Two New Azulene Pigments from the Fruiting Bodies of the Basidiomycete *Lactarius hatsudake*

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Two new azulene pigments, 7-(1-hydroxy-1-methylethyl)-4-methylazulene-1-carbaldehyde (**1**) and 4-methyl-7-(1-methylethyl)azulene-1-carboxylic acid (**2**), were isolated from the fruiting bodies of the basidiomycete *Lactarius hatsudake*, together with one known azulene pigment (**3**). Their structures were determined by spectroscopic means (including 2D-NMR) and by HR-TOF-MS experiments.

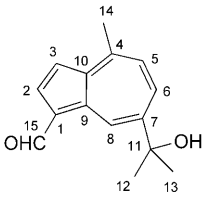
Introduction. – The mushrooms belonging to the genus *Lactarius* (family Russulaceae, Basidiomycotina) form a milky juice when their fruiting bodies are injured. In the great majority of *Lactarius* species, different kinds of sesquiterpenes play important biological roles, being responsible for the pungency and bitterness of the milky juice, and for changes in latex color when exposed to air.

Lactarius hatsudake is an edible, slightly bitter mushroom widely distributed in China, Japan, Europe, and North America. Similar to *L. deliciosus*, the latex of the fruiting bodies is at the beginning orange-colored, but then slowly darkens (within minutes), and eventually turns blue-green when the fruiting bodies are cut or broken [1]. These colors in *L. deliciosus* have previously been shown to be due to guaiane sesquiterpenes. Lactaroviolin [2], the free dihydroazulene alcohol and its stearic acid ester [3], lactarazulene [4], and lactarofulvene [5] have been isolated from specimens of *L. deliciosus* in different areas of the world.

During our search of bioactive metabolites of *Lactarius* and *Russula* species from Yunnan Province, China [6–10], we recently isolated two new azulene pigments from the fruiting bodies of *L. deliciosus* [11]. Herein, we report the isolation and structure elucidation of two the new azulene pigments **1** and **2** from the fruiting bodies of *L. hatsudake*.

Results and Discussion. – The fresh fruiting bodies of *L. hatsudake* (4.65 kg) were extracted with acetone, and the combined extracts were evaporated to give a deep-brown syrup, which was partitioned between H_2O and AcOEt. The organic layer was concentrated *in vacuo* to afford a residue (40 g), which was subjected to repeated column chromatography to afford compounds **1**–**3**.

Compound **1** was obtained as a red-purple solid. Its molecular formula was determined as $C_{15}H_{16}O_2$ on the basis of HR-TOF-MS data ($[M+H]^+$ at m/z 229.1229;



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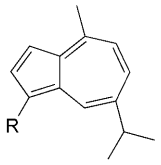


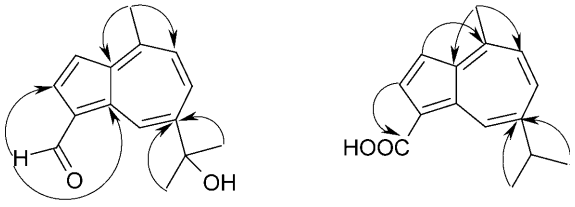
2 R = COOH
3 R = CHO

Table. *^{1}H- and ^{13}C-NMR Data for* **1**–**3**. At 500 and 125 MHz, resp., in $CDCl_3$; δ in ppm, J in Hz.

Position	**1**		**2**		**3**	
	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)
1	126.2 (*s*)	–	115.7 (*s*)	–	125.4 (*s*)	–
2	141.6 (*d*)	8.16 (*d*, J=4.2)	140.1 (*d*)	8.40 (*d*, J=4.2)	141.4 (*d*)	8.11 (*d*, J=4.2)
3	116.0 (*d*)	7.30 (*d*, J=4.2)	114.2 (*d*)	7.23 (*d*, J=4.2)	115.4 (*d*)	7.19 (*d*, J=4.2)
4	148.8 (*s*)		146.8 (*s*)	–	147.7 (*s*)	–
5	130.9 (*d*)	7.57 (*d*, J=10.8)	130.1 (*d*)	7.42 (*d*, J=10.6)	131.2 (*d*)	7.43 (*d*, J=10.6)
6	135.4 (*d*)	8.19 (*dd*, J=10.8, 1.8)	136.7 (*d*)	7.67 (*dd*, J=10.6, 1.4)	137.3 (*d*)	7.66 (*dd*, J=10.6, 1.8)
7	149.4 (*s*)	–	147.7 (*s*)	–	149.5 (*s*)	
8	135.3 (*d*)	9.93 (*d*, J=1.8)	137.9 (*d*)	9.82 (*d*, J=1.4)	137.5 (*d*)	9.72 (*d*, J=1.8)
9	139.1 (*s*)	–	140.7 (*s*)	–	139.5 (*s*)	–
10	144.1 (*s*)	–	143.0 (*s*)	–	144.1 (*s*)	–
11	74.2 (*s*)	–	38.7 (*d*)	3.22 (*sept.*, J=6.9)	38.4 (*d*)	3.19 (*sept.*, J=6.9)
12	32.5 (*q*)	1.77 (*s*)	24.7 (*q*)	1.39 (*d*, J=6.9)	24.4 (*q*)	1.38 (*d*, J=6.9)
13	32.5 (*q*)	1.77 (*s*)	24.7 (*q*)	1.39 (*d*, J=6.9)	24.4 (*q*)	1.38 (*d*, J=6.9)
14	24.7 (*q*)	2.92 (*s*)	24.8 (*q*)	2.92 (*s*)	24.6 (*q*)	2.86 (*s*)
15	186.8 (*d*)	10.28 (*s*)	170.5 (*s*)	–	186.3 (*d*)	10.30 (*s*)

calc. 229.1228). The ^{1}H-NMR spectrum of **1** (*Table*) exhibited signals at δ(H) 1.77 (*s*, 2 Me) due to a substituted i-Pr group, and at 2.92 (*s*, Me–C(4)) due to a Me group attached to an aromatic ring. An aldehyde H-atom was present at δ(H) 10.28 (*s*), together with five aromatic H-atoms (δ(H) 8.16 (*d*, J=4.2, H–C(2)); 7.30 (*d*, J=4.2, H–C(3)); 7.57 (*d*, J=10.8, H–C(5)); 8.19 (*dd*, J=10.8, 1.8, H–C(6)); 9.93 (*d*, J=1.8, H–C(8)). ^{1}H,^{1}H-COSY Cross-peaks indicated coupling between H–C(2) and H–C(3), H–C(5) and H–C(6), and H–C(6) and H–C(8), respectively.

The carbon skeleton of **1** was clearly shown to be the same as that of **3**, a known guaiane sesquiterpene [15], by comparison of their ^{1}H- and ^{13}C-NMR spectra (*Table*). The obvious difference between **1** and **3** was that the quaternary C-atom of **1** appeared at δ(C) 74.2 instead of the methine at 38.4 of **3**, which suggested the presence of an OH substituent at C(7) of the side chain of **1**, as corroborated by an IR band at 3444 cm^{-1}. From these data, in combination with analysis of key HMBC correlations (*Figure*), the structure of **1** was elucidated as 7-(1-hydroxy-1-methylethyl)-4-methylazulene-1-carbaldehyde.



1 **2**

Figure. *Key HMBC correlations for* **1** *and* **2**

Compound **2** was obtained as a purple solid. It was more polar than **3**, and its molecular formula was established as $C_{15}H_{16}O_2$ on the basis of HR-TOF-MS data ($[M+Na]^+$ at *m*/*z* 251.1044; calc. 251.1047). The ^{1}H- and ^{13}C-NMR spectra of **2** (*Table*) were very similar to those of **3**, except that a COOH group (δ(C) 170.5) replaced the CHO function (δ(H) 10.30, δ(C) 186.3), which indicated that **2** shared the azulene skeleton of **3**. Comparison of the physicochemical properties with those reported previously [12–15] allowed us to identify **2** and **3**, respectively, as 4-methyl-7-(1-methylethyl)azulene-1-carboxylic acid and the (known) corresponding aldehyde. Note that **2** had been obtained by organic synthesis some years ago [12][13].

To show that compound **2** is a true natural product rather than an artifact formed by air oxidation of **3** during purification, the fresh fruiting bodies of *L. hatsudake* were re-extracted under *anaerobic* conditions, and the resulting organic AcOEt extract was immediately analyzed by HPLC. Indeed, compounds **1**–**3** were all detected, which supports our assumption.

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Experimental Part

General. Melting points (m.p.) were determined on an *XRC-1* micro-melting point apparatus; uncorrected. TLC: silica-gel plates; visualization by spraying with 10% H_2SO_4 in EtOH, followed by heating. UV Spectra: *Shimadzu UV-2411 PC* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Perkin-Elmer 577* spectrometer, with KBr pellets; in cm^{-1}. ^{1}H- and ^{13}C-NMR Spectra: *Bruker DRX-500* spectrometers; δ in ppm, *J* in Hz. MS: *VG Autospec-3000* spectrometer; in *m*/*z* (rel. %).

Fungal Material. The fresh fruiting bodies of *L. hatsudake* were collected at the Wuding mushroom market in Yunnan Province, P. R. China, in August 2005. A voucher specimen was deposited at the Herbarium of the Kunming Institute of Botany, the Chinese Academy of Sciences.

Extraction and Isolation. The fresh fruiting bodies of *L. hatsudake* (4.65 kg) were extracted with acetone (6×). The combined extracts were evaporated to give a deep-brown syrup, which was partitioned between H_2O and AcOEt. The org. layer was concentrated *in vacuo*, and the residue (40 g) was subjected to repeated column chromatography (CC) on SiO_2 employing a gradient elution with petroleum ether and acetone. The fraction (7.28 g) eluted with petroleum ether/acetone 100 : 20 (*v*/*v*) was further purified by repeated CC (SiO_2; $CHCl_3$/petroleum ether/acetone 30 : 10 : 1) and by reverse-phase chromatography (*RP-8*; H_2O/MeOH 1 : 4) to afford **1** (13.1 mg), **2** (7 mg), and **3** (40 mg).

7-(1-Hydroxy-1-methylethyl)-4-methylazulene-1-carbaldehyde (**1**). Red-purple solid. M.p. 72–73° (acetone). TLC ($CHCl_3$/MeOH 15 : 1): R_f 0.69. UV/VIS (MeOH): 226.5 (4.09), 311 (4.14), 380 (3.70), 513.5 (2.69), 883 (1.94). IR (KBr): 3444, 2924, 2852, 1727, 1633, 1408, 1385, 1119. ^{1}H- and ^{13}C-NMR:

see *Table*. EI-MS: 228 (56, M^+), 213 (100, $[M-Me]^+$), 195 (3, $[M-Me-H_2O]^+$), 185 (30), 171 (17), 152 (11), 141 (28), 128 (26), 115 (34), 99 (4), 91 (8), 77 (6), 69 (7), 63 (5), 57 (4). HR-TOF-MS: 299.1229 ($[M+H]^+$, $C_{15}H_{17}O_2^+$; calc. 299.1228).

4-Methyl-7-(1-methylethyl)azulene-1-carboxylic Acid (**2**). Purple solid. M.p. 70–71° (acetone). TLC ($CHCl_3$/MeOH 15:1): R_f 0.62. UV/VIS (MeOH): 220 (4.12), 237.5 (4.13), 295.5 (4.26), 370.5 (3.64), 521.5 (2.62), 564 (2.59), 643 (2.20), 756.5 (1.90), 862 (1.98). IR (KBr): 3452, 2925, 2854, 1641, 1654, 1094. ^{1}H- and ^{13}C-NMR: see *Table*. EI-MS: 228 (85, M^+), 213 (100, $[M-Me]^+$), 195 (9, $[M-Me-H_2O]^+$), 185 (8), 167 (26), 152 (31), 141 (14), 128 (18), 115 (18), 102 (3), 91 (5), 83 (8), 76 (7), 69 (4), 63 (5), 55 (4). HR-TOF-MS: 251.1044 ($[M+Na]^+$, $C_{15}H_{16}NaO_2^+$; calc. 251.1047).

4-Methyl-7-(1-methylethyl)azulene-1-carbaldehyde (**3**) [15]. Red-purple solid. M.p. 59–60° (acetone). TLC ($CHCl_3$/MeOH 100:1): R_f 0.70. ^{1}H- and ^{13}C-NMR: see *Table*.

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