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Note

Novel Bioactive Compound from the *Sparassis crispa* Mushroom

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A novel compound (2) and a known one (1) were isolated from the mushroom, *Sparassis crispa*. Both compounds inhibited melanin synthesis and MRSA growth.

Key words: anti-melanin synthesis; anti-methicillin-resistant *Staphylococcus aureus*; mushroom; *Sparassis crispa*

The mushroom, *Sparassis crispa* (Hanabiratake in Japanese and cauliflower mushroom in English), grows on acicular trees and is widespread in northern temperate zones throughout the world. This mushroom has been used as a food and a home remedy in Asian countries. There are some reports on the isolation of β-glucan and antifungal benzoate derivatives.[1–6] We examined the biological activity of an extract of the mushroom, and found inhibitory activity against melanin synthesis by mouse B16 melanoma cells and against the growth of methicillin-resistant *Staphylococcus aureus* (MRSA). We report here the isolation, structural determination of a novel compound (**2**) and a known one (**1**), and their biological activities.

General. ^{1}H-NMR spectra (one- and two-dimensional) were recorded by a Jeol lambda-500 spectrometer at 500 MHz, while ^{13}C-NMR spectra were recorded on the same instrument at 125 MHz. The FABMS data were recorded by a Jeol DX-303HF instrument and the HRESIMS spectra were measured by a JMS-T100LC mass spectrometer. A Jasco grating infrared spectrophotometer was used to record the IR spectra, and CD spectra were measured by a Jasco J-820 spectropolarimeter. MPLC was done with a Yamazen MPLC system and an UltraPak ODS-S50D column (50 × 300 mm, Yamazen, Japan). HPLC separation was performed with a Jasco Gulliver system, using an ODS column (Wakopak Wakosil ODS, 20 × 300 mm, Wako Pure Chemicals, Japan) or a C30 column (Develosil C30-UG-5, 20 × 250 mm, Nomura Chemicals, Japan). Silica gel plates (Merck F_{254}) and silica gel 60 N (Merck 100–200 mesh) were respectively used for analytical TLC and for flash column chromatography.

Extraction and isolation. Dried fruiting bodies of *Sparassis crispa* (10.0 kg) were successively extracted with hexane (4-liter, twice), EtOAc (4-liter, three times) and then MeOH (8-liter). The residue (75.1 g) obtained after removing EtOAc was fractionated by silica gel flash column chromatography (90%, 70%, 60%, 50% hexane/EtOAc, 90%, 50% EtOAc/EtOH, MeOH, each 2-liter) to obtain twelve fractions. Fraction 8 (6.501 g) was further separated by reversed-phase MPLC (90% MeOH), and ten fractions were obtained. Fraction 8–2 (212.1 mg) was separated by reversed-phase HPLC (70% MeOH) to afford compound **2** (4.0 mg). while fraction 8–3 (31.1 mg) was subjected to reversed-phase HPLC (70% MeOH) to provide compound **1** (8.2 mg).

Anti-MRSA. Methicillin-resistant *Staphylococcus aureus* 2932 (a clinical isolate of MRSA) was kindly supplied by Prof. Michio Ohta (Nagoya University Graduate School of Medicine, Nagoya, Japan). Each sample at various concentrations was added to each well in a 96-well micro-plate. The pre-incubated MRSA suspension was then added to the wells. After incubating at 37 °C for 16 h, inhibition was evaluated on the basis of the turbidity of the culture medium in the wells. The minimum inhibitory concentration (MIC) of each sample is defined as the minimum concentration that gave no turbidity of the culture medium.

Inhibition of melanin production in B16 melanoma cells. B16 mouse melanoma cells were purchased from American Type Culture Collection (ATCC) and grown in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were placed in flat-bottomed 6-well plates (Asahi Techno Glass, Tokyo, Japan) at a density of 1.0×10^5 cells/well in an RPMI 1640 medium (Wako Pure Chemicals, Japan) containing 10% (v/v) fetal bovine

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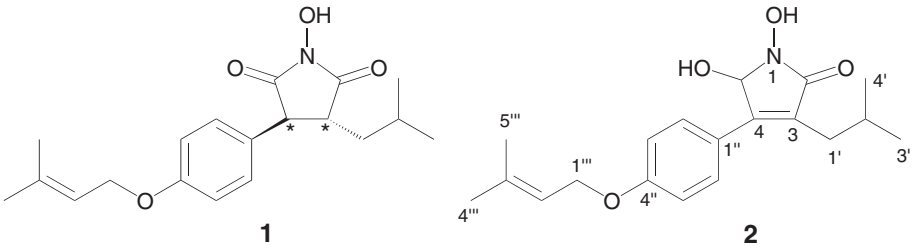
serum (FBS) and 0.5% (v/v) penicillin (10000 U/ml)-streptomycin (10 mg/ml). After 24 h of cultivation, the medium was replaced with a new RPMI 1640 medium containing each sample (25 μg/ml final conc.). After further cultivation for 48 h, the medium was again replaced by a new quantity of the same medium containing the test samples, and cultivation was continued for 24 h. The adherent cells were washed with 10 mM phosphate-buffered saline (pH 7.4) (PBS) and detached from the wells by trypsinization. The cells were collected in a test tube by centrifugation at 3000 rpm for 3 min, and the cell pellet was washed with PBS twice. In order to extract melanin from the B16 mouse melanoma cells, the cell pellet was suspended in 0.5 ml of 3 N NaOH and stored at 37 °C for 2 h. After mixing well, the melanin content was determined by measuring value of the absorbance at 450 nm minus that at 690 nm by using a microplate reader (Viento XS, Dainippon Pharma Co., Osaka, Japan. Arubutin was used as the positive control.

The dried fruiting bodies of *Sparassis crispa* were successively extracted with $CHCl_3$, EtOAc, and MeOH. Since the EtOAc-soluble fraction showed significant anti-melanin-synthesis and anti-MRSA activity, this fraction was repeatedly chromatographed as guided by the bioassay. As a result, two compounds (**1** and **2**) were purified as active principles.



1 **2**

All the spectral data for **1** were identical with those of a cytotoxic compound isolated from the mycelium of *Antrodia camphorata*.[7)]

Compound **2** was isolated as colorless oil. IR ν_{max} (neat) cm^{-1}: 3502, 1792. FABMS of **2** showed the ion peak at m/z 332 ($[M + H]^+$). Its molecular formula was determined as $C_{19}H_{25}NO_4$ by HRESIMS [m/z 330.1718 $[M - H]^-$ (calcd. for $C_{19}H_{25}NO_4$, 330.1705)], indicating the presence of eight degrees of unsaturation in the molecule. The ^{1}H- and ^{13}C-NMR spectra of **2** (Table 1) together with DEPT, COSY and HMQC data suggested the presence of an isobutyl attached at an sp^2 carbon [δ_H 0.80 (3H, d, $J = 6.7$), δ_C 22.5; 0.85 (3H, d, $J = 6.7$), 22.8; 1.93 (1H, m), 27.6; 2.31 (2H, m), 32.9], a methine [5.73 (1H, s), 84.2], a *p*-substituted phenyl [6.93 (2H, d, $J = 8.9$), 114.8; 7.40 (2H, d, $J = 8.9$), 130.2], $-CH_2-CH=$ [4.52 (2H, d, $J = 7.0$), 64.9, 5.47 (1H, m), 119.2], and two methyls attached at sp^2 carbon(s) [1.73 (3H, s), 18.2; 1.78 (3H, s), 25.8]. These NMR data were similar to those of **1**, and the molecular formulas of the two compounds were the same. However, the structure of the five-membered ring of **2** was different from that of **1**. The complete structure was determined by interpretating the HMBC correlations (Fig. 1). Significant cross peaks to connect each partial structure and to determine the complete structure were as follows: H1′/C2, H1′/C3, H1′/C4, H5/C3, H5/C4, H2″H6″/C4 and H1‴/C4″. This compound had an asymmetric carbon, but was racemic since the CD spectrum of it showed a plane line.

Compounds **1** and **2** were evaluated in anti-MRSA and melanin-production inhibition assays. MIC values of **1** and **2** in the anti-MRSA assay were 0.5 and 1.0 mM, respectively. IC_{50} values of **1** and **2** in the melanin

Table 1. ^{1}H-NMR [δ_H (number of protons, multiplicity, J in Hz)] and ^{13}C-NMR (δ_C) Data for **1** and **2** in $CDCl_3$[a]

Position	**1**		**2**	
2	—	174.7	—	170.1
3	2.85 (1H, m)	44.6	—	130.2
4	3.50 (1H, d, 4.3)	49.8	—	148.9
5	—	173.1	5.73 (1H, s)	84.2
1′	1.49 (1H, m)	40.4	2.31 (2H, m)	32.9
	1.80 (1H, m)			
2′	1.75 (1H, m)	25.4	1.93 (1H, m)	27.6
3′	0.69 (3H, d, 6.4)	21.3	0.80 (3H, d, 6.7)	22.5
4′	0.88 (3H, d, 6.1)	23.0	0.85 (3H, d, 6.7)	22.8
1″	—	127.9	—	124.1
2″, 6″	7.05 (2H, d, 8.9)	128.8	7.40 (2H, d, 8.9)	130.2
3″, 5″	6.86 (2H, d, 8.9)	115.4	6.93 (2H, d, 8.9)	114.8
4″	—	158.7	—	159.8
1‴	4.46 (2H, d, 6.7)	64.8	4.52 (2H, d, 7.0)	64.9
2‴	5.45 (1H, m)	119.4	5.47 (1H, m)	119.2
3‴	—	138.4	—	138.7
4‴	1.77 (3H, s)	25.8	1.78 (3H, s)	25.8
5‴	1.71 (3H, s)	18.2	1.73 (3H, s)	18.2

[a]These assignments were established by COSY, DEPT, HMQC and HMBC experiments.

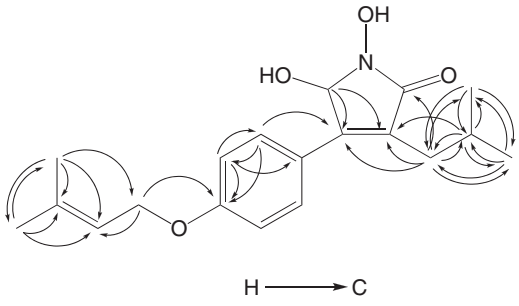


Fig. 1. HMBC Correlations in **2**.

production inhibition assay were 33 μM and 12 μM, respectively. On the other hand, IC_{50} value of the positive control, arubutin, was 1.32 mM.

Acknowledgment

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