 127-131.

[9] Zhang L, Liu MS, Huang J, *et al*. A new 3, 4-seco-labdane diterpenoid with inhibitory activity against the production of nitric oxide from the leaves of *Callicarpa nudiflora* [J]. *J Asian Nat Prod Res*, 2014, **16**(2): 216-221.

[10] Sun X, Liu F, Yang X, *et al*. Seco-labdane diterpenoids from the leaves of *Callicarpa nudiflora* showing nitric oxide inhibitory activity [J]. *Phytochemistry*, 2018, **149**: 31-41.

[11] Wu AZ, Zhai YJ, Zhao ZX, *et al*. Phenylethanoid glycosides from the stems of *Callicarpa peii* (hemostatic drug) [J]. *Fitoterapia*, 2013, **84**: 237-241.

[12] Luo Y, Zhou Z, Ma S, *et al*. Three new antioxidant furofuran lignans from *Callicarpa nudiflora* [J]. *Phytochem Lett*, 2014, **7**: 194-197.

[13] Chen YH, Feng F, Ren DC, *et al*. Chemical constituents from the aerial part of *Callicarpa kwangtungensis* Chun [J]. *Chin J Nat Med*, 2008, **6**(2): 120-122.

[14] Gao JB, Yang SJ, Yan ZR, *et al*. Isolation, characterization, and structure-activity relationship analysis of abietane diterpenoids from *Callicarpa bodinieri* as spleen tyrosine kinase inhibitors [J]. *J Nat Prod*, 2018, **81**(4): 998-1006.

[15] Wang W, Ali Z, Li XC, *et al*. New clerodane diterpenoids from *Casearia sylvestris* [J]. *Fitoterapia*, 2009, **80**(7): 404-407.

[16] Luo GY, Ye Q, Du BW, *et al*. Iridoid glycosides and diterpenoids from *Caryopteris glutinosa* [J]. *J Nat Prod*, 2016, **79**(4): 886-893.

[17] Bisio A, Schito AM, Ebrahimi SN, *et al*. Antibacterial compounds from *Salvia adenophora Fernald* (Lamiaceae) [J]. *Phytochemistry*, 2015, **110**: 120-132.

[18] Raha P, Das AK, Adityachaudhuri N, *et al*. Cleroinermin, aneoclerodane diterpenoid from *Clerodendron inermi* [J]. *Phytochemistry*, 1991, **30**(11): 3812-3814.

[19] Mora S, Castro V, Poveda L, *et al*. Two new 3, 4-seco-entkaurenes and other constituents from the costa rican endemic species *Croton megistocarpus* [J]. *Helv Chim Acta*, 2011, **94**(10): 1888-1892.

[20] Jaensch M, Jakupovic J, Sanchez H, *et al*. Diterpenes from *Hoffmannia strigillosa* [J]. *Phytochemistry*, 1990, **29**(11): 3587-3590.

[21] Costa M, Tanaka CMA, Imamura PM, *et al*. Isolation and synthesis of a new clerodane from *Echinodorus grandifloras* [J]. *Phytochemistry*, 1999, **50**(1): 117-122.

[22] Avila D, Medina, José D, Deeming AJ. A new clerodane-type diterpenoid from *Eperua leucantha* [J]. *J Nat Prod*, 1992, **55**(7): 845-850.

[23] Gao Y, Fang YD, Hai P, *et al*. Isoprenylated flavonoids and clerodane diterpenoids from *Dodonaea viscosa* [J]. *Nat Prod Bioprospect*, 2013, **3**(5): 250-255.

[24] Wu TH, Cheng YY, Chen CJ, *et al*. Three new clerodane diterpenes from *Polyalthia longifolia* var. *pendula* [J]. *Molecules*, 2014, **19**(2): 2049-2060.

[25] Vainio MJ, Johnson MS. Generating conformer ensembles using a multiobjective genetic algorithm [J]. *J Chem Inf Model*, 2007, **47**(9): 2462–2474.

[26] Diaz C, Frazer A, Morales A, *et al*. Structural identification of a novel axially chiral binaphthyl fluorene based salen ligand in solution using electronic circular dichroism: A theoretical–experimental analysis [J]. *J Phy Chem A*, 2012, **116**(10): 2453-2465.

[27] Bruhn T, Schaumlöffel A, Hemberger Y, *et al*. SpecDis: quantifying the comparison of calculated and experimental electronic circular dichroism spectra [J]. *Chirality*, 2013, **25**(4): 243-249.

[28] Zhang XJ, Lu LH, Wang RR, *et al*. DB-02, a C-6-cyclohexylmethyl substituted pyrimidinone HIV-1 reverse transcriptase inhibitor with nanomolar activity, displays an improved sensitivity against K103N or Y181C than S-DABOs [J]. *PLoS One*, 2013, **8**(11): e81489.

[29] Zhang D, Liu R, Sun L, *et al*. Anti-inflammatory activity of methyl salicylate glycosides isolated from *Gaultheria yunnanensis* (Franch.) Rehder [J]. *Molecules*, 2011, **16**(5): 3875-3884.

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