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NOTE

Lactariolines A and B: new guaiane sesquiterpenes with a modulatory effect on interferon-γ production from the fruiting bodies of *Lactarius hatsudake*

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Interferons (IFNs) are cytokines that have a complex and central role in the resistance of mammalian hosts to pathogens. IFN-γ, also known as immune or type II IFN, is a homodimer produced predominantly by activated T cells and natural killer (NK) cells, and it is crucial for innate and adaptive immunity against intracellular pathogens and for tumor control.[1,2] Aberrant IFN-γ expression is associated with a number of autoinflammatory and autoimmune diseases. The importance of IFN-γ in the immune system stems in part from its ability to inhibit viral replication directly, however, most importantly, IFN-γ possesses immunostimulatory and immunomodulatory effects.[3,4]

Lactarius hatsudake, belonging to the family Russulaceae of Basidiomycete, is a common edible mushroom widely distributed in Korea, China, Japan, Europe and North America, and biological activity (for example, antitumor and antiviral effects) of *L. hatsudake* has been reported.[5] Previous investigation on the bioactive constituents of *L. hatsudake* resulted in the isolation of several ergosterol derivatives[6] and azulene pigments.[7] During the investigation of novel bioactive metabolites from *L. hatsudake*, we identified two new guaiane sesquiterpenes, lactariolines A (**1**) and B (**2**) (Figure 1), along with four known related compounds, 4-methyl-7-isopropyl-azulene-1-carboxylic acid (**3**), 1-formyl-4-methyl-7-isopropyl azulene (**4**), lactaroviolin (**5**) and 1-formyl-4-methyl-7-(1-hydroxy-1-methylethyl) azulene (**6**).[8,9] In this study, we describe the isolation, structure elucidation and biological activity of two new compounds.

The fruiting bodies of *L. hatsudake* (6 kg) provided by the National Agrobiodiversity Center (Suwon, Korea) in July 2009 were extracted three times with MeOH at room temperature over 3 days (for each extraction step). The combined MeOH solution was concentrated under reduced pressure to produce a residue (240 g), which was suspended in H_2O and successively partitioned with *n*-hexane, EtOAc and *n*-BuOH. The *n*-hexane layer (17 g) was loaded on a silica gel column and eluted with CH_2Cl_2–MeOH in a gradient mode (20:1 → 1:1) to yield nine fractions (LH1-9). The fraction LH2 (1.5 g) was applied to a Lichroprep RP-18 column (Merck Ltd, Seoul, Korea) and eluted with MeOH–H_2O (3:1 → 1:0) to give three fractions (LH21–23), and the fraction LH21 was subjected to Sephadex LH-20 column (GE Healthcare Bio-Sciences, Uppsala, Sweden) chromatography and eluted with MeOH to afford **4** (12 mg) and **5** (5 mg). The fraction LH5 (1.8 g) was subjected to Sephadex LH-20 column chromatography and eluted with CH_2Cl_2–MeOH (1:1) to give four fractions (LH51–54), and the subfraction LH52 was further separated by preparative HPLC on a YMC-Pack ODS column (20×250 mm, MeOH–H_2O=7:3, flow rate=4 ml min^{-1}, detection at 254 nm; YMC Co Ltd, Kyoto, Japan) to afford **1** (4 mg, t_R=28 min) and **2** (3 mg, t_R=35 min). The fraction LH53 was purified by a Sephadex LH-20 column eluting with 70% MeOH to yield **3** (2 mg). The fraction LH8 (300 mg) was further purified by preparative HPLC (MeOH–H_2O=4:1) to afford **6** (15 mg, t_R=18 min).

Compound **1** was obtained as a blue solid. UV (MeOH) λ_{max} nm (log ε): 250 (3.96), 291 (4.40, sh), 302 (4.48), 392 (3.58); IR (KBr) ν_{max} (cm^{-1}): 3439, 2925, 1671, 1594, 1424, 1367, 1239, 1094; ESI-MS *m/z*: 251 $[M+Na]^+$; high-resolution electrospray mass spectrometry (HR-ESI-MS) *m/z*: 251.1076 $[M+Na]^+$ (calcd for $C_{15}H_{16}NaO_2$, 251.1048). For ^1H and ^{13}C NMR data, see Table 1. The UV spectrum of **1** showed absorption maxima at 250, 302 and 392 nm, indicating an azulene skeleton.[10] The IR spectrum revealed the characteristic absorption band for a conjugated carbonyl group at 1671 cm^{-1}. The molecular formula of **1**, $C_{15}H_{16}O_2$, was established by HR-ESI-MS. The ^1H NMR

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spectrum of **1** showed two AB type signals at δ_H 7.86 and 7.55 (each 1H, d, J=4.0 Hz, H-2, 3), three ABX type signals at δ_H 9.19 (1H, d, J=1.6 Hz, H-8), 8.29 (1H, dd, J=10.4, 1.6 Hz, H-6) and 7.21 (1H, d, J=10.4 Hz, H-5), one oxygen-bearing methylene group at δ_H 5.01 (2H, s, H-15), one methoxy group at δ_H 3.46 (3H, s), two methyl group at δ_H 2.93 and 2.77 (each 3H, s, H-12, 14). The ^{13}C NMR spectrum of **1** showed 15 carbon signals including one carbonyl group, five quaternary aromatic carbons, five olefinic methines, one oxygenated methylene, two methyl groups and one methoxy group. These spectral data suggest that **1** possessed a structure similar to that of 7-acetyl-4-methylazulene-1-carbaldehyde, a known guaiane sesquiterpene,[11] and the gross structure was confirmed by HMBC experiments (Table 1). In the HMBC spectrum of **1**, the methylene group was long-range coupled to C-1 at δ_C 135.0, C-2 at δ_C 137.2 and C-9 at δ_C 132.9 indicating that the oxygenated methylene group was located at C-1. The acetyl group was assigned at C-7 by the HMBC correlations of H-12 with C-7 at δ_C 129.5 and C-11 at δ_C 199.1. In addition, HMBC correlations of H-14 with C-4 at δ_C 150.9, C-5 at δ_C 125.7 and C-10 at δ_C 138.5 were observed. Therefore, the structure of **1** was elucidated as 7-acetyl-4-methyl-1-methoxymethyl azulene, and the compound was named 'lactariolines A'.

Compound **2** was obtained as a red solid. UV (MeOH) λ_{max} nm (log ε): 229 (4.11), 238 (4.06, sh), 311 (4.35), 380 (3.44); IR (KBr) V_{max} (cm^{-1}): 3435, 2977, 2928, 1648, 1499, 1407, 1234, 1070, 788; ESI-MS *m/z*: 265 $[M+Na]^+$; HR-ESI-MS *m/z*: 243.1373 $[M+H]^+$ (calcd for $C_{16}H_{19}O_2$, 243.1385). For ^1H and ^{13}C NMR data, see Table 1. The UV and IR spectra of **2** revealed obvious similarities to those of **1**, suggesting that **2** also possessed an azulene skeleton. The molecular formula of **2**, $C_{16}H_{18}O_2$, was established by HR-ESI-MS data. The ^1H NMR spectrum of **2** showed resonances for one aldehyde proton at δ_H 10.37, two methyl groups at δ_H 1.74 (6H, s, H-12, 13) due to a substituted isopropyl group and five aromatic protons at δ_H 9.91 (1H, d, J=2.0 Hz, H-8), 8.21 (1H, d, J=4.0 Hz, H-2), 8.09 (1H, dd, J=11.2, 2.0 Hz, H-6), 7.58 (1H, d, J=11.2 Hz, H-5) and 7.34 (1H, d, J=4.0 Hz, H-3). Its ^{13}C NMR spectrum showed 16 carbon resonances consisting of one carbonyl group, five quaternary aromatic carbons, five olefinic methines, one quaternary aliphatic carbon, three methyl groups and one methoxy group. The above-mentioned spectral data suggest that **2** had the same gross structure as an analogue, 7-(1-hydroxy-1-methylethyl)-4-methylazulene-1-carbaldehyde,[9] except for the presence of an additional methoxy group at δ_H 3.17 (3H, s) and δ_C 51.1. The observed HMBC correlation between the methoxy group and C-11 at δ_C 79.1 indicated the location of the methoxy group was at C-11. Thus, the structure of **2** was elucidated as 1-formyl-4-methyl-7-(1-methoxy-1-methylethyl) azulene, and the compound was named 'lactariolines B'.

Compounds **1** and **2** were evaluated for their effects on the modulation of IFN-γ in NK92 cells. The interleukin (IL)-2-dependent NK cell line NK92 (human NK lymphoma) was obtained from the American Type Culture Collection. NK92 cells were maintained in α-MEM (Life Technologies, Karlsruhe, Germany) containing 20% fetal calf serum (HyClone, Logan, UT, USA), 2 mM L-glutamate, 100 μg ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (Life Technologies) and supplemented with 100 U ml^{-1} IL-2 (Chiron, Emeryville, CA, USA). NK92 cell culture was performed at 37 °C in 5% CO_2-humidified atmosphere. Quantification of human IFN-γ was performed according to the supplier's protocol, using commercially available mAb pairs (Endogen, Woburn, MA, USA). Cell-free supernatants were collected after 18 h of incubation at 37 °C. For the

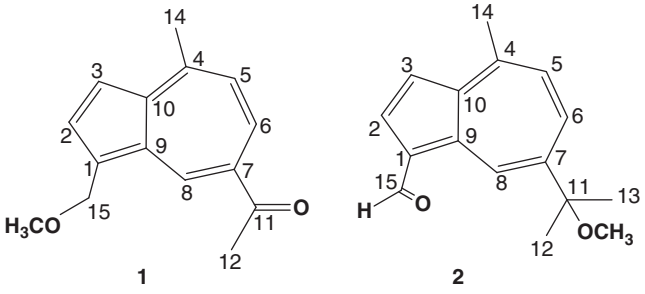

Figure 1 Structures of lactariolines A (**1**) and B (**2**).

Table 1 ^1H and ^{13}C NMR data for compounds 1 and 2 in $CDCl_3$

	1			*2*		
Position	δ_C	δ_H *(J in Hz)*	*HMBC (H→C)*	δ_C	δ_H *(J in Hz)*	*HMBC (H→C)*
1	135.0			126.6		
2	137.2	7.86 d (4.0)	C-3, 9	141.5	8.21 d (4.0)	C-1, 9, 10
3	119.0	7.55 d (4.0)	C-2, 10	116.3	7.34 d (4.0)	C-2, 4, 9, 10
4	150.9			149.5		
5	125.7	7.21 d (10.4)	C-7, 10, 14	131.2	7.58 d (11.2)	C-7, 10, 14
6	136.6	8.29 dd (10.4, 1.6)	C-4, 8	136.9	8.09 dd (11.2, 2.0)	C-4, 8
7	129.5			146.7		
8	133.9	9.19 d (1.6)	C-6, 9, 10, 11	136.3	9.91 d (2.0)	C-6, 10, 11
9	132.9			139.2		
10	138.5			144.2		
11	199.1			79.1		
12	27.2	2.77 s	C-7, 11	28.9	1.74 s	C-7, 11
13				28.9	1.74 s	C-7, 11
14	24.8	2.93 s	C-4, 5, 10	25.0	2.98 s	C-4, 5, 10
15	68.3	5.01 s	C-1, 2, 9, OCH_3	186.9	10.37 s	C-1, 9
OCH_3	58.3	3.46 s	C-15	51.1	3.17 s	C-11

^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were acquired on Varian Unity Inova 400 spectrometer (Varian Inc, Palo Alto, CA, USA); TMS was used as internal standard.

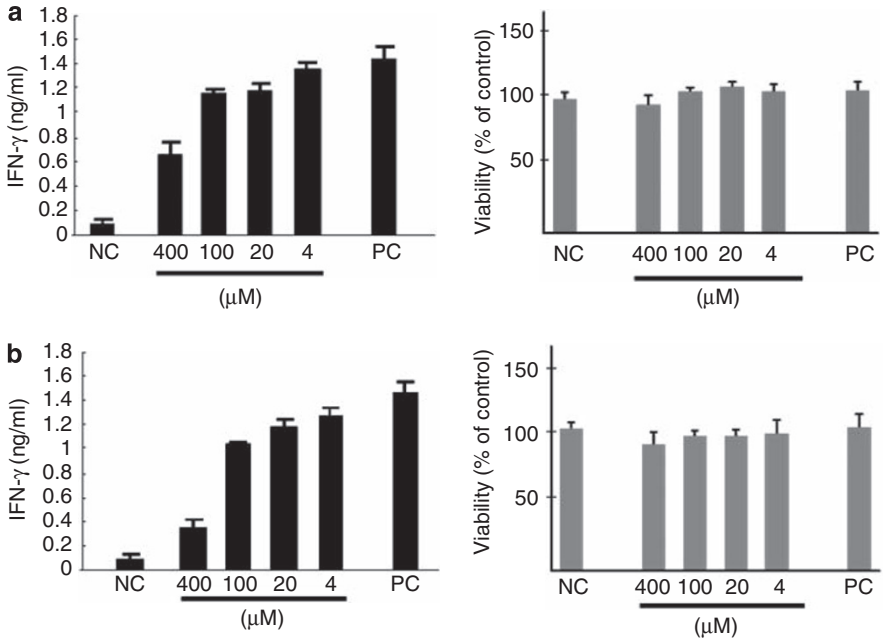

Figure 2 The results of ELISA for IFN-γ evaluation in compounds **1** (**a**) and **2** (**b**)-treated NK92 cells and cytotoxicity. NK92 cells were cultured in DMEM medium containing 20% FCS and IL-2. For evaluation of the released IFN-γ, the washed 5.0×10^5 cell per ml was distributed into 48-well culture plates. The modulation of IFN-γ was assayed by ELISA method with culture supernatant in compounds **1-** and **2**-treated cells. As negative control (NC) and positive control (PC) was used released IFN-γ in the culture supernatant originated from culture medium only and culture medium containing 100 ng of PMA treated cell, respectively. For evaluation of cell viability, we distributed 2.5×10^3 cell per well into 96-well plates. The WST-1 cell proliferation assay was carried out in compounds **1-** and **2**-treated cells with dose-dependent manner.

detection of IFN-γ, we used enzyme-linked immunosorbent assay (ELISA) kits from Endogen. Results were shown as the means of triplicate wells ± s.e.m. Cell viability and proliferation were assessed using Cell Proliferation Reagent WST-1 (catalog no. 11644807001; Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. The experiments were performed in 96-well plates at two cell densities. NK92 cells were plated at 1.5×10^3 and 2.5×10^3 cells per well. Absorbance was measured on the microplate reader after incubation for 12 h. All treatment experiments were performed in quadruplicate.

As shown in Figure 2, compound **1** inhibited IFN-γ production in NK92 cells in a dose-dependent manner, corresponding to 56.7% inhibition at 400 μM and 21.4% at 100 μM, respectively. Compound **2** also showed a dose-dependent effect, with 80.9% inhibition at 400 μM and 31.2% at 100 μM. The WST-1 cell proliferation assay showed that no cytotoxicity was observed in the NK92 cells with both cell density up to a concentration of 400 μM.

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