Two New Highly Oxidized Humulane Sesquiterpenes from the Basidiomycete *Lactarius mitissimus*

by **Du-Qiang Luo***[a])[b]), **Yuan Gao**[b]), **Xiao-Long Yang**[b]), **Jian-Guo Tang**[b]), **Li-Yan Zhao**[a]), and **Ji-Kai Liu***[a])

[a]) College of Life Science, Hebei University, Baoding, 071002, China (e-mail: duqiangluo@163.com)
[b]) State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China

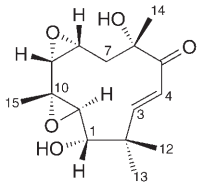
Two new highly oxidized humulane sesquiterpenes, mitissimols F (**1**) and G (**2**), were isolated from the fruiting bodies of *Lactarius mitissimus*. Their structures were elucidated by using extensive spectroscopic techniques including 1D- and 2D-NMR experiments. The absolute configuration of mitissimol F (**1**) was determined by ^{1}H-NMR resolution of its diastereoisomeric α-methoxy-α-(trifluoromethyl)benzeneacetates (MTPA). It was shown to be (1*S*,3*E*,6*S*,8*R*,9*R*,10*S*,11*R*)-8,9 : 10,11-diepoxy-1,6-dihydroxyhumul-3-en-5-one (= (1*S*,2*R*,4*R*,6*S*,8*E*,11*S*,12*R*)-6,11-dihydroxy-1,6,10,10-tetramethyl-3,13-dioxatricyclo[10.1.0.0^{2,4}]tridec-8-en-7-one).

Introduction. – The fungal Basidiomycotina produce 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 for 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 (= 1,1,4,8-tetramethylcycloundecane) [1]. Fungi of the genus *Lactarius* have been shown to be a good source of bioactive secondary metabolites. Many sesquiterpenes with the lactarane skeleton are formed by *Lactarius* species [2–5].

Humulane derivatives, metabolites from *Zingiber aromaticum* and *Z. jerumbet*, were reported that had diverse activities such as a potent inhibitory activity against CYP3A4 [6], a potent inhibitor of tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate-induced *Epstein–Barr* virus activation [7], inhibitory lipopolysaccharide-induced nitric oxide production in murine macrophage RAW 264.7 cells [8], antitumor activity [9], and inhibitory HIV activity [10].

However, there are rare highly oxidized humulane sesquiterpenes isolated from higher fungi. In the previous paper, we have reported five new humulane sesquiterpenoids from mushrooms of *Lactarius mitissimus* occurring in Yunnan Province of China [11]. Further investigations of the mushroom material led to the isolation of two new, highly oxidized humulane sesquiterpenoids; herein, we report their isolation and structure elucidation.

Results and Discussion. – The 95% aqueous EtOH extract prepared from the fresh fruiting bodies of *L. mitissimus* was partitioned between AcOEt and H_2O. The AcOEt layer was subjected repeatedly to column chromatography (*Sephadex LH-20* and silica gel) to afford two new compounds, mitissimols F (**1**) and G (**2**).



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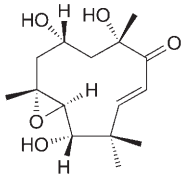

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Mitissimol F (**1**) was obtained as a white powder. The molecular formula was determined to be $C_{15}H_{22}O_5$ on the basis of HR-ESI-MS ($[M + Na]^+$ at m/z 305.1373).

The IR spectrum of **1** showed bands at 3441 (OH), 1676, and 1617 (C(=O)C=C) cm^{-1}. The ^{1}H- and ^{13}C-NMR spectra (*Table*) of **1** were similar to those of mitissimol B (=(1*R*,4*E*,7*E*,10*S*,11*S*)-10-hydroxy-1,5,9,9-tetramethyl-12-oxabicyclo[9.1.0]dodeca-4,7-dien-6-one), which suggested these compounds possess the same humulene skeleton [11]. The structure of **1** was established as 8,9:10,11-diepoxy-1,6-dihydroxyhumul-3-en-5-one[1]).

Table. *^{1}H- and ^{13}C -NMR Data* (400 MHz, CD_3OD) *of Mitissimols F* (**1**) *and G* (**2**)[1])

	1		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
H–C(1)	3.34 (*d*, *J* = 10.0)	72.9 (*d*)	3.62 (*d*, *J* = 9.5)	74.6 (*d*)
C(2)		43.2 (*s*)		42.1 (*s*)
H–C(3)	6.20 (*d*, *J* = 16.1)	150.7 (*d*)	6.77 (*d*, *J* = 16.2)	150.8 (*d*)
H–C(4)	7.11 (*d*, *J* = 16.1)	129.8 (*d*)	7.58 (*d*, *J* = 16.2)	127.2 (*d*)
C(5)		209.5 (*s*)		204.1 (*s*)
C(6)		79.4 (*s*)		77.6 (*s*)
CH_2(7)	2.52 (*dd*, *J* = 13.4, 9.2), 1.23 (*dd*, *J* = 13.4, 5.2)	44.8 (*t*)	2.67 (br. *d*, *J* = 14.6), 2.57 (*dd*, *J* = 14.6, 6.8)	51.6 (*t*)
H–C(8)	2.43 (*ddd*, *J* = 9.2, 5.2, 2.4)	52.4 (*d*)	3.80 (*dd*, *J* = 9.7, 6.8)	64.6 (*d*)
H–C(9) or CH_2(9)	2.68 (*d*, *J* = 2.4)	60.3 (*d*)	2.54 (br. *d*, *J* = 13.1), 1.80 (*dd*, *J* = 13.1, 9.7)	52.9 (*t*)
C(10)		61.8 (*s*)		60.7 (s)
H–C(11)	2.81 (*d*, *J* = 10.0)	62.3 (*d*)	3.25 (*d*, *J* = 9.5)	65.1 (*d*)
Me(12)	1.22 (*s*)	27.1 (*q*)	1.43 (*s*)	26.7 (*q*)
Me(13)	1.13 (*s*)	18.3 (*q*)	1.32 (*s*)	17.6 (*q*)
Me(14)	1.40 (*s*)	27.9 (*q*)	2.05 (*s*)	22.6 (*q*)
Me(15)	0.95 (*s*)	11.9 (*q*)	1.34 (*s*)	18.8 (*q*)

[1]) Arbitrary numbering; for systematic names, see the *Exper. Part.*

The ^{13}C-NMR spectrum of **1** showed signals for a carbonyl (COC=C; δ(C) 209.5) and three quaternary C-atoms (δ(C) 79.4, 61.8, and 43.2) and for two olefinic CH (δ(C) 150.7 and 129.8), four CH (δ(C) 72.9, 62.3, 60.3, and 52.4), one CH_2 (δ(C) 44.8), and four Me groups (δ(C) 27.9, 27.1, 18.3, and 11.9). Its molecular formula indicated a sesquiterpene skeletone containing 5 degrees of unsaturation. The structure was suggested to be a tricyclic sesquiterpene. The distinct differences between compound **1** and mitissimol B [11] were: *a*) the epoxide ring at C(8)–C(9)[1]) (δ(H) 2.43 (*ddd*, *J* = 9.2, 5.2, 2.4, H–C(8)), δ(C) 52.4 (*d*, C(8)); δ(H) 2.68 (*d*, *J* 2.4, H–C(9)), δ(C) 60.3 (*d*, C(9))) in **1** was absent in mitissimol B; *b*) the double bond at C(6)=C(7) (δ(C) 139.2 (*s*, C(6)); δ(H) 6.05 (br. *d*, *J* = 8.7, H–C(7)), δ(C) 147.0 (*d*, C(7))) of mitissimol B was replaced by an OH group (δ(C) 79.4 (*s*, C(6)); δ(H) 2.52 (*dd*, *J* = 13.4, 9.2, 1 H–C(7)), 1.23 (*dd*, *J* = 13.4, 9.2, 1 H–C(7)), δ(C) 44.8 (*t*, C(7))) at C(6) of **1**. The location of the epoxide ring and the OH group were further determined by the COSY, HMQC, and HMBC (*Fig. 1*). The geometry of the C(3)=C(4) bond was determined as (*E*) from the coupling constant *J*(3,4) = 16.1 Hz in the ^{1}H-NMR. The ROESY (*Fig. 2*) of **1** showed the following significant correlations: H–C(1)/H–C(3) and Me(12), H–C(4)/Me(13), Me(14)/H–C(3) and H–(8), and Me(15)/H–C(1), H–C(8) and H–(9).

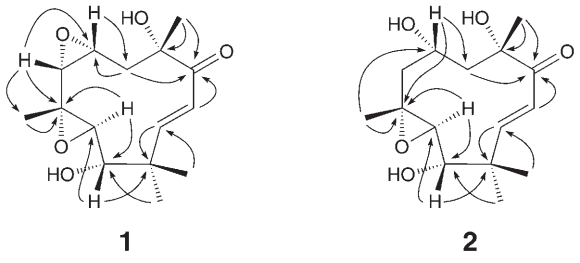

Fig. 1. *Key HMBC Correlations of Compounds* **1** *and* **2**

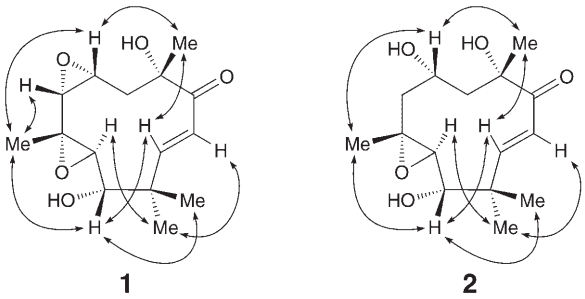

Fig. 2. *Key ROESY Correlations of Compounds* **1** *and* **2**

The absolute configuration at C(1) of **1** was determined by the advanced *Mosher*'s method [12]. In the ^{1}H-NMR spectra of the α-methoxy-α-(trifluoromethyl)benzeneacetates (MTPAs), H–C(3), H–C(4), CH_2(7), H–C(8), Me(12), Me(13), and Me(14) of (*R*)-MTPA **1a** appeared at higher field than those of (*S*)-MTPA **1b**, whereas H–C(9), H–C(11), and Me(15) of **1a** were at lower field than those of **1b** (*Fig. 3*). Thus, the configuration at C(1) should be (*S*). From these data, compound **1** was determined as (1*S*,3*E*,6*S*,8*R*,9*R*,10*S*,11*R*)-8,9 : 10,11-diepoxy-1,6-dihydroxyhumul-3-en-5-one[1]), named mitissimol F.

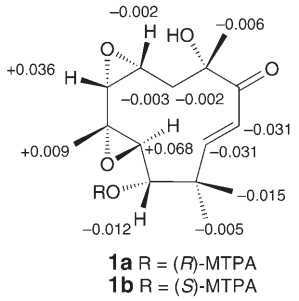

Fig. 3. *Chemical-shift differences* ($\Delta\delta(H) = \delta(S) - \delta(R)$) *obtained from the MTPA ester in* CD_3OD

Mitissimol G (**2**) was obtained as a white powder, whose molecular formula was determined to be $C_{15}H_{24}O_5$ on the basis of its HR-ESI-MS ($[M + Na]^+$ at m/z 307.1522). The ^{1}H- and ^{13}C-NMR were similar to those of **1**, which suggested that this compound possessed the same humulane skeleton. Comparison of the NMR spectra suggested that there was an epoxy ring in compound **1** in **2**. Based on further spectral data, compound **2** was determined as (1*S*,3*E*,6*S*,8*R*,10*R*,11*S*)-10,11-epoxy-1,6,8-trihydroxy-humul-3-en-5-one[1]), named mitissimol G.

C(9) gave rise to a *t* in the ^{13}C-NMR of **2**, and two proton signals, were present for CH_2(9) of **2** instead of the *d* (1 H) in **1**. Both compounds **1** and **2** showed a *d* for C(8), *i.e.*, only one proton was attached to it, and both compounds were oxygenated at C(8). The location of the additional OH group of **2** was confirmed to be C(8) by the COSY, HMQC, and HMBC data (*Fig. 1*). The geometry of the C(3)=C(4) bond was determined as (*E*) from the coupling constant $J(3,4) = 16.2$ Hz in the ^{1}H-NMR. The conformation of **2** was further determined by the ROESY experiments (*Fig. 2*) exhibiting the following significant correlations: H−C(1)/H−C(3), Me(12), and Me(15), H−C(11)/H_α−C(9), and Me(13), H(8)/Me(14), Me(15), and H_β−C(9), and H−C(4)/Me(13). The absolute configuration at C(1) could not be determined because of the unsufficient amount of isolated **2**, but it was assumed to be (*S*) because **1** and **2** were isolated from the same extract.

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Experimental Part

General. Column chromatography (CC): silica gel (200–300 mesh, *Qingdao Marine Chemical Inc.*, P. R. China) and *Sephadex LH-20* (*Amersham Biosciences*, Sweden); TLC monitoring, visualization by heating the silica gel plates sprayed with 10% H_2SO_4 in EtOH. M.p.: *XRC-1* micro-melting-point apparatus; uncorrected. Optical rotations: *Horiba-SEPA-300* polarimeter. UV Spectra: *Shimadzu 210-A* double-beam spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Nicolet Avatar-360* FT-IR spectrometer; KBr pellets; in cm^{-1}. NMR Spectra: *Bruker AV-400* and *DRX-500* spectrometers; δ in ppm with $SiMe_4$ as internal standard, *J* in Hz. MS: *VG Autospec-3000* and *API QSTAR-Pulsar-1* spectrometer.

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). The voucher specimen was deposited in the Herbarium of the Kunming Institute of Botany, CAS.

Extraction and Isolation. The fresh fruiting bodies of *L. mitissimus* (2.3 kg) were extracted with 95% aq. EtOH (18 l). The EtOH soln. was concentrated to give the extract (123 g), which was suspended in

H_2O and extracted with AcOEt. The AcOEt extract was concentrated and the residue (55 g) subjected to CC ($CHCl_3$/MeOH 100:0 → 50:50 (*v*/*v*)): 8 fractions. The fraction eluted with $CHCl_3$/MeOH 95:5 was further subjected to CC (petroleum ether/acetone 10:1, 8:1, 6:1, 4:1, 2:1, 1:1, and 1:5): *Frs. 1–3* (40 mg each). *Fr. 1*, eluted with petroleum ether/acetone 10:1, was further purifed by prep. TLC (petroleum ether/acetone 7:1) and CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1): **2** (4 mg; TLC (petroleum ether/acetone 2:1): R_f 0.5). *Fr. 2*, eluted with petroleum ether/acetone 6:1, was further purifed by prep. TLC ($CHCl_3$/MeOH 14:1) and CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1): **1** (17 mg; TLC (petroleum ether/AcOEt 1:1); R_f 0.7).

Mitissimol F (=*(1*S*,2*R*,4*R*,6*S*,8*E*,11*S*,12*R*)-6,11-Dihydroxy-1,6,10,10-tetramethyl-3,13-dioxatricyclo[10.1.0.0^{2,4}]tridec-8-en-7-one*; **1**): Colorless powder. M.p. 148–150° (MeOH). $[\alpha]_D^{25.1} = -37.7$ (c = 0.55, MeOH). UV (MeOH): 235 (3.48). IR (KBr): 3441, 2968, 2927, 1676, 1617, 1453, 1395, 1150, 1116, 1064, 994, 901. ^{1}H- and ^{13}C-NMR (CD_3OD): *Table*. ESI-MS (pos.): 305 ($[M+Na]^+$), 587 ($[2\,M+Na]^+$). HR-ESI-MS (pos.): 305.1373 ($C_{15}H_{22}O_5Na^+$; calc. 305.1364).

Mitissimol G (=*(1*R*,2*S*,4*E*,7*S*,9*R*,11*R*)-2,7,9-Trihydroxy-3,3,7,11-tetramethyl-12-oxabicyclo[9.1.0]dodec-4-en-6-one*; **2**): Colorless powder. M.p. 205–207° (MeOH). $[\alpha]_D^{24.2} = -2.4$ (c = 0.11, $CHCl_3$). UV ($CHCl_3$): 241 (3.63). IR (KBr): 3552, 3391, 2990, 2967, 2927, 1696, 1630, 1464, 1387, 1338, 1127, 1081, 1049, 999, 911. ^{1}H- and ^{13}C-NMR (CD_3OD): *Table*. ESI-MS (pos.): 307 ($[M+Na]^+$), 591 ($[2\,M+Na]^+$). HR-ESI-MS (pos.): 307.1522 ($C_{15}H_{24}NaO_5^+$; calc. 307.151).

MTPA Esterification of **1**. To a soln. of **1** (2.82 mg) in dry $CHCl_2$ (10 ml), (*S*)-MTPA (11.2 mg), *N,N′*-dicyclohexylcarbodiimide (DCC; 9.9 mg) and *N,N*-dimethylpyridin-4-amine (DMAP; 2 mg) were added, and the mixture was stirred overnight at r.t. The mixture was directly subjected to prep. TLC (petroleum ether/AcOEt 2:1) to give (*R*)-MTPA ester **1a** (3.1 mg). By the same procedure, (*S*)-MPTA ester **1b** (2.6 mg) was prepared.

*(*R*)-MTPA Ester* **1a**: Colorless amorphous solid. ^{1}H-NMR (CD_3OD)[1]): 7.180 (*d*, J = 16.3, H–C(4)); 6.231 (*d*, J = 16.1, H–C(3)); 5.087 (*d*, J = 10.0, H–C(1)); 2.947 (*d*, J = 10.0, H–C(11)); 2.696 (*d*, J = 2.4, H–C(9)); 2.540 (*dd*, J = 13.2, 4.9, H_α–C(7)); 1.224 (br. *d*, J = 9.7, 1 H, H_β–C(7)); 2.474 (br. *d*, J = 8.2, H–C(8)); 1.403 (*s*, Me(14)); 1.244 (*s*, Me(12)); 1.117 (*s*, Me(13)); 0.896 (*s*, Me(15)). FAB-MS: 499 ($[M+H]^+$).

*(*S*)-MTPA Ester* **1b**: Colorless amorphous solid. ^{1}H-NMR (CD_3OD)[1]): 7.149 (*d*, J = 16.2, 1 H–C(4)); 6.197 (*d*, J = 16.2, H–C(3)); 5.075 (*d*, J = 10.1, H–C(1)); 3.015 (*d*, J = 10.1, H–C(11)); 2.732 (br. *d*, J = 2.4, H–C(9)); 2.538 (*dd*, J = 13.3, 5.0, H_α–C(7)); 1.221 (br. *d*, J = 9.2, 1 H, H_β–C(7)); 2.472 (br. *d*, J = 8.9, H–C(8)); 1.397 (*s*, Me(14)); 1.239 (*s*, Me(12)); 1.112 (*s*, Me(13)); 0.905 (*s*, Me(15)); FAB-MS: 499 ($[M+H]^+$).

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