

Rufuslactone, a New Antifungal Sesquiterpene from the Fruiting Bodies of the Basidiomycete *Lactarius rufus*

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Abstract A new lactarane sesquiterpene, rufuslactone (**1**), was isolated from the fruiting bodies of the basidiomycete *Lactarius rufus*. Rufuslactone (**1**) is an isomer of a previously described lactarane 3,8-oxa-13-hydroxylactar-6-en-5-oic acid γ -lactone (**2**) from *Lactarius necatar*. Its structure was elucidated on the basis of spectroscopic data. Rufuslactone (**1**) showed antifungal properties against plant pathogenic fungi.

Keywords *Lactarius rufus*, basidiomycete, lactarane sesquiterpene, rufuslactone, antifungal agent

Introduction

Large fungi of the genus *Lactarius* belong to subdivision Basidiomycotina, order Agaricales, family Russulaceae in Whittaker's kingdom of fungi [1]. They are important symbionts, forming mycorrhiza with higher plants which explains in some cases their preference for growing among certain kinds of trees. The name *Lactarius* has its origin in the fact that when the fruiting bodies are damaged, they exude a milky cellular juice, namely, lactate. This feature easily allows one to distinguish a *Lactarius* species from a congener *Russula* species or other similar mushrooms.

The fungal subdivision Basidiomycotina produces many toxic sesquiterpenes derived from the protoilludane skeleton. Rearrangements and transformation of this skeleton result in a multitude of compounds. Fungal sesquiterpenes formed *via* the humulane-protoilludane biosynthetic pathway are characteristic for the subdivision

Basidiomycotina [2]. Fungi of the genus *Lactarius* have been shown to be a good source of bioactive secondary metabolites. Uvidin A, a new fatty acid ester of a drimane sesquiterpene from *L. uvidus* showed insect antifeedant and cytotoxic activities [3]. The esterification of various sesquiterpenoid alcohols of *Lactarius* origin with *N*-benzoyl-[2*R*,3*S*]-phenylisoserine (side chain of Taxol) produced compounds whose antifeedant properties against storage pests *Tribolium confusum*, *Trogoderma granarium* and *Sitophilus granarius* were demonstrated [4]. Daniewski *et al.* also reported that 3,8-oxa-13-hydroxylactar-6-en-5-oic-acid γ -lactone was a good deterrent against insects [5]. Antiviral activities *in vitro* were reported for *N*-benzoylphenylisoserinates of *Lactarius* sesquiterpenoid alcohols [6]. 2-Geranylgeranyl-1,4-dihydroxybenzene isolated from the fruiting bodies of *Lactarius lignyotus* was highly active in the brine shrimp test. It showed significant inhibitory activity on DNA, RNA, and protein synthesis in HeLa and HL-60 cell lines [7]. Recently, a green pigment blennione and a red pigment lilacinone were isolated from *Lactarius blennius* and *Lactarius lilacinus*, respectively [8, 9].

During continuing research on bioactive metabolites of *Lactarius* and *Russula* sp. in Yunnan Province of China [10~14], the chemical constituents of the fruiting bodies of *Lactarius rufus* were investigated. This report deals with the isolation and structure elucidation of a new lactarane sesquiterpene, rufuslactone (**1**) and its antifungal activities against phytopathogenic fungi.

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Experimental

General

Optical rotations were measured on a Horiba SEPA-300 polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrophotometer. IR spectra were obtained with a Tensor 27 with KBr pellets. NMR spectra were recorded on Bruker AV-400 and Bruker DRX-500 spectrometers in CDCl_3 with TMS as an internal standard. 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., 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.

Material

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

Extraction and Isolation

The fresh fruiting bodies of *L. rufus* (7 kg) were extracted with 95% aq. EtOH (30 liters). The EtOH soln. was evaporated *in vacuo* to give the extract (512 g). The EtOH extract was extracted with CHCl_3 , and $\text{CHCl}_3/\text{MeOH}$ (1 : 1, v/v) three times, respectively, at room temperature. The combined CHCl_3 and $\text{CHCl}_3/\text{MeOH}$ (1 : 1, v/v) extracts were concentrated *in vacuo* to yield crude residues weighing 84 g and 30 g respectively. The CHCl_3 extract was subjected to column chromatography eluting with $\text{CHCl}_3/\text{MeOH}$ from 100 : 0 (v/v) to 50 : 50 (v/v) to give 10 fractions. The fraction eluted by $\text{CHCl}_3/\text{MeOH}$ (98 : 2, v/v) was concentrated to give a solid (150 mg) which was further purified by Sephadex LH-20 column chromatography, eluting with $\text{CHCl}_3/\text{MeOH}$ (1 : 1, v/v) to afford compound **1** (20 mg).

Physico-chemical Properties

Rufuslactone (**1**): colorless crystals, m.p. 154~156°C (CHCl_3). $[\alpha]_D^{22.6} -5.87$ (*c* 0.24, CHCl_3). UV λ_{max} (CHCl_3) 239 nm. IR (KBr) 3417, 3331, 2965, 2936, 2907, 1732, 1684, 1464, 1382, 1365, 1347, 1256, 1233, 1142, 1113, 1032 cm^{-1} . EI-MS *m/z* 248 (M, 100), 233 (M-Me, 37),

230 (M-H₂O, 17), 219 (8), 215 (11), 206 (33), 204 (51), 191 (27), 187 (20), 175 (14), 170 (47), 161 (25), 159 (28), 152 (64), 122 (93). HRESI-MS *m/z* 249.1487 [(M+1)⁺, calcd. for $\text{C}_{15}\text{H}_{21}\text{O}_3$ 249.1490].

Mycelial Growth Inhibition Test

Rufuslactone dissolved in DMSO was tested for antifungal activity *in vitro* by a Poison Food Technique. Potato dextrose agar (PDA) medium was used as the medium for all test fungi. The test pathogenic fungi were *Alternaria brassicae* Cav, *Botrytis cinerea* Pers. ex Tris., *Fusarium graminearum* Schw and *Alternaria alternata* (Fries) Keissler.

The medium incorporating test compound **1** at concentration of 100 $\mu\text{g}/\text{ml}$ (DMSO concentration 1%) was inoculated at the centre with agar discs of the test fungi (4 mm diameter). Three replicate plates for each fungus were incubated at 26 (± 2)°C. Control plates containing media mixed with DMSO (DMSO concentration 1%) were included. After incubation for 2~6 days until the fungal growth in the control dishes was almost complete, the mycelial growth of fungi (mm) in both treated (*T*) and control (*C*) Petri dishes was measured diametrically in three different directions. The percentage of growth inhibition (*I*) was calculated using the formula:

$$I(\%) = [(C - T)/C] \times 100$$

The corrected inhibition (IC) was then calculated as follow:

$$IC = [(I - CF)/(100 - CF)] \times 100$$

Where $CF = [(90 - C_0)/C_0] \times 100$; 90 is the diameter (mm) of the Petri dish, and the C_0 is the growth (mm) of the fungus in the control.

Analysis of variance was performed on the data with the PROCGLM procedure (SAS Institute, Cary, NC, USA). If $P > F$ was less than 0.01, means were separated with the least significant different (LSD) test at the $P = 0.05$ level.

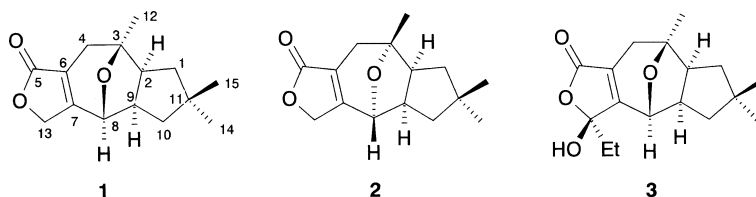
Results and Discussion

Compound **1** was obtained as colorless crystals. The molecular formula of **1** was determined to be $\text{C}_{15}\text{H}_{20}\text{O}_3$ on the basis of HRESI-MS $[\text{M}+1]^+$ *m/z* 249.1487 (calcd. for $\text{C}_{15}\text{H}_{21}\text{O}_3$ 249.1490) and its ¹³C-NMR (DEPT) spectrum including signals for a carbonyl carbon (δ 175.8), two quaternary carbons (δ 74.7, 36.8), two olefinic carbons (δ 160.1, 123.4), three methine carbons (δ 67.1, 49.1, 46.0), four methylene carbons (δ 71.7, 45.3, 45.1, 34.7) and three methyl carbons (δ 31.0, 29.1, 26.4). Its IR spectrum showed bands 3417 cm^{-1} (OH) and 1732 cm^{-1} , typical for a

Table 1 The ^1H - and ^{13}C -NMR data for **1**, **2** and **3**

No.	1		2 ⁵⁾		3 ¹⁵⁾	
1	1.60 (dd, 11.6, 8.8)	45.1 (t)	1.40 (dd, 12.0, 8.0)	31.2 (t)	1.60 (m)	45.9 (t)
	1.09 (t, 11.8)		0.90 (m)		1.23 (m)	
2	2.62 (m)	49.1 (d)	2.82 (td, 11.6, 11.6, 8.0)	54.1 (d)	2.65 (m)	50.0 (d)
3	—	74.7 (s)	—	80.7 (s)	—	74.9 (s)
4	2.59 (brd, 18.7)	34.7 (t)	2.44 (d, 18.0)	42.0 (t)	2.61 (d, 19.2)	35.2 (t)
	2.48 (brd, 18.7)		2.34 (d, 18.0)		2.46 (d, 19.2)	
5	—	175.8 (s)	—	172.6 (s)	—	172.5 (s)
6	—	123.4 (s)	—	124.8 (s)	—	126.3 (s)
7	—	160.1 (s)	—	165.6 (s)	—	159.6 (s)
8	4.04 (d, 3.3)	67.1 (d)	4.61 (d, 7.1)	74.6 (d)	4.21 (d, 3.2)	66.1 (d)
9	2.82 (m)	46.0 (d)	3.33 (m)	55.9 (d)	2.86 (m)	47.8 (d)
10	1.46 (dd, 11.5, 6.3)	45.3 (t)	1.44 (m)	40.0 (t)	1.39 (m)	45.4 (t)
	0.97 (overlapped)		0.90 (m)		1.23 (m)	
11	—	36.8 (s)	—	47.0 (s)	—	37.3 (s)
12	1.22 (s)	31.0 (q)	1.48 (s)	29.1 (q)	1.24 (s)	34.1 (q)
13	4.88 (brd, 17.4)	71.7 (t)	4.83 (d, 17.6)	71.5 (t)	—	108.1 (s)
	4.53 (brd, 17.4)		4.74 (d, 17.6)			
14	0.95 (s)	26.4 (q)	1.00 (s)	27.0 (q)	0.99 (s)	29.3 (q)
15	0.98 (s)	29.1 (q)	0.98 (s)	27.2 (q)	0.97 (s)	26.8 (q)
16	—	—	—	—	1.98 (m)	30.3 (t)
17	—	—	—	—	0.82 (t, 7.3)	7.9 (q)

1 and **2** were measured in CDCl_3 , **3** in acetone- d_6 . Coupling constants are given in Hz.

**Fig. 1** Structures of **1**, **2** and **3**.

lactone. The ^1H - and ^{13}C -NMR spectra (Table 1) of **1** were similar to those of **2** and **3** [5, 15], which suggested these compounds possess the same lactarane skeleton. The key differences were that δ_{C} for carbons 3 and 8 in the spectrum of **2** (80.7 and 74.6 ppm) are shifted downfield compared to those of **1** (74.7 and 67.1 ppm, respectively) [5]. This characteristic difference was caused by different configurations of the internal ether between C-3 and C-8 in **1** and **2**. **1** is an isomer of a previously described lactarane 3,8-oxa-13-hydroxylactar-6-en-5-oic acid γ -lactone (**2**) from *Lactarius necatar*. The distinct differences between **1** and **3** are that: the hydroxy and the ethyl groups at C-13 of **3** [δ_{H} 1.98 (2H, m, $-\text{CH}_2-$, H-16), 0.82 (3H, t, $-\text{CH}_3$, H-17); δ_{C} 30.3 (t, C-16), 7.9 (q, C-17), 108.1 (s, C-13)] are absent in **1** [δ_{H} 4.88 (brd, $J=17.4$ Hz, H-13a), 4.53 (brd,

$J=17.4$ Hz, H-13b); δ_{C} 71.7 (t, C-13)] [15]. The HMBC spectra (Fig. 2) of **1** demonstrated the following key correlations: H-8 and C-2, C-6, C-13; H-12 and C-2, C-4; H-9 and C-7; H-13 and C-6, which were well consisted with the lactarane skeleton. From a Dreiding model, it was evident that the formation of an internal ether between C-3 and C-8 required the bridge-head protons at C-2 and C-9 to be *cis*. The proposed biosynthesis [16] of the lactarane skeleton indicated the protons at C-2 and C-9 to be α configuration. The small value of the coupling constant ($J=3.3$ Hz) demonstrated a *syn* quasiequatorial orientation of H-8 and H-9 [17], as judged from the Karplus curves. The NOE spectra (Fig. 2) of **1** showed significant correlations between H-8 and H-9, H-2 and H-12, which further confirmed these protons were to be *syn*. Thus, H-8,

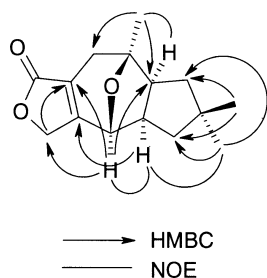


Fig. 2 Key correlations of HMBC and NOE.

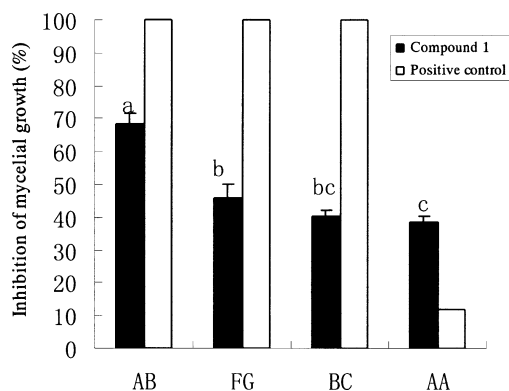


Fig. 3 Antifungal activity spectra of compound **1** and positive control (carbendazim) at 100 $\mu\text{g/ml}$ against four phytopathogenic fungi. AB: *Alternaria brassicae*; FG: *Fusarium graminearum*; BC: *Botrytis cinerea*; AA: *Alternaria alternata*. Error bars represent the standard error of the mean of three replicates. Means followed by the same letter are not significantly different ($P=0.05$) according to the least significantly difference test.

H-9, and H-2 were all *syn* to each other but *anti* to the internal ether. In light of the evidences mentioned above, the structure of **1** was therefore elucidated as shown in Fig. 1 and named rufuslactone.

Compound **1** was found to inhibit the mycelial growth of some plant pathogenic fungi *in vitro* (Fig. 3). *Alternaria brassicae* was the most sensitive to compound **1**, and its mycelial growth inhibition was 68.3 at 100 $\mu\text{g/ml}$. To evaluate the fungicidal activity of compound **1**, this compound and the commercial fungicide carbendazim were compared under the same assay conditions at 100 $\mu\text{g/ml}$. We found that carbendazim was more effective at inhibiting the mycelial growth of test fungi except for *A. alternata*. The growth of *A. alternata* was almost unaffected by carbendazim, while 100 $\mu\text{g/ml}$ of compound **1** inhibited the growth by 38.9%.

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