J. Antibiot. 59(6): 351–354, 2006

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THE JOURNAL OF ANTIBIOTICS

Hypocrellin D, a Cytotoxic Fungal Pigment from Fruiting Bodies of the Ascomycete *Shiraia bambusicola*

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Received: April 18, 2006 / Accepted: May 25, 2006

Abstract A fungal pigment, hypocrellin D (**1**), together with three known perylenequinone derivatives hypocrellin A (**2**), B (**3**) and C (**4**), was isolated from the fruiting bodies of *Shiraia bambusicola*. Its structure was elucidated on the basis of spectral data including 2D NMR experiments. Hypocrellin D (**1**) significantly inhibited the growth of tumor cell lines Bel-7721, A-549 and Anip-973 with IC_{50} values of 1.8, 8.8, 38.4 μg/ml, respectively.

Keywords hypocrellin D, perylenequinone, *Shiraia bambusicola*, pigment, ascomycete, cytotoxicity

Introduction

Shiraia bambusicola P. Hennigs (Hypocreaceae), an ascomycete parasitic on bamboo twigs, is recorded only in China and Japan. It has been commonly used as medicinal fungi under the name of "Zhu Huang" in China for treatment of rheumatism and pneusomia in traditional Chinese medicine (TCM) [1]. Previously new perylenequinone pigments hypocrellin A~C and shiraiachrome A~C have been isolated from *S. bambusaicola* as fungal metabolites which exert photodynamic activity towards bacteria and fungi [2, 3]. Lately the methanolic extract of the mycelium of the fungus *S. bambusicola* was found to show significant cytotoxicity in the A-549 and HCT-8 solid tumor cells. Subsequent bioassay-guided fractionation in HCT-8 *in vitro* led to the isolation and characterization of shiraiachromes A and B as two major cytotoxic principles [4]. A series of new perylene derivatives related to shiraiachrome-A and -B as well as calphostin-C have been synthesized and evaluated for their cytotoxicities, antiviral activities, and inhibitory activities against protein kinase C [4]. In our continual investigation of the bioactive metabolites from higher fungi [5~9], a pigment, named hypocrellin D (**1**), was isolated from *S. bambusaicola*, together with hypocrellins A (**2**), B (**3**) and C (**4**). This report deals with the isolation, structural elucidation and cytotoxic activity of hypocrellin D (**1**).

Materials and Methods

General

Melting points were determined by the micro hot-plate method and are uncorrected. Optical rotations were measured on a Horiba SEPA-300 polarimeter. IR spectra were obtained with a Tensor 27 with KBr pellets. UV spectra were recorded on a Shimadzu UV-2411 PC spectrophotometer. NMR spectra were recorded on Bruker AV-400 and Bruker DRX-500 spectrometers in $CDCl_3$ solvent with TMS as an internal standard. EI-MS were recorded with a VG Autospec-3000 spectrometer. ESI-MS and HRESI-MS were recorded with an API QSTAR Pulsar I spectrometer.

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Silica gel (200~300 mesh, Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (Amersham Bioscience, Sweden) were used for column chromatography. Preparative TLC was performed on glass plates with silica gel 60 F_{254} (Qingdao Marine Chemical Inc., China). Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol.

Mushroom Material

The fresh fruiting bodies of *S. bambusicola* were collected in Shangri-La of Yunnan Province, China, in July 2004. The voucher specimen was deposited at the Herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences.

Extraction and Isolation

Fruiting bodies of *S. bambusicola* (1.5 kg) were pulverized and extracted with $CHCl_3/CH_3OH$ (1 : 1, v/v) six times at room temperature. The combined extracts were concentrated *in vacuo* to give syrup. The syrup (87 g) was subjected to silica gel column chromatography employing a gradient elution with $CHCl_3/CH_3OH$ (100 : 0 to 50 : 100, v/v) to give five fractions. Fractions II (163.3 mg), eluted with $CHCl_3/CH_3OH$ (100 : 0 to 100 : 10, v/v), was separated by preparative TLC with petroleum ether/acetone (3 : 2, v/v) and purified on Sephadex LH-20 with acetone to afford **1** (15.5 mg) and a dark red mixture. The mixture was further separated by repeated preparative TLC with $CHCl_3/CH_3OH$ (9 : 1, v/v) to yield **2** (42.3 mg), **3** (21.5 mg), and **4** (19.6 mg).

Physico-chemical Properties

Hypocrellin D (**1**): orange-red crystal, m.p. 109~110°C (acetone), $[\alpha]_D^{20}$ +2465.9 (*c* 0.71, acetone). UV $\lambda_{max}^{CHCl_3}$ nm (ε) 438 (6,725), 293 (22,729), 254.5 (19,223), 239.5 (21,913). IR (KBr) 3453, 1633 cm^{-1}. EI-MS *m/z* 578 (M, 18), 563 (M−CH_3, 2), 546 (M−CH_3−OH, 14), 521 (21), 503 (15), 487 (8), 479 (47), 461 (20), 447(9), 419 (8), 368 (6), 321 (7), 307 (8), 281 (10), 265 (12), 236 (9), 221 (15), 181 (15), 169 (16), 149 (55), 131 (28), 111 (44), 97 (59), 83 (70), 69 (98), 57 (100) cm^{-1}. HRESI-MS *m/z* 601.1331 (M+Na, Calcd. for $C_{30}H_{26}O_{12}Na$ 601.1321).

Cell Lines and Culture

All cell lines were grown in RPMI-1640 medium (GIBCO) supplemented with 10% heat-inactivated bovine serum, 2 nM L-glutamine, 10^5 IU/liter penicillin G, 100 mg/liter streptomycin and 10 mM HEPES, pH 7.4. Cells were kept at 37°C in a humidified 5% CO_2 incubator.

Cell Growth Inhibition Assay

Growth inhibition of hypocrellin D (**1**) on tumor cells was measured by the microculture [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [10, 11] with minor modification [12]. Briefly, adherent tumor cells were seeded into 96-well microculture plates and allowed to adhere for 24 hours before drug addition, while suspended cells were seeded just before drug addition. Each tumor cell line was exposed to compound at 0.01, 0.1, 1, 10, 100 μg/ml concentrations for different periods (adhere cells for 72 hours, suspended cells for 48 hours) and each concentration was tested in triplicate. At the end of exposure, 20 μl of 5 g/liter MTT (Sigma Chemical Co.) was added to each well and the plates were incubated for 4 hours at 37°C, then "triplex solution (10% SDS - 5% isobutanol - 0.012 M HCl)" was added and the plates were incubated for 12~20 hours at 37°C. The optical density (OD) was read on a plate reader at a wavelength of 570 nm. Media and DMSO control wells, in which **1** was absent, were included in all the experiments in order to eliminate the influence of DMSO. The inhibitory rate of cell proliferation was calculated by the following formula:

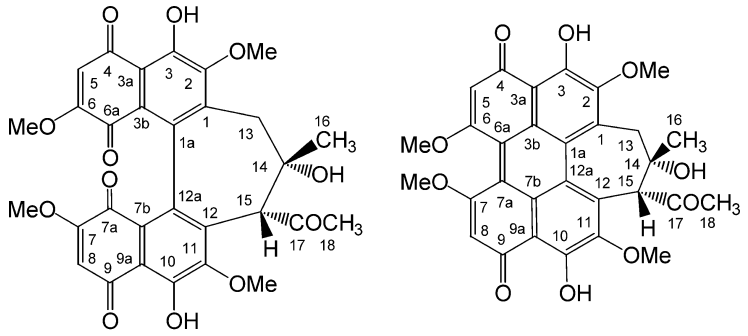
$$\text{Growth inhibition (\%)} = [OD_{control} - OD_{treated}/OD_{control}] \times 100\%$$

The cytotoxicity of **1** on tumor cells was expressed as IC_{50} values and was calculated by LOGIT method.

Results and Discussion

Compounds **2**, **3** and **4** were obtained as dark red crystals with metallic luster and displayed molecular (EI-MS) ion at *m/z* 546, 546 and 528, which were in agreement with molecular formula $C_{30}H_{26}O_{10}$, $C_{30}H_{26}O_{10}$ and $C_{30}H_{24}O_9$, respectively. These compounds were determined to be hypocrellins A, B and C by comparing the ^{1}H- and ^{13}C-NMR spectral data with those of hypocrellins in literature [2]. The relative configuration of hypocrellin A was already confirmed by X-ray analysis [13], and its absolute configuration was also established by comparing its CD spectrum with those of isocercisporin and cercisporin [2, 14, 15]. The axial chirality of **2** is *P* (*S*), and the absolute configurations at C-14 and C-15 of **2** are *S* and *R*, respectively (Fig. 1).

Compound **1** was obtained as orange-red crystals. The molecular formula of **1** was determined to be $C_{30}H_{26}O_{12}$ on the basis of HR-ESI-MS *m/z* 601.1331 (M+Na, Calcd. for $C_{30}H_{26}O_{12}Na$ 601.1321) and ^{13}C-NMR spectra (DEPT: six methyls, one methylene, three methines and twenty quaternary carbons). IR spectrum of **1** showed absorption



1 **2**

Fig. 1 Structures of hypocrellin D (**1**) and A (**2**).

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

Position	**1**		**2**	
1		131.7 (s)		133.1 (s)
1a		135.3 (s)		127.7 (s)
2		152.0 (s)		150.9 (s)[a]
3		154.6 (s)		172.0 (s)[b]
3a		114.7 (s)		106.7 (s)
3b		124.0 (s)		125.0 (s)
4		191.3 (s)		180.2 (s)[c]
5	6.04 (1H, s)	108.8 (d)	6.53 (1H, s)	102.0 (d)
6		161.4 (s)		167.4 (s)
6a		179.2 (s)		118.1 (s)
7		161.1 (s)		167.4 (s)
7a		178.9 (s)		117.7 (s)
7b		122.6 (s)		125.0 (s)
8	6.02 (1H, s)	108.5 (d)	6.59 (1H, s)	102.0 (d)
9		191.1 (s)		179.7 (s)[c]
9a		115.4 (s)		106.9 (s)
10		154.5 (s)		171.1 (s)[b]
11		151.6 (s)		150.7 (s)[a]
12		134.6 (s)		133.9 (s)
12a		135.4 (s)		128.6 (s)
13H[a]	3.15 (1H, d, 12.6)	38.0 (t)	3.52 (1H, d, 12.0)	41.9 (t)
H[b]	1.93 (1H, d,12.6)		2.63 (1H, d, 12.0)	
14		78.1 (s)		78.7 (s)
15	3.02 (1H, s)	57.8 (d)	3.46 (1H, s)	60.7 (d)
16	1.31 (3H, s)	26.0 (q)	1.70 (3H, s)	26.9 (q)
17		207.6 (s)		207.5 (s)
18	2.09 (3H, s)	30.6 (q)	1.89 (3H, s)	30.0 (q)
2-OCH_3	4.07 (3H, s)	61.3 (q)	4.11 (3H, s)	62.0 (q)
11-OCH_3	3.90 (3H, s)	60.9 (q)	4.11 (3H, s)	61.7 (q)
6-OCH_3	3.81 (3H, s)	56.6 (q)	4.06 (3H, s)	56.5 (q)
7-OCH_3	3.80 (3H, s)	56.6 (q)	4.06 (3H, s)	56.4 (q)
3-OH	13.19 (1H, s)		15.92 (1H, s)	
10-OH	13.25 (1H, s)		15.97 (1H, s)	

[a, b, c]: Assignments in each column may be interchangeable.

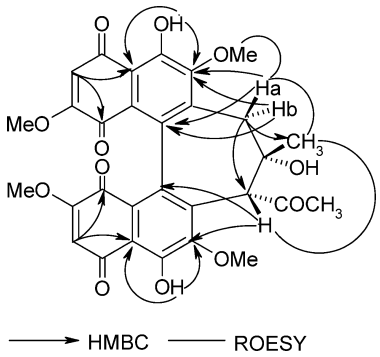


Fig. 2 Key HMBC and ROESY correlations of **1**.

at 1633 cm^{-1}, which was consistent with the presence of H-bonded carbonyl groups. The pair of singlet peaks at δ 13.25 and 13.19 in ^{1}H-NMR indicated the presence of chelating protons. According to ^{1}H- and ^{13}C-NMR spectra, the carbon skeleton of **1** was similar to that of hypocrellin A (**2**). The most distinct differences between **1** and **2** were that: (a) The signals of two carbons at δ 179.2 and 178.9 of **1** appeared instead of those at δ 118.1 and 117.7 of **2**; (b) The carbonyl signals at C-4 and C-9 (δ 180.2 and 179.7) in **2** markedly shifted downfield (δ 191.3 and 191.1) in **1**.

Such changes suggested the presence of conjugated carbonyl groups in **1**. Remaining signals of ^{1}H- and ^{13}C-NMR spectra of **1** were consistent with a seven-membered ring, which was similar to that of hypocrellin A (**2**). To establish the relative stereostructure of **1**, the ROESY experiment was carried out. Observed correlations between 2-OCH_3 and 13-Ha, 13-Ha and 16-CH_3, 16-CH_3 and 15-H indicated that 13-Ha 16-CH_3 and 15-H are *cis* form.

1 exhibited to possess cytotoxic activities against tumor cell lines, Bel-7721, A-549 and Anip-973, with IC_{50} values, 1.8, 8.8, 38.4 μg/ml, respectively. It is noted that **1** possessing a *seco*-3b,7b perylenequinone structure is firstly reported from nature though it was already reported as a photochemically converted compound [16, 17].

Acknowledgements This project was supported by the National Natural Science Foundation of China (30470027 and 30225048) and Natural Science Foundation of Yunnan Province (2000C0001P).

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