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•Research article•

3, 4-*seco*-Isopimarane and 3, 4-*seco*-pimarane diterpenoids from *Callicarpa nudiflora*

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[ABSTRACT] A phytochemical investigation was carried out on the extract of a medicinal plant *Callicarpa nudiflora*, resulting in the characterization of five new 3, 4-*seco*-isopimarane (**1**−**5**) and one new 3, 4-*seco*-pimarane diterpenoid (**6**), together with four known compounds. The structures of the new compounds were fully elucidated by extensive analysis of MS, 1D and 2D NMR spectroscopic data, and time-dependent density functional theory (TDDFT) calculation of electronic circular dichroism (ECD) spectra, and DFT calculations for NMR chemical shifts and optical rotations.

[KEY WORDS] *Callicarpa nudiflora*; 3, 4-*seco*-Isopimarane; 3, 4-*seco*-Pimarane; TDDFT-ECD; DFT calculation
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Introduction

The genus *Callicarpa*, which belongs to the Verbenaceae family, is comprised of about 140 species, mainly distributed in East Asia, Southeast Asia, Australia, the southeast of North America and Central America [1]. Plants of this genus have long been used as ethnomedicines for the treatment of hepatitis, rheumatism, fever, headache, indigestion, and other disorders [2, 3]. About 48 *Callicarpa* species grow in China, and nearly half of them have been documented in ancient medicinal classics with therapeutic effects [2]. Previous investigations revealed that terpenoids and flavonoids were two major types of compounds identified from this genus, and many of them were reported to exhibit cytotoxic, antibacterial, anti-inflammatory, anti-tubercular, hemostatic, analgesia, neuroprotective, and other activities [2].

Chinese Pharmacopeia documents several preparations containing the *Callicarpa* species, e.g. *Luo-Hua-Zi-Zhu-Pian*, a tablet made from the extract of *Callicarpa nudiflora* Hook. & Arn. *Callicarpa nudiflora* is mainly distributed in the area south of the Yangtze River, and its leaves have long been used to treat trauma and swelling, injure pain, rheumatalgia, and gastrointestinal bleeding. In recent years, increasing attention has been drawn towards its chemical constituents, such as diterpenoids, triterpenoids, flavonoids, iridoids, and phenylpropanoid glycosides from the plant, as well as their various bioactivities like anti-oxidant and anti-inflammatory effects, and anti-platelet aggregation [4-17].

In our continuing effort to search for bioactive terpenoids from natural sources, a systematic investigation focusing on the diterpenoids of *C. nudiflora* was carried out, obtaining five new 3, 4-*seco*-isopimarane (**1**−**5**) and one new 3, 4-*seco*-pimarane diterpene (**6**), together with four known compounds including two 3, 4-*seco*-labdane diterpenes, one sesquiterpene, and one triterpene (Fig. 1). The structures of the new compounds were determined by extensive analysis of MS and NMR data, especially 2D NMR spectra, in combination with time-dependent density functional theory (TDDFT) calculations of electronic circular dichroism (ECD), and DFT calculations for NMR chemical shifts and optical rotations. Herein, we describe the isolation and structural elucidation of these new compounds.

Results and Discussion

Compound **1** was obtained as a light yellow oil. HR-ESI-MS data (*m*/*z* 357.2045 $[M + Na]^+$, Calcd. for $C_{20}H_{30}O_4Na$,

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These authors have no conflict of interest to declare.

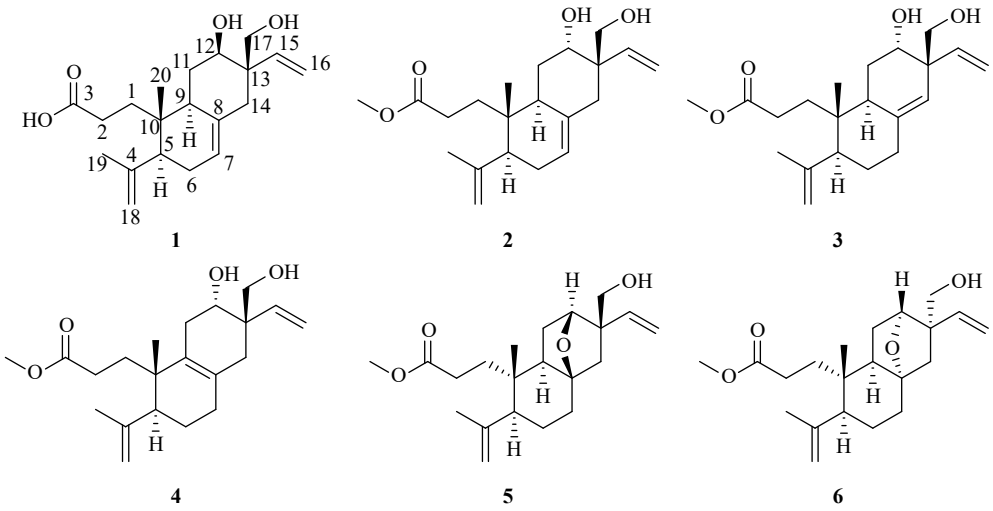

Fig. 1 Structures of new compounds 1–6 from *C. nudiflora*

Table 1 ^{13}C NMR spectroscopic data (125 MHz) for compounds 1–6 (δ in ppm)

Position	1 [b]	2 [a]	3 [a]	4 [a]	5 [a]	6 [a]
1	33.5, CH_2	31.9, CH_2	32.4, CH_2	31.6, CH_2	36.6, CH_2	36.8, CH_2
2	29.9, CH_2	28.8, CH_2	28.4, CH_2	29.6, CH_2	29.6, CH_2	29.2, CH_2
3	178.2, C	175.0, C	174.4, C	174.7, C	174.8, C	174.5, C
4	148.6, C	147.2, C	147.1, C	146.9, C	147.9, C	145.9, C
5	50.2, CH	49.7, CH	50.8, CH	46.9, CH	53.1, CH	52.2, CH
6	30.4, CH_2	29.3, CH_2	28.8, CH_2	24.4, CH_2	25.1, CH_2	26.5, CH_2
7	123.2, CH	122.3, CH	35.6, CH_2	30.9, CH_2	31.1, CH_2	34.4, CH_2
8	135.4, C	134.9, C	140.6, C	129.4, C	86.3, C	86.5, C
9	43.9, CH	37.7, CH	41.5, CH	128.2, C	50.6, CH	54.1, CH
10	38.5, C	37.4, C	41.1, C	41.0, C	38.8, C	39.4, C
11	31.2, CH_2	27.5, CH_2	26.9, CH_2	31.6, CH_2	28.9, CH_2	26.1, CH_2
12	77.2, CH	69.4, CH	70.7, CH	72.2, CH	81.3, CH	82.1, CH
13	46.5, C	47.9, C	48.5, C	44.7, C	54.3, C	55.0, C
14	41.3, CH_2	34.1, CH_2	121.6, CH	32.3, CH_2	44.8, CH_2	37.1, CH_2
15	144.4, CH	142.0, CH	138.3, CH	140.0, CH	139.4, CH	138.6, CH
16	114.3, CH_2	117.9, CH_2	118.5, CH_2	115.4, CH_2	117.1, CH_2	117.7, CH_2
17	63.9, CH_2	65.6, CH_2	70.1, CH_2	69.3, CH_2	69.4, CH_2	69.9, CH_2
18	114.6, CH_2	115.0, CH_2	114.1, CH_2	114.2, CH_2	114.0, CH_2	114.4, CH_2
19	24.1, CH_3	23.8, CH_3	24.1, CH_3	23.1, CH_3	24.3, CH_3	23.3, CH_3
20	17.3, CH_3	16.8, CH_3	18.5, CH_3	22.6, CH_3	15.9, CH_3	18.3, CH_3
21		51.8, CH_3	51.8, CH_3	51.7, CH_3	51.7, CH_3	51.8, CH_3

[a] in $CDCl_3$; [b] in CD_3OD

357.2042), in combination with its ^{13}C NMR data (Table 1), gave a molecular formula of $C_{20}H_{30}O_4$ with six degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl (3402 cm^{-1}), carboxyl (1707 cm^{-1}), and double bond groups (1638 cm^{-1}). The ^{1}H NMR data (Table 2) showed signals for two methyls [δ_H 0.97 (3H, s, Me-20), 1.83 (3H, s, Me-19)], one oxymethylene [δ_H 3.55 (d, J = 10.9 Hz, H-17a), 3.83 (d, J = 10.9 Hz, H -17b)], one oxymethine [δ_H 3.69 (dd,

Table 2 ^{1}H NMR spectroscopic data (600 MHz) for compounds 1–6 (δ in ppm, *J* in Hz)

Position	1 [b]	2 [a]	3 [a]	4 [a]	5 [a]	6 [a]
1a	1.70 (m, 2H)	1.69 (m, 2H)	1.74 (m, 2H)	1.65 (m)	1.49 (m, 2H)	1.59 (m, 2H)
1b				1.78 (m)		
2a	2.32 (m)	1.85 (m)	2.27 (m)	1.27 (m)	2.27 (m, 2H)	2.32 (m)
2b	2.32 (m)	2.34 (m)	2.41 (m)	2.28 (s)		2.17 (m)
5	2.24 (m)	2.22 (m)	2.18 (m)	2.19 (m)	1.88 (m)	2.01 (m)
6a	1.80 (m)	1.83 (m)	1.53 (m)	1.53 (m)	1.38 (m)	1.65 (m)
6b	2.28 (m)	2.22 (m)	1.55 (m)	1.56 (m)	1.91 (m)	1.70 (m)
7a	5.45 (m)	5.47 (m)	2.10 (m)	1.76 (m)	1.73 (m)	1.74 (m)
7b			2.33 (m)	2.10 (d, 17.6)	2.18 (m)	2.03 (m)
9	2.16 (m)	2.36 (m)	2.12 (m)		1.50 (m)	1.54 (m)
11a	1.53 (dd, 12.5, 12.5)	1.49 (dd, 14.8, 14.8)	1.66 (m)	1.82 (m)	1.72 (m)	1.50 (m)
11b	1.84 (m)	1.52 (s)	1.78 (m)	2.02 (m)	1.82 (dd, 13.2, 8.8)	1.72 (m)
12	3.68 (dd, 11.9, 4.5)	3.89 (s)	4.00 (dt, 9.1, 4.3)	3.98 (s)	4.21 (d, 5.9)	4.37 (d, 5.9)
14a	1.99 (m)	2.07 (d, 14.2)	5.13 (s)	1.95 (d, 17.8)	1.52 (d, 9.7)	1.48 (m)
14b	2.24 (m)	2.47 (d, 14.3)		2.31(m)	1.57 (d, 12.5)	2.28 (d, 12.9)
15	6.14 (dd, 17.8, 11.1)	5.85 (dd, 17.8, 11.0)	6.00 (dd, 17.7, 10.7)	5.63 (dd, 17.6, 10.9)	5.82 (dd, 17.6, 10.8)	5.82 (dd, 17.6, 10.8)
16a	5.17 (dd, 11.1, 1.3)	5.25 (dd, 17.8, 1.1)	5.16 (dd, 17.6, 1.7)	5.0 7 (d,17.6)	5.10 (dd, 17.6, 1.2)	5.13 (dd, 17.6, 1.2)
16b	5.21 (dd, 17.8, 1.3)	5.43 (dd, 10.9, 1.1)	5.37 (dd, 10.7, 1.6)	5.17 (m)	5.26 (dd, 10.8, 1.2)	5.31 (dd, 10.8, 1.2)
17a	3.55 (d, 10.9)	3.40 (d, 10.6)	3.63 (m)	3.56 (m)	3.43 (d, 10.4)	3.50 (d,10.4)
17b	3.83 (d,10.9)	3.45 (m)	3.64 (m)	3.70 (d, 11.6)	3.50 (d, 10.4)	3.44 (d, 10.4)
18a	4.83 (d, 2.1)	4.78 (s)	4.70 (d, 1.8)	4.68 (s)	4.72 (d, 1.7)	4.68 (m)
18b	4.90 (m)	4.87 (s)	4.90 (t, 1.8, 1.8)	4.92 (s)	4.86 (t, 1.7, 1.7)	4.89 (t, 1.7, 1.7)
19	1.83 (s, 3H)	1.80 (s, 3H)	1.76 (s, 3H)	1.76 (s, 3H)	1.78 (s, 3H)	1.73 (s, 3H)
20	0.97 (s, 3H)	0.89 (s, 3H)	0.85 (s, 3H)	0.95 (s, 3H)	0.88 (s, 3H)	0.88 (s, 3H)
21		3.65 (s, 3H)	3.66 (s, 3H)	3.64 (s, 3H)	3.64 (s, 3H)	3.64 (s, 3H)

[a] in $CDCl_3$; [b] in CD_3OD

J = 11.9, 4.5 Hz, H-12)], and six olefinic protons ascribed to a trisubstituted [δ_H 5.45 (1H, m, H-7)], a disubstituted [δ_H 4.83 (d, *J* = 2.1 Hz, H-18a), 4.90 (m, H-18b)], and a monosubstituted double bond [δ_H 6.14 (dd, *J* = 17.8, 11.1 Hz, H-15), 5.17 (dd, *J* = 11.1, 1.3 Hz, H-16a), 5.21 (1H, dd, *J* = 17.8, 1.3 Hz, H-16b)]. The ^{13}C NMR data (Table 1), in combination with the DEPT 135 spectrum, illustrated 20 carbon resonances comprised of two methyl (δ_C 24.1, C-19; 17.3, C-20), one oxygenated methylene (δ_C 63.9, C-17), one oxygenated methine (δ_C 77.2, C-12), six olefinic (δ_C 148.6, C-4; 123.2, C-7; 135.4, C-8; 144.4, C-15; 114.3, C-16; 114.6, C-18), and a carboxyl carbon (δ_C 178.2, C-3). Since the above-mentioned double bonds and carbonyl group accounted for four degrees of unsaturation, the left two, combined with the spectroscopic features, especially the 20 carbon resonances in the ^{13}C NMR spectrum, suggested the presence of a bicyclic diterpenoid.

The ^{1}H-^{1}H COSY and HMBC experiments further enabled the elucidation of compound **1**. The ^{1}H-^{1}H COSY correlations exhibited the connections of four moieties, –CH_2(1)-CH_2(2)–, –CH(5)-CH_2(6)-CH(7)–, –CH(9)-CH_2(11)-CH(12)–, and –CH(15)-CH_2(16) (Fig. 2). In the HMBC spectrum, the long-rang correlations were observed from the olefinic H-15 to C-12, C-13, C-14, and C-17, from the olefinic H-7 to C-5, C-6, C-8, C-9, and C-14, and from H-9 to C-11 and C-12, which permitted the construction of a six-membered ring C with a hydroxymethyl (-CH_2OH) and a vinyl group (–CH=CH_2) attaching to the same C-13, and a hydroxyl group to C-12. In addition, the HMBC correlations from the Me-20 to C-1, C-5, C-9, C-10, together with the above-mentioned correlations starting from H-7, constructed another six-membered ring, which fused to the ring C through C-8 and C-9. Moreover, an isopropenyl located at C-5 was deduced from the HMBC correlation from the olefinic protons H_2-18 to C-

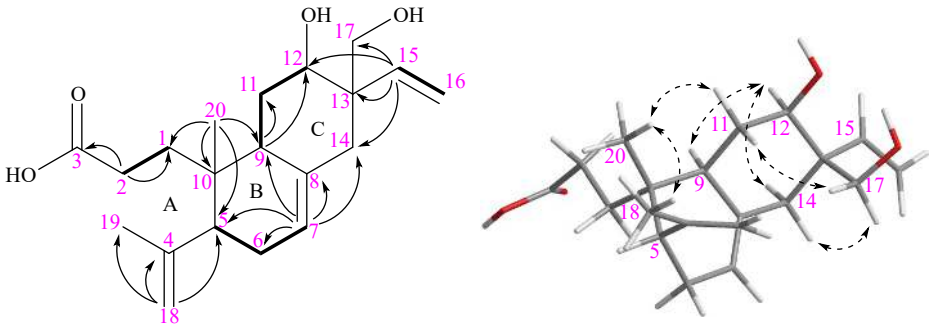

Fig. 2 Key ^{1}H–^{1}H COSY (bold line), HMBC (H→C) and ROESY correlations (dashed double-head arrow) of compound 1

4, C-5, and C-19. Besides CH_3-20, a propanoic acid group was attached to C-10, which was established by the HMBC correlations from H_2-2 to C-1 and the carbonyl C-3, and from CH_3-20 to C-1. Thus, the planar structure was established as a pimarane skeleton *seco* at C-3 and C-4 of ring A (Fig. 1). It is a rare type of primarane derivatives previously reported from *Salvia cinnobarina* [18, 19] and *Trigonostemon heterophyllus* [20].

The relative configuration of compound **1** was inferred from the ROESY experiment. The key ROESY correlations of H_3-20/H-11a, H_3-20/H-18a, H_2-17/ H-11a, H_2-17/H-14b, H-12/H-14a, and H-12/H-9 implied that the Me-20, the isopropenyl group (H_2-18), and the hydroxymethyl group (H_2-17) were on the same face while the vinyl group (H-15), H-5, H-9, and H-12 on the other face.

Compound **2** was obtained as a white powder. Its molecular formula was determined to be $C_{21}H_{32}O_4$ with six degrees of unsaturation by HR-ESIMS. The IR spectrum showed the presence of a hydroxyl (3463 cm^{-1}), a carbonyl (1736 cm^{-1}), and double bond groups (1632 cm^{-1}). The ^{1}H and ^{13}C NMR data of **2** (Tables 1 and 2) showed high similarities to those of compound **1**, suggesting that they may share the same nucleus skeleton. A detailed comparison of their NMR data revealed that an additional singlet of a methoxy group (δ_C 51.8; δ_H 3.65, s, 3H) was presented for **2**, which was attached to C-3 by the HMBC correlation from the oxymethyl to the carbonyl carbon at δ_C 175.0. Such elucidation was consistent with its molecule formula, which has one C and two H more than that of compound **1**. In addition, the major differences of chemical shift were observed for C-9, C-11, C-12, C-13, C-14, and C-16, suggesting that the configuration of ring C may differ from that of **1** (NMR data recorded in $CDCl_3$ for compound **1** was presented as Table S1 in Supporting information). The relative configuration of **2** was further fixed by the ROESY experiment (Fig. S18). The key ROESY correlations of H_3-20/H-18, H_3-20/H-11a, H-17/H-11a, H-17/H-12, and H-17/H-14a indicated that the relative configurations of C-5, C-9, C-10, and C-13 were the same with those of compound **1** except for that of C-12. Thus, compound **2** was proposed as a methyl ester of **1** with an isomerized C-12.

In order to assign the absolute configurations of compounds **1** and **2**, their ECD spectra were measured (Fig. 3). Both compounds showed a positive Cotton effect (CE) around 204 nm, similar with that reported for the known compound heterophypene B [20], suggesting that compounds **1** and **2** may possess a 3, 4-*seco*-isopimarane skeleton. To substantiate such elucidation, time-dependent density functional theory (TDDFT) calculation of ECD spectra was performed. Since the side chain of C-1-C-3 was expected to have a negligible impact on the CE around 204 nm, we decided to use truncated structures of (5*S*, 9*S*, 10*S*, 12*R*, 13*S*)-**1a** and (5*S*, 9*S*,

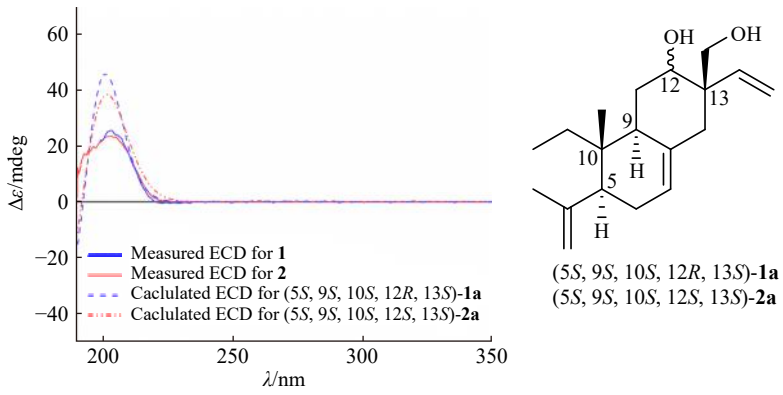

Fig. 3 Experimental ECD spectra of compounds 1 and 2 in MeOH compared with the Boltzmann-weighted M062X/TZVP SMD/MeOH ECD spectra of (5*S*, 9*S*, 10*S*, 12*R*, 13*S*)-1a and (5*S*, 9*S*, 10*S*, 12*S*, 13*S*)-2a computed for the B3LYP/6-31G (d) optimized conformers

10*S*, 12*S*, 13*S*)-**2a** for computational calculation. Conformational search of **1a** and **2a** was conducted using the Conflex in a 5.0 kcal·mol^{-1} energy window, yielding 27 and 18 conformers above 0.5% Boltzmann population, respectively. The conformers were re-optimized at the B3LYP/6-31G (d) *in vacuo*, resulting in 21 and 14 conformers. The TDDFT calculation of ECD spectra were calculated at the level of M06-2X/TZVP with SMD solvent model for methanol. The results (Fig. 3) clearly showed that the calculated spectra also exhibited positive CE around 204 nm, which matched well with the experimental spectra.

Although the experimental and computational spectra were well matched, it is noteworthy that the stereochemistry of C-12 had a negligible impact on the CE pattern of the whole structure. To figure out the orientation of H-12, DFT calculation of ^{1}H and ^{13}C NMR chemical shifts was carried out on the optimized conformers of **1a** and **2a** at the level of B3LYP/6-311G (d, p) with PCM model for chloroform. The Boltzmann-weighted average NMR data of **1a** and **2a** were compared with the experimental data of compounds **1** and **2**, respectively, using the improved statistical method DP4+ [21]. Compound **1** gave 100% possibilities (H data, C data, and all data) for **1a** while compound **2** showed 100% possibilities (H data, C data, and all data) for **2a**. Thus, the whole structures of compounds **1** and **2** were proposed as shown and named callinudins A and B.

Compound **3**, obtained as a colorless oil, gave a molecular formula of $C_{21}H_{32}O_4$ by HR-ESIMS, which was corresponding to six degrees of unsaturation. The IR absorption bands suggested the presence of hydroxyl (3421 cm^{-1}), carbonyl (1734 cm^{-1}), and double bond groups (1630 cm^{-1}). The ^{1}H and ^{13}C NMR data of **3** (Tables 1 and 2) were very close to those of compounds **1** and **2**, implying that **3** might be a derivative sharing the same skeleton. A detailed analysis of 1D and 2D NMR spectra also constructed a 3, 4-*seco*-isopimarane diterpenoid, in which the double bond shifted to C-8 and C-14 compared with the one at C-7 and C-8 in **1** and **2**. Such elucidation was supported by the HMBC correlations from the olefinic proton (δ_H 6.00, dd, J = 17.8, 11.0 Hz, H-15) and the methylene (δ_H 2.10, m, H-7a; 2.33, m, H-7b) to the tertiary carbon (δ_C 121.6, C-14). The ROESY experiment fixed the relative configuration (Fig. S27). The correlation of H_3-20/H_3-19, H_3-20/H-12, and H-17/H-12 clearly indicated that they were co-facial while the hydroxy group attached to C-12 was on the same face with the H-5 and the vinyl group. The ECD spectrum of **3** showed a big negative CE around 200 nm (Fig. 4), which was opposite to the positive CE of compounds **1** and **2**. To further determine the absolute configuration, the TDDFT calculation of ECD spectra was employed for a truncated structure (5*S*, 9*S*, 10*S*, 12*S*, 13*R*)-**3a**, and the theoretical spectrum gave an agreeable negative CE around 200 nm (Fig. 4). The good agreement supported the proposal of the full structure, and compound **3** was named callinudin C.

The molecular formula of compound **4** was designated as $C_{21}H_{32}O_4$ by HR-ESIMS. Its IR spectrum and NMR data (Tables 1 and 2) showed high similarities to those of compounds **1**−**3**. HMBC correlations from H_3-20 to a quaternary carbon resonating at δ_C 128.2, and from the methylenes (H_2-6, H_2-7, H_2-11, H_2-14) to another quaternary carbon at δ_C 129.4 suggested a double bond at C-8 and C-9. The ROESY experiment (Fig. S36) showed correlations of H_3-20/H-7b, H_3-20/H_2-18, H-7b/H-12, H-7b/H-14a, H_2-17/H-12, and H_2-17/H-14a, indicating that the orientations of H_3-20, H-5, H-12, and the hydroxymethyl group were the same with those of compound **3**. The ECD spectrum of **4** exhibited a big positive CE around 195 nm and a small negative CE at 214 nm (Fig. 5). The TDDFT calculation of ECD spectra was also carried out for a truncated structure (5*S*, 10*S*, 12*S*, 13*S*)-**4a** (Fig. 5). The comparison showed a good matching between the experimental and calculated ECD spectra. Accordingly, the structure of compound **4** was proposed and named callinudin D.

Compound **5** was obtained as a colorless oil. The molecular formula was designated as $C_{21}H_{32}O_4$ by HR-ESIMS, corresponding to six degrees of unsaturation. The IR spectrum showed the absorption bands of hydroxy (3453 cm^{-1}), carbonyl (1734 cm^{-1}), and double bond groups (1635 cm^{-1}). The ^{1}H and ^{13}C NMR data displayed the characteristic sig-

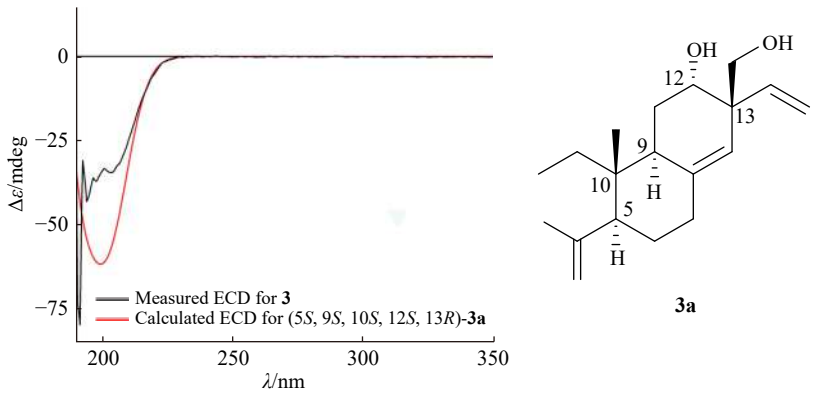

Fig. 4 Experimental ECD spectra of compound 3 in MeOH compared with the Boltzmann-weighted M062X/TZVP SMD/MeOH ECD spectra of (5*S*, 9*S*, 10*S*, 12*S*, 13*R*)-3a computed for the B3LYP/6-31G (d) optimized conformers

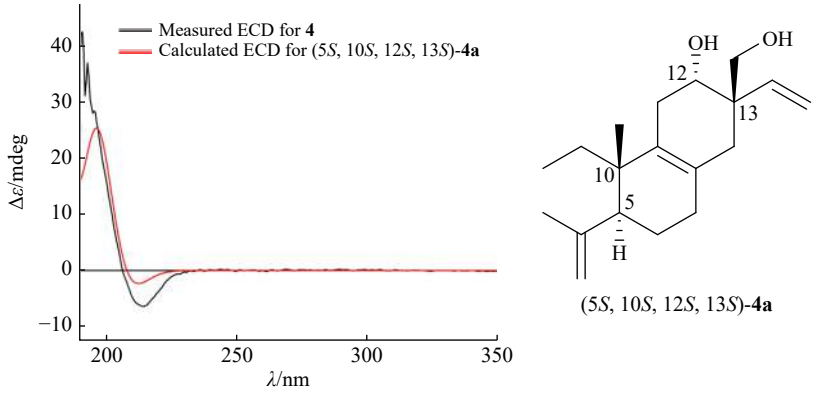

Fig. 5 Experimental ECD spectra of compound 4 in MeOH compared with the Boltzmann-weighted M062X/TZVP SMD/MeOH ECD spectra of (5*S*, 10*S*, 12*S*, 13*S*)-4a computed for the B3LYP/6-31G (d) optimized conformers

nals of one oxymethine [δ_H 4.21 (1H, d, J = 5.9 Hz, H-12)], one oxymethylene [δ_H 3.50 (1H, d, J = 10.4 Hz, Ha-17), 3.43 (1H, d, J = 10.4 Hz, Hb-17)], two methyls [δ_H 0.88 (3H, s, H_3-20), 1.78 (3H, s, H_3-19)], one methoxy [δ_H 3.64 (3H, s, H_3-21)], one vinyl [(δ_H 5.82 (dd, J = 17.6, 10.8 Hz, H-15), 5.10 (dd, J = 17.6, 1.2 Hz, Ha-16), 5.26 (dd, J = 10.8, 1.2 Hz, Hb-16)], and two other olefinic protons [δ_H 4.72 (d, J = 1.7 Hz, Ha-18), 4.86 (t, J = 1.7, 1.7 Hz, Hb-18)], suggesting that compound **5** was also a 3, 4-*seco*-isopimarane diterpene. A detailed NMR data comparison further revealed that one oxygenated quaternary carbon (δ_C 86.3, C-8) and one methine carbon (δ_C 50.6, C-9) were presented for **5** rather than two olefinic carbons (δ_C 129.4, 128.2) for compound **4**. To the oxygenated quaternary carbon (C-8), a key HMBC correlation from H-12 was clearly observed, suggesting an oxygen atom bridged between C-8 and C-12.

The molecular formula of compound **6** was determined by HR-ESIMS to be $C_{21}H_{32}O_4$ with six degrees of unsaturation. Its IR absorption bands showed the presence of hydroxy (3446 cm^{-1}), carbonyl (1736 cm^{-1}), and double bond groups (1635 cm^{-1}). The ^1H and ^{13}C NMR data of **6** were very close to those of **5** except for some small chemical shift changes for C-7, C-9, C-11, C-14, and C-20, and for H-7, H-11, H-12, and H-14. The key HMBC correlation from H-12 to C-8 was also observed. All evidence suggested that compounds **5** and **6** might share the same planar structure but differ at the stereochemistry of ring C.

The ROESY experiments were used to fix the relative configurations of compounds **5** and **6**. The ROESY spectrum of **5** showed correlations of H_3-20/H-18, H_3-20/Hb-6, H-12/H-15, H-17/Hb-14, Ha-14/Ha-7, Ha-14/H-15, and Ha-7/H-5, suggesting that Me-20, the isopropenyl, and the hydroxymethyl group was on the same face while H-12, H-5 and the vinyl were on the other face (Fig. S45). The ROESY correlation pattern of **6** was quite different. The methyl at C-20 was observed to correlate to Hb-14, H-18, and H-11, the H_2-17 correlate to Ha-14, the H-16 correlate to H_3-20 and Hb-14, and the H-9 correlated to H-5. These correlations supported that Me-20, the isopropenyl, and the vinyl were on the same face while the hydroxymethyl group, H-9 and H-5 were on the other face (Fig. S54). Therefore, these two compounds differed not only in the orientation of the oxygen bridge but also the stereochemistry at C-13.

The ECD spectrum of **5** was measured, which showed a negative CE at 209 nm and a positive CE at 197 nm (Fig. 6). The TDDFT calculation of ECD spectra was performed for (5*S*, 8*R*, 9*R*, 10*S*, 12*R*, 13*S*)-**5**, and the calculated ECD spectrum exhibited similar CE with the experimental one (Fig. 6).

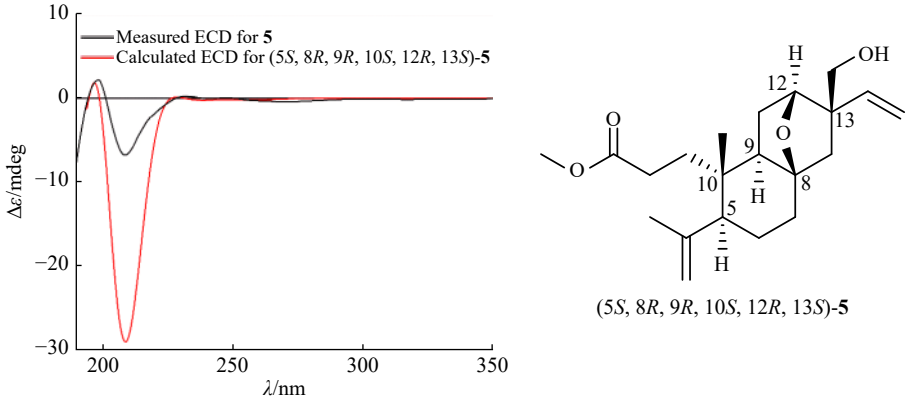

Fig. 6 Experimental ECD spectra of compound 5 in MeOH compared with the Boltzmann-weighted M062X/TZVP SMD/MeOH ECD spectra of (5*S*, 8*R*, 9*R*, 10*S*, 12*R*, 13*S*)-5 computed for the B3LYP/6-31G (d) optimized conformers

Accordingly, the whole structure of **5** was proposed as shown and named callinudin D.

The relative configuration of **6** was proposed different from that of **5** at C-8, C-12, and C-13, based on the ROESY experiment. That means, **6** might be a primarane diterpene rather than a isoprimarane derivative. To further confirm such elucidation, we first used the TDDFT calculation of ECD spectra to predict the spectra of two possible ^{13}C-epimers, (5*S*, 8*S*, 9*R*, 10*S*, 12*S*, 13*R*)-**6** and (5*S*, 8*S*, 9*R*, 10*S*, 12*S*, 13*S*)-**6** (Fig. 7). Both spectra showed an obvious negative CE around 197 nm, very similar to that observed in the experimental ECD spectrum of **6** (Fig. 7). TDDFT ECD spectra verified the absolute configurations of other chiral carbons except for C-13. Therefore, NMR calculation of these two isomers were conducted at the level of B3LYP/6-311G (d, p) with PCM model on chloroform. The results were analyzed using the DP4+ statistical method, showing 100% possibility (H data, C data, and all data) for the 13*R* isomer. Consequently, the whole structure of **6** was proposed as shown and named callinudin F.

Optical rotations were also calculated using DFT based on the established structures of compounds **5** and **6**. The results showed that the calculated values (−38.0 for **5**; +88.0 for **6**) were consistent with the corresponding experimental data (+3.3 for **5**; +49.5 for **6**), which further supported the proposed structures.

Notable, five of the six new compounds were methyl esters, while only compound **1** contained a group of carboxylic acid. The co-occurrence of compounds with carboxylic acid and methyl ester group implied that the methyl ester compounds **2**−**6** might be artifacts due to the use of methanol during purification.

In addition to the six new compounds (**1**−**6**), another four known compounds were identified from *C. nudiflora* as nudiflopene H [15], methylcallicarpate [4], (−)-clovane-2, 9-diol [22], and astragalin [23] through comparison with previous spectroscopic data (see Fig. S1). Both nudiflopene H and methylcallicarpate are 3, 4-*seco*-labdane diterpene.

In summary, a total of ten compounds especially six new compounds were characterized from *C. nudiflora*. Eight of these compounds were diterpenes with a common structural feature, a 3, 4-*seco* ring A. The feature was also described in previously reported labdane diterpenoids from *C. nudiflora* [4, 6, 7, 9, 13, 15], and abietane diterpenoids from *C. pilosissima* [24] and *C. longissima* [25]. Isopimarane diterpenoids have not been well studied. So far, only several isopimaranes were sporadically reported from different *Callicarpa* species, such as isopimaric acid, isopimarol, akhdarenol, and calliphyllin [26-29]. The current study is a first systematic investigation of this type of diterpenoids, and the resultant six new compounds provide better understanding of chemical constitution of *C. nudiflora*. These findings also reveal the common 3, 4-*seco* ring A that exists in isopimarane diterpenoids, which may be considered as a chemotaxonomic feature.

Experimental

General experimental procedures

Optical rotations were measured on a Rudolph Research Analytical Autopol VI 90079 polarimeter (Hackettstown, NJ, USA). UV spectra were recorded on a Varian Cary 50 UV spectrometer (Varian Inc., USA). ECD spectra were measured with a J-815150 spectropolarimeter (JASCO, Japan). IR spectra were collected on a Thermo Nicolet FTIR IS5 spectrometer (Thermo Fisher, USA). HR-ESIMS spectra were collected with a Waters Synapt G2-Si Q-Tof mass detector (Waters, USA). 1D and 2D NMR spectra were recorded using a Bruker AVANCE III 500 or 600 MHz instrument (BRUKER BIOSPIN AG, Switzerland). Chemical shifts were reported in ppm (δ) with coupling constants (*J*) in hertz. The residual signals of $CDCl_3$ and CD_3OD were used as references. Analytical HPLC and ESIMS were performed on a Waters 2695 instrument with a 2998 PAD coupled with a Waters Acquity ELSD and a Waters 3100 SQDMS detector (Waters, USA). Preparative HPLC was performed on a Varian PrepStar instrument with an Alltech 3300 ELSD detector (Columbia, MD, USA) using a Waters SunFire RP C_{18}

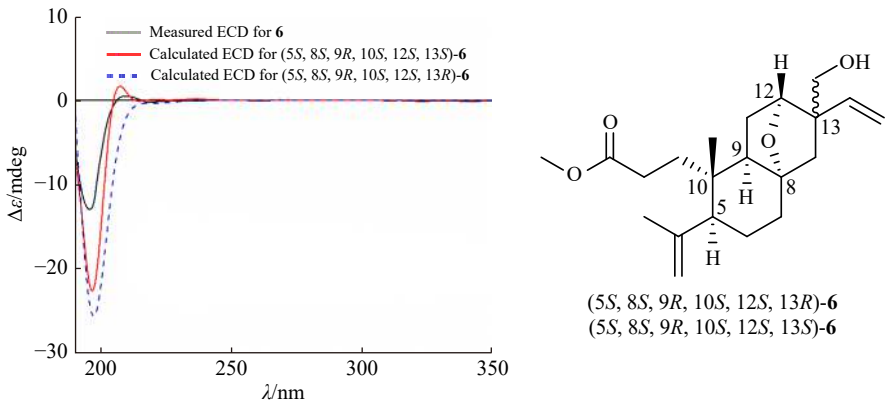

Fig. 7 Experimental ECD spectra of compound 6 in MeOH compared with the Boltzmann-weighted M062X/TZVP SMD/MeOH ECD spectra of (5*S*, 8*S*, 9*R*, 10*S*, 12*S*, 13*R*)-6 and (5*S*, 8*S*, 9*R*, 10*S*, 12*S*, 13*S*)-6 computed for the B3LYP/6-31G (d) optimized conformers

column (5 μm, 30 mm × 150 mm, at a flow rate of 30 mL·min^{-1}, acetonitrile−water). MPLC (medium pressure liquid chromatography, Soochow High Tech Chromatorgraphy Co., Ltd., Suzhou, China) was used for further purification, equipped with an infusion system (HT7200A constant flow pump), a UV detector (EasySep TM-1010 spectrophotometer), and an automatic collection device (SmartCollect-3060 automatic fraction collector). AB-8 macroporous resin (Shandong Lukang Chemical Co., Ltd., China), MCI gel CHP20P (75−150 μm, Mitsubishi Chemical Industraies, Japan), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), ODS (50 μm, DIKMA, China), and silica gel (200−300 and 300−400 mesh, Qingdao Ocean Chemical Co., Ltd., China) were used for column chromatography (CC). TLC was performed on pre-coated silica gel 60 F_{254} aluminum sheets (Merck, Germany), and the TLC spots were observed at 254 nm and visualized by 5% sulfuric acid in alcohol containing 10 mg·mL^{-1} of vanillin. All reagents and solvents used in the experiments are of analytical grade.

Plant materials

The extract of *C. nudiflora* was provided by the PROZIN pharmaceutical Co., Ltd. (Jian, Jiangxi Province, China). The plant was identified by Professor WU Yong-Zhong of the PROZIN pharmaceutical Co., Ltd. (Jiangxi, China). The leaves of *C. nudiflora* was macerated in water, and then extracted at 100 °C twice (first 2 h, and then 1 h). The volume of the water was 12 times the weight of the leaves. The percolates were combined and condensed under reduced pressure until the relative density of the extract reached to 1.30−1.35 mg·mL^{-1}.

Extraction and isolation

The extract of *C. nudiflora* (1.5 kg) was suspended in water and sequentially extracted with petroleum ether (PE), dichloromethane, and ethyl acetate (three times, 10 L each), yielding the PE extract A (450 g), the dichloromethane part B (500 g), and the ethyl acetate part C (260 g). Part B was subjected to column chromatography (CC) over AB-8 macroporous resin eluted with aqueous ethanol in a step-wise manner (0, 30%, 50%, 70%, 95%), affording five fractions (B1−B5). The B3 fraction (195 g) was chromatographed over a CHP20P MCI column before gradient elution with aqueous ethanol (50 : 50−100 : 0, *V/V*), resulting in eleven sub-fractions (B3A−B3J). The B3E (17.5 g) sub-fraction was separated by CC over Sephadex LH-20 eluted with CH_3Cl/MeOH (1 : 1, *V/V*) to obtain three sub-fractions (B3E1−B3E3). The B3E2 (14.4 g) sub-fraction was applied to CC over reversed-phase ODS before gradient elution with MeOH/H_2O (45 : 55−100 : 0, *V/V*) to afford 11 sub-fractions (B3E2A−B3E2K). The B3E2I (2.2 g) sub-fraction was subjected to silica gel column chromatography using a mixture of CH_2Cl_2 and acetone (gradient elution 70 : 1−1 : 1, *V/V*) as the eluting solvent to obtain fifteen sub-fractions (B3E2I1−B3E2I15). The B3E2I5 (21 mg) and B3E2I10 (28 mg) sub-fractions were further purified by preparative HPLC to give compounds **2** (1.4 mg), **3** (1.3 mg), **4** (0.8 mg), **5** (2.0 mg), and **6** (2.8 mg). Similarly, the B3E2H sub-fraction (1.5 g) was subjected to repeated CC over silica gel, Sephadex LH-20, ODS, and finally preparative HPLC to afford compounds **1** (4.7 mg), **7** (2.3 mg), **8** (2.0 mg), **9** (8.3 mg), and **10** (6.7 mg).

Callinudin A (**1**): a light yellow oil, $[\alpha]_D^{25}$ +41.7 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3402, 2922, 1707, 1638, 1381, 1195, 1035 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 1 and 2; ESI-MS *m/z* 333.0 $[M - OH]^+$; HR-ESIMS *m/z* 357.2045 $[M + Na]^+$ (Calcd. for $C_{20}H_{30}O_4Na$, 357.2042).

Callinudin B (**2**): a white powder, $[\alpha]_D^{25}$ +50.0 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3463, 2922, 1736, 1632, 1383, 1198, 1077 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 1 and 2; ESI-MS *m/z* 371.2 $[M + Na]^+$; HR-ESIMS *m/z* 371.2203 $[M + Na]^+$ (Calcd. for $C_{20}H_{30}O_4Na$, 371.2198).

Callinudin C (**3**): a colorless oil, $[\alpha]_D^{25}$ −13.3 (*c* 0.1, MeOH), IR (KBr) ν_{max} 3421, 2925, 1734, 1630, 1385, 1195, 1074 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 1 and 2; ESI-MS *m/z* 331.2 $[M - OH]^+$; HR-ESIMS *m/z* 371.2197 $[M + Na]^+$ (Calcd. for $C_{20}H_{30}O_4Na$, 371.2198).

Callinudin D (**4**): a colorless oil, $[\alpha]_D^{25}$ +55.2 (*c* 0.1, MeOH), IR (KBr) ν_{max} 3434, 2920, 1739, 1637, 1385, 1195, 1131 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 1 and 2; ESI-MS *m/z* 331.2 $[M - OH]^+$; HR-ESIMS *m/z* 331.2278 $[M - OH]^+$ (Calcd. for $C_{21}H_{31}O_3$, 331.2273).

Callinudin E (**5**): a colorless oil, $[\alpha]_D^{25}$ +3.3 (*c* 0.1, MeOH), IR (KBr) ν_{max} 3453, 2925, 1734, 1635, 1437, 1380, 1195, 1044 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 1 and 2; ESI-MS, *m/z* 331.1 $[M - OH]^+$; HR-ESIMS *m/z* 331.2282 $[M - OH]^+$ (Calcd. for $C_{21}H_{31}O_3$, 331.2273).

Callinudin F (**6**): a colorless oil, $[\alpha]_D^{25}$ +49.5 (*c* 0.1, MeOH), IR (KBr) ν_{max} 3446, 2927, 1736, 1635, 1383, 1190, 1126, 1037 cm^{-1}; ^{1}H and ^{13}C NMR data see Tables 1 and 2; ESI-MS, *m/z* 331.1 $[M - OH]^+$; HR-ESIMS *m/z* 331.2275 $[M - OH]^+$ (Calcd. for $C_{21}H_{31}O_3$, 331.2273).

Computational section

The TDDFT calculation of DFT spectra were performed using the Gaussian 09 program (Version Revision E.01, Gaussian, Inc., Wallingford CT). Conformational search was carried out by the Conflex 8.0 software (Version 8.0, CONFLEX Corporation, Tokyo, Japan) using the MMFF force field within an energy window of 5.0 kcal·mol^{-1}. The conformers with the Boltzmann population above 0.5% were re-optimized at the B3LYP/6-31G (d) level *in vacuo*. The ^{1}H and ^{13}C NMR chemical shifts were calculated at the B3LYP/6-311G (d, p) level with the implicit PCM solvent model for chloroform. Computed data were unscaled and unreferenced Boltzmann-weighted NMR shielding tensors, and used for the improved DP4+ probabilities [21]. Optical rotations (λ 598 nm) were calculated at the PBE1PBE/ma-TZVP level with the implicit PCM model for methanol. The TDDFT calculation of ECD spectra were run at the M06-2X/TZVP level with the SMD solvent model for methanol. ECD spectra were generated using the SpecDis Version 1.71 with 0.3 sigma/gamma (eV) after UV correction [30, 31].

Supporting information

Supplementary materials were provided in supporting information, and is available on requrest from the corresponding author.

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