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Two Androstane Derivatives from the Cultures of Fungus *Marasmiellus ramealis* (Bull.) Singer

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A new androstane derivative, 4*β*-methyl-15-oxa-14*β*-androstane-7-ene-4*α*-carboxylic acid (**1**) and a known one 4*β*-methyl-15-oxa-14*β*-androstane-7-ene-4*α*-hydroxyl (**2**) were isolated from the EtOAc extract of the cultures of the fungus *Marasmiellus ramealis* (Bull.) Singer. Their structures were elucidated on the basis of 1D and 2D NMR as well as MS spectroscopic data analysis. The inhibitory activity of two isolates against acetylcholinesterase (AChE) revealed that compound **1** exhibited definitely inhibitory activity.

Key Words : *Marasmiellus ramealis* (Bull.) Singer, Fermentation, Androstane derivative, AChE inhibitory activity

Introduction

Marasmiellus ramealis (Bull.) Singer, belonging to the family Marasmiaceae, is a small, thin, white mushroom, usually growing on the deadwood. The fungi is an edible fungus with a wide distribution in most parts of China, especially in Hainan, Hunan,Yunnan, and Tibet province.[1] So far, a few early researches on chemical constituents of fungus *M. ramealis* were reported and showed the presence of several natural products such as marasin and isocoumarins.[2-4] In order to make full use of the *M. ramealis*, the deep chemical investigation was thus undertaken to find the bioactive metabolites from the cultures of this fungus, which led to the isolation of a new androstane derivative 4*β*-methyl-15-oxa-14*β*-androstane-7-ene-4*α*-carboxylic acid (**1**) and a known one, 4*β*-methyl-15-oxa-14*β*-androstane-7-ene-4*α*-hydroxyl (**2**). The two compounds belong to the type of isopimaric diterpenes. In this paper, the isolation and structural elucidation as well as their inhibitory activity against AChE of two isolates were described.

Experimental

General Experimental Procedures. Melting points were measured on a SGW X-4 micro-melting point apparatus and were uncorrected. The IR spectra were obtained on a Nicolet 380 FT-IR instrument, as KBr pellets (Thermo, Pittsburgh, PA, USA). Optical rotations were measured with a Rudolph Autopol III polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). Shimadzu UV-2550 spectrometer (Beckman, Brea, CA, USA) was used for scanning UV spectroscopy. HR-ESI-MS were performed on an API QSTAR Pulsar mass spectrometer (Bruker, Bremen, Germany). 1D and 2D NMR spectra were recorded on AV-500 spectrophotometers (Bruker, Bremen, Germany) with tetramethylsilane (TMS) as the internal standard. Column chromatography was performed with Si gel (200-300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), Lichroprep RP-18 gel (40-63 μm; Merck, Darmastadt, Germany). The fractions were monitored by thin layer chromatography (TLC), and spots were visualized by heating Si gel plats sprayed with 5% H_2SO_4 in EtOH.

Fungus Materials. The fungus *M. ramealis* was collected in Jianfengling Mountain, Hainan province of China, in June 2012, and identified by Associate Professor Nian-kai Zeng, Hainan Medical College. The mycelium was isolated from the cap of *M. ramealis* and its strain was maintained on potato dextrose agar (PDA) slant at 4 °C. A voucher specimen (No. HUANG 201201) was deposited at the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, Haikou, China.

Fermentation, Extraction and Isolation. The fungus was cultured on PDA at room temperature for a week. Two pieces of mycelial agar plugs (0.5 cm × 0.5 cm) were inoculated into 1 L Erlenmeyer flasks containing 500 mL potato dextrose broth (PDB). The fermentation was carried out on a shaker at 25 °C and 150 $r\cdot min^{-1}$ for 7 days, and then kept in still at room temperature for 23 days. The culture broth (90 L) was filtered to give the filtrate and mycelia. The filtrate was evaporated *in vacuo* to small volume and then partitioned in succession between H_2O, EtOAc and *n*-BuOH. The EtOAc solution was evaporated under reduced pressure to give a crude extract (15.6 g), which was separated into 9 fractions on a silica gel column chromatography (CC) using a step gradient elution of petroleum ether–EtOAc (20:1, 15:1, 10:1, 8:1, 5:1, 1:1, 1:5, 0:1, *v*/*v* each 1 L). Fr. 3 (3.0 g) was submitted to Sephadex LH-20 CC ($CHCl_3$-MeOH 1:1, *v*/*v*, 600 mL) and further separated by silica gel CC with

petroleum ether–EtOAc (3:1, *v/v*, 800 mL), yielding compound **1** (3.0 mg). Fr. 4 (2.5 g) was submitted to silica gel CC with petroleum ether–EtOAc (1:2, *v/v*, 1.2 L) as eluent and further purified by Sephadex LH-20 CC with $CHCl_3$/MeOH (1:1, *v/v*, 600 mL) as eluent, yielding compound **2** (3.0 mg).

4*α*-Methyl-15-oxa-androstane-7-ene-4*β*-carboxylic acid (1): White powder; mp 185-189 °C; $[\alpha]_D^{32}$ –2.0 (*c* 0.025, MeOH); UV (MeOH) λ_{max} (log ε) 196 (4.94), 198 (4.87), 201 (4.83), 273 (4.60), 306 (4.17) nm; IR (KBr) ν_{max} 2925, 1615, 1419, 1115 cm^{-1}; HREIMS *m/z* $[M]^+$ 318.2196 (calcd. for $C_{20}H_{30}O_3$, 318.2195) ; ^1H and ^{13}C NMR see Table 1.

4*α*-Methyl-15-oxa-androstane-7-ene-4*β*-hydroxyl (2): White powder; mp 164-167 °C; $[\alpha]_D^{32}$ –4.4 (*c* 0.025, MeOH); UV (MeOH) λ_{max} (log ε) 190 (4.37), 200 (4.12), 277 (3.77), 306 (3.62), 315 (3.57), 353 (3.51) nm; IR (KBr) ν_{max} 3424, 2924, 1628, 1450, 1112 cm^{-1}; HRESIMS *m/z* $[M+Na]^+$ 313.2140 (calcd. for $C_{19}H_{30}NaO_2$, 313.2143); ^1H and ^{13}C NMR see Table 1.

Bioassay of AChE Inhibitory Activity: AChE inhibitory activity of these compounds was assayed by the spectrophotometric method developed by Ellman.[5] Acetylthiocholine iodide (Sigma, St. Louis, MO, USA) was used as substrate in the assay. Compounds were dissolved in DMSO. The reaction mixture, consisting of 110 μL phosphate buffer (pH 8.0), 10 μL of tested compounds solution (2000 μmol L^{-1}), and 40 μL AChE solution (0.04 U/100 μL), was mixed and incubated for 20 min (30 °C). The reaction was initiated by the addition of 20 μL 5,5-dithiobis-2-nitrobenzoic acid (6.25 mmol L^{-1}) and 20 μL acetylthiocholine. The hydrolysis of acetylthiocholine was monitored at 405 nm after 30 min. Tacrine (Sigma-Aldrich 99%) was used as positive control. All the reactions were done in triplicate. The percentage inhibition was calculated as follows: % inhibition = $(E - S)/E \times 100$ (E is the activity of the enzyme without test compound and S is the activity of enzyme with test compounds).

Results and Discussion

The culture broth (90 L) of the fungus *Marasmiellus ramealis* (Bull.) Singer was filtered to give the filtrate and mycelia. The filtrate was evaporated *in vacuo* to small volume and then partitioned in succession between H_2O, EtOAc and n-BuOH. The EtOAc extract was subjected to repeated CC over silica gel, Sephadex LH-20, and RP-18 to give two androstane derivatives (Fig. 1) including a new one (**1**). Their structures were identified by spectroscopic means including 1D and 2D NMR combined comparing their spectra with those in literatures.

Compound **1**, white powder, possessed the molecular formula $C_{20}H_{30}O_3$ from its HREIMS peak at *m/z* 318.2196 $[M]^+$ (calcd. 318.2195) and NMR data (Table 1), indicating six degrees of unsturation. The IR spectrum displayed the presence of double bond (1615 cm^{-1}). The ^{13}C NMR and DEPT spectra (Table 1) revealed 20 carbon resonances, including three methyls, eight methylenes, four methines, five quaternary carbons (one carboxyl), suggestive of diterpene skeleton. The ^1H and ^{13}C NMR spectra of **1** exhibited

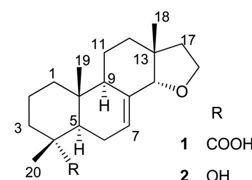

Figure 1. Structures of compounds **1** and **2**.

Table 1. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of **1** and **2** (δ in ppm, J in Hz) in $CDCl_3$

No.	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
1*α*	39.8	1.08 m	39.1	1.05 m
β	–	1.87 m	–	1.86 m
2*α*	19.7	1.45 m	18.1	1.45 m
β	–	1.85 m	–	1.77 m
3*α*	38.3	1.04 m	41.5	1.68 m
β	–	2.15 m	–	1.45 m
4	43.8	–	71.7	–
5	50.8	1.49 m	48.2	1.34 m
6*α*	24.7	2.26 m	22.8	2.04 m
β	–	2.45 m	–	2.14 m
7	129.6	5.79 d (*J* = 5.6)	129.5	5.81 d (*J* = 5.5)
8	133.5	–	134.5	–
9	47.4	1.96 m	47.5	1.93 m
10	35.6	–	34.9	–
11*α*	21.5	1.62 m	20.6	1.52 m
β	–	1.18 m	–	1.25 m
12*α*	32.5	1.55 m	32.3	1.55 m
β	–	1.33 m	–	1.32 m
13	40.5	–	40.5	–
14	88.9	3.60 br s	88.9	3.61 br s
15	–	–	–	–
16*α*	65.3	3.91 dd (*J* = 8.5, 8.0)	65.3	3.90 dd (*J* = 8.5, 8.0)
β		3.85 dt (*J* = 8.5, 3.5)	41.3	3.84 dt (*J* = 8.5, 3.5)
17*α*	41.5	1.75 m	21.3	1.70 m
β		1.82 m	14.4	1.81 m
18	21.2	0.98 s	30.9	0.99 s
19	14.1	0.72 s	–	0.94 s
20	29.1	1.22 s		1.14 s
COOH	182.1	–		–

similarities with those of 4*β*-methyl-15-oxa-8*α*,14*β*-androstane-4*α*-carboxylic acid.[6] The major difference of ^{13}C NMR data was that one double bond signals (δ_C 129.6, δ_C 133.5) in **1** replaced two saturated carbon signals (δ_C 39.5, δ_C 36.9) at C-7 and C-8 in 4*β*-methyl-15-oxa-8*α*,14*β*-androstane-4*α*-carboxylic acid. The double bond located at C-7 and C-8 in **1** was confirmed by ^1H, ^1H-COSY correlation of δ_H 5.79 (H-7) and 2.45 (H-6) and HMBC correlations from H-7 (δ_H 5.79) to C-5 (δ_C 50.8) and C-14 (δ_C 88.9) (Fig. 2). The relative configuration of compound **1** was determined by NOESY experiment (Fig. 2). The *β*-orientations of C-18 (δ_C 21.2) and C-19 (δ_C 14.1) were hypothetically assigned to be the same as that of 4*β*-methyl-15-oxa-8*α*,14*β*-androstane-4*α*-carboxylic acid. The *β*-orientations of H-14 and CH_3-4 were elucidated by clear NOE of H-14 (δ_H 3.60)/H-18 (δ_H 0.98) and H-19 (δ_H 0.72)/H-20 (δ_H 1.22). The *α*-orientation of H-5 was deduced

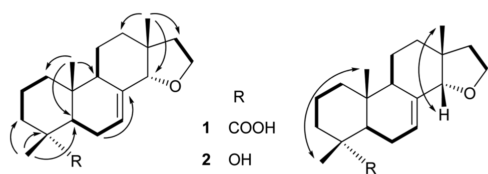

Figure 2. Selected ^{1}H,^{1}H-COSY (—), HMBC (→) and NOESY (↔) correlations of **1** and **2**.

by the key NOE of H-5 [δ_H 1.49 (1H, m)]/H-9 [δ_H 1.96 (1H, m)]. Thus, the new structure of **1** was assigned as shown and was named 4*β*-methyl-15-oxa-14*β*-androstane-7-ene-4*α*-carboxylic acid.

Compound **2** was isolated as white powder, and its molecular formula, $C_{19}H_{30}O_2$ requiring five degrees of unsaturation, was determined by the HRESIMS positive (*m/z*) $[M+Na]^+$ 313.2140 (calcd. for $C_{19}H_{30}O_2$, 313.2143) and NMR spectroscopic data (Table 1). The IR spectrum displayed the presence of double bond (1628 cm^{-1}) and hydroxyl group (3424 cm^{-1}). The ^{13}C NMR and DEPT spectrum (Table 1) showed 19 carbon resonances, including three methyls, eight methylenes, four methines, and four quaternary carbons. The NMR spectroscopic data of **2** were extremely similar to those of **1** except for the loss of signal for a carboxyl at C-4 and the chemical shift δ_C 71.7 of C-4 in **2** replaced signal δ_C 43.8 in **1** which suggested that **2** was derived from **1** by the loss of carboxyl group and the attachment of hydroxyl at C-4. The hydroxyl group at C-4 was supported by HMBC correction from H-20 (δ_H 1.14) to C-4 in compound **2**. Compound **2** had the same relative configuration as those of **1** with *β*-orientations of H-14 and CH_3-4 by their similar NOESY experiment (Fig. 2) and NMR data (Table 1). Thus, the structure of compound **2**, named 4*β*-methyl-15-oxa-14*β*-androstane-7-ene-4*α*-hydroxyl, was elucidated as shown. Compound **2** was reported as 4*α*,16-epoxy-18-norisopimar-7-en-4*α*-ol in the patent.[7]

The AChE inhibitory activities of compounds **1** and **2** were tested by existed method as described in previous. As a result, compound **1** showed definite inhibitory activity with the percentage inhibition 40.80% (positive control tacrine was 56.3%), while compound **2** showed no inhibitory activitiy against AChE. The anti-tumor and anti-fungal activities of the isopimaric diterpenes were reported, while the reports of inhibitory activities against AChE were few.[7,8] Compound **1** showed significant inhibitory activity of AChE, while compound **2** showed no activitiy. We may infer that the key point was carboxyl group at the C-4 of compound **1** which showed a close relationship with the activitiy.

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References

1. Mao, X. L. *Macromycetes of China*; Science Press: Beijing, China, 2009; Vol. 1, p 190.
2. Jarrah, M. Y.; Thaller, V. *J. Chem. Soc. Perkin Trans.* **1983**, *1*, 1719.
3. Holroyde, J. K.; Orr, A. F.; Thaller, V. *J. C. S. Chem. Comm.* **1976**, *7*, 242.
4. Holroyde, J. K.; Orr, A. F.; Thaller, V. *J. Chem. Soc. Perkin Trans. 1* **1978**, *12*, 1490.
5. Ellman, G. L.; Courtney, K. D.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88.
6. Martín, M. B.; Mateos, A. F.; González, R. R. *J. Chem. Soc. Perkin Trans. 1* **1995**, *5*, 569.
7. Wu, S. H.; Chen, Y. W.; Miao, C. P. *Isopimaric Diterpene Compounds and its Application*: 201310298815.8, 2013.
8. Wang, X. N.; Bashyal, B. P.; Wijeratne, E. M.; U'Ren, J. M.; Liu, M. X.; Gunatilaka, M. K.; Arnold, A. E.; Gunatilaka, A. A. *J. Nat. Prod.* **2011**, *74*, 2052.