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Alkaloids from the stems of *Clausena lansium* and their neuroprotective activity†

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Eight new alkaloids, including three pairs of enantiomers (+)-(2'S,3'R)-clauselansine A (**1a**) and (–)-(2'R,3'S)-clauselansine A (**1b**); (+)-(2'S,3'R)-clauselansine B (**2a**) and (–)-(2'R,3'S)-clauselansine B (**2b**); (+)-(3S,4R,5S,6S)-clauselansine C (**3a**) and (–)-(3R,4S,5R,6R)-clauselansine C (**3b**), (+)-(1'R,2'R,6'R)-clauselansine B (**4a**), and (+)-(1'R,2'R)-clauselansine D (**5a**), together with twelve known alkaloids (**4b**, **5b**, **6a**, **6b**, **7a**, **7b** and **8–13**) were isolated from the stems of *Clausena lansium*. Their structural elucidation and stereochemistry determination were achieved by spectroscopic and chemical methods including 1D and 2D NMR experiments, especially the employment of electronic circular dichroism calculation spectra, Mosher's method, and Snatzke's method expressed by the induced circular dichroism spectrum. Compounds **1b**, **2a**, **3b**, **5a**, and **5b** inhibited PC12 cell damage induced by Okadaic Acid, and increased cell viability from 70.5 ± 5.4% to 83.4 ± 4.1%, 91.2 ± 10.1%, 83.5 ± 7.8%, 89.7 ± 4.8%, 83.3 ± 5.9% at 10 μM, respectively.

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Introduction

Clausena is a small genus within the family Rutaceae, which is distributed throughout the south of the Yangtze River in China, such as the Yunnan, Guangxi and Guangdong regions.¹ Previous phytochemical studies have revealed that the *Clausena* genus contains a wide variety of carbazole alkaloids, amide alkaloids, coumarins, and limonoids, many of which possess various pharmacological properties such as antibacterial, anti-inflammatory, anti-HIV, cytotoxic, hepatoprotective and neuroprotective effects.^{2–10} The leaves and fruits of *Clausena lansium*, *Clausena excavata*, *Clausena emarginata*, and *Clausena anisum-molens* are not only eaten as food but also used for traditional medicine.

Clausena lansium (Lour.) Skeels (Rutaceae), a fruit tree, was widely distributed in southern China. In traditional Chinese medicine, the leaves and roots of *C. lansium* were used for cough, asthma, dermatological disease, viral hepatitis, and gastro-intestinal diseases; the seeds are used to treat acute and chronic gastro-intestinal inflammation, ulcers, etc.¹¹ Various bioactive constituents including coumarins, carbazole alkaloids

and amide alkaloids have been isolated and identified from this plant.^{12–14} Previously, twenty new natural products including thirteen new carbazole alkaloids,^{10,15} eight alkaloid glycosides,¹⁶ four new coumarins,¹⁷ a new amide and a new megastigmane glucoside¹⁸ from the leaves and stems of *C. lansium* were reported by our research group, and some of these alkaloids showed selective neuroprotective effects. In order to investigate the potential neuroprotective constituents from different parts of *C. lansium*, *n*-BuOH extract of the stems of this plant were selected for investigation. This paper reported further investigation of *n*-BuOH extract from the stems of *C. lansium* which led to the isolation and characterization of four new indole alkaloids (**1a**, **1b**, **2a**, **2b**), two new amide alkaloids (**3a**, **3b**), and two carbazole alkaloids (**4a**, **5a**) along with twelve known compounds (**4b**, **5b**, **6a**, **6b**, **7a**, **7b**, **8–13**) from the stems of *C. lansium*. The neuroprotective activities of **1–13** were also evaluated. We present herein the isolation and structural characterization of **1–13**, as well as their bioactivities (Fig. 1).

Results and discussion

Compound **1** (**1a/1b**) was obtained as colourless oil. Its molecular formula was assigned as C₁₈H₂₅NO₄ based on the ¹³C NMR spectroscopic data and HRESIMS (*m/z* 320.1854 [*M* + *H*]⁺, calcd for C₁₈H₂₆NO₄, 320.1856), implying seven indices of hydrogen deficiency. The IR spectrum displayed absorptions characteristic of amino (3313 cm^{–1}), amide (1640 cm^{–1}) and aromatic ring (1671, 1612, and 1439 cm^{–1}) groups, and the UV spectrum showed absorptions at λ_{max} 202, 223, 282 nm. The ¹H NMR (Table 1) spectrum showed a set of signals for 1,2,3-

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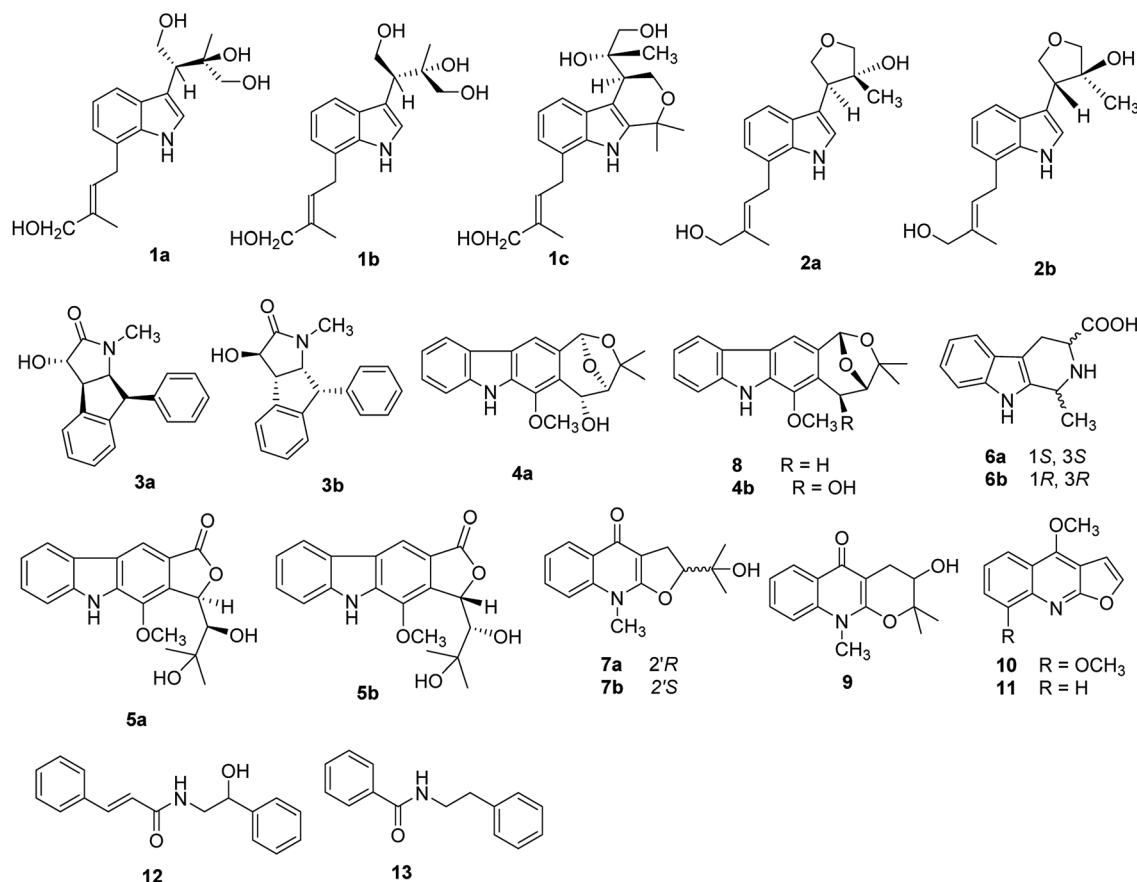


Fig. 1 Alkaloid derivatives (1–13) obtained from the stems of *C. lansium*.

trisubstituted benzene ring at δ_{H} 7.43 (1H, d, $J = 7.6$ Hz, H-4), 6.90 (1H, t, $J = 7.4$ Hz, H-5), 6.85 (1H, d, $J = 6.8$ Hz, H-6), together with a doublet at δ_{H} 7.18 (1H, d, $J = 2.4$ Hz, H-2) and a broad signal at δ_{H} 10.80 (1H, NH), which indicated a 3,7-disubstituted indole moiety. In addition, a double bond group at δ_{H} 5.66 (1H, t, $J = 7.3$ Hz, H-2''), a methylene at 3.55 (2H, d, $J = 7.3$ Hz, H-1'') and two methyls at δ_{H} 1.70 (3H, s, H-5''), 1.00 (3H, s, H-5') were also exhibited in the ^1H NMR spectrum. The ^{13}C NMR and DEPT spectra exhibited a double bond, and four methylenes, two methines, two methyls and the remaining eight in indole moiety. The above information indicated **1** was a diprenylated indole. A comparison of the ^1H and ^{13}C NMR of **1** with those of hexalobines¹⁹ suggested that their structures are closely related, except for 1,3,4-trihydroxy-3-methylbut-2-yl and 4-hydroxy-3-methyl-2-butenyl at C-3 and C-7 in **1**. In the HMBC spectrum, the cross-peaks between H-1''/C-3 (δ_{C} 114.2), C-3' (δ_{C} 75.0), H-2'/C-2 (δ_{C} 123.0), C-3a (δ_{C} 128.3), C-5' (δ_{C} 22.0), H-4'/C-3' (δ_{C} 75.0), C-5' (δ_{C} 22.0) demonstrated 1,3,4-trihydroxy-3-methylbut-2-yl group attached to C-3 of the indole moiety (Fig. 2); the cross-peaks between H-1''/C-6 (δ_{C} 119.7), C-7a (δ_{C} 134.4), C-3'' (δ_{C} 136.3), H-2''/C-7 (δ_{C} 123.7), C-4'' (δ_{C} 66.3), C-5'' (δ_{C} 13.7) demonstrated 4-hydroxy-3-methyl-2-butenyl group attached to C-7 of the indole moiety (Fig. 2). The NOE difference experiment displayed that a strong enhancement of H-2'' was observed when H-4'' was irradiated while H-2'' was no enhanced on

irradiation of H-5'', indicating an *E* configuration of the double bond. Thus, the structure of **1** was elucidated. The specific rotation of **1** approached zero, and no Cotton effect was found in the electronic circular dichroism (ECD) spectrum of **1**, indicating a racemic mixture. Subsequent chiral resolution of **1** afforded the anticipated enantiomers **1a** and **1b**, which showed mirror image-like ECD curves and specific rotations {**1a**: $[\alpha]_{\text{D}}^{20} +18.7$ (c 0.1 MeOH); **1b**: $[\alpha]_{\text{D}}^{20} -17.0$ (c 0.1 MeOH)}. In order to confirm the absolute configuration of the 1',3',4'-triol of **1b**, **1b** was treated with 2,2-dimethoxypropane and pyridinium *p*-toluene sulfonate and converted into its acetonide **1c** (Fig. 3). According to 1D and 2D NMR, **1c** was determined similar to pyrido[3,4-*b*]pyrano[3,4-*b*]indoles.²⁰ In order to confirm the absolute configuration of **1c**, the ECD calculations were also performed for the four configurations 2'*R*,3'*S*-, 2'*R*,3'*R*-, 2'*S*,3'*R*-, and 2'*S*,3'*S*-**1c** using the time-dependent density functional theory (TDDFT) method at the B₃LYP/6-31G (d) level.^{21,22} The calculated ECD spectrum for 2'*R*,3'*S*- and 2'*R*,3'*R*-**1c** enantiomer agreed with the experimental ECD data (Fig. 4) of **1c**. Thus, the absolute configuration at C-2' of **1c** was 2'*R*. In addition, the absolute configuration of the 3',4'-diol moiety of **1c** was determined using induced CD spectra by Sneath's method.^{23,24} A positive Cotton effect at 323 nm (Fig. 5) in the induced CD spectrum indicated the 3'*S* configuration for **1c** by means of the empirical helicity rule. According to the above information, the



Table 1 ^1H and ^{13}C NMR spectroscopic data of compounds **1**, **1c**, and **2** (δ in ppm, J in Hz)

Position	1		1c		2	
	δ_{H}^a	δ_{C}^b	δ_{H}^a	δ_{C}^b	δ_{H}^a	δ_{C}^b
2	7.18, d (2.4)	123.0 d		140.5	7.23, d (2.4)	123.9 d
3		114.2 s		106.1		108.4 s
3a		128.3 s		127.3		128.3 s
4	7.43, d (7.4)	116.8 d	7.58, d (7.5)	117.1	7.42, d (7.4)	116.5 d
5	6.90, t (7.4)	118.3 d	6.86, t (7.5)	118.7	6.90, t (7.4)	118.5 d
6	6.85, d (7.4)	119.7 d	6.80, d (7.5)	119.6	6.85, d (7.4)	119.9 d
7		123.7 s		123.4		123.5 s
7a		134.4 s		134.4		134.7 s
1'	4.02, m; 3.76, m	62.6 t	4.28, d (11.6); 3.68, dd (11.6, 3.6)	61.0 t	4.02, m	71.8 t
2'	3.21, m	43.6 d	2.87, d (3.6)	38.9 d	3.42, t (9.7)	46.3 d
3'		75.0 s		74.8 s		76.8 s
4'	3.19, m	68.4 t	3.41, m; 3.26, m	68.4 t	3.79, s	80.5 t
5'	1.00, s	22.0 q	1.02, s	22.0 q	1.18, s	22.9 q
1''	3.55, d (7.3)	28.6 t	3.57, d (7.4)	28.7 t	3.56, d (7.4)	28.5 t
2''	5.66, t (7.3)	121.2 d	5.67, t (7.4)	121.2 d	5.65, t (7.4)	121.1 d
3''		136.3 s		136.3 s		136.3 s
4''	3.84, d (5.6)	66.3 t	3.85, s	66.2 t	3.83, s	66.2 t
5''	1.70, s	13.7 q	1.70, s	13.7 q	1.69, s	13.6 q
NH	10.80, br s		10.60, s		10.90, s	
1'''			1.56, s	28.3		
2'''				71.7		
3'''			1.45, s	26.0		

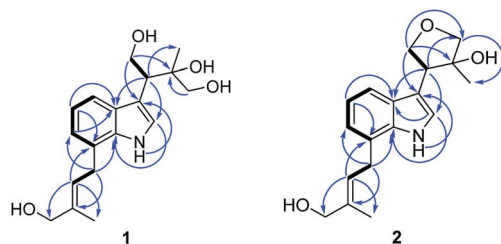
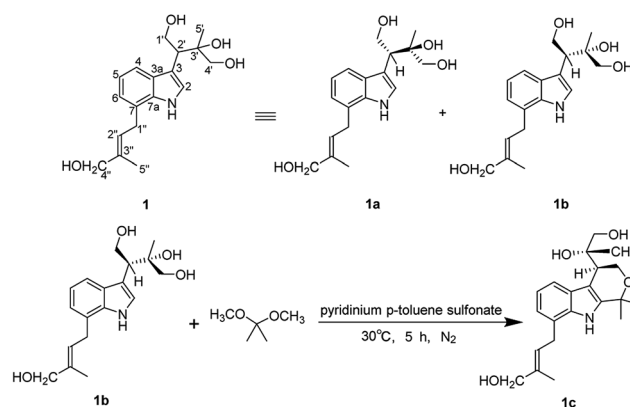
^a In DMSO- d_6 (600 MHz). ^b In DMSO- d_6 (150 MHz). Coupling constants (J) in Hz are given in parentheses. The assignments were based on HSQC and HMBC experiments.

absolute configuration of **1b** was $2'R,3'S$, and then the absolute configuration of **1a** was $2'S,3'R$. Therefore, compounds **1a** and **1b** were given the trivial names (+)-(2' $S,3'R$)-clauselansine A and (–)-(2' $R,3'S$)-clauselansine A, respectively.

Compound **2** (**2a/2b**) was obtained as a white powder. Its molecular formula $\text{C}_{18}\text{H}_{23}\text{NO}_3$ was deduced from the HRESIMS (m/z 302.1758 [$\text{M} + \text{H}$]⁺, calcd for $\text{C}_{18}\text{H}_{24}\text{NO}_3$, 302.1751) and ^{13}C NMR spectroscopic data, corresponding with eight indices of hydrogen deficiency. The ^1H and ^{13}C NMR of **2** displayed signals characteristic of diprenylated indole, which were similar to these reported for compound **1**. The only difference between **2** and **1** was that OH-1' and OH-4' of 1,3,4-trihydroxy-3-methylbut-2-yl group in **1** is formed five-membered oxygen ring in **2**. This was supported further by the HMBC correlations between H-1' (δ_{H} 4.02) and C-4' (δ_{C} 80.5) and H-4' (δ_{H} 3.79) and C-1' (δ_{C} 71.8). The NOE difference experiment displayed that a strong enhancement of H-2'' was observed when H-4'' was irradiated, while H-2'' was no enhanced on irradiation of H-5'', indicating

an *E* configuration of the double bond. Thus, the structure of **2** was elucidated.

The specific rotation of **2** approached zero, and no Cotton effect was found in the electronic circular dichroism (ECD) spectrum of **2**, indicating a racemic mixture. Subsequent chiral resolution of **2** afforded the anticipated enantiomers **2a** and **2b**, which showed mirror image-like ECD curves and specific rotations {**2a**: $[\alpha]_{\text{D}}^{20} +28.0$ (c 0.1 MeOH); **2b**: $[\alpha]_{\text{D}}^{20} -32.6$ (c 0.1 MeOH)}. In order to confirm the absolute configuration of the enantiomers **2a** and **2b**, a systematic conformational analysis and optimization were performed for **2a** and **2b** using the same method applied to **1a** and **1b**. A comparison of the theoretically

Fig. 2 Key ^1H , ^1H -COSY and HMBC correlations of compound **1** and **2**.Fig. 3 The isolation of **1** to **1a** and **1b** and the action of compound **1b** to its acetonide derivative **1c**.

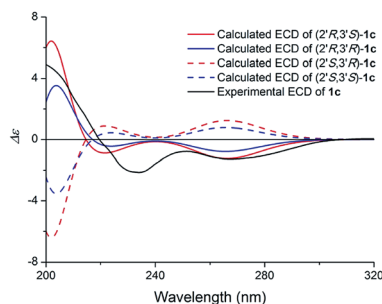


Fig. 4 Calculated ECD spectra of (2'*R*,3'*S*)-1c, (2'*R*,3'*R*)-1c, (2'*S*,3'*R*)-1c, (2'*S*,3'*S*)-1c and the experimental ECD spectrum of 1c in MeOH.

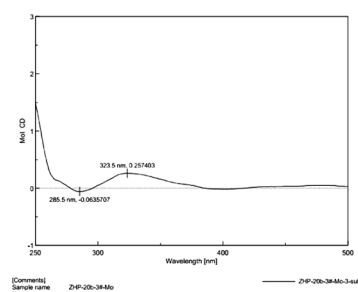


Fig. 5 CD spectrum of compound 1c in a DMSO of dimolybdenum tetraacetate (the inherent CD of the diol was subtracted).

calculated and experimental ECD curves (Fig. 6) demonstrated that the configuration of **2a** was 2'*S*,3'*R* and the configuration of **2b** was 2'*R*,3'*S*. According to the structures of **1a** and **2a**, we speculate that **2a** was possibly generated by dehydration of **1a**, which means the configuration of **2a** was the same as **1a**. And **2b** was also possibly generated by dehydration of **1b**, which means the configuration of **2b** was the same as **1b**. Thus, **2a** has a 2'*S*,3'*R*-configuration and **2b** has a 2'*R*,3'*S*-configuration. Therefore, compounds **2a** and **2b** were given the trivial names (+)-(2'*S*,3'*R*)-clauselansine B and (+)-(2'*R*,3'*S*)-clauselansine B, respectively.

Compound **3** (**3a/3b**) was obtained as a white solid. Its molecular formula was assigned as C₁₈H₁₇NO₂ based on the ¹³C NMR spectroscopic data and HRESIMS (*m/z* 280.1333 [M + Na]⁺, calcd for C₁₈H₁₇NO₂Na, 280.1332), implying eleven indices of hydrogen deficiency. The IR spectrum displayed absorptions

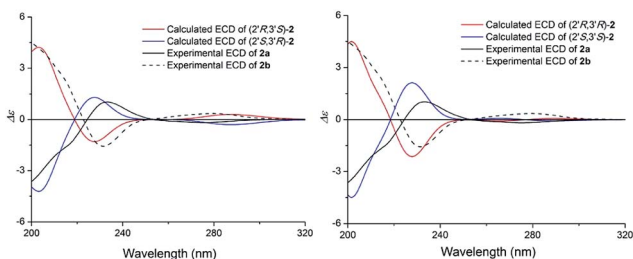


Fig. 6 Calculated ECD spectra of (2'*R*,3'*S*)-2, (2'*S*,3'*R*)-2, (2'*R*,3'*R*)-2, (2'*S*,3'*S*)-2 and the experimental ECD spectrum of (+)- and (-)-clauselansine B (**2a/2b**) in MeOH.

characteristic of hydroxyl (3320 cm⁻¹), carbonyl (1678 cm⁻¹) and aromatic groups (1601, 1483, and 1454 cm⁻¹). The ¹H NMR spectrum showed nine aromatic protons [ring A: δ_H 6.92 (1H, d, *J* = 7.6 Hz, H-3'), 7.23 (1H, overlapped, H-4'), 7.31 (1H, m, H-5') and 6.23 (1H, d, *J* = 7.6 Hz, H-6'); ring B: δ_H 6.98 (2H, d, *J* = 7.1 Hz, H-2'', 6''), 7.27 (2H, m, H-3'', 5''), 7.23 (1H, overlapped, H-4'')], together with four methine groups at δ_H 4.11 (1H, d, *J* = 8.3 Hz, H-3), 3.77 (1H, dd, *J* = 8.3, 5.3 Hz, H-4), 4.66 (1H, t, *J* = 8.3 Hz, H-5), 4.77 (1H, d, *J* = 8.3 Hz, H-6), and a hydroxyl group at δ_H 5.97 (1H, s). The ¹³C NMR spectrum exhibited twelve aromatic carbons, one carbonyl δ_C 174.1, one oxymethine δ_C 75.0, three methines (δ_C 52.3, 53.3, 65.2), and one methyl (δ_C 29.6). The ¹H, ¹H-COSY correlations (Fig. 7) suggested one OCH-CH-CH-CH fragment. The ¹H and ¹³C NMR data (Table 2) also indicated 1,2-disubstituted and monosubstituted aromatic units (ring A and B) and one methyl group. The HMBC correlations (Fig. 6) of H-4 with C-2' (δ_C 144.7) and C-6' (δ_C 124.3); H-5 with C-2' (δ_C 144.7) and C-1'' (δ_C 140.7); H-6 with C-3' (δ_C 125.8) and C-2''/C-6'' (δ_C 129.6) showed that ring A was connected at C-4 and C-6, and ring B was connected at C-6. The above information coupled with biogenetic considerations and literature references indicated **3** was similar to the dehydro-derivative of neoclausenamide.^{25,26} Its relative configuration was established on the basis of NOESY correlations (Fig. 7). The NOESY correlations of H-4 with H-5, H-5 with H-4, H-6, N-CH₃ and H-6 with H-5, H-6'', H-3 with H-2'' showed that H-4, H-5 and H-6 were β orientation, while H-3 was α orientation. Thus, compound **3** (clauselansine C) was fully identified.

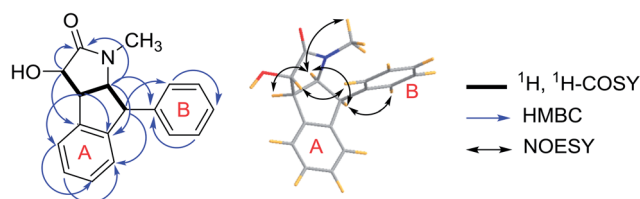


Fig. 7 Key ¹H, ¹H-COSY, HMBC and NOESY correlations of compound **3**.

Table 2 ¹H and ¹³C NMR spectroscopic data of compounds **3** (δ in ppm, *J* in Hz)

3			3		
Position	δ _H ^a	δ _C ^b	Position	δ _H ^a	δ _C ^b
2		174.1 s	5'	7.31, m	127.7
3	4.11, d (8.3)	75.0 d	6'	7.45, d (7.6)	124.3
4	3.77, dd (8.3, 5.3)	52.3 d	1''		140.7
5	4.66, t (8.3)	65.2 d	2'', 6''	6.98, d (7.1)	129.6
6	4.77, d (8.3)	53.3 d	3'', 5''	7.27, m	128.2
1'		142.8 s	4''	7.23 ^c	126.9
2'		144.7 s	N-CH ₃	2.10, s	29.6
3'	6.92, d (7.6)	125.8 d	OH	5.97, s	
4'	7.23 ^c	128.0 d			

^a In DMSO-*d*₆ (600 MHz). ^b In DMSO-*d*₆ (150 MHz). ^c Signal overlapped. Coupling constants (*J*) in Hz are given in parentheses. The assignments were based on HSQC and HMBC experiments.



The specific rotation of **3** approached zero, and no Cotton effect was found in the electronic circular dichroism (ECD) spectrum of **3**, indicating a racemic mixture. Subsequent chiral resolution of **3** afforded the anticipated enantiomers **3a** and **3b**, which showed mirror image-like ECD curves and specific rotations {**3a**: $[\alpha]_D^{20} +17.9$ (c 0.1 MeOH); **3b**: $[\alpha]_D^{20} -22.0$ (c 0.1 MeOH)}. Compound **3a** was treated with (*R*)- and (*S*)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) in anhydrous CH_2Cl_2 to afford the **3a**-(*S*)-MTPA ester (**3aa**) and **3a**-(*R*)-MTPA ester (**3ab**), respectively. The $\Delta\delta_{\text{H}}^{\text{SR}}$ values were calculated as shown in Fig. 8. Application of Mosher's rule²⁷ revealed that **3a** had the 3*S*,4*R*,5*S*,6*S* configuration. Meanwhile, the absolute configuration of **3b** was assigned with the 3*R*,4*S*,5*R*,6*R*-configuration. In order to confirm the absolute configuration of the enantiomers **3a** and **3b**, the ECD calculations were also performed for the two configurations 3*S*,4*R*,5*S*,6*S*- and 3*R*,4*S*,5*R*,6*R*-**3** using the time-dependent density functional theory (TDDFT) method at the B₃LYP/6-31G (d) level.^{21,22} The calculated ECD spectrum for 3*S*,4*R*,5*S*,6*S*-**3** agreed with the experimental ECD data (Fig. 9) of **3a**. The calculated ECD spectrum for 3*R*,4*S*,5*R*,6*R*-**3** was in good accordance with the experimental spectrum of **3b** (Fig. 9). Thus, **3a** has a 3*S*,4*R*,5*S*,6*S* configuration and **3b** has a 3*R*,4*S*,5*R*,6*R* configuration. Therefore, compounds **3a** and **3b** were given the trivial names (+)-(3*S*,4*R*,5*S*,6*S*)-clauselansine C and (−)-(3*R*,4*S*,5*R*,6*R*)-clauselansine C, respectively.

Compound **4** (**4a/4b**) was obtained as a white powder. Its molecular formula was determined as $\text{C}_{19}\text{H}_{19}\text{NO}_4$ on the basis of its ^{13}C NMR and HRESIMS (m/z 326.1398 $[\text{M} + \text{H}]^+$, calcd for $\text{C}_{19}\text{H}_{20}\text{NO}_4$ 326.1387), corresponding with eleven indices of hydrogen deficiency. Its NMR data (Table 3) was almost identical to those of claulansine B.¹⁰ Chiral separation of **4** afforded a pair of enantiomer **4a** $\{[\alpha]_D^{20} +142.7$ (c 0.1 MeOH)} and **4b** $\{[\alpha]_D^{20} -99.5$ (c 0.1 MeOH)}, which had opposite ECD curves. By

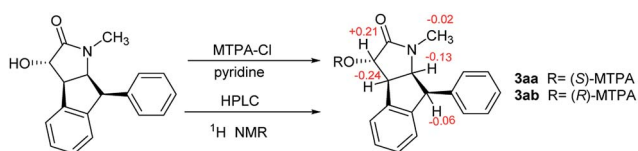


Fig. 8 The Mosher's method of **3a**.

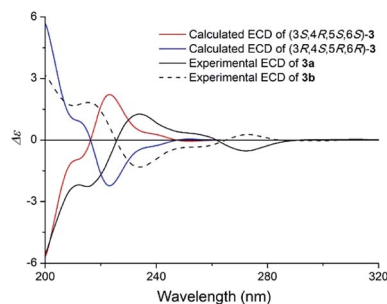


Fig. 9 Calculated ECD spectra of (3*S*,4*R*,5*S*,6*S*)-**3** and (3*R*,4*S*,5*R*,6*R*)-**3** and the experimental ECD spectra of (+)- and (−)-clauselansine C (**3a/3b**) in MeOH.

Table 3 ^1H and ^{13}C NMR spectroscopic data of compounds **4a** and **5a** (δ in ppm, J in Hz)

Position	4a		5a	
	δ_{H}^a	δ_{C}^b	δ_{H}^a	δ_{C}^b
1		144.3		138.6
1a		132.6 s		136.3
2		123.2 s		135.1
3		129.1 s		118.8
4	7.62, s	111.2 s	8.37, s	112.7
4a		122.7 d		122.6
5	8.04, d (7.5)	120.1 d	8.27, d (7.5)	121.0
5a		123.6		126.3
6	7.14, t (7.5)	118.8	7.22, t (7.5)	119.6
7	7.38, t (7.5)	125.8	7.46, t (7.5)	126.7
8	7.50, t (7.5)	111.4	7.55, d (7.5)	111.7
8a		140.1		140.8
1'	4.77, d (7.5)	61.2	6.16, s	77.6
2'	4.30, s	86.0	3.89, d (7.5)	75.8
3'		76.9		71.6
4'	1.27, s	29.7	1.22, s	28.5
5'	1.04, s	23.2	1.26, s	24.7
6'	6.09, s	100.3		170.8
1-OCH ₃	3.91, s	61.0	4.06, s	60.3
NH	11.37, br s		11.80 br s	
1'-OH	5.34, d (7.5)			
2'-OH			5.71, d (6.0)	

^a In DMSO- d_6 (600 MHz). ^b In DMSO- d_6 (150 MHz). Coupling constants (J) in Hz are given in parentheses. The assignments were based on HSQC and HMBC experiments.

comparison of the calculated ECD spectra of the 1'*R*,2'*R*,6'*R* and 1'*S*,2'*S*,6'*S* configurations of **4** with the experimental data of **4a** and **4b** (Fig. 10), the absolute configurations of **4a** and **4b** were assigned as 1'*R*,2'*R*,6'*R* and 1'*S*,2'*S*,6'*S*. Thus, **4a** was defined as (+)-(1'*R*,2'*R*,6'*R*)-claulansine B, and **4b** was identified as the known compound (−)-(1'*S*,2'*S*,6'*S*)-claulansine B.

Compound **5** (**5a/5b**) was obtained as a white powder. The HRESIMS displayed m/z 364.1163 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{19}\text{H}_{19}\text{NO}_5\text{Na}$ 364.1155), which was consistent with a molecular formula of $\text{C}_{19}\text{H}_{19}\text{NO}_5$ with eleven indices of hydrogen deficiency. Its NMR data (Table 3) was almost identical to those of claulansine D.¹⁰ Chiral isolation of **5** afforded the enantiomer **5a** $\{[\alpha]_D^{20} +62.0$ (c 0.11 MeOH)} and **5b** $\{[\alpha]_D^{20} -99.2$ (c 0.1 MeOH)},

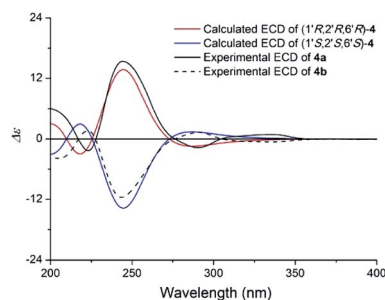


Fig. 10 Calculated ECD spectra of (1'*R*,2'*R*,6'*R*)-**4** and (1'*S*,2'*S*,6'*S*)-**4** and the experimental ECD spectra of (+)- and (−)-claulansine B (**4a/4b**) in MeOH.



which had opposite ECD curves (Fig. 11). The absolute configurations of **5a** and **5b** were determined using the same methods as described in **4a** and **4b**. Thus, **5a** was defined as (+)-(1*R*,2*R*)-claulansine D, and **5b** was identified as the known compound (–)-(1*S*,2*S*)-claulansine D.

The structures of nine known compounds were also identified by comparing their spectroscopic data to those found in the literature. The known compounds isolated were claulansine B (**4b**),¹⁰ claulansine D (**5b**),¹⁰ (–)-(1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**6a**),²⁸ (+)-(1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**6b**),²⁸ (*R*)-isoplatydesmine (**7a**),²⁹ (*S*)-isoplatydesmine (**7b**),³⁰ claulansine A (**8**),¹⁰ ribalinine (**9**),³¹ γ-fagarine (**10**),³² dictamnine (**11**),³³ *N*-(2-hydroxy-2-phenylethyl)-cinnamamide (**12**),³⁴ *N*-phenylethylbenzamide (**13**).³⁴

Compounds **1–13** were evaluated for their neuroprotective effect on neuron-like PC12 cells induced by Aβ_{25–35}, and Okadaic Acid (OKA) *in vitro* using the MTT method. The neuron growth factor (NGF) was used as a positive control. At 10 μM, **1b**, **2a**, **3b**, **5a**, **5b** increased the cell survival rate of the Okadaic acid-treated group, other compounds were inactive, while all the compounds failed to protect cells from Aβ_{25–35}.

Clausenamide is one of novel compounds isolated from *Clausena lansium* (Lour) skeels. Clausenamide is unusual in that it contains 4 chiral centers yielding 8 pairs of enantiomers. In

pharmacological studies numerous models and indicators showed that (–)-clausenamide is the active enantiomer, while (+)-clausenamide is inactive and elicits greater toxicity than (–)-clausenamide.³⁵ Compounds **3** and clausenamide are very similar in structure and therefore have the same biological activity. Similarly, **3b** is the active enantiomer, while **3a** is inactive. The carbazole and quinolone alkaloids having neuroprotective effects were exhibited in previous researches,^{10,36} and carbazole alkaloids may derived from indole alkaloids. As we all know, thalidomide as a chiral racemic compound, its *R*-configuration has inhibitory activity of pregnancy, while *S*-configuration has teratogenic. One of the isomers of the enantiomer is highly active and the other isomer is inactive, or both isomers are active, or both isomers are inactive. Thus, it's reasonable that compound **1b**, **2a**, **5a**, and **5b** are active while **1a**, **2b** are inactive. All in all, alkaloids isolated from *C. lansium* are worthy of study to find more potential effects in the further (Table 4).

Conclusions

In summary, we have reported the isolation, identification and biological study of twenty compounds (**1–13**) including three new enantiomers (**1a**, **1b**, **2a**, **2b**, **3a**, **3b**), two new ones (**4a**, **5a**), and several of them inhibited PC12 cell damage induced by Okadaic Acid. Furthermore, compounds **6–7** were isolated from the genus *Clausena* for the first time. The occurrence of alkaloids derivatives from *C. lansium* is in agreement with the previous findings, **1–7** indicating that the isolation of these enantiomer compounds might be a useful chemotaxonomic for screening activity. The results of preliminary neuroprotective effect assays suggested that several isolated alkaloids derivatives showed moderate activity. Moreover, previous findings exhibited that the carbazole, amide and quinolone alkaloids metabolites have neuroprotective effects and others biological activities such as anti-inflammatory, hepatoprotective, cytotoxic^{9,15–17} which indicating that the alkaloid compounds and their biological activities of *C. lansium* are worth studying in order to find compounds with potential activity.

Experimental

Optical rotations were measured on a JASCO P2000 automatic digital polarimeter. UV spectra were recorded on a JASCO V-650 spectrophotometer, CD spectra were measured on a JASCO J-815 spectropolarimeter. IR spectra were recorded on a Nicolet 5700 spectrometer using an FT-IR microscope transmission method. NMR spectra were acquired with Bruker AVIIHD 600, VNS-600, and Mercury-400 spectrometers in DMSO-*d*₆ and MeOH-*d*₆. HRESIMS spectra were collected on an Agilent 1100 series LC/MSD ion trap mass spectrometer. MPLC system was composed of two C-605 pumps (Büchi), a C-635 UV detector (Büchi), a C-660 fraction collector (Büchi), and an ODS column (450 mm × 60 mm, 50 μm, 400 g; YMC). Semi-preparative HPLC was conducted using a Shimadzu LC-6AD instrument with an SPD-20A detector and a Daicel Chiralpak AD-H column (250 × 10 mm, 5 μm). Preparative HPLC was also performed on

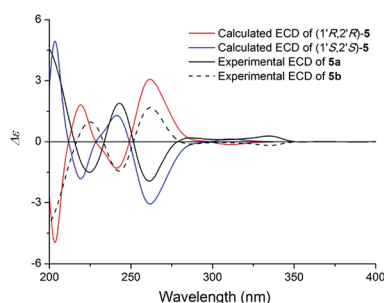


Fig. 11 Calculated ECD spectra of (1*R*,2*R*)-**5** and (1*S*,2*S*)-**5** and the experimental ECD spectra of (+)- and (–)-claulansine D (**5a/5b**) in MeOH.

Table 4 Neuroprotective effects of compounds **1–13** against Okadaic acid-induced injury in PC12 Cells (10 μM, means ± SD, *n* = 6)^a

Control	10 μM	100.0 ± 2.64
Model	10 μM	70.5 ± 5.4 ^{###}
1a	10 μM	65.4 ± 6.2
1b	10 μM	83.4 ± 4.1 ^{**}
2a	10 μM	91.2 ± 10.1 ^{***}
2b	10 μM	69.5 ± 5.3
3a	10 μM	71.7 ± 5.9
3b	10 μM	83.5 ± 7.8 ^{**}
4a	10 μM	74.4 ± 4.6
4b	10 μM	76.6 ± 7.5
5a	10 μM	89.7 ± 4.8 ^{***}
5b	10 μM	83.3 ± 5.9 ^{***}

^a ^{###}*p* < 0.001 vs. control, ^{***}*p* < 0.001 vs. model, ^{**}*p* < 0.01 vs. model, ^{*}*p* < 0.1 vs. model.



a Shimadzu LC-6AD instrument with a YMC-Pack ODS-A column (250 × 20 mm, 5 μm). Column chromatography (CC) was performed with silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, People's Republic of China), SF-PRP 512A (100–200 mesh, Beijing Sunflower and Technology Development Co., Beijing, People's Republic of China), ODS (50 μm, YMC, Japan), and Sephadex LH-20 (GE, Sweden). TLC was carried out on glass precoated silica gel GF254 plates. Spots were visualized under UV light or by spraying with 10% sulfuric acid in EtOH followed by heating. PC12 cells (adrenal gland; pheochromocytoma) were purchased from the American Type Culture Collection. Dimethyl sulphoxide (DMSO), Aβ_{25–35}, Okadaic acid, 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and equine serum were purchased from Gibco BRL (New York, NY, USA). All other chemicals were of analytical grade and were commercially available.

Plant material

The stems of *C. lansium* were collected in Liuzhou, Guangxi, China, in December 2008 and identified by Engineer Guangri Long, Forestry of Liuzhou. A voucher specimen has been deposited at the Herbarium of Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (ID-S-2320).

Extraction and isolation. Air-dried, powdered stems of *C. lansium* (200 kg) were macerated for 3 h with 20 L of 95% EtOH (aq) and refluxed for 4 h (20 L × 2). The filtrate was evaporated under reduced pressure to yield a dark brown residue (640 g). The residue was suspended in water (2000 mL) and then partitioned with CHCl₃ (3 × 2000 mL), EtOAc (3 × 2000 mL), and *n*-BuOH (3 × 2000 mL), successively. After removing the solvent, the *n*-BuOH-soluble portion (850 g) was fractionated *via* macroporous adsorbent resin (HPD-100) column with H₂O, 30% EtOH, 60% EtOH, and 95% EtOH to yield four corresponding fractions A–D.

Fraction B (180.6 g) was fractionated *via* silica gel column chromatography, eluting with CHCl₃–MeOH–H₂O (10 : 1 : 0.05, 9 : 1 : 0.1, 8 : 2 : 0.2, 7 : 3 : 0.3, 6 : 4 : 0.4) to afford twelve fractions B₁–B₁₂ on the basis of TLC analysis. Fraction B₄ (5.9 g) was further separated by reversed-phase silica MPLC with MeOH–H₂O (20–50%, 50 mL min^{−1}, 6 h) to afford B₄₋₁–B₄₋₇ fractions. Fractions B₄₋₃₈–B₄₋₄₂ was successively separated using preparative HPLC (detection at 210 nm, 18% CH₃CN, 8 mL min^{−1}) to yield **1** (20.3 mg, *t*_R 37.50 min). Compound **1** was further separated by semipreparative chiral HPLC (*n*-hexane-2-propanol, 6 : 1, 3 mL min^{−1}) to give **1a** (9.1 mg, *t*_R 57.63 min) and **1b** (8.7 mg, *t*_R 67.99 min). Fraction B₁₀ (5.1 g) was further separated by reversed-phase silica MPLC with MeOH–H₂O (20–50%, 50 mL min^{−1}, 7 h) to afford B₁₀₋₁–B₁₀₋₆₅ fractions. Fractions B₁₀₋₁₅–B₁₀₋₂₀ were successively separated using preparative HPLC (detection at 210 nm, 8% CH₃CN, 8 mL min^{−1}) to yield **6a** (8.2 mg, *t*_R 58.35 min) and **6b** (2.4 mg, *t*_R 68.93 min).

Fraction C (197.3 g) was fractionated *via* silica gel column chromatography, eluting with CHCl₃, EtOAc, *n*-BuOH,

CH₃COCH₃, MeOH to afford five fractions C₁–C₅. Fraction C₁ was further separated by PRP-512A with MeOH–H₂O (35–70%) to afford C₁₋₁–C₁₋₆ fractions. Fraction C₁₋₃ was further separated by reversed-phase silica MPLC with MeOH–H₂O (35–55%, 50 mL min^{−1}, 6 h) to afford C₁₋₃₋₁–C₁₋₃₋₁₂ fractions. Fraction C₁₋₃₋₁₁ was successively separated by Sephadex LH-20 and then using preparative HPLC (detection at 210 nm, 30% CH₃CN, 8 mL min^{−1}) to yield **5** (6.2 mg, *t*_R 42.87 min), **7** (11.2 mg, *t*_R 62.14 min) and **9** (24.4 mg, *t*_R 51.17 min). Compound **5** was further separated by semipreparative chiral HPLC (*n*-hexane-2-propanol, 3 : 1, 3 mL min^{−1}) to give **5a** (2.8 mg, *t*_R 36.37 min) and **5b** (2.1 mg, *t*_R 45.78 min). Compound **7** was further separated by semipreparative chiral HPLC (*n*-hexane-2-propanol, 7 : 1, 3 mL min^{−1}) to give **7a** (4.8 mg, *t*_R 19.85 min) and **7b** (5.3 mg, *t*_R 25.86 min). Fraction C₁₋₃₋₁₂ was successively separated by Sephadex LH-20 and then using preparative HPLC (detection at 210 nm, 29% CH₃CN, 8 mL min^{−1}) to yield **2** (5.2 mg, *t*_R 48.14 min). Compound **2** was further separated by semipreparative chiral HPLC (*n*-hexane-2-propanol, 8 : 1, 3 mL min^{−1}) to give **2a** (2.8 mg, *t*_R 25.81 min) and **2b** (2.1 mg, *t*_R 34.17 min). Fraction C₁₋₅ was further separated by reversed-phase silica MPLC with MeOH–H₂O (35–55%, 50 mL min^{−1}, 6 h) to afford C₁₋₅₋₁–C₁₋₅₋₈ fractions. Fraction C₁₋₅₋₆ was successively separated *via* silica gel column chromatography and then using preparative HPLC (detection at 210 nm, 32% CH₃CN, 8 mL min^{−1}) to yield **3** (10.7 mg, *t*_R 53.75 min), **4** (9.2 mg, *t*_R 39.05 min), **10** (3.1 mg, *t*_R 52.10 min), **12** (2.4 mg, *t*_R 49.16 min) and **13** (3.2 mg, *t*_R 65.64 min). Compound **3** was further separated by semipreparative chiral HPLC (*n*-hexane-2-propanol, 9 : 1, 3 mL min^{−1}) to give **3a** (4.1 mg, *t*_R 19.87 min) and **3b** (3.9 mg, *t*_R 25.12 min). Compound **4** was further separated by semipreparative chiral HPLC (*n*-hexane-2-propanol, 7 : 1, 3 mL min^{−1}) to give **4a** (1.5 mg, *t*_R 21.98 min) and **4b** (4.3 mg, *t*_R 40.07 min). Fraction C₁₋₆ was further separated by reversed-phase silica MPLC with MeOH–H₂O (35–55%, 50 mL min^{−1}, 6 h) to afford C₁₋₆₋₁–C₁₋₆₋₁₀ fractions. Fraction C₁₋₆₋₂ was successively separated *via* silica gel column chromatography and then using preparative HPLC (detection at 210 nm, 30% CH₃CN, 8 mL min^{−1}) to yield **8** (5.2 mg, *t*_R 33.31 min) and **11** (7.1 mg, *t*_R 23.17 min).

(+)-(2'*S*,3'*R*)-Clauselansine A (**1a**). Colourless oil; [α]_D²⁰ +18.7 (c 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 202.8 (4.29), 222.6 (4.38), 281.8 (3.48) nm; ECD (MeOH) λ_{max} (Δε) 268 (+0.75), 226 (+4.01) nm; IR (microscope) ν_{max} 3313, 2925, 1671, 1612, 1439, 1379, 1109, 1040, 749 cm^{−1}; ¹H NMR (DMSO-*d*₆, 400 MHz) and ¹³C NMR (DMSO-*d*₆, 100 MHz), see Table 1; HRESIMS *m/z* 320.1854 [M + H]⁺ (calcd for C₁₈H₂₆NO₄, 320.1856).

(−)-(2'*R*,3'*S*)-Clauselansine A (**1b**). Colourless oil; [α]_D²⁰ −17.0 (c 0.1 MeOH); ECD (MeOH) λ_{max} (Δε) 268 (−0.23), 226 (−0.79) nm; UV, IR, NMR, and HRESIMS were the same as those of **1a**.

(+)-(2'*S*,3'*R*)-Clauselansine B (**2a**). White powder; [α]_D²⁰ +28.0 (c 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 203.0 (4.37), 220.0 (4.37), 281.6 (3.53) nm; ECD (MeOH) λ_{max} (Δε) 276 (−0.18), 233 (+1.03) nm; IR (microscope) ν_{max} 3358, 2917, 2851, 1615, 1377, 1039, 1002, 739 cm^{−1}; ¹H NMR (DMSO-*d*₆, 600 MHz) and ¹³C NMR (DMSO-*d*₆, 150 MHz), see Table 1; HRESIMS *m/z* 302.1758 [M + H]⁺ (calcd for C₁₈H₂₄NO₃, 302.1751).



(–)-(2′R,3′S)-Clauselansine B (**2b**). White powder; $[\alpha]_D^{20}$ –32.6 (c 0.1 MeOH); ECD (MeOH) λ_{\max} ($\Delta\epsilon$) 280 (+0.35), 232 (–1.56) nm; UV, IR, NMR, and HRESIMS were the same as those of **2a**.

(+)-(3S,4R,5S,6S)-Clauselansine C (**3a**). White powder; $[\alpha]_D^{20}$ +17.9 (c 0.1 MeOH); UV (MeOH) λ_{\max} (log ϵ) 203.8 (4.28) nm; ECD (MeOH) λ_{\max} ($\Delta\epsilon$) 272 (–0.53), 234 (+1.27), 215 (–2.27) nm; IR (microscope) ν_{\max} 3320, 2923, 1678, 1483, 1454, 1403, 1202, 1132, 1078, 753, 703 cm^{-1} ; ^1H NMR (DMSO- d_6 , 400 MHz) and ^{13}C NMR (DMSO- d_6 , 100 MHz), see Table 2; HRESIMS m/z 280.1333 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{18}\text{H}_{17}\text{NaNO}_2$, 280.1332).

(–)-(3R,4S,5R,6R)-Clauselansine C (**3b**). White powder; $[\alpha]_D^{20}$ –22.0 (c 0.1 MeOH); ECD (MeOH) λ_{\max} ($\Delta\epsilon$) 272 (+0.27), 234 (–1.31), 215 (+1.84) nm; UV, IR, NMR, and HRESIMS were the same as those of **3a**.

(+)-(1′R,2′R,6′R)-Claulansine B (**4a**). White powder; $[\alpha]_D^{20}$ +142.7 (c 0.1 MeOH); UV (MeOH) λ_{\max} (log ϵ) 201.6 (4.40), 241.8 (4.24), 294.8 (3.68) nm; ECD (MeOH) λ_{\max} ($\Delta\epsilon$) 335 (+0.90), 290 (–1.71), 244 (+15.40), 223 (–2.31) nm; IR (microscope) ν_{\max} 3319, 2971, 1613, 1573, 1503, 1456, 1383, 1248, 1081, 1024, 745 cm^{-1} ; ^1H NMR (DMSO- d_6 , 600 MHz) and ^{13}C NMR (DMSO- d_6 , 150 MHz), see Table 3; HRESIMS m/z 326.1398 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{19}\text{H}_{20}\text{NO}_4$, 326.1387).

(+)-(1′R,2′R)-Claulansine D (**5a**). White powder; $[\alpha]_D^{20}$ +62.0 (c 0.11 MeOH); UV (MeOH) λ_{\max} (log ϵ) 195.8 (3.71), 231.0 (3.87), 278.2 (4.11) nm; ECD (MeOH) λ_{\max} ($\Delta\epsilon$) 335 (+0.28), 261 (–1.94), 243 (+1.89), 225 (–1.50) nm; IR (microscope) ν_{\max} 3379, 2919, 2850, 1737, 1613, 1462, 1358, 1242, 1099, 729 cm^{-1} ; ^1H NMR (DMSO- d_6 , 600 MHz) and ^{13}C NMR (DMSO- d_6 , 150 MHz), see Table 3; HRESIMS m/z 364.1163 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{19}\text{H}_{19}\text{NNaO}_5$, 364.1155).

Preparation of acetone derivative (**1c**)

To determine the relative configuration of **1b**, compound **1b** (3 mg) was treated with 2,2-dimethoxypropane (1 mL) and pyridinium *p*-toluene sulfonate (1 mg), then stirred at 30 °C for 5 h under N_2 circumstance. The reaction solution was evaporated *in vacuo* and purified by reversed-phase preparative HPLC using $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (60 : 40 v/v) to yield the acetone derivative **1c** (2.47 mg).

Compound 1c. White amorphous powder; $[\alpha]_D^{20}$ +12.0 (c 0.2 MeOH); UV (MeOH) λ_{\max} (log ϵ) 206 (6.24), 225 (6.40), 277 (5.75) nm; ECD (MeOH) λ_{\max} ($\Delta\epsilon$) 267 (–1.28), 251 (–0.78), 234 (–2.14) nm; $\text{Mo}_2(\text{OAc})_4$ -induced CD (DMSO) 323 ($\Delta\epsilon$ +0.26) nm; IR (microscope) ν_{\max} 3307, 2923, 2854, 1645, 1542, 1452, 1400, 1240, 1099, 660 cm^{-1} ; ^1H NMR (DMSO- d_6 , 600 MHz) and ^{13}C NMR (DMSO- d_6 , 150 MHz), see Table 1; HRESIMS m/z 382.1993 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{21}\text{H}_{29}\text{NNaO}_4$, 382.1989).

Determination of absolute configurations of the 3′,4′-diol unit in **1c**

According to the published procedure, a 1 : 1.2 mixture of diol/ $\text{Mo}_2(\text{OAc})_4$ for **1c** was subjected to CD measurements at a concentration of 0.1 mg mL^{-1} in anhydrous DMSO. The first CD spectrum was recorded immediately after mixing, and its time evolution was monitored until stationary (about 10 min after mixing). The inherent CD was subtracted. The observed

sign of the diagnostic band at around 310 nm in the induced CD spectrum was correlated to the absolute configuration of the 3′,4′-diol unit.

Preparation of (R)- and (S)-MTPA esters of **3a**

A solution of **3a** (1.31 mg) in dehydrated CH_2Cl_2 (2 mL) was treated with (R)-(+)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride [(R)-MTPA-Cl (10 mg)] in the presence of anhydrous pyridine, and the mixture was stirred at room temperature for 13 h. After cooling, the reaction mixture was poured into ice-water and extracted with EtOAc. The EtOAc extract was successively washed with 5% aqueous HCl, saturated aqueous NaHCO_3 , and brine, then dried over Na_2SO_4 and filtered. The solvent was removed from the filtrate under reduced pressure to afford a residue. The residue was purified by semi-preparative HPLC (C18 column, 3.0 mL min^{-1} , UV 210 nm, 80% $\text{CH}_3\text{CN}-\text{H}_2\text{O}$) to yield (S)-MTPA ester derivative of **3a** (compound **3aa** 1.03 mg). (R)-MTPA ester derivative of **3a** (compound **3ab** 0.92 mg) was obtained from **3a** (1.05 mg).

Compound 3aa. Colourless oil; ^1H NMR (600 MHz, CDCl_3) δ_{H} 5.73 (1H, d, J = 4.2 Hz, H-3), 3.83 (1H, dd, J = 8.1, 4.2 Hz, H-4), 4.62 (1H, d, J = 8.1 Hz, H-5), 4.69 (1H, d, J = 8.1 Hz, H-6), 2.17 (3H, s, N- CH_3), 3.69 (3H, s, OCH_3), 6.99–7.68 (14H, Ar-H).

Compound 3ab. Colourless oil; ^1H NMR (600 MHz, CDCl_3) δ_{H} 5.52 (1H, d, J = 4.2 Hz, H-3), 4.07 (1H, dd, J = 8.1, 4.2 Hz, H-4), 4.75 (1H, m, H-5), 4.75 (1H, overlapped, H-6), 2.19 (3H, s, N- CH_3), 3.59 (3H, s, OCH_3), 7.00–7.70 (14H, Ar-H).

Neuroprotection bioassays

Pheochromocytoma (PC12) cells were incubated in DMEM supplied with 5% fetal bovine serum and 5% equine serum as basic medium. PC12 cells in logarithmic phase were cultured at a density of 5000 cells per well in a 96-well microtiter plate. After 24 h incubation, the medium of the model group was changed to DMEM or basic medium with 15 μM $\text{A}\beta_{25-35}$ for 48 h or basic medium with 50 nM OKA for 24 h. Test compounds dissolved in dimethyl sulfoxide (DMSO) were added to each well for >1000-fold dilution in the model medium at the same time. Each sample was tested in triplicate. After the incubation at 37 °C in 5% CO_2 for 24 h, 10 μL of MTT (5 mg mL^{-1}) was added to each well and incubated for another 4 h; then liquid in the wells was removed. DMSO (100 μL) was added to each well. The absorbance was recorded on a microplate reader (Bio-Rad model 550) at a wavelength of 570 nm.³⁷ Analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test were performed to assess the differences between the relevant control and each experimental group. *p*-Values of <0.05, <0.01, and <0.001 were regarded as statistically significant. Data were expressed as mean \pm SEM as indicated.

Conflict of interest

There are no conflicts of interest to declare.



Acknowledgements

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