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MELANOGENESIS-MODULATING DIKETOPIPERAZINE DERIVATIVES FROM *HYPOCREA* SPP.

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Abstract – A new dioxopiperazine derivative, hypocreasin (**1**), and five known (**2**–**6**) compounds were isolated from the fungus *Hypocrea* spp. The structure of **1** was determined by 1D/2D NMR and MS spectroscopic analyses. Compound **2** increased melanogenesis but **3** inhibited it in cultured murine melanoma B16F1 cells.

Diketopiperazines are the secondary metabolites of cyclic peptides produced mainly by various fungi.[1-5] Diketopiperazine analogs exhibit antitumor,[6,7] antimicrobial,[7] and anti-inflammatory activities.[8,9]

To further investigate the beneficial effects of diketopiperazines, we tested the effects of various microbial metabolites and their constituents on melanogenesis using B16F1 murine melanoma cells. We report here that a new dioxopiperazine derivative, hypocreasin (**1**), along with five known compounds, *cis*-bis (methylthio) silvatin (**2**),[10] 3-[(4-hydroxyphenyl)methyl]-1,4-dimethyl-3,6-bis(methylthio)-2,5-piperazinedione (**3**),[11] *trans*-bis (methylthio) silvatin (**4**),[11] 3-[4-(3-methyl-2-butenyl)phenylmethyl]-6-methylthio-2,5-piperazinedione (**5**)[12] and fusaperazine E (**6**)[13] were isolated from *Hypocrea* spp.

We found that some extracts inhibited melanogenesis in cultured melanoma cells. Among them, the extract of *Hypocrea* spp from the shellfish inhibited melanogenesis without any significantly cytotoxicity. The fungus strain of *Hypocrea* spp. was isolated from shellfish. A MeOH extract of culture plates was partitioned between EtOAc and H_2O. The EtOAc layer was partitioned between *n*-hexane and 90% MeOH–H_2O. The 90% MeOH–H_2O layer showed inhibitory activity against melanogenesis. The 90%

MeOH–H_2O layer was subjected to column chromatography on silica gel and ODS. Compounds **1**–**6** were obtained by HPLC.

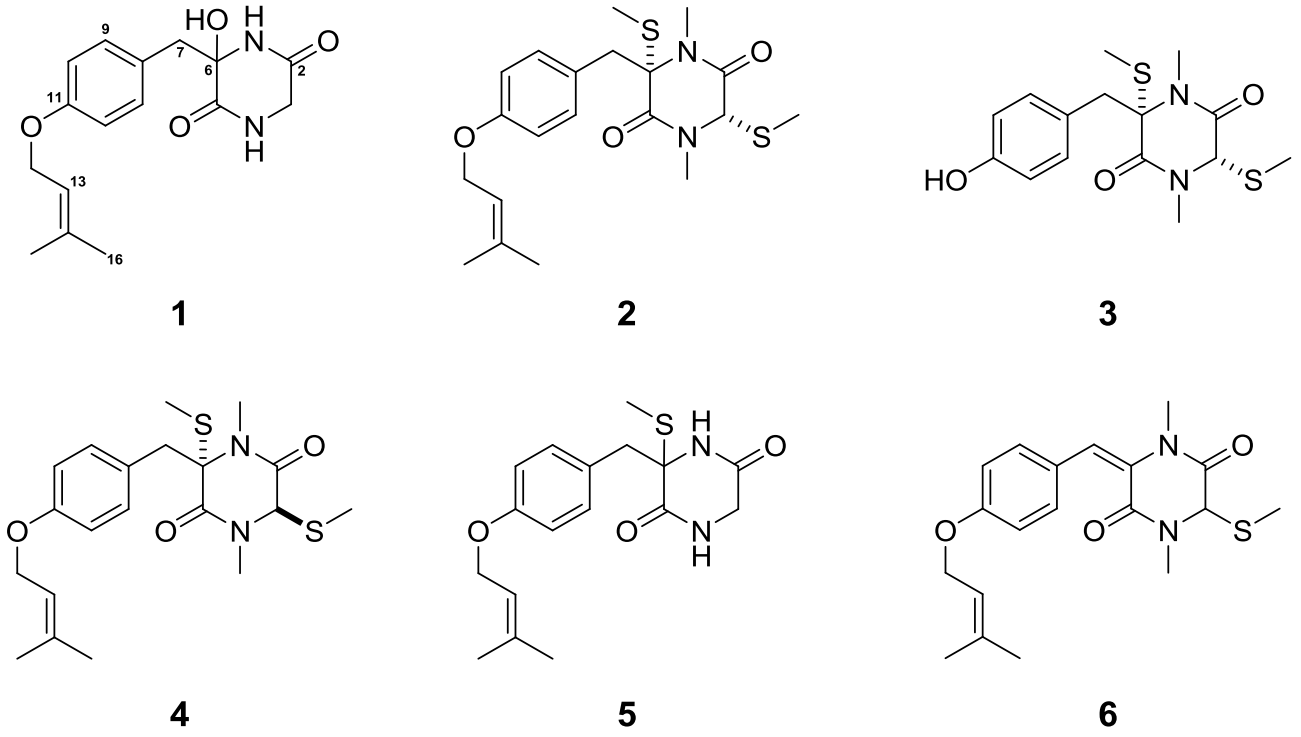

Figure 1. Structures of compounds **1**–**6**.

Compound **1** was obtained as white amorphous powder, and the molecular formula $C_{16}H_{20}O_4N_2$ was deduced by HRFABMS at *m/z* 303.1343 $[M-H]^-$. The ^1H NMR spectrum of **1** showed two methyl proton signals [δ_H 1.67 and δ_H 1.72], four aromatic proton signals of the AA'XX' spin system [δ_H 6.78 (2H, d, J = 8.6 Hz) and δ_H 7.13 (2H, d, J = 8.6 Hz)] assigned to a *para*-disubstituted aromatic ring. An olefinic proton signal δ_H 5.39 (1H, t, J = 6.8 Hz), six methylene proton signals [δ_H 2.68 (1H, d, J = 8.6 Hz), δ_H 3.06 (1H, d, J = 17.9 Hz), δ_H 3.20 (1H, d, J = 8.6 Hz), δ_H 3.62 (1H, d, J = 17.9 Hz) and δ_H 4.45 (2H, d, J = 6.8 Hz)], and three exchangeable proton signals [δ_H 6.60 (1H, s), δ_H 7.86 (1H, s) and δ_H 8.64 (1H, s)] were noted.

HMBC correlations were observed between: 1-NH and C-6; H-3 and C-2; 4-NH/C-3, C-5, H-7 and C-9; H-9 and C-11; H-10 and C-8; H-12 and C-13; H-15 and C-14; H-16 and C-13 as well as C-14. In addition, a NOE key correlation was also observed between H-10 and H-12 (Figure 2). C-6 was observed as a deshielded chemical shift compared with the sulfur-substituted carbons in **4**[11] (**1**: C-6, δ_C 81.8, in DMSO-d_6 **4**: C-6, δ_C 44.3 in DMSO-d_6), suggesting a hydroxy group attachment at this position. Therefore, **1** was defined as a diketopiperazine ring including tyrosine moiety in the structure. Known compounds **2**–**6** were identified by comparison of 1D NMR data with those in the literature.[10-13]

After establishing their structures, the effect of isolated compounds (**2**–**5**) on melanogenesis in cultured murine melanoma cells was tested. We measured the melanin production in the culture medium of B16F1 cells after stimulation of α-melanocyte-stimulating hormone (α-MSH). Kojic acid was used as the positive control. Melanogenesis was inhibited by compounds **3**–**5** in a concentration-dependent manner (Figure 3A). Further, cytotoxic activity was observed if cells were treated with compounds **4** or **5** but not with compound **3** (Figure 3B). Compounds **3**–**5** did not suppress tyrosinase activity, suggesting that these compounds inhibit melanin synthesis by a tyrosinase-independent mechanism. Interestingly, compound **2** enhanced the production of melanin at concentrations of 25 and 50 μM, whereas melanogenesis was inhibited at higher concentrations of this compound (Figure 3A). This compound was also cytotoxic at higher concentration up to 100 μM. This indicates compound **2** is a stimulator for melanin synthesis within a no toxic concentration. Taken together, these compounds could be new cosmetic agents.

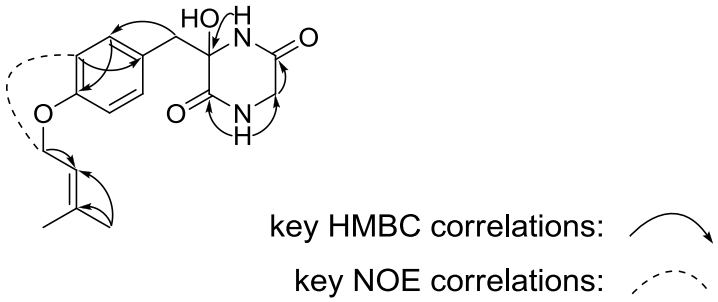

Figure 2. Selected HMBC and NOE correlations of **1**.

Table 1. ^{1}H and ^{13}C NMR data for **1**[a)]

Position	δ_H (*J* Hz)	δ_C	Position	δ_H (*J* Hz)	δ_C
1			11		157.2
2		165.8*	12	4.45 (2H, d, 6.8)	64.5*
3	3.06 (d, 17.9)	44.5*	13	5.39 (t, 6.8)	119.4*
	3.62 (d, 17.9)		14		136.9*
4			15	1.72 (3H, s)	25.4
5		166.8	16	1.67 (3H, s)	18.0
6		81.8*	6-OH	6.60 (s)	
7	2.68 (d, 13.1)	43.3	1-NH	7.86 (s)	
	3.20 (d, 13.1)		4-NH	8.64 (s)	
8		126.8*			
9	7.13 (2H, d, 8.6)	131.6			
10	6.78 (2H, d, 8.6)	113.9			

[a)] Values in parentheses indicate coupling constants in Hz
* These positions were assigned by correlations of HMQC and HMBC keys

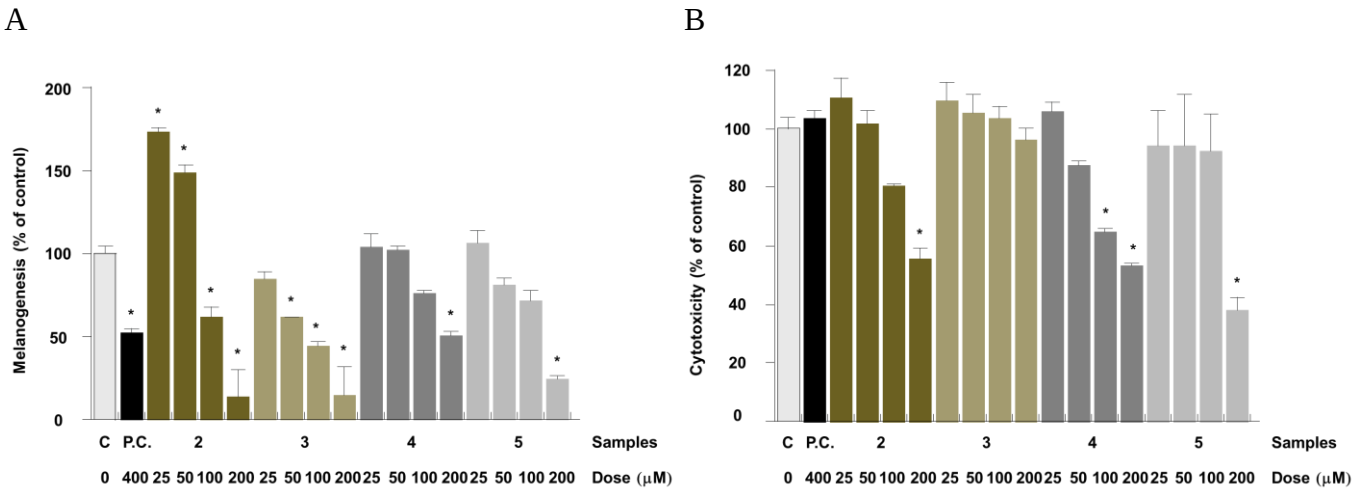

Figure 3. Effects of compounds **2**–**4** and kojic acid (P.C.) on melanogenesis (A) and cytotoxicity (B) in cultured murine B16F1 melanoma cells. Melanin content measured 72 h after α-MSH stimulation with or without tested samples. Data are mean ± SD of quadruplicate cultures. **P* < 0.001 compared with control cultures. C: Control, P.C.: Positive Control

EXPERIMENTAL

Optical rotations were measured using a Horiba SEPA-3000 High-sensitivity Polarimete (Horiba, Kyoto, Japan). UV spectra were measured using a Shimadzu UV-1600 UV–visible Spectrometer. NMR spectra were obtained using a JEOL ECA-600 Spectrometer in $CDCl_3$ and DMSO-d_6. Chemical shifts were referenced to the residual solvent peaks (δ_H 7.24 and δ_C 77.0 for $CDCl_3$, δ_H 2.49 and δ_C 39.5 for DMSO-d_6). Mass spectra were measured on a JEOL JMS-700 Mass Spectrometer. Reverse-phase HPLC was carried out on RP-23 (5 μm, Waters Corp., Milford, MA, U.S.A.). Silica gel (63–210 μm, Kanto Kagaku, Tokyo, Japan) and ODS (63–212 μm, Wako Pure Chemical, Osaka, Japan) were used for open-column chromatography. TLC was carried out on silica gel 60 F_{254} (Merck) and RP-18 F_{254S} (Merck). Agar was purchased from Wako Pure Chemical (Osaka, Japan). Fungal culture plate (90φ×15 mm) was from Sansei Medical (Kyoto, Japan).

Fungal strain

The fungus was isolated from shellfish collected at the shore of Tokumitsu (Hakusan, Ishikawa) in the Sea of Japan. The collected fungus was identified by Bex Co., Ltd (Tokyo, Japan).

Extraction and Isolation

The MeOH extracts from thriving colonies of marine fungi (approx. 300 species) were tested for their capacity to inhibit melanogenesis in cultured murine B16F1 melanoma cells assay system. The fungus was cultivated on agar medium (24 L; composed of 1.5% agar, 2.0% malt extract, 0.5% peptone in 100%

seawater) in fungal culture plate (1,200 plates in total, 20 mL of medium/plate) for 14 days at 25 °C. After cultivation, agar media were collected and extracted thrice with MeOH (15 L × 3). The MeOH extract was then partitioned between EtOAc and H_2O. The EtOAc layer was partitioned between *n*-hexane and 90% MeOH–H_2O, which showed anti-melanogenic activity. A part of the MeOH–H_2O layer (7.0 g/7.3 g) was subjected to column chromatography on silica gel with gradient mixtures of *n*-hexane/EtOAc (4/1, 2/1, 1/1) and $CHCl_3$/MeOH (20/1, 10/1, 1/1, 0/1) to afford 13 fractions (A01–A13). Fraction A03 (20.9 mg) was purified by ODS HPLC with 70% MeOH/H_2O to afford **1** (0.2 mg) and **5** (0.9 mg). The fractions mixed in A06, A07 and A08 were purified by ODS HPLC with 50% MeOH/H_2O to afford **2** (23.0 mg) and one fraction (B01). Fraction B01 was purified further by ODS HPLC with 70% MeOH/H_2O to afford **3** (1.4 mg) and **4** (2.5 mg). Fraction A09 was also purified by ODS HPLC with 30% MeCN/H_2O to afford **6** (0.2 mg).

Hypocreasin (1)

Amorphous powder, $[\alpha]_D^{23}$ +84.5° (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ε): 276 (3.43), 228 (4.16), 202 (4.58) nm; ^1H and ^{13}C NMR spectral data are shown in Table 1; HRFABMS *m/z* 303.1343 $[M-H]^-$ (calcd. for $C_{16}H_{19}O_4N_2$, 303.1345).

Inhibitory effects on melanogenesis in cultured murine melanoma B16F1 cells

Cultured B16 mouse melanoma cells were trypsinized (0.05% trypsin/EDTA at 37 °C for 5–10 min). Cells (5×10^4 cells/mL in 400 μL Dulbecco's modified Eagle's medium (DMEM)) were seeded onto 24-well plates and incubated for 24 h at 37 °C in a CO_2 incubator. After 24 h incubation, 50 μL of each sample (final concentration: sample media in 0.2% DMSO) solution and α-MSH (final concentration: 10 ng/mL) was added to each well, and the 24-well plate incubated for 48 h at 37 °C in a CO_2 incubator. In the control group, DMSO solution diluted with DMEM to 0.2% of the final concentration of DMSO was used instead of the sample solution. The positive control group was kojic acid (final concentration: 400 μM in 0.2% DMSO). After incubation, the culture medium was removed by pipette. The remaining melanoma cells were washed with phosphate-buffered saline (PBS) and added to 100 μL of 0.05% trypsin/EDTA solution. Cells were dissolved by the addition of 200 μL 1N NaOH solution. After 2 h, the resultant cell lysates were removed from the 96-well plates (each solution, 280 μL). The optical density at 475 nm of the resulting solution was measured, and the amount of intracellular melanin calculated.

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