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# Highly Oxidized Humulane Sesquiterpenes from the Basidiomycete *Lactarius mitissimus*

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**Abstract** Two new highly oxidized humulane sesquiterpenes, mitissimols D (**1**) and E (**2**) were isolated from the fruiting bodies of *Lactarius mitissimus*. Their structures were elucidated using extensive spectroscopic techniques including 1D and 2D NMR spectra.

**Keywords** *Lactarius mitissimus*, highly oxidized humulane sesquiterpenes, mitissimols D and E

## Introduction

The Russulaceae is one of the largest family in the subdivision Basidiomycotina in Whittaker's Kingdom of Fungi and comprises hundreds of species [1]. In the great majority of *Lactarius* species, different kinds of sesquiterpenes play an important biological role, being responsible for the pungency and bitterness of the milky juice, the atmospheric change in color of the latex [2], and a chemical defense system against various predators such as bacteria, fungi, animals, insects [3]. The fungal subdivision Basidiomycotina produces many toxic sesquiterpenes derived from the protoilludane skeleton. This skeleton is transformed and rearranged to a multitude of compounds. Fungal sesquiterpenes formed *via* the humulane-protoilludane biosynthetic pathway are characteristic of the subdivision Basidiomycotina. The largest group of sesquiterpenes belonging to the classes of lactaranes, secolactaranes, marasmanes isolactaranes, norlactaranes, and caryophyllanes were believed to be biosynthesized from humulane [4]. Fungi of the genus *Lactarius* have been shown to be a good source of bioactive secondary metabolites [5~8]. Humulane sesquiterpenoids were reported that had diverse activity such as potent inhibitory activity against CYP3A4 [9], a potent inhibitor of tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus activation [10], inhibitory lipopolysaccharide-induced nitric oxide production in murine macrophage RAW 264.7 cells [11], antitumor activity [12]. However, there are rare humulane sesequiterpenes isolated from higher fungi. In the previous paper, we have reported five new humulane

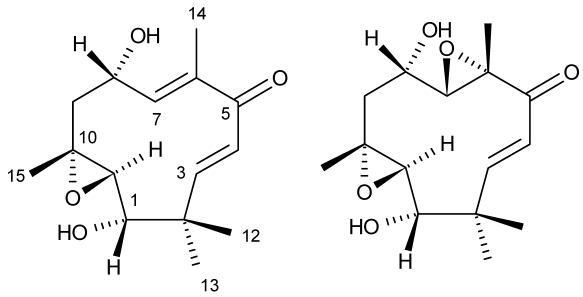


**Fig. 1** Structures of mitissimols D (**1**) and E (**2**).

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sesquiterpenoids from mushrooms of *Lactarius mitissimus* in Yunnan Province of China [13]. Further investigation of the mushroom material led to the isolation of two new highly oxidized humulane sesquiterpenoids. Here we report on the isolation and the structure elucidation of two new highly oxidized humulane sesquiterpenes (**1** and **2**, Fig. 1) from the fruiting bodies of *L. mitissimus*.

## Experimental

### General

Optical rotations were measured with a Horiba SEPA-300 polarimeter. IR spectra (KBr) were obtained with a Tensor 27. NMR spectra were recorded with Bruker AV-400 and Bruker DRX-500 spectrometers. FAB-MS and EI-MS were recorded with a VG Autospec-3000 spectrometer. HRESI-MS were recorded with an API QSTAR Pulsar 1 spectrometer. Silica gel (200～300 mesh, Qingdao Marine Chemical Inc., P. R. China) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for column chromatography. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% $H_2SO_4$ in ethanol.

### Fungus Material

The fresh fruiting bodies of *L. mitissimus* were collected at Ailao Mountain, Yunnan Province, China in July 2003 and identified by Prof. Mu Zang, Kunming Institute of Botany, Chinese Academy of Sciences (CAS). A voucher specimen was deposited in the Herbarium of Kunming Institute of Botany, CAS.

### Extraction and Isolation

The fresh fruiting bodies of *L. mitissimus* (1.6 kg) were extracted with 95% aq. EtOH (15 liters). The EtOH soln. was evaporated *in vacuo* to give the extract (101 g), which was suspended in water and extracted with EtOAc. The EtOAc extracts were evaporated under red. Press., giving 33.5 g of a residue which was subjected to column chromatography eluting with $CHCl_3$/MeOH from 100 : 0 (v/v) to 50 : 50 (v/v) to give 8 fractions. The fraction eluted by $CHCl_3$/MeOH (95 : 5, v/v) was further subjected to column chromatography eluting with petroleum ether/acetone from 10 : 1, 8 : 1, 6 : 1, 4 : 1, 2 : 1, 1 : 1, 1 : 5 (v/v) to give fractions 1, 2 and 3. Fraction 2 eluted with petroleum ether/acetone (2 : 1, v/v) was further purified by preparative-TLC and Sephadex LH-20 column chromatography which eluted by $CHCl_3$/MeOH (1 : 1, v/v) to afford **1** (3 mg) and **2** (4 mg), respectively.

### Physico-chemical Properties

Mitissimol D (**1**): white powder; m.p. 193～196°C (MeOH); $[\alpha]_D^{25}$+138.0 (*c* 0.51, MeOH); UV(MeOH) $\lambda_{max}$ (log $\varepsilon$) 230 (3.93); IR (KBr) $\nu_{max}$ 3338 (OH), 3039, 2961, 2928, 1665 (C=CCOC=C), 1388, 1359, 1126, 1031, 982, 909 $cm^{-1}$; $^1$H-NMR (500 MHz, $CD_3OD$) and $^{13}$C-NMR (125 MHz, $CD_3OD$), see Table 1; ESI-MS (pos) *m/z*

**Table 1** $^1$H and $^{13}$C -NMR data for **1** and **2**

| Position | **1** | | **2** | |
|---|---|---|---|---|
| 1 | 3.25 (d, 9.7) | 76.9 (d) | 3.39 (d, 9.8) | 76.1 (d) |
| 2 | | 41.9 (s) | | 41.8 (s) |
| 3 | 6.12 (d, 16.4) | 160.1 (d) | 6.08 (d, 17.2) | 158.9 (d) |
| 4 | 6.28 (d, 16.4) | 130.5 (d) | 6.80 (d, 17.2) | 128.1 (d) |
| 5 | | 205.3 (s) | | 200.4 (s) |
| 6 | | 142.8 (s) | | 67.0 (s) |
| 7 | 5.92 (br d, 9.9) | 146.8 (d) | 2.81 (d, 9.1) | 66.9 (d) |
| 8 | 4.53 (ddd, 12.1, 9.9, 5.3) | 65.2 (d) | 3.77 (d, 11.6, 9.1, 3.6) | 66.0 (d) |
| 9 | 2.51 (dd, 12.7, 5.3) | 48.4 (t) | 2.44 (d, 13.4, 3.6) | 46.7 (t) |
| | 1.27 (dd, 12.7, 12.1) | | 1.40 (dd, 13.4, 11.6) | |
| 10 | | 61.8 (s) | | 60.6 (s) |
| 11 | 2.79 (d, 9.7) | 66.7 (d) | 2.77 (d, 9.8) | 66.0 (d) |
| 12 | 1.22 (s) | 27.2 (q) | 1.20 (s) | 27.5 (q) |
| 13 | 1.11 (s) | 17.7 (q) | 1.18 (s) | 18.1 (q) |
| 14 | 1.95 (br s) | 12.5 (q) | 1.69 (s) | 16.7 (q) |
| 15 | 1.29 (s) | 18.0 (q) | 1.46 (s) | 18.9 (q) |

**1** and **2** were measured in $CD_3OD$, Coupling constants are given in Hz. Assignments made on the basis of $^1$H, $^1$H-COSY, HMQC and HMBC experiments.

$[M+Na]^+$ 289, $[2M+Na]^+$ 555; HRESI-MS (pos) *m/z* 289.1408 (calcd for $C_{15}H_{22}O_4Na$ 289.1415).

Mitissimol E (**2**): white powder; m.p. 80~82°C; $[\alpha]_D^{18}$−41.5 (*c* 0.5, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 233 (3.84); IR (KBr) $\nu_{max}$ 3442 (OH), 2967, 2929, 1687, 1637 (C=OC=C), 1467, 1389, 1365, 1055, 966, 916 $cm^{-1}$; $^1$H-NMR (400 MHz, $CD_3OD$) and $^{13}$C-NMR (100 MHz, $CD_3OD$), see Table 1; ESI-MS (pos) *m/z* $[M+Na]^+$ 305, $[2M+Na]^+$ 587; HRESI-MS (pos) *m/z* 305.1365 (calcd. for $C_{15}H_{22}O_5Na$ 305.1364).

## Results and Discussion

The EtOH extract prepared from the fresh fruiting bodies of *L. mitissimus* was partitioned between EtOAc and water. The EtOAc layer was subjected repeatedly to column chromatography on Sephadex LH-20 and silica gel to afford two new compounds mitissimol D (**1**) and E (**2**).

**1** was obtained as white powder. The molecular formula of **1** was determined to be $C_{15}H_{22}O_4$ on the basis of HR-ESI-MS $[M+Na]^+$ *m/z* 289.1408 (calcd. for $C_{15}H_{24}O_4Na$ 289.1415) and its $^{13}$C-NMR (DEPT) spectrum including signals for a carbonyl carbon (C=CCOC=C) ($\delta_C$ 205.3), one olefinic quaternary carbon ($\delta_C$ 142.8), two quaternary carbons ($\delta_C$ 41.9, 61.8), three olefinic methine carbons ($\delta_C$ 130.5, 146.8, 160.1), three methine carbons ($\delta_C$ 65.2, 66.7, 76.9), one methylene carbons ($\delta_C$ 48.4) and four methyl carbons ($\delta_C$ 12.5, 17.7, 18.0, 27.2). Its molecular formula indicated a sesquiterpene skeletone containing 5 degees of unsaturation. The structure was suggested to be sesquiterpene. Its IR spectrum also showed bands 3338 $cm^{-1}$ (OH), 1665 (COC=C) $cm^{-1}$. The $^1$H- and $^{13}$C-NMR spectra (Table 1) of **1** were similar to those of mitissimol B which suggested these compounds possess the same humulene skeleton [13]. The key difference was that $\delta_C$ for carbon 8 in the spectrum of **1** ($\delta_C$ 65.2) are shifted downfield compared to those of mitissimol B ($\delta_C$ 24.5). This characteristic difference was caused by a proton at position 8 of mitissimol B being displaced by an OH group in **1**. The location of the OH group was further determined to be at C-8 by the key HMBC (Fig. 2). The geometry of the 3, 4 double bond was determined as *E* from the proton coupling constant ($J_{3,4}$=16.4 Hz) displayed in its $^1$H-NMR. The relative stereo structure of **1** was further determined by the ROESY experiment (Fig. 3), which showed significant correlations between H-1 and H-3, H-12 and H-15; H-11 and H-7, H-9$\alpha$; H-8 and H-14, H-15; and H-4 and H-13. From these data, **1** was determined to be 10,11-epoxy-3*E*,6*E*-humuladien-1$\alpha$,8$\alpha$-diol-5-one, named mitissimol D.

**2** was obtained as a white powder, whose molecular formula was determined to be $C_{15}H_{22}O_5$ by the HR-ESI-MS $[M+Na]^+$ *m/z* 305.1365 (calcd. for $C_{15}H_{22}O_5Na$ 305.1364). The $^1$H- and $^{13}$C-NMR spectra of **2** (Table 1) were similar to those of **1**, which suggested that this compound possessed the same humulane skeleton. Comparision of the NMR spectra data suggested that the only difference between compound **2** and **1** was that an epoxide ring at C-6/C-7 of **2** was absent in **1**. According to HMQC, $^1$H-$^1$H COSY and HMBC (Fig. 2) experiments, the characteristic $^{13}$C NMR signals at $\delta_C$ 67.0 and $\delta_C$ 66.9 were ascribable to C-6 and C-7, respectively. The conformation of **2** was determined by the ROESY experiments (Fig. 3), which showed significant correlations between H-1 and H-3, H-12 and H-15; H-11 and H-9$\alpha$; H-7 and H-9$\alpha$; H-8 and H-14, H-15; and H-4 and H-13. The geometry of the 3, 4 double bond was further determined as *E* from the proton coupling constant ($J_{3,4}$=17.2 Hz) displayed in its $^1$H-NMR. Thus, **2** was determined to be 6,7:10,11-diepoxy-3*E*-humulen-1$\alpha$,8$\alpha$-diol-5-one, named mitissimol E (**2**).

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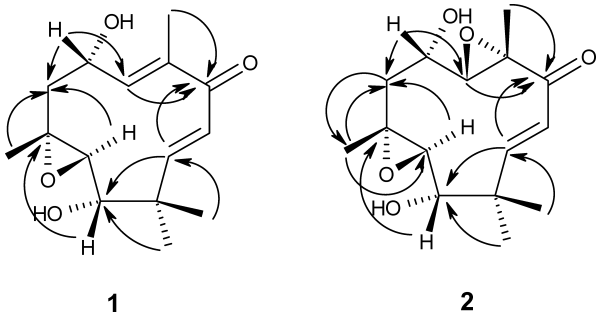


**Fig. 2** Key HMBC correlations of **1** and **2**.

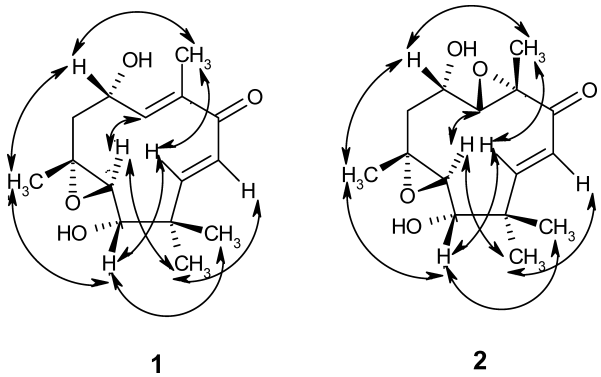


**Fig. 3** Key ROESY correlations of **1** and **2**.

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