Sesquiterpenes from Cultures of the Basidiomycete *Clitocybe conglobata* and Their 11β-Hydroxysteroid Dehydrogenase Inhibitory Activity

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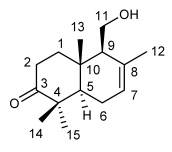
A new drimane sesquiterpenoid, 3-keto-drimenol (1), were isolated from cultures of the basidiomycete *Clitocybe conglobata* together with 3β-hydroxy-11-acetyldrimene (2), 3β-hydroxydrimenol (3), 11,12-dihydroxydrimene (4), 3β-hydroxy-11,12-*O*-isopropyldrimene (5), and 3β,11,12-trihydroxydrimene (6). Their structures were established from MS and NMR experiments. Compounds 1, 3, and 6 showed inhibitory activities against two isozymes of 11β-hydroxysteroid dehydrogenases with IC_{50} 1.7—8.0 μg/ml (human 11β-HSD1), 10.7—24.1 μg/ml (mouse 11β-HSD1); 177.0—220.0 μg/ml (human 11β-HSD2), 250.5—500.2 μg/ml (mouse 11β-HSD2), respectively, which catalyze the interconversion of active cortisol and inactive cortisone.

Key words drimane sesquiterpene; *Clitocybe conglobata*; basidiomycete; 11β-hydroxysteroid dehydrogenase

Yunnan Province, southwest of China, is one of the areas with the richest and most diverse bioresources in the world. Among these bioresources, fungi produce a broad variety of sencondary metablites. Basidomycetes of genus *Clitocybe* are known to produce many types of biologically active compounds. Previous studies on some species of this genus revealed that a large number of sesquiterpenes, such as protoilludene,[1] allenes,[2] marasmanes,[3] acetylenes[4] and calvilactones[5] showed pharmacological properties, including antibacterial, antifungal and antitumor activities. Recent research showed that a novel diterpene alkaloid was isolated from the cultures of *C. concave*.[6] But little work has been done on the chemical constituents of *C. conglobata*. During our search for naturally occurring bioactive secondary metabolite from higher fungi in China, we investigated the cultures of *C. conglobata* which led to the isolation of a new drimane-type sesquiterpenoid **1**, and known drimene sesquiterpenoids **2**—**6** (Fig. 1). This paper deals with the isolation and structure elucidation of **1**. Among them, compounds **1**, **3**, and **6** showed inhibitory activities against two isozymes of 11β-hydroxysteroid dehydrogenases (11β-HSD1 and 11β-HSD2), which catalyze the interconversion of active cortisol and inactive cortisone.

3-Keto-drimenol (**1**) was isolated as colorless oil. The molecular formula was determined to be $C_{15}H_{24}O_2$ by a combination of positive EI-MS, ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra, and further confirmed by HR-ESI-MS. The IR spectrum of **1** showed characteristic absorption for a hydroxyl group (3402 cm^{-1}) and carbonyl function (1704 cm^{-1}). The ^{1}H-NMR spectrum of **1** (Table 1) displayed signals due to four tertiary methyl groups (δ_H 1.09, s, H-15; 1.10, s, H-14; 1.06, s, H-13; 1.78, d, J=1.5 Hz, H-12), an oxymethylene (δ_H 3.87, dd, J=10.9, 3.9 Hz, H-11α; 3.82, dd, J=10.9, 3.9 Hz, H-11β) and an olefinic proton (δ_H 5.58, m, H-7). The ^{13}C-NMR spectrum of **1** together with the DEPT, HMQC and ^{1}H-NMR spectra revealed 15 carbon resonances, including four methyl carbons (δ_C 14.5, C-15; 22.3, C-14; 25.2, C-13; 21.7, C-12), three methylene carbons (δ_C 23.8, C-6; 34.5, C-1, 38.5, C-2), two methines (δ_C 51.1, C-5; 56.0, C-9), two quaternary carbons (δ_C 35.8, C-10; 47.5, C-4), two olefinic carbons (δ_C 123.7, C-7; 132.9, C-8), one oxygenated methylene carbon at δ_C 60.6 (C-11), and one keto carbon at δ_C 216.7 (C-3). The above-mentioned data exhibited similarities with those of the known compound 3β-hydroxydrimenol (**3**),



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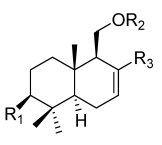

2: R_1=OH, R_2=Ac, R_3=CH_3
3: R_1=OH, R_2=H, R_3=CH_3
4: R_1=H, R_2=H, R_3=CH_2OH
6: R_1=OH, R_2=H, R_3=CH_2OH

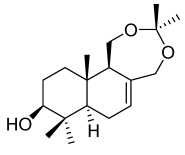

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Fig. 1. The Structures of Compounds **1**—**6**

Table 1. NMR Spectral Data for Compounds **1** and **3**[a)]

Position	**1**		**3** (literature data)
	^{1}H ($CDCl_3$)	^{13}C ($CDCl_3$)	^{13}C (CD_3OD)
1	2.74 (dd, 6.1, 13.9) 2.28 (m)	34.5 t	38.9
2	1.56 (m) 2.30 (m)	38.5 t	28.1
3		216.7 s	79.6
4		47.5 s	39.7
5	1.60 (m)	51.1 d	51.0
6	1.94 (m) 2.08 (m)	23.8 t	24.2
7	5.58 (m)	123.7 d	123.7
8		132.9 s	134.9
9	1.92 (m)	56.0 d	58.2
10		35.8 s	36.8
11	3.82 (dd, 10.9, 3.9) 3.87 (dd, 10.9, 3.9)	60.6 t	61.2
12	1.78 (d, 1.5)	21.7 q	22.2
13	1.06 (s)	25.2 q	28.7
14	1.10 (s)	22.3 q	15.9
15	1.09 (s)	14.5 q	14.9

a) Chemical shift values δ in ppm, coupling constants J in Hz (in parentheses).

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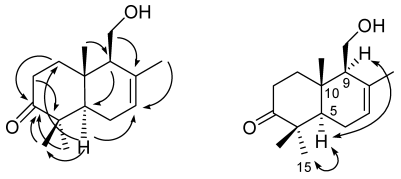

Fig. 2. The Key HMBC and ROESY Correlations of Compound **1**

which suggested compound **1** possessing drimane sesquiterpenoid skeleton. The notable difference between **3** and **1** was that the oxygenated methine carbon at δ_C 79.6 in **3** was replaced by the keto carbon at δ_C 216.7, which caused that the downfield shifts of C-4 from δ=39.7 in **3** to 47.5 in **1**, and C-2 from 28.1 in **3** to 38.5 in **1**. The above assignment was further supported by the HMBC correlations from H-1, H-2, H-14 and H-15 with C-3 (Fig. 2). From a biogenetic point of view, **3** seemed to be the precursor of **1**, suggesting the relative configuration at C-10 and C-5 in **1** was the same as those in **3** and that C-13 was β-oriented and H-5 was α-oriented. The α-orientation of H-9 was deduced from the ROESY cross-peak between H-9 and H-5α. Hence, compound **1** was determined as 3-keto-drimenol (Fig. 1). The four known sesquiterpenes were identified as 3β-hydroxydrimenol (**3**), 11,12-dihydroxydrimene (**4**), 3β,11,12-trihydroxydrimene (**6**),[8] 3β-hydroxy-11-acetyldrimene (**2**),[9] by comparison of their spectroscopic data with literature values. 3β-Hydroxy-11,12-*O*-isopropylidenedrimene (**5**) might be not a natural product. This acetonide is likely to be produced by reaction of the corresponding diol with the solvent during the isolation process, since compound **5** has not been detected from original CH_2Cl_2 extract of *C. conglobata* with RP-8 HPLC.

Glucocorticoid hormones play important roles in many biological and physiological processes, including regulation of energy metabolism; inflammatory, immune and stress responses; and cardiovascular homeostasis. The action of glucocorticoid on target tissue is not inevitable dependent on the circulating levels, but is regulated in a tissue-specific manner by the enzyme of 11β-hydroxysteroid dehydrogenases (11β-HSD1 and 11β-HSD2), which catalyze the interconversion of active 11-hydroxy-glucocorticoids (cortisol in human and corticosterone in rodent) and their respective inert 11-keto forms (cortisone in human and 11-dehydrocorticosterone in rodent).[10] 11β-HSD1 is highly expressed in liver, gonad, adipose tissue and brain, where it acts as a reductase regenerating the active glucocorticoids from its inactive forms, thus amplifies local glucocorticiod action.[11] 11β-HSD2 is predominantly expressed in aldosterone target cells such as kidney and colon, where it catalyses the inactivation of glucocorticiods, thereby preventing excessive activation of the mineralocorticoid receptor and sequelae including sodium retention, hypokalemia, and hypertension. We tested the inhibitory effect of compounds on both human and mouse 11β-HSD1 and 11β-HSD2. All tests were done triplicate with glycyrrhizininc acid as positive control. IC_{50} (X$\pm$S.D., n=3) values were calculated by using Prism Version 4 (GraphPad Software, San Diego, CA, U.S.A.). IC_{50} of glycyrrhizininc acid (positive control) are 29.5, 18.6, and 0.71 nM for mouse 11β-HSD1, human 11β-HSD1, and human 11β-HSD2, respectively. Compound **1** showed inhibitory activities against 11β-HSD1 (human IC_{50}=1.7 μg/ml; mouse IC_{50}=10.7 μg/ml) and 11β-HSD2 (human IC_{50}=200.0 μg/ml; mouse IC_{50}=500.2 μg/ml). Compound **3** showed inhibitory activities against 11β-HSD1 (human IC_{50}=8.0 μg/ml; mouse IC_{50}=19.4 μg/ml) and 11β-HSD2 (human IC_{50}=220.0 μg/ml; mouse IC_{50}=330.3 μg/ml). Compound **6** showed inhibitory activities against 11β-HSD1 (human IC_{50}=3.2 μg/ml; mouse IC_{50}=24.1 μg/ml) and 11β-HSD2 (human IC_{50}=177.0 μg/ml; mouse IC_{50}=250.5 μg/ml). Therefore, compounds **1**, **3**, and **6** showed inhibitory activities against 11β-HSD1 and 11β-HSD2, and provide possibility for modulating local cortisone/cortisol availability *in vivo*. Compounds **2**, **4**, and **5** were not tested in this experiment.

Experimental

General Experimental Procedures Optical rotations were measured on a Horiba SEPA-300 polarimeter. IR spectra were recorded on a Bruker Tensor 27 spectrometer with KBr pellets. Both 1D and 2D NMR experiments were performed on a Bruker AM-400 or DRX-500 spectrometer with tetramethylsilane (TMS) as the internal standard. The EI-MS was recorded with a VG Autospec-3000 spectrometer. The HR-ESI-MS was recorded with an API QSTAR Pulsar 1 spectrometer. Column chromatography was performed on silica gel (200—300 mesh; Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China) and Sephadex LH-20 (Amersham Biosciences, Sweden). TLC analysis was carried out on silica gel GF_{254} precoated plates (0.20—0.25 mm; Qingdao) with detection by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol.

Fungal Material The basidiomycete *C. conglobata* was collected from the campus of Kunming Institute of Botany, Chinese Academy of Sciences, China, in August 2007 and identified by Prof. Mu Zang, Kunming Institute of Botany, Chinese Academy of Sciences (CAS). Voucher specimens were deposited at the Herbarium of the Kunming Institute of Botany, CAS.

Cultivation The culture medium consisted of potato (peel off) (200 g), glucose (20 g), $KHPO_4$ (3 g), $MgSO_4$ (1.5 g), citric acid (0.1 g), and thiamin hydrochloride (10 mg) in 1 l of deionized H_2O. The fungus was grown in reagent bottles (500 ml; media of 300 ml). The pH was adjusted to 6.5 before autoclaving. Fermentation was carried out on a shaker at 22 °C and 150 rpm for 25 d.

Extraction and Isolation The whole culture broth of *C. conglobata* (18 l) was filtered, and the filtrate was extracted three times with EtOAc. The organic layer was concentrated under reduced pressure to give an oil residue (4.1 g) that was subjected to column chromatography over silica gel (200—300 mesh) eluting with $CHCl_3$/MeOH (from 100 : 0 to 0 : 100, v/v) to afford fractions A—I. Fraction D was rechromatographed on Sephadex LH-20 column eluting with $CHCl_3$/MeOH (1 : 1) to give fractions D_1—D_4. Subfraction D_3 was further subjected to column chromatography on silica gel with petroleum ether/EtOAc (8 : 1) to afford compound **2** (12.0 mg). Subfraction D_4 was separated further by column chromatography on silica gel using petroleum ether/EtOAc (3 : 1) and Sephadex LH-20 to give compounds **1** (4.4 mg) and **3** (42.6 mg). Fraction E was passed over a Sephadex LH-20 column and repeatedly applied to silica gel column eluted with petroleum ether/EtOAc (2 : 1) to yield compound **4** (9.2 mg). Fraction F was purified by a Sephadex LH-20 and repeated chromatography over silica gel using petroleum ether/acetone (4 : 1) as the eluent to yield compounds **5** (3.3 mg) and **6** (47.5 mg).

Biological Testing Inhibition of compounds on human or mouse 11β-HSD1 and 11β-HSD2 enzymatic activities were determined by the scintillation proximity assay (SPA) using microsomes containing 11β-HSD1 or 11β-HSD2 according to our previous studies.[7] Briefly the full-length cDNAs of human or murine 11β-HSD1 and 11β-HSD2 were isolated from the cDNA libraries provided by NIH Mammalian Gene Collection and cloned into pcDNA3 expression vector. HEK-293 cells were transfected with the pcDNA3-derived expression plasmid and selected by cultivation in the presence of 700 μg/ml of G418. The microsomal fraction overexpressing 11β-HSD1 or 11β-HSD2 was prepared from the HEK-293 cells stable transfected with either 11β-HSD1 or 11β-HSD2 and used as the enzyme source for SPA. Microsomes containing human or mouse 11β-HSD1 was incubated with NADPH and [^{3}H]cortisone, then the product, [^{3}H]cortisol was specifically captured by a monoclonal antibody coupled to protein A-coated SPA beads. The 11β-HSD2 screening was performed by incubating 11β-HSD2 microsomes with [^{3}H]cortisol and NAD^+ and monitoring substrate disap-

pearance. IC_{50} (X±S.D., $n=3$) values were calculated by using Prism Version 4 (GraphPad Software, San Diego, CA, U.S.A.) with glycyrrhizininc acid as positive control.

3-Keto-drimenol (**1**): Colorless oil. $[\alpha]_D^{26.9}$ −55.4° ($c=0.04$, $CHCl_3$). IR (KBr) cm^{-1}: ν_{max} 3402, 2926, 2938, 2858, 1704, 1453, 1385, 1009. ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$): see Table 1. EI-MS *m*/*z*: (%) 236 (86) $[M]^+$, 218 (10), 205 (43), 187 (46), 163 (57), 139 (98), 133 (50), 123 (63), 119 (89), 107 (84), 105 (52), 96 (100). HR-ESI-MS *m*/*z*: 259.1669 (Calcd 259.1673 for $C_{15}H_{24}O_2Na$, $[M+Na]^+$).

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