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

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A new androstane derivative, 4β-methyl-15-oxa-14β-androstane-7-ene-4α-carboxylic acid (1) and a known one 4β-methyl-15-oxa-14β-androstane-7-ene-4α-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. 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. 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 4β-methyl-15-oxa-14β-androstane-7-ene-4α-carboxylic acid (1) and a known one, 4β-methyl-15-oxa-14β-androstane-7-ene-4α-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, Darmstadt, Germany). The fractions were monitored by thin layer chromatography (TLC), and spots were visualized by heating Si gel plats sprayed with 5% H2SO4 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/min 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 H2O, 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 (CHCl3-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 CHCl₃/MeOH (1:1, v/v, 600 mL) as eluent, yielding compound 2 (3.0 mg).

4α-Methyl-15-oxa-androstane-7-ene-4β-carboxylic acid (1): White powder; mp 185-189 °C; [α]D²⁰ −2.0 (c 0.025, MeOH); UV (MeOH) λmax (log e) 196 (4.94), 198 (4.87), 201 (4.83), 273 (4.60), 306 (4.17) nm; IR (KBr) νmax 2925, 1615, 1419, 1115 cm⁻¹; HRESIMS m/z [M]⁺ 318.2196 (calcd. for C₂₀H₃₀O₃, 318.2195); ¹H and ¹³C 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), 40 µL of 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 percent 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).

Results and Discussion

The culture broth (90 L) of the fungus Marasmiellus ramealis (Bull.) Singer was filtered to give the filtrate and mycelia. Androstane derivatives (Fig. 1) including a new one, were purified by Sephadex LH-20 CC with CHCl₃/MeOH, silica gel, Sephadex LH-20, and RP-18 to give two fractions. Their structures were identified by spectroscopic means including 1D and 2D NMR comparing their spectra with those in literatures.

Compound 1, white powder, possessed the molecular formula C₂₀H₃₀O₃ from its HREIMS peak at m/z 318.2196 [M]⁺ (calcd. 318.2195) and NMR data (Table 1), indicating six degrees of unsaturation. The IR spectrum displayed the presence of double bond (1615 cm⁻¹). The ¹³C NMR and DEPT spectra (Table 1) revealed 20 carbon resonances, including three methyls, eight methylenes, four methines, five quaternary carbons (one carbonyl), suggestive of diterpene skeleton. The ¹H and ¹³C NMR spectra of 1 exhibited similarities with those of 4β-methyl-15-oxa-8α,14β-androstane-4α-carboxylic acid.⁶ The major difference of ¹³C NMR data was that one double bond signals (δC 39.5, δC 36.9) at C-7 and C-8 in 4β-methyl-15-oxa-8α,14β-androstane-4α-carboxylic acid. The double bond located at C-7 and C-8 in 1 was confirmed by ¹H, ¹H-COSY correlation of δH 5.79 (H-7) and 2.15 m (H-20) (δH 1.33 m). The ¹H-orientations of H-14 and CH-20 were elucidated by clear NOE of H-14 (δH 3.61) and H-20 (δH 1.33 m). The α-orientation of H-5 was deduced.

![Figure 1. Structures of compounds 1 and 2.](image-url)
by the key NOE of H-5 $[^1]H$, 1.49 (1H, m)/H-9 $[^1]H$, 1.96 (1H, m)]. Thus, the new structure of 1 was assigned as shown and was named 4β-methyl-15-oxa-14β-androstane-7-ene-4α-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 13C 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 δC 71.7 of C-4 in 2 replaced signal δC 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 (δH 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 4β-methyl-15-oxa-14β-androstane-7-ene-4α-hydroxy, was elucidated as shown. Compound 2 was reported as 4α,16-epoxy-18-norisopimar-7-en-4α-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 activity 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 activity. We may infer that the key point was carboxyl group at the C-4 of compound 1 which showed a close relationship with the activity.

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