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Aspochalasin I, a Melanogenesis Inhibitor from *Aspergillus* sp.

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In the course of screening for the melanogenesis inhibitors, aspochalasin I was isolated from solid-state culture of *Aspergillus* sp. Fb020460. Its structure was determined by spectroscopic analysis including mass spectroscopy and NMR analysis. Aspochalasin I potently inhibited melanogenesis in Mel-Ab cells with an IC_{50} value of 22.4 μM without cytotoxicity.

Keywords: Melanogenesis, aspochalasin I, *Aspergillus* sp.

Although melanins protect the skin from harmful environments and sunlight, increased production of melanin can induce skin disorders, including acquired hyperpigmentation, such as melasma, postinflammatory melanoderma, and solar lentigo [3]. Melanins are synthesized within the melanosome in melanocytes. Tyrosinase catalyzes two rate-limiting steps of melanogenesis; that is the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to dopaquinone [6]. For this reason, most popular whitening agents such as hydroquinone (HQ), kojic acid, and arbutin (HQ β-D-gluconopyranoside) act as inhibitors of this enzyme. However, because of the hazardous side-effects of such whitening agents, the use of HQ in cosmetics has been banned by the European Committee [9]. Kojic acid was also restricted because of the carcinogenic potential [5]. Thus, attention has recently been focused on the use of natural products in cosmetics [1, 10]. In order to discover new whitening agents, we have screened melanin biosynthesis inhibitors from microbial metabolites and identified the active molecule, terrein, from *Penicillium* sp. [8]. Terrein inhibits melanogenesis in Mel-Ab cells by down-regulating microphthalmia-associated transcription factor (Mitf) *via* extracellular signal-regulated kinase (ERK) activation leading to the inhibition of tyrosinase production [12].

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In the continued search for melanogenesis inhibitors from microbial metabolites, we found that the solid-state culture of *Aspergillus* sp. Fb020460 inhibited melanogenesis in Mel-Ab melanocytes. The active compound was isolated and identified as aspochalasin I, which was previously reported to be a metabolite of *Aspergillus flavipes*. Aspochalasins are a subset of the fungal metabolites known as the cytochalasins and defined by the 2-methyl-propyl group at the C-3 position of the perhydroisoidol-1-one moiety [2]. The majority of aspochalasins have been isolated from the species of *Aspergillus* [14, 15]. In this study, we describe the isolation, structure determination, and the biological activity of aspochalasin I.

The fermentation of fungal strain Fb020460 was carried out in a solid state of moistened wheat-bran [11]. A piece of the strain Fb020460 from a mature plate culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium containing glucose 2%, yeast extract 0.2%, polypeptone 0.5%, $MgSO_4$ 0.05%, and KH_2PO_4 0.1% (pH 5.7). The strain was cultured on a rotary shaker (140 rpm) at 28°C for 3 days. For the production of aspochalasin I, 5 ml of the seed culture was transferred into fifty 500-ml Erlenmeyer flasks containing 100 g of moistened wheat-bran each, and cultured at 28°C for 6 days. An active compound was purified by melanogenesis inhibitory activity-guided fractionation using Mel-Ab melanocytes. The entire cultured medium was extracted with 80% acetone and the extract was concentrated *in vacuo* to provide an aqueous solution, which was then extracted three times with equal volumes of EtOAc. The concentrated EtOAc extract was subjected to silica gel column chromatography eluted with $CHCl_3$-MeOH (50:1–5:1, stepwise). The active fractions were combined and concentrated *in vacuo* to give an oily residue. The residue dissolved in MeOH was further purified by Sephadex LH-20 column chromatography eluted with MeOH. An active fraction was rechromatographed on a Sephadex LH-20 column with MeOH:H_2O (7:3, v/v), followed by preparative reversed-phase TLC using MeOH:H_2O (75:25,

v/v) to afford aspochalasin I (5.2 mg) with a Rf value of 0.13.

Aspochalasin I was isolated as a white solid. Its molecular formula was established as $C_{24}H_{35}NO_5$ from electron spray ionization (ESI) mass and NMR spectral data. Its positive ESI mass provided quasimolecular ion peaks at *m/z* 418.60 $[M+H]^+$ and *m/z* 440.61 $[M+Na]^+$, suggesting a molecular mass of 417. Its ^1H and ^{13}C NMR spectral data were as follows: δ_H [CD_3OD] 7.24 (1H, dd, *J*=2.4, 15.0 Hz, H-19), 6.14 (1H, d, *J*=10.8 Hz, H-13), 5.91 (1H, dd, *J*=2.4, 15.0 Hz, H-20), 5.25 (1H, br s, H-7), 4.46 (1H, br s, H-18), 3.82 (1H, d, *J*=10.8 Hz, H-8), 3.75 (1H, br d, *J*=5.4 Hz, H-17), 3.20 (1H, dt, *J*=3.3, 9.0 Hz, H-3), 3.05 (1H, br s, H-5), 2.89 (1H, dd, *J*=3.3, 5.4 Hz, H-4), 2.33 (1H, m, H-15a), 2.19 (1H, m, H-15b), 2.19 (1H, m, H-16a), 1.75 (3H, br s, H-12), 1.71 (1H, m, H-10a), 1.62 (1H, m, H-22), 1.52 (1H, m, H-10b), 1.43 (1H, m, H-16b), 1.37 (3H, br s, H-25), 1.25 (3H, d, *J*=7.2 Hz, H-11), 0.94 (3H, d, *J*=6.0. H-23), 0.95 (3H, d, *J*=6.0, H-24); δ_C [CD_3OD] 175.3 (C-1), 169.3 (C-21), 154.6 (C-19), 141.6 (C-6), 140.5 (C-14), 125.2 (C-7), 123.8 (C-13), 120.5 (C-20), 90.3 (C-9), 79.2 (C-17), 74.3 (C-18), 53.5 (C-4), 52.7 (C-3), 49.9 (C-10), 41.0 (C-8), 40.7 (C-15), 35.5 (C-5), 28.4 (C-16), 25.7 (C-22), 24.1 (C-24), 21.9 (C-23), 19.7 (C-12), 15.4 (C-25), 14.2 (C-11). Based on the ESI-MS, ^1H and ^{13}C NMR, ^1H-^1H-COSY, HMQC, and HMBC data, the structure of the purified compound was determined. The ^1H NMR spectrum showed the presence of three methyl doublets (δ 0.94, 0.95, and 1.25), two methyl singlets (δ 1.37 and 1.75), and four olefinic (δ 5.25, 5.91, 6.14, and 7.24), two oxygenated methine (δ 3.75 and 4.46) and one nitrogenated methine (δ 3.2) protons. The ^{13}C NMR spectrum displayed signals for 24 carbon atoms including an α,β-unsaturated ester/lactone carbonyl (δ 169.3) and an amide carbonyl (δ 175.3). The combined analysis of ^{13}C NMR and HMQC spectra revealed the presence of five methyl, three methylene, eleven methine, and three quaternary carbons in addition to the two carbonyls. This spectral data suggested that this compound was very similar to aspochalasin I. The chemical structure was confirmed by the ^1H-^1H COSY and HMBC experiments. The ^1H-^1H COSY revealed three partial structures, and these partial structures were unambiguously connected by the HMBC, as shown in Fig. 1B. On the basis of the above NMR spectral data and ROESY spectrum, the active substance was identified as aspochalasin I, 17α,18α-dihydroxy-10-isopropyl-14-methyl[12]cytochalasa-6,13,19-trien-1-one-21-(9)-lactone [15].

Mel-Ab is a mouse-derived spontaneously immortalized melanocyte cell line that produces a large amount of melanin [3]. In the present study, Mel-Ab cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1 nM cholera toxin (CT), 50 U/ml penicillin, and 50 mg/ml streptomycin at 37°C in 5% CO_2. To analyze any possible cytotoxic effects of aspochalasin I, Mel-Ab cells were treated with various concentrations of aspochalasin I at

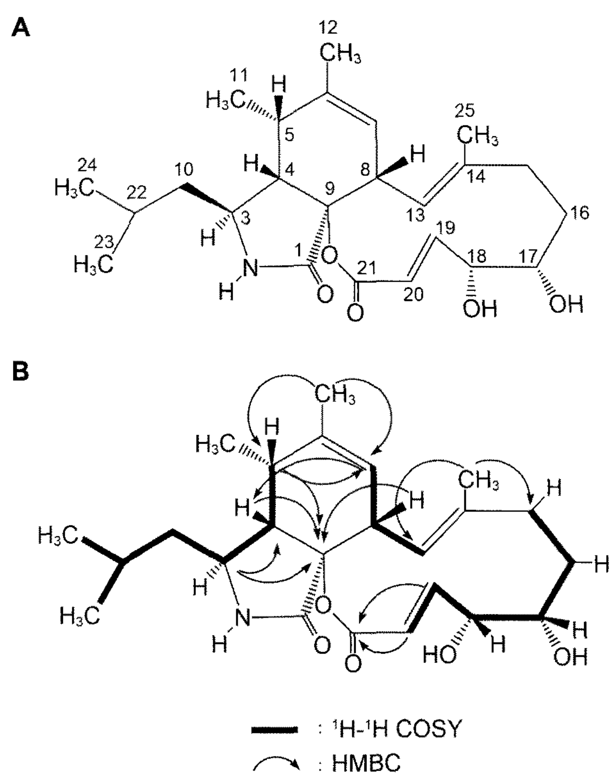

Fig. 1. **A.** The structure of aspochalasin I. **B.** ^1H-^1H COSY and HMBC correlations for aspochalasin I.

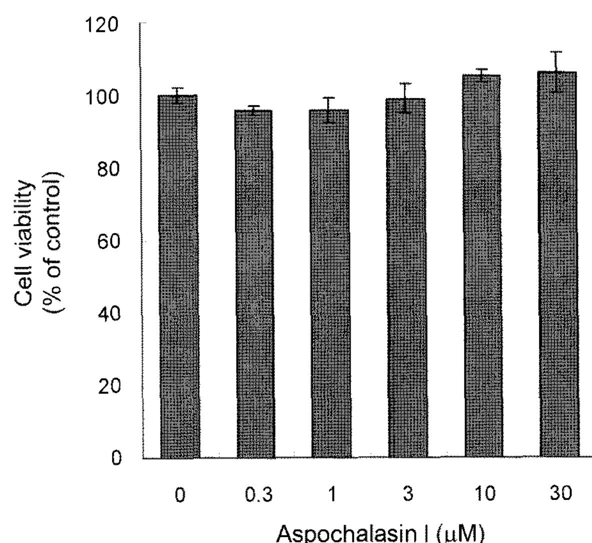

Fig. 2. Effect of aspochalasin I on Mel-Ab cell viability.
Cells were treated with 0.3–30 μM of aspochalasin I for 24 h. Cell viabilities were determined by crystal violet assay. Each determination was made in triplicate and data shown are means±SD.

various concentrations for 24 h, and cell viability was estimated using the crystal violet assay. Aspochalasin I had no cytotoxic effect on Mel-Ab cells at the concentrations used, as shown in Fig. 2. However, this compound showed cytotoxicity at 100 μM or over. To investigate the melanogenic inhibition effect of aspochalasin I, Mel-Ab cells were cultured at an initial cell density of 1×10^5 cells/well in 24-well plates. After 24 h, the medium was replaced with DMEM supplemented with 2% FBS containing various concentrations of aspochalasin I. After incubation for an additional 4 days, cell pellets were then dissolved in 1 ml of 1 N NaOH at 100°C for 30 min and centrifuged for 20 min at 16,000 ×*g*. Optical densities (OD) of the supernatants were measured at 400 nm using an ELISA reader. As shown in Fig. 3, the melanin contents of cells treated with aspochalasin I were significantly downregulated, with an IC_{50} value of 22.4 μM. Aspochalasin I showed higher activity than kojic acid, which inhibited melanin synthesis by 20% at 100 μM [8]. In mammalian melanocytes, tyrosinase plays a key role in melanogenesis [7]. Thus, we investigated any possible cell-based tyrosinase inhibitory activity of aspochalasin I. Mel-Ab cells were cultured with various concentrations of aspochalasin I for 4 days, and the cells were washed with ice-cold PBS and lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. The cells were then disrupted by sonication, and the lysates were clarified by centrifuging at 10,000 ×*g* for 5 min. After quantifying the protein levels and adjusting

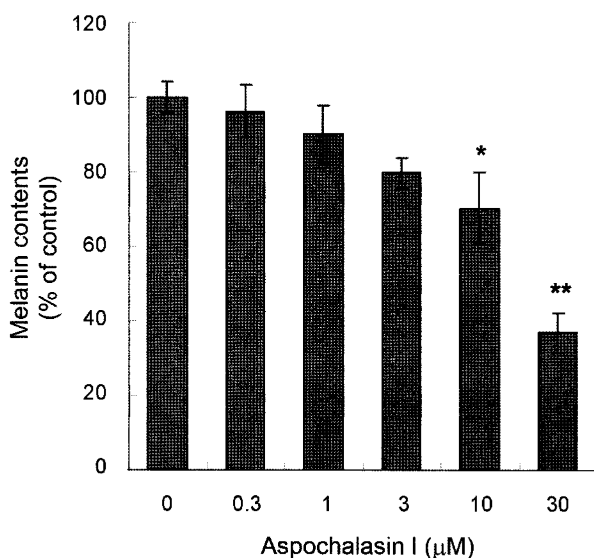

Fig. 3. Effect of aspochalasin I on melanogenesis in Mel-Ab cells.
Cells were cultured for 4 days in medium containing 0.3–30 μM of aspochalasin I and then harvested, and cell pellets were then dissolved in 1 ml of 1 N NaOH at 100°C for 30 min and centrifugated for 20 min. The OD of the supernatants were measured at 400 nm using an ELISA reader. Each determination was made in triplicate and data shown are means±SD. *$P<0.01$ and **$P<0.001$: Statistically significant *vs.* value of control group.

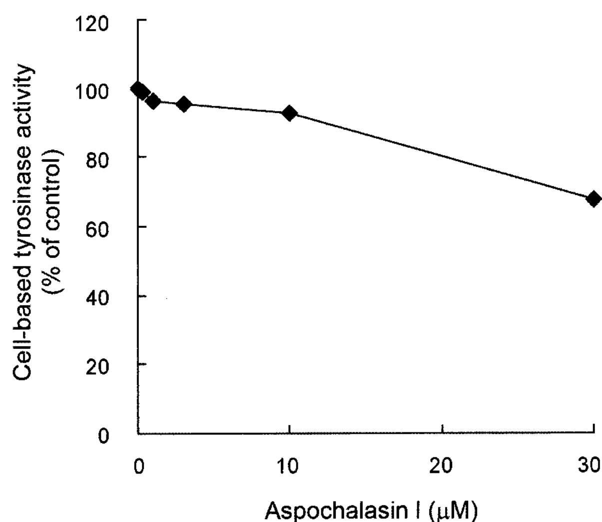

Fig. 4. Effect of aspochalasin I on cell-based tyrosinase inhibitory activity.
Mel-Ab cells were cultured for 4 days in medium containing 0.3–30 μM of aspochalasin I and then harvested, and tyrosinase was extracted from the cell. The cellular tyrosinase was then reacted with 10 μl of 10 mM L-DOPA at 37°C for 1 h and the dopachrome contents were measured at 475 nm using an ELISA reader at every 10 min for 1 h.

final concentrations with lysis buffer, 90 μl of each lysate was placed in a well of a 96-well plate, and 10 μl of 10 mM L-DOPA was then added. During incubation at 37°C, absorbance was measured at 475 nm using an ELISA reader per every 10 min for 1 h. The results of this study revealed that aspochalasin I inhibited tyrosinase activity by 30% at 30 μM (Fig. 4).

A variety of biological activities have been reported for the cytochalasins. The conjugated α,β-unsaturated carbonyl group was shown to possess antibacterial activity against Gram-positive bacteria [4] and cytotoxic activity against a panel of three sentinel cancer cell lines [15]. More recently, aspochalasin L was shown to possess activity against HIV integrase with an IC_{50} of 71.7 μM [14]. This is the first report on the melanogenesis inhibitory activity of aspochalasins on the melanogenic process.

In conclusion, we found that aspochalasin I from the *Aspergillus* sp. Fb020460 had an inhibitory activity on melanogenesis without cytotoxic effects in Mel-Ab melanocytes. Aspochalasin I also inhibited the cell-based tyrosinase activity in a dose-dependent manner. Thus, the present study suggests that aspochalasin I has the potential to be used as a skin-lightening agent.

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