Chemical Constituents from the Fungus *Amauroderma amoiensis* and Their *In Vitro* Acetylcholinesterase Inhibitory Activities

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Abstract

One new compound named amauroamoienin (**1**), together with thirteen known compounds (**2**–**14**), was isolated from the EtOAc extract of *Amauroderma amoiensis*. The structures of these compounds were elucidated by the analysis of 1D and 2D spectroscopic data and the MS technique. The bioassays of inhibitory activities of these isolates against acetylcholinesterase were evaluated, and compounds **1**, **3**, and **5** exhibited acetylcholinesterase inhibitory activities.

Key words

Amauroderma amoiensis · Ganodermataceae · amauroamoienin · acetylcholinesterase inhibitory activity

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The family Ganodermataceae comprising more than 200 fungal species is mainly distributed in the tropical and subtropical areas of Asia, Australia, Africa, and America [1]. More than 100 species of this family grow in China, of which 78 wild species were found in Hainan Province [2]. *Ganoderma* and *Amauroderma* are two genera of the Ganodermataceae family. A great deal of work has been carried out on the genus *Ganoderma* [3]. Two of its species, recorded in the Chinese Pharmacopoeia, *G. lucidum* and *G. sinense,* have been used for the treatment of migraine, hypertension, arthritis, bronchitis, asthma, gastritis, diabetes, nephritis, and hepatitis problems for centuries [4,5]. Recent research on the chemical constituents of *Ganoderma* species showed the presence of natural products including triterpenes, steroid, alkaloids, flavonoids, polysaccharides, and fatty acids [6–8]. Previous screening of acetylcholinesterase (AChE) inhibitors from fungal extracts showed that many fungi, including the genera of the Ganodermataceae family, exhibited inhibitory activities against AChE [9]. At present, chemical constituents from many *Amauroderma* species, including *Amauroderma amoiensis* (Zhao) found in Fujian and Hainan Province of China [10], and their biological activities are still not reported.

In order to study the bioactive constituents of *A. amoiensis*, a chemical investigation was carried out and led to the isolation of one new compound, named amauroamoienin (**1**), along with thirteen known compounds (**2**–**14**) (**Fig. 1**), 4-hydroxy-17-methylincisterol (**2**) [11], (l7R)-17-methylincisterol (**3**) [12], 1,5-dihydroxy-6′,6′-dimethylpyrano[2′,3′:3,2] xanthone (**4**) [13], jacareubin (**5**) [13], ergosterol peroxide (**6**) [14], ergosta-7,22-dien-3*β*-ol (**7**) [15,16], (22*E*,24R)-ergosta-8,22*E*-diene-3*β*,5*α*,6*β*,7*α*-tetraol (**8**) [17,18], 22E-7*α*-methoxy-5*α*,6*α*-epoxyergosta-8(14),22-dien-3*β*-ol (**9**) [19], 3*β*,5*α*,9*α*-trihydroxyergosta-7,22-dien-6-one (**10**) [20,21], 1H-indole-3-carboxylic acid (**11**) [22], *p*-hydroxybenzoic acid (**12**) [23], methyl 3,4-dihydroxybenzoate (**13**) [24], and 7,8-dimethylalloxazine (**14**) [25]. Herein, we describe the isolation and structural elucidation of the new compound (**1**), as well as the AChE inhibitory activities of all isolates.

Compound **1** was obtained as a yellow amorphous powder, and its molecular formula was assigned to be $C_{46}H_{54}O_7$ from its HREIMS (*m/z* 718.3871 $[M]^+$, calcd. for $C_{46}H_{54}O_7$, 718.3870) and NMR data (**Table 1**), indicating twenty degrees of unsaturation. The IR spectrum displayed the presence of hydroxyl (3353 cm^{-1}), carbonyl (1726 cm^{-1}), and double bond (1621 cm^{-1}) absorptions. Analysis of ^{13}C NMR and DEPT spectra (**Table 1**) showed 46 carbon resonances including eight for methyls, six for methylenes, sixteen for methines (two oxygenated), and sixteen for quaternary carbons (including one carbonyl). The ^{13}C NMR spectra showed resonances characteristic of the structure consisting of an ergostane moiety and a xanthone moiety. Comparison of 18 carbon signals of compound **1** with those of jacareubin (**5**) [14] hinted that **1** had a xanthone moiety with the same structure of that of **5**, which was also confirmed by olefinic and aromatic protons signals [δ_H 6.71 (1H, d, J = 10.1 Hz), 5.57 (1H, d, J = 10.1 Hz), δ_H 6.39 (1H, s), 6.88 (1H, d, J = 8.9 Hz), and 7.67 (1H, d, J = 8.9 Hz)], and two resonance signals [δ_H 1.45 and 1.46 (each 1H, s)] for methyl groups similar to those of jacareubin. The ^1H-^1H COSY correlations of H-7′/H-8′ and H-13′/H-14′ and HMBC correlations (**Fig. 2**) further deduced the presence of a jacareubin moiety in **1**. The remaining 28 carbon signals in compound **1** were reasonably characteristic of an ergostane steroid skeleton which was the basic natural product isolated from this genus. The steroid moiety possessed six olefinic carbons [δ_C 115.9 (d), 124.0 (d), 132.5 (d), 135.6 (d), 140.0 (s), 141.0 (s)], two oxygenated methines [δ_C 66.8, 73.6], and one oxygenated quaternary carbon [δ_C 77.3], among which an epoxy group was formed based on analysis of its molecular formula. Comparison of these ^{13}C NMR data with those of (22*E*,24R)-ergosta-7,9(11),22-triene-3*β*,5*α*,6*β*-triol [26] suggested that they might have identical skeletons except for the difference of three oxygenated carbons. The key HMBC correlations from H-6 (δ_H 4.73, s) to C-6′, C-7, and C-8 indicated that the steroid and xanthone moieties were connected via an oxygen at C-6 and C-6′, which also confirmed the epoxy group formed at two other oxygenated carbons. The HMBC correlation from H-19 (δ_H 1.24, s) to C-5 (δ_C 77.3) showed that the epoxy group was at C-4 and C-5. The stereochemistry of the chiral centers (C-10, C-13, C-14, C-17) and the side chain at C-20 and C-24 of the steroid moiety in **1** were proposed to be the same as those of (22*E*,24R)-ergosta-7,9(11),22-triene-3*β*,5*α*,6*β*-triol by comparison of the their ^{13}C NMR data, with the possible *β*-orientations of CH_3-18 and CH_3-19 and *α*-orientations of H-14 and H-17 [26]. The *β*-orientation of H-6 was determined by the key NOE of H-6/H-19 (**Fig. 2**). The weak correlations of H-6 and H-4 (δ_H 3.93, m) with the same proton H-2b (δ_H 1.61, m) proposed the *β*-orientation of H-4, which accordingly hinted the *α*-orientation of the epoxy group in **1**. NOE of H-18/H-20 further confirmed

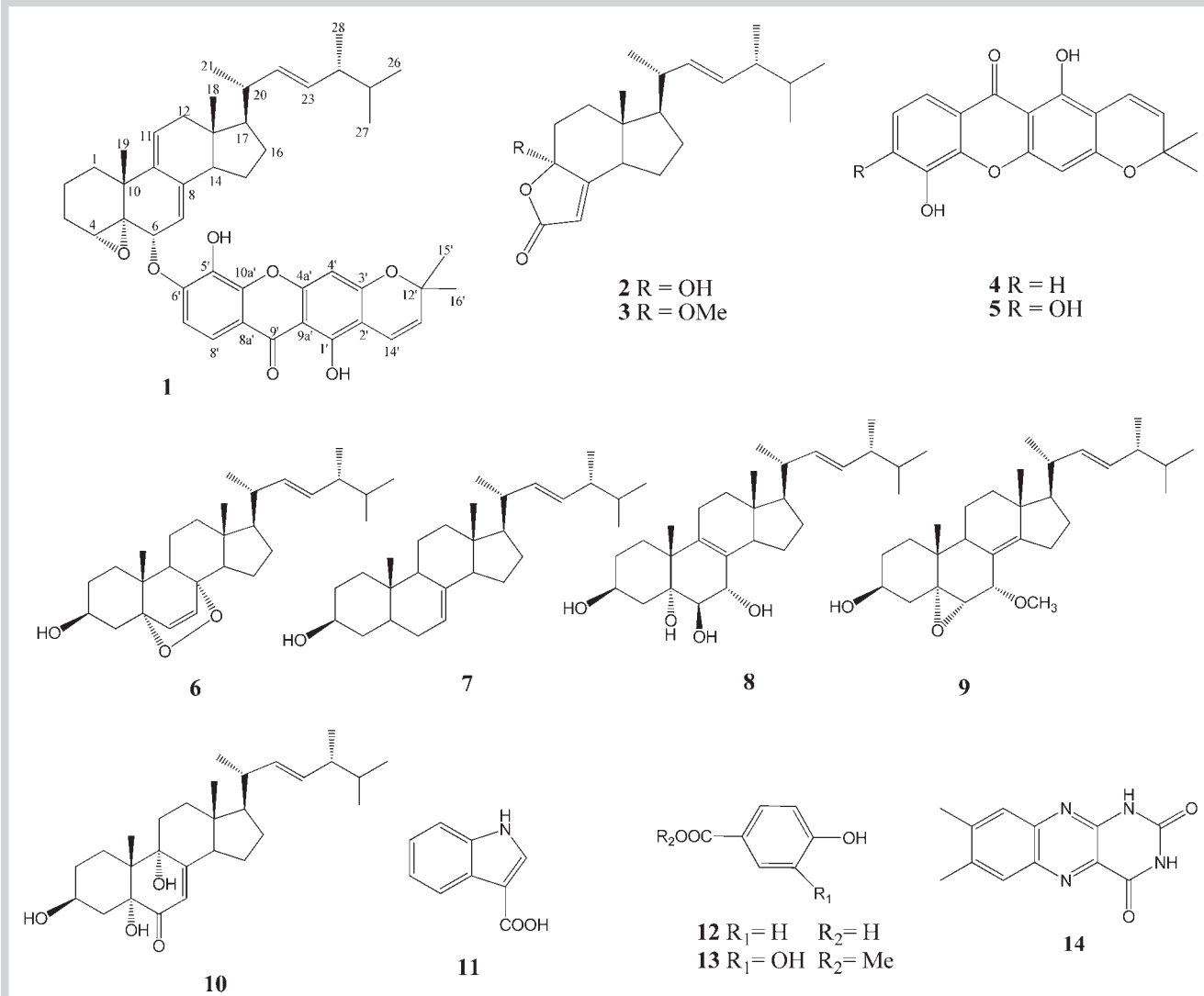

Fig. 1 Structures of compounds **1**–**14**.

the β-orientation of CH_3-18 and the α-orientation of H-17. Thus, the structure of compound **1** was assigned as shown in **Fig. 1**, and this compound was named amauroamoienin.

The AChE inhibitory activities for compounds **1**–**11** were tested. The purities of all compounds were more than 98% by HPLC analyses. Compound **3** showed a certain inhibitory activity (inhibition percentage was 46.33%) at the concentration of 100 µM, and compounds **1** and **5** showed weak inhibitory activity (inhibition percentages were 14.63% and 25.49%, respectively) (**Table 2**). Meanwhile, the other compounds were inactive with inhibition ratios less than 10%.

Materials and Methods

The fruiting bodies of *A. amoiensis* were collected in Lingshui County, Hainan Province, People's Republic of China in June 2011 and identified by Prof. Xing-Liang Wu of the Hainan University. A voucher specimen (No. 2011JZ01) was deposited at the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences.

Extraction and isolation: The dried and powdered fruiting bodies of *A. amoiensis* (6.0 kg) were extracted at room temperature with 95% EtOH (3 × 20 L, 7 d each). The extract was concentrated and suspended in water followed by successive partition with EtOAc and *n*-BuOH, respectively. The EtOAc extract (70.8 g) was separated by silica gel column chromatography (CC) (10 × 40 cm, 560.0 g) under vacuum using a gradient eluent mixture of petroleum ether/EtOAc (20 : 1–3 : 1, 3 L each) to afford nine fractions (Fr 1–Fr 9). Fraction 4 (3.2 g) was subjected to silica gel CC (4 × 30 cm, 30.0 g) under vacuum eluted with petroleum ether/EtOAc (10 : 1–3 : 1, 1 L each) to give 6 subfractions 4a–4 f. Subfraction 4 d (350.0 mg) was subjected to CC with Sephadex LH-20 (2 × 100 cm, $CHCl_3$/MeOH 1 : 1, 1 L) to yield compound **4** (5.0 mg). Fraction 5 (7.3 g) was subjected to silica gel CC (5.5 × 30 cm, 70.0 g) eluted with petroleum ether/EtOAc (4 : 1–1 : 1, 3 L each) to give 5 subfractions 5a–5e. Subfraction 5a (447.0 mg) was repeatedly purified by silica gel CC (2 × 45 cm, 24.0 g) eluted with petroleum ether/EtOAc (7 : 1, 3 L) and Sephadex LH-20 (2 × 100 cm, $CHCl_3$/MeOH 1 : 1, 500 mL) to yield compound **1** (11.9 mg). Subfraction 5b (2.4 g) was chromatographed on silica gel CC (3 × 30 cm, 20.0 g) eluted with petroleum ether/EtOAc

Table 1 ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compounds **1** and **5** in $CDCl_3$ (δ_H in ppm, *J* in Hz).

	1					5	
No	δ_H, *J* (Hz)	δ_C	No	δ_H	δ_C	δ_H, *J* (Hz)	δ_C
1	1.69 (m), 1.90 (m)	30.0	1′		160.5		159.5
2	1.28 (m), 1.61 (m)	23.2	2′		104.8		103.7
3	1.59 (m), 2.16 (m)	35.4	3′		157.9		156.9
4	3.93 (m)	66.8	4′	6.39 (s)	95.6	6.37 (s)	94.6
5		77.3	4a’		157.2		156.4
6	4.73 (s)	73.6	5′		130.2		132.5
7	5.05 (s)	115.9	6′		146.7		152.1
8		141.0	7′	6.88 (d, 8.9)	114.1	6.93 (d, 8.7)	113.2
9		140.0	8′	7.67 (d, 8.9)	117.2	7.50 (d, 8.7)	115.9
10		40.6	8a’		115.5		112.9
11	5.69 (d, 6.4)	124.0	9′		180.7		179.9
12	2.22 (m), 2.35 (m)	42.3	9a’		103.6		102.2
13		42.8	10a’		147.1		146.0
14	2.19 (m)	51.1	12′		78.3		78.1
15	1.71 (m), 2.24 (m)	28.9	13′	5.57 (d, 10.1)	127.6	5.71 (d, 10.1)	128.1
16	1.69 (m), 2.05 (m)	31.0	14′	6.71 (d, 10.1)	115.8	6.57 (d, 10.1)	114.5
17	1.27 (m)	56.2	15′	1.45 (s)	28.7	1.41 (s)	27.9
18	0.56 (s)	12.0	16′	1.46 (s)	28.6	1.41 (s)	27.9
19	1.24 (s)	24.5					
20	2.01 (m)	40.6					
21	1.00 (d, 6.6)	21.0					
22	5.11 (dd, 8.3, 15.3)	135.6					
23	5.20 (dd, 7.7, 15.3)	132.5					
24	1.92 (m)	43.1					
25	2.06 (m)	33.3					
26	0.79 (d, 6.8)	19.9					
27	0.81 (d, 6.8)	20.2					
28	0.89 (d, 6.8)	17.9					

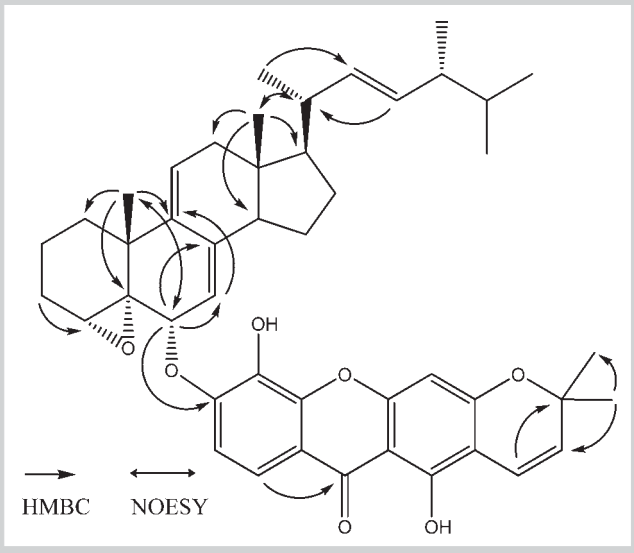

Fig. 2 Key HMBC and NOESY correlations of compound **1**.

(4 : 1, 4 L) to yield compound **6** (19.6 mg). Subfraction 5c (169.2 mg) was separated by silica gel CC (1.2 × 34 cm, 9.0 g) eluted with petroleum ether/EtOAc (5 : 1, 3 L) to yield compound **7** (3.8 mg). Fraction 6 (3.7 g) was separated by silica gel CC (4 × 30 cm, 32 g) using as eluent petroleum ether/EtOAc (4 : 1–1 : 1, 2 L each) to afford subfractions 6a–6e. Subfraction 6e (671.7 mg) was gel-filtrated on Sephadex LH-20 (2 × 100 cm, $CHCl_3$/MeOH 1 : 1, 1.2 L) to yield compound **2** (10.9 mg). Subfraction 6 d (332.0 mg) was gel-filtrated on Sephadex LH-20 (2 × 100 cm, $CHCl_3$/MeOH 1 : 1, 500 mL) and then separated by silica gel column (1.2 × 34 cm, 8.0 g) using as eluent petroleum ether/EtOAc (15 : 1, 1 L) to yield compound **3** (8.9 mg). Fraction 7 (4.7 g) was separated by vacuum liquid column (4 × 30 cm, 50.0 g) using as eluent petroleum ether/EtOAc (3 : 2–1 : 2, 2 L) to afford subfractions 7a–7 g. Subfraction 7c (520 mg) was subjected to Sephadex LH-20 (2 × 100 cm, $CHCl_3$/MeOH 1 : 1, 800 mL) to yield compound **11** (5.2 mg). Subfractions 7a and 7b were separated by silica gel column to yield compounds **12** (8.1 mg), using as eluent $CHCl_3$/MeOH (50 : 1, 800 mL), and **13** (8.1 mg) (2.5 × 40 cm, $CHCl_3$/MeOH 22 : 1, 600 mL) from 7a (830 mg), as well as compounds **8** (21.8 mg), **9** (12.9 mg), and **10** (24.3 mg) (2.5 × 40 cm, $CHCl_3$/EtOAc 50 : 1–14 : 1, 600 mL each) from 7b (790 mg). Fraction 8 (7.7 g) was separated by vacuum liquid column (5.5 × 30 cm, 90 g) using as eluent $CHCl_3$/MeOH (35 : 1–10 : 1, 1.5 L each) to afford subfractions 8a–8 g. Subfractions 8 d (55.3 mg) and 8e (880.3 mg) were gel-filtrated on Sephadex LH-20 (2 × 100 cm, $CHCl_3$/MeOH 1 : 1, 400 mL and 700 mL) to yield compounds **5** (13.0 mg) and **14** (5.3 mg), respectively.

Isolate: *Amauroamoienin* (**1**): Yellow amorphous powder; $[\alpha]_D^{27}$ + 12.5 (*c* 3.2, MeOH); UV (MeOH) λ_{max} (log ε) 365 (1.35), 315 (1.56), 257 (3.02), 241 (2.77); IR (KBr) ν_{max}·cm^{-1} 3353 cm^{-1}, 2885, 2270, 1726, 1621, 1590, 1490, 1407, 1296, 1253, 1195, 1093, 884, 888, 670; ^{1}H and ^{13}C NMR data, see **Table 1**; positive ESIMS *m/z* $[M + H]^+$ 719 (100); HREIMS *m/z* $[M]^+$ 718.3871 (calcd. for $C_{46}H_{54}O_7$, 718.3870).

Bioassay of AChE inhibitory activity: Acetylcholinesterase inhibitory activity of all compounds was assayed by the spectrophoto-

Compound	Percentage of inhibition	Compound	Percentage of inhibition
1	14.6	**7**	< 10
2	< 10	**8**	< 10
3	46.3	**9**	< 10
4	< 10	**10**	< 10
5	25.5	**11**	< 10
6	< 10	Tacrine*	53.0

* Positive control (0.333 µM)

Table 2 AChE inhibitory activity of compounds **1–11** (%) at 50 µM.

metric method developed by Ellman et al. [27] with slight modifications. *S*-Acetylthiocholine iodide, 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB, Ellman's reagent), and acetylcholinesterase derived from human erythrocytes were purchased from Sigma Chemical. Compounds were dissolved in DMSO. The reaction mixture (totally 200 µL), containing phosphate buffer (pH 8.0), test compound (50 µM), and acetylcholinesterase (0.02 U/mL), was incubated for 20 min (30 °C). The reaction was initiated by the addition of 20 µL of DTNB (0.625 mM) and 20 µL acetylthiocholine iodide (0.625 mM) for the AChE inhibitory activity assay. The hydrolysis of acetylthiocholine was monitored at 405 nm after 30 min. Tacrine (Sigma-Aldrich 99 %) was used as a positive control with final concentration of 0.333 µM, and DMSO was used as negative control with final concentration of 0.1 %. All the reactions were performed in triplicate. The percentage of inhibition was calculated as follows: % inhibition = (E–S)/E × 100 (E is the activity of the enzyme without test compound and S is the activity of enzyme with test compounds).

Supporting information

The original spectra of NMR and HREIMS data for the new compound (**1**) and general experimental procedures are available as Supporting Information.

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Conflict of Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of the manuscript entitled.

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