Mitissimolone, a New Sesquiterpene with a Novel Carbon Skeleton from the Basidiomycete *Lactarius mitissimus*

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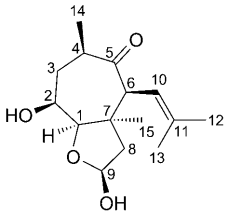
Mitissimolone, a new regular sesquiterpene possessing a novel carbon skeleton, was isolated from the EtOH extract of fruiting bodies of *Lactarius mitissimus*. Its structure was determined by extensive spectroscopic analyses including 1D- and 2D-NMR spectra. Mitissimolone inhibited moderately the growth of HeLa cells lines, with an IC_{50} value of 29.8 μg/ml.

Introduction. – The Russulacea family is one of the largest in the subdivision Basidiomycotina in *Whittaker*'s kingdom of Fungi and comprises hundreds of species [1]. Mushrooms that belong to the genus Lactarius (family Russulaceae, Basidiomycotina) contain a milky juice, which can be observed when the fruiting bodies are injured. Sesquiterpenes play an important role in the great majority of *Lactarius* species, being responsible for the pungency and bitterness of the milky juice and the change in color of the latex on exposure to air [2] and constituting a chemical defense system against various predators such as bacteria, fungi, animals, and insects [3][4]. The largest group of sesquiterpenes belonging to the classes of lactaranes, secolactaranes, marasmanes, isolactaranes, norlactaranes, and caryophyllanes were assumed to be biosynthesized from humulane [5]. 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 the *Lactarius* species [6–9].

In previous publications, we reported nine new humulane sesquiterpenoids from mushrooms of *Lactarius mitissimus* in the Yunnan Province of the P. R. China [10–12]. Continuing a program seeking novel naturally occurring bioactive metabolites from higher fungi, we have carried out a further investigation on the fruiting bodies of *L. mitissimus*, which led to the isolation of a sesquiterpene possessing a novel C-skeleton. Here, we describe the isolation and structure elucidation of an unusual sesquiterpene, named mitissimolone (**1**), from the fruiting bodies of *L. mitissimus*.

Results and Discussion. – The 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 on *Sephadex LH-20* and silica gel to afford **1**.

Compound **1** was obtained as a white powder. The molecular formula was determined to be $C_{15}H_{24}O_4$ on the basis of HR-ESI-MS peak at m/z 269.1643 ($[M+1]^+$,



$C_{15}H_{25}O_4^+$: calc. 269.1647). The ^{13}C-NMR spectrum (*Table*) including signals for a CO group (δ(C) 215.4), two quaternary C-atoms (δ(C) 135.3, 47.5), one olefin CH group (δ(C) 120.6), five additional CH groups (δ(C) 97.9, 89.8, 70.5, 60.5, 41.9), two CH_2 groups (δ(C) 51.0, 41.4) and four Me groups (δ(C) 26.2, 18.5, 17.8, 17.0). The IR spectrum also showed bands at 3440 cm^{-1} (OH) and 1709 cm^{-1} (C=O). Cross-peaks between H–C(1) and H–C(2), H–C(3) and H–C(2)/H–C(4), H–C(4) and Me(14), H–C(6) and H–C(10), H–C(8) and H–C(9) were observed in the ^{1}H,^{1}H-COSY spectrum (*Fig. 1*). We also observed a vinylic H-atom at δ(H) 5.38 (*d*, $J = 9.8$) having long-range correlations with the two Me groups at δ(H) 1.61 and 1.71. It allowed establishment of three H-atom systems, one at C(1) through C(2) and C(3) and C(44), others at C(6) through C(10), and C(8) through C(9), respectively. Since two out of four unsaturation equivalents were accounted for by the ^{13}C-NMR data, **1** was inferred to have two rings (ring *A* as a five-membered and ring *B* as a seven-membered ring). In addition, ring *A* was further determined as a tetrahydrofuran by the HMBC correlations of H–C(8) and C(1), C(6), C(7), C(9) and C(15); H–C(9) and C(1) and C(8); H–C(1) and C(2), C(6), C(15). Ring *B* as a seven-membered ring was confirmed by the HMBC from H–C(1) to C(2), C(6), C(15); H–C(2) to C(1), C(4); H–C(3) to C(2), C(4) and C(5); H–C(4) and C(2), C(3), C(5) and C(14); H–C(6)

Table. *NMR Spectral Data for Mitissimolone.* In CD_3OD, *J* in Hz. Assignments made on the basis of ^{1}H,^{1}H-COSY, HMQC, and HMBC experiments.

	^{13}C	^{1}H	^{1}H,^{1}H-COSY	HMBC
1	89.8 (*d*)	4.37 (*d*, $J = 8.2$)	H–C(2)	C(2), C(6), C(15)
2	70.5 (*d*)	3.75 (*t*, $J = 6.4$)	H–C(1), CH_2(3)	C(1), C(4)
3	41.4 (*t*)	1.97 (*d*, $J = 2.67$), 1.70 (br. *s*)	H–C(2), H–C(4)	C(2), C(4), C(5)
4	41.9 (*d*)	3.01–3.05 (*m*)	CH_2(3), Me(14)	C(2), C(3), C(5), C(14)
5	215.4 (*s*)			
6	60.5 (*d*)	3.55 (*d*, $J = 10.0$)	H–C(10)	C(1), C(5), C(7), C(11), C(15)
7	47.5 (*s*)			
8	51.0 (*t*)	2.02 (*d*, $J = 1.26$), 1.70 (br. *s*)	H–C(9)	C(1), C(6), C(7), C(9), C(15)
9	97.9 (*d*)	5.43 (*t*, $J = 5.6$)	CH_2(8)	C(1), C(8)
10	120.6 (*d*)	5.38 (*d*, $J = 9.8$)	H–C(6), Me(12), Me(13)	C(12), C(13)
11	135.3 (*s*)			
12	18.5 (*q*)	1.61 (br. *s*)	H–C(10)	C(10), C(11), C(13)
13	26.2 (*q*)	1.71 (overlapped)	H–C(10)	C(10), C(11), C(12)
14	17.8 (*q*)	0.99 (br. *d*, $J = 6.7$)	H–C(4)	C(4), C(5)
15	17.0 (*q*)	0.82 (*s*)		C(1), C(6), C(7), C(8)

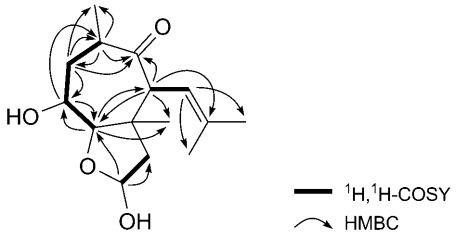


Fig. 1. *Key correlations of ^{1}H,^{1}H-COSY and HMBC of compound* **1**

and C(1), C(5), C(7), C(15) (*Fig. 1*). The location of two OH groups were further determined to be at C(9) and C(2), respectively, based on (δ(H) 5.43 (*t*, $J = 9.8$, H–C(9)), δ(C) 97.9 (*d*, C(9)) and δ(H) 3.75 (*t*, $J = 6.4$, H–C(2)), δ(C) 70.5 (*d*, C(2))) assigned by the COSY, HMQC, and HMBC spectra. The presence of a 2-methylprop-2-ene moiety was suggested by the HMBC from H–C(6) to C(11); from Me(12) to C(10), C(11) and C(13); and from Me(13) to C(10), C(11), and C(12). This group is located at C(6) (δ(H) 3.55 (*d*, $J = 10.0$, H–C(6)), δ(C) 60.5 (*d*, C(6))), which was established by the HMBC experiment (*Table*). The relative configuration was determined on the basis of NOEs observed in a difference NOE experiment (*Fig. 2*). The key correlations are found between H–C(1) and H–C(4), H–C(6) and H$_\alpha$–C(8); between H–C(2) and H–C(6), H$_\alpha$–C(3), and Me(15); between H–C(4) and H–C(1), H–C(6); between H–C(6) and H–C(1) and H–C(2) and H–C(4); between H–C(9) and Me(15); between Me(15) and H–C(2) and H–C(10). From these data, compound **1** was determined to be rel-(2*R*,3a*S*,4*R*,6*S*,8*R*,8a*S*)-2,8-dihydroxy-3a,6-dimethyl-4-(2-methylprop-1-en-1-yl)octahydro-5*H*-cyclohepta[*b*]furan-5-one, named mitissimolone.

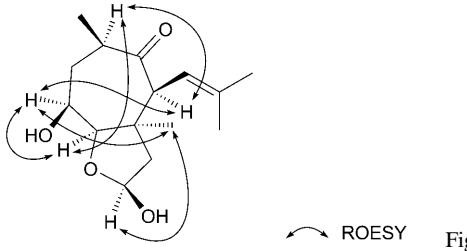


Fig. 2. *Key correlations of ROESY of compound* **1**

Mitissimolone also exhibited cytotoxic activity against HeLa cells with an IC_{50} value of 23.0 μg/ml.

Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh, *Qingdao Marine Chemical Inc.*, P. R. China) and *Sephadex LH-20* (*Amersham Biosciences*, Sweden). Fractions were monitored by TLC and spots were visualized by heating SiO_2 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. IR Spectra: *Nicolet AVATR360FT-IR* spectrometer, with KBr pellets. NMR Spectra: *Bruker DRX-500* spectrometers with TMS as internal standard, MS spectra: *VG Autospec-3000* spectrometer and *API QSTAR Pulsar 1* spectrometer.

Fungus Material. The fresh fruiting bodies of *L. mitissimus* were collected at Ailao Mountain, Yunnan Province, P. R. China in July 2006 and identified by Prof. *M. Zang*, Kunming Institute of Botany, Chinese Academy of Sciences (CAS). The voucher specimen was deposited with the Herbarium of Kunming Institute of Botany, CAS.

Extraction and Isolation. The fresh fruiting bodies of *L. mitissimus* (3.0 kg) were extracted with 95% aq. EtOH (20 l). The EtOH soln. was evaporated *in vacuo* to give the extract (140 g), which was suspended in H_2O and extracted with AcOEt. The AcOEt extracts were evaporated under reduced pressure, giving 55 g of a residue which was subjected to CC eluting with $CHCl_3$/MeOH from 100 : 0 (*v*/*v*) to 50 : 50 (*v*/*v*) to give eight fractions. The fraction eluted by $CHCl_3$/MeOH 95 : 5 (*v*/*v*) was further subjected to CC eluting with $CHCl_3$/MeOH 10 : 1, 8 : 1, 6 : 1, 4 : 1, 2 : 1, 1 : 1, 1 : 5 (*v*/*v*) to give 40 mg as *Frs. 1–4*. *Fr. 2*, eluted with petroleum ether (PE)/acetone 4 : 1 (*v*/*v*) was further purifed by prep. TLC (PE/acetone 5 : 1 (*v*/*v*)), and *Sephadex LH-20* CC, eluted with $CHCl_3$/MeOH 1 : 1 (*v*/*v*) to afford compound **1** (7 mg).

Mitissimolone (= rel-*(2*R*,3a*S*,4*R*,6*S*,8*R*,8a*S*)-2,8-Dihydroxy-3a,6-dimethyl-4-(2-methylprop-1-en-1-yl)octahydro-5*H*-cyclohepta[*b*]furan-5-one*; **1**). White powder. M.p. 63–65° (MeOH). $[\alpha]_D^{19.5} = +3.08$ ($c = 0.06$, MeOH). UV (MeOH): 202 (3.54). IR (KBr): 3440, 2932, 1709, 1456, 1015, 975, 911. ^{1}H- and ^{13}C-NMR (CD_3OD): *Table*. FAB-MS (pos.): 361 ($[M + Gly]^+$), 453 ($[M + 2\,Gly]^+$). HR-ESI-MS (pos.): 269.1643 ($C_{15}H_{25}O_4^+$; calc. 269.1647).

HeLa Cell Lines and Culture. HeLa lines were grown in RPMI-1640 (*GIBCO*) supplemented with 10% heat-inactivated bovine serum, 2 nM L-glutamine, 10^5 IU/liter penicillin, 100 mg/liter streptomycin and 10 mM HEPES, pH 7.4. Cells were kept at 37° in a humidified 5% CO_2 incubator.

Cell-Growth Inhibition Assay. Growth inhibition of compound **1** on tumor cells was measured by the microculture tetrazolium (MTT) as previously reported by our group [13].

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