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CJ-19,784, a New Antifungal Agent from a Fungus, *Acanthostigmella* sp.

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A new antifungal agent, CJ-19,784 (**I**), was isolated from the fermentation broth of a fungus, *Acanthostigmella* sp. CL12082. Based on spectroscopic analyses, structure of **I** was determined to be 3′-bromo-2′,5-dihydroxy-3,7,8-trimethoxy-flavone. Compound **I** inhibits the growth of pathogenic fungi, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* with IC_{50} values of 0.11, 20 and 0.54 μg/ml, respectively.

The clinical need for the discovery and development of specific, fungicidal and safe new antifungal agents has been increasing in the past decade due to an expanding population of immunocompromised patients as results of transplantation, cancer and infection with HIV[1,2]. Today, amphotericin B and azole antifungals have been commonly used for systemic and local fungal infections. Amphotericin B shows a broad antifungal spectrum and fungicidal activity, but the use of the agent is being limited due to the nephrotoxic activity and the intravenous administeration[3]. Concerning the azole antifungals, their fungistatic activity and emergence of resistant microorganisms are significant problems[4].

In the course of our screening program for discovery of antifungal agents, three antifungal flavones were found to be produced by a fungus, *Acanthostigmella* sp. CL12082. In this paper, we describe fermentation, isolation, structure elucidation and biological properties of the flavones.

Results

Isolation

The detection of the antifungal flavones was monitored by HPLC using an ODS column as described in the experimental section. The fermentation broth (1.5 liters) was filtered after the addition of 1.5 liters of EtOH. The filtrate was concentrated to an aqueous solution (500 ml) and extracted 3 times with EtOAc (1 liter each). The EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated to dryness *in vacuo*. The resulting residue (1 g) was applied to a silica gel column (Kieselgel60, 230~400 mesh, Merck Co. Ltd.) which had been equilibrated with 500 ml of *n*-hexane - EtOAc (8 : 1). The active entity was eluted with *n*-hexane - EtOAc (1 : 1). The eluate was concentrated to dryness *in vacuo* to yield a brown residue. The residue was dissolved in MeOH (10 ml) and subjected to an ODS column (J'sphere ODS H80, 20×250 mm, YMC Co., Ltd.) with MeOH - H_2O (65 : 35) to afford **I** (7.4 mg), chlorflavonin[5] (**II**, 3.7 mg) and dechlorochlorflavonin[6] (**III**, 1.4 mg).

Physico-chemical Properties

The physico-chemical properties of **I**, **II** and **III** are summarized in Table 1. Compound **I** is soluble in $CHCl_3$ and MeOH, but insoluble in H_2O. Compound **I** showed the same UV spectrum as those of **II** and **III** with absorption maxima at 265 and 351 nm.

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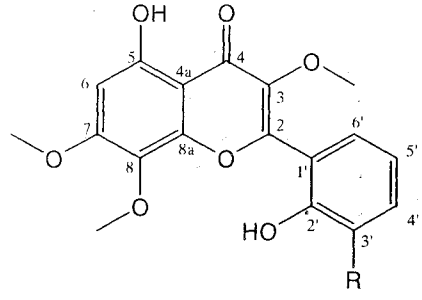
Table 1. Physico-chemical properties of CJ-19,784 (**I**), chlorflavonin (**II**) and dechlorochlorflavonin (**III**).

	CJ-19,784 (**I**)	Chlorflavonin (**II**)	Dechlorochlorflavonin (**III**)
Appearance	Yellow powder	Yellow powder	Yellow powder
Molecular formula	$C_{18}H_{15}BrO_7$	$C_{18}H_{15}ClO_7$	$C_{18}H_{16}O_7$
Molecular weight	423.2	378.8	344.3
HRFAB-MS (m/z) $(M+H)^+$			
Found:	423.0046	379.0583	345.0946
Calcd.:	423.0063	379.0585	345.0974
UV λmax (nm)	265, 351	265, 351	265, 351

Structure Elucidation

The structure of **I** was determined by comparison of its spectral properties with those of **II**[7]. The molecular formula, $C_{18}H_{15}BrO_7$ was assigned based on its HRFAB-MS (*m/z* found: 423.0046, calcd. 423.0063 for $C_{18}H_{16}BrO_7$ $[M+1]^+$). Comparison with its molecular formula with $C_{18}H_{16}ClO_7$ of **II** suggested that **I** would be a bromo derivative instead of the chlorine atom of **II**. As shown in Table 2, the ^1H NMR spectrum of **I** revealed three double doublets (H-4′: δ 7.67, H-5′: δ 6.97 and H-6′: δ 7.72) and a singlet (H-6: δ 6.43) in the benzene proton area, and three methoxy protons (3-OMe: δ 3.89, 7-OMe: δ 3.93, 8-OMe: δ 3.83). These chemical shifts were very similar to those of **II**, indicating the similar structure to **II**. The coupling patterns of H-4′, H-5′ and H-6′ ($J_{H4'\text{-}H5'}$ and $J_{H5'\text{-}H6'}$=7.8 Hz, $J_{H4'\text{-}H6'}$=1.5 Hz) showed 1,2,3-tri-substituted benzene type. The higher chemical shift of H-5′ than those of H-4′ and H-6′ indicated that the hydroxy group should be attached to be C-2′ because of its electron donating effect. The ^{13}C NMR spectrum of **I** was also similar to that of **II** except for the signal of the quaternary carbon at C-3′ (δ 113.54). The higher shift of C-3′ of **I** than the corresponding C-3′ (δ 123.91) of **II** indicated that the bromine atom was attached to C-3′ instead of the chlorine atom of **II**. The UV maxima of **I** supported the presence of the flavon nuclei. These data demonstrated that **I** was a new analog of **II** with the replacement of the chlorine atom by a bromine atom in the molecule (Fig. 1).

Fig. 1. Structures of CJ-19,784 (**I**), chlorflavonin (**II**) and dechlorochlorflavonin (**III**).



R = Br CJ-19,784 (**I**)
= Cl Chlorflavonin (**II**)
= H Dechlorochlorflavonin (**III**)

Biological Activities

Table 3 shows the antifungal activities of **I**, **II** and **III**. These compounds inhibited the growth of *Candida. albicans* and *Aspergillus fumigatus* with IC_{50} values of 0.1~0.93 μg/ml. They did not exhibit significant cytotoxic activity against HeLa cells.

Discussion

Three antifungal flavones, **I**, **II** and **III**, were isolated

Table 2. ^{1}H and ^{13}C chemical shifts of CJ-19,784 (**I**) and chlorflavonin (**II**).

Position	CJ-19,784 (**I**)		Chlorflavonin (**II**)*	
	^{13}C Chemical shift δ	^{1}H Chemical shift δ (multiplicity, J = Hz)	^{13}C Chemical shift δ	^{1}H Chemical shift δ (multiplicity, J = Hz)
2	154.76		154.71	
3	138.00		137.93	
4	177.96		177.93	
4a	105.36		105.31	
5	157.51		157.48	
6	95.91	6.43 (s)	95.87	6.46 (s)
7	158.97		158.94	
8	129.46		129.02	
8a	149.01		148.98	
1'	119.42		119.57	
2'	151.74		150.90	
3'	113.54		123.91	
4'	136.29	7.67 (dd, 7.8, 1.5)	133.11	7.58 (dd, 7.8, 1.5)
5'	121.70	6.97 (dd, 7.8, 7.8)	121.23	7.07 (dd, 7.8, 7.8)
6'	129.08	7.72 (dd, 7.8, 1.5)	128.61	7.65 (dd, 7.8, 1.5)
3-OMe	62.15	3.89 (s)	62.17	3.91 (s)
7-OMe	56.45	3.93 (s)	56.45	3.96 (s)
8-OMe	61.71	3.83 (s)	61.72	3.86 (s)

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Table 3. Biological activities of CJ-19,784 (**I**) and its related compounds.

Compound	IC_{50} (μg/ml)			
	C. albicans	*C. neoformans*	*A. fumigatus*	HeLa cells
CJ-19,784 (**I**)	0.11	20	0.54	81
Chlorflavonin (**II**)	0.035	12	0.10	20
Dechlorochlorflavonin (**III**)	0.42	16	0.93	79
Amphotericin B	0.076	0.57	0.14	50

from the fermentation broth of a fungus, *Acanthostigmella* sp. CL12082. These flavones showed potent antifungal activities against *C. albicans* and *A. fumigatus*, but less potent against *C. neoformans*. Compounds **I** (3'-Br) and **II** (3'-Cl) having a halogen atom at C-3' position showed more potent anti-*Candida* and -*Aspergillus* activities than **III** (3'-H), suggesting that the presence of a halogen atom at C-3' position is important for the activity.

It is well known that the production of halogenated microbial metabolites depends on the presence of halogen atoms in the fermentation medium. For example, chlortetracyclin[8] and chloromonilicin[9] are produced well under the fermentation of chlorine-containing media, and the chlorine atom is easily exchanged for a bromine

atom by replacing the media with bromine-containing media. It is very interesting that **I** (3′-Br) was produced as a major product rather than **II** (3′-Cl) and **III** (3′-H) under the fermentation of chlorine-containing medium (0.2% sodium chloride). This suggests that the fungus *Acanthostigmella* sp. CL12082 would have unknown biosynthetic mechanisms enabling prior use of the bromine atom.

Experimental

General

Spectral and physico-chemical data were obtained on the following instruments: UV, JASCO Ubest-30; NMR, JEOL JNM-GX270 updated with an LSI-11/73 host computer, TH-5 tunable probe and version 1.6 software; and LRFAB- and HRFAB-MS, JEOL MS-700 with a mastation data processing system. All NMR spectra were measured in $CDCl_3$ unless otherwise indicated and peak positions are expressed in parts per million (ppm) based on the internal standard of the $CHCl_3$ peak at 7.24 ppm for ^1H NMR and 77.0 ppm for ^{13}C NMR. All FAB-MS spectra were measured using glycerol-matrix.

Producing Microorganism

The fungal strain, *Acanthostigmella* sp. CL12082 was obtained from the New York Botanical Garden, New York, USA.

Fermentation

Acanthostigmella sp. CL12082 was maintained on a plate of potato dextrose agar medium (Difco). Vegetative cell suspension from the plate (in 2 ml sterile H_2O) was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of a seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). After incubation at 26°C on a rotary shaker (2-cm throw at 90 rpm) for 9 days, 5 ml aliquots were inoculated into fifteen 500-ml Erlenmeyer flasks containing 100 ml of a production medium (glucose 1%, glycerol 3%, peptone 0.5%, NaCl 0.2% and agar 0.1%, pH 7.0) and 30 g of rye flake (Nisshin Flour Milling). Static incubation was carried out at 26°C for 23 days.

HPLC Analysis

HPLC analysis was performed on a Hewlett Packard HP1090 system. Samples were subjected to an ODS column (YMC-Pack J'sphere H80, 4.6×150 mm, YMC Co., Ltd.) maintained at 42°C and eluted with MeOH - H_2O (70 : 30) at a flow rate of 0.9 ml/minute. Compound **I** was monitored by absorbance at 220 nm. Under these conditions, **I** was eluted at the retention time of 5.8 minutes.

Test Strains

Test strains, *Candida albicans* Y01.06, *Cryptococcus neoformans* Y16.03 and *Aspergillus fumigatus* H06.03 were all clinical isolates in Pfizer culture collection.

Antifungal Activity

Antifungal assay was performed on 96-well microtiter plates containing a 100 μl of Sabouraud agar medium (Nissui; 10 g peptone, 40 g glucose, 15 g agar, per liter of distilled water) with fungal cells per well. A 10 μl aliquot of each test sample was added to each well, and amphotericin B was used as a positive control. The plates were incubated at 28°C for 15 hours (*Candida albicans*), 18 hours (*C. neoformans*) and 24 hours (*A. fumigatus*). Antifungal activities of the compounds were determined by measuring absorbance at 650 nm.

Cytotoxicity

The HeLa cell line was cultured with EAGLE's minimum essential medium (Nissui) containing 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin. A 180 μl aliquot of cell suspension (5.5×10^4 cells/ml) was dispensed into each well of 96-well microtiter plate, and incubated with 20 μl of test sample in a humidified 5% CO_2 at 37°C. After 72 hours, the medium was discarded. Following wash with PBS once, cells were dyed with 50 μl of 0.4% crystal violet solution for 30 minutes. The plate was washed with tapped water 10 times by repeating of fill-and-decant, and dried. To determine the absorbance, pigment was eluted thoroughly with 50% MeOH and absorbance at 490 nm was measured. Percent inhibition of HeLa proliferation is calculated by formula below:

$$\%\text{Inhibition} = 100\times[A_{490}\text{ (no drug control)} - A_{490}\text{ (sample)}/A_{490}\text{ (no drug control)} - A_{490}\text{ (no growth control)}]$$

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