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# Melanocins A, B and C, New Melanin Synthesis Inhibitors Produced by *Eupenicillium shearii*

## I. Taxonomy, Fermentation, Isolation and Biological Properties

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New melanin synthesis inhibitors, melanocins A, B and C, were isolated from the fermentation broth and mycelium extract of *Eupenicillium shearii* F80695. Melanocin A, an isocyanide compound, inhibited mushroom tyrosinase and melanin biosynthesis of B16 melanoma cells with $IC_{50}$ value of 9.0 nM and MIC value of 0.9 $\mu$M, respectively. Melanocin A also inhibited growth of *Streptomyces bikiniensis*. While, the structurally very related but non-isocyanide compounds melanocins B and C did not show inhibitory activity in these assays. Melanocins A, B and C showed potent antioxidant activity with scavenging activity of DPPH radical and superoxide anion radical.

Melanin biosynthesis inhibitors are useful not only for the material used in cosmetics as skin-whitening agents but also as a remedy for disturbances in pigmentation. Melanin synthesis inhibitors are used topically for treatment of localized hyper-pigmentation in humans such as lentigo, nevus, ephelis, post-inflammatory state and melanoma of pregnancy.

Tyrosinase (phenol oxidase)[1] is known to be a key enzyme for melanin biosynthesis in plants, microorganisms and mammalian cells. Tyrosinase catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and L-DOPA to DOPA-quinone[2]. These reactions are the initial steps of melanin biosynthesis pathway which has been the target of screening for anti-hyperpigmentation agents. As cosmetics and pharmaceuticals, many tyrosinase inhibitors have been tested and evaluated for the prevention of melanin overproduction in epidermal layers. For example, kojic acid and albutin have been utilized as whitening agents of cosmetics[3]. Several melanin biosynthesis inhibitors of microbial origin, feldamycin[4], melanostatin[5], albocycline and its derivatives[6,7], MR304A[8], MR566A and B[9,10], melanoxadin[11] and melanoxazol[12] have been reported.

During the course of searching for melanin biosynthesis inhibitors, new melanin synthesis inhibitors designated as melanocins A (**1**), B (**2**) and C (**3**) (Fig. 1) were isolated from a fungus[13]. Taxonomical study indicated that the producing organism was *Eupenicillium shearii*. In this paper we describe taxonomical studies of the producing strain, fermentation, isolation and biological properties of **1**~**3**.

### Materials and Methods

#### Microorganisms and Taxonomic Studies

The melanocins (**1**~**3**) producing strain, *Eupenicillium shearii* F80695, was isolated from a soil sample collected

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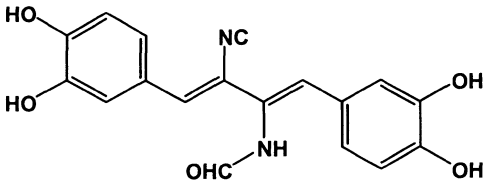
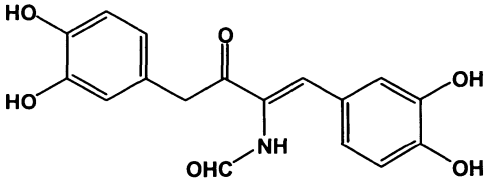
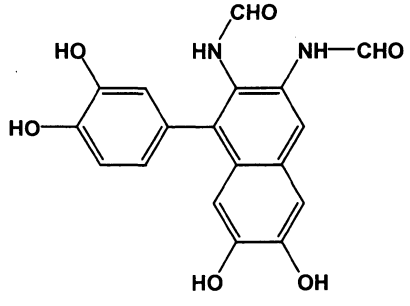
Fig. 1. Structures of melanocins A (**1**), B (**2**) and C (**3**).




**1**




**2**




**3**

in Daejeon, Korea. The taxonomical identification for the strain was examined according to PITT[14,15] and RAMIREZ[16]. Colors of morphological structures and colonies were determined using the charts of KORNERUP and WANSCHER[17]. Cultural characteristics were determined using various agar media as follows; Malt Extract Agar (MEA, malt extract 1%, peptone 0.1%, glucose 2.0%, agar 2.0%), Czapek's Yeast Extract Agar (CYA, $NaNO_3$ 0.3%, $K_2HPO_4$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, sucrose 3.0%, agar 2.0%), Czapek's Solution Agar (CzA) and Glycerol Nitrate Agar (G25N). The morphological characteristics were determined using an optical microscope (Nikon Microphot FXA, Japan) and a scanning electron microscope (Philips SEM 515, Netherlands). For SEM, sporulating material from agar media was fixed and dehydrated using the methods of NAKAGIRI[18].

Fermentation

A loopful fungus strain F80695 was inoculated from a PDA (Difco Co.) slant culture into the 500-ml Erlenmeyer flasks containing 120 ml of a medium consisting of 1% glucose, 0.3% malt extract, 0.5% Bacto-tryptone, 0.3% yeast extract. The flasks were shaken at 28°C on a rotary shaker at 160 rpm for 3 days. The seed culture (5 ml) was inoculated into fifty 500-ml Erlenmeyer flasks each containing 120 ml of the same culture medium. The flasks were placed on a rotary shaker at 200 rpm, at 28°C for 5 days.

Melanin Synthesis Inhibitory Activity

Melanin synthesis inhibitory activity was determined by the paper-disc agar diffusion method using the inhibition of melanin production in *Streptomyces bikiniensis*, mushroom tyrosinase (Sigma Chemical Co.) inhibitory activity determination[19] and inhibition of melanin formation in B16 melanoma cells[6]. The reaction mixture for the mushroom tyrosinase activity determination consisted of 15 $\mu$l of sample solution, 150 $\mu$l of 0.1 M phosphate buffer (pH 6.5), 25 $\mu$l of 1.5 mM L-tyrosine and 7 $\mu$l of mushroom tyrosinase (2100 unit/ml, 0.05 M phosphate buffer, pH 6.5) in a 96-well microplate and was incubated at 37°C for 10 minutes. The optical density at 490 nm was determined by a microplate reader (Molecular Devices, CA, USA).

DPPH Radical Scavenging Activity

Antioxidant activity was assessed on the basis of the scavenging activity of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH, from Sigma) free radical and superoxide anion radical.

DPPH radical scavenging activity was determined according to a modified version of the method of BRAND-WILLIAMS *et al.*[20] Ten microliters of diluted samples, standard antioxidant solutions or ethanol (control) was added to 190 $\mu$l of a $1.5\times10^{-4}$ M ethanolic solution of DPPH· in a well of 96-well plate. The absorbance of the reaction mixture at 517 nm was measured at steady state after 20 minutes of incubation at room temperature (25°C) using a microplate reader. The concentration-dependent scavenged DPPH· was calculated from absorptions at steady state by

$$\text{scavenged DPPH (\%)} = (1 - A_{\text{test at steady state}}/A_{\text{control at time 0}}) \times 100$$

The scavenged DPPH· (%) in a series of dilution of one test compound was used to calculate the effective relative concentration $EC_{50}$, at which 50% of DPPH· has been scavenged.

Superoxide Anion Radical Scavenging Activity

Superoxide radical scavenging activity was assayed by the modified irradiated riboflavin/EDTA/Nitroblue tetrazolium (NBT) system[21]. The reaction mixture contained 140 $\mu$l of 0.03 mM riboflavin, 1 mM EDTA, 0.6 mM methionine and 0.03 mM NBT solution in 50 mM potassium phosphate buffer (pH 7.8) and 10 $\mu$l of methanolic solutions of various concentrations of test compounds or standard compounds in a well of 96-well plate. The photoinduced reactions were performed in an aluminum foil-lined box with two 20 W fluorescent lamps. The distance between reactant and lamp was adjusted until the intensity of illumination reached 1000 lux. The reactant was illuminated at 25°C for 7 minutes. The photochemically reduced riboflavin generated $O_2^-$, which reduced riboflavin generated $O_2^-$, which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. Reduction of NBT was measured at 560 nm before and after irradiation using a microplate reader. Scavenging activity was calculated from the absorbance changes of control and tested samples.

$$\text{Scavenging activity (\%)} = (1 - \Delta A_{sample}/\Delta A_{control}) \times 100$$

The effective relative concentration $EC_{50}$ is the concentration of compound that inhibits 50% of NBT reduction. The assays were performed in triplicate and the absorptions were averaged before calculation. $\alpha$-Tocopherol and butylated hydroxyanisole (BHA) were used as standards.

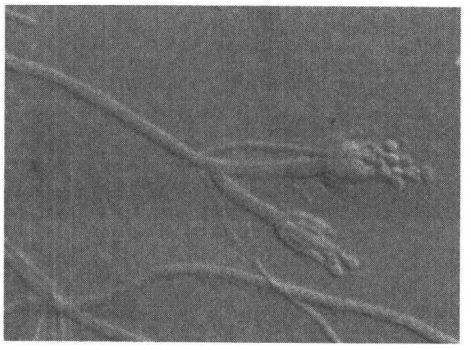
## Results and Discussion

Taxonomic Studies of the Producing Strain

For the taxonomic studies of the fungus, the strain was incubated in MEA, CYA, CzA and G25N for 7 days at 5, 25 or 37°C.

The growth of the strain was good on all the tested media except G25N at 25°C. No colonies were formed at 5°C and on G25N, and small colony zones (less than 1.0 cm) were formed at 37°C. Colonies on CYA after 7 days at 25°C were 3.8 cm in diameter and radially sulcate with dense white mycelium overlaid except in marginal areas by a contiguous layer of gray cleistothecia. Reverse of the colonies was pale yellow. Exudate developed abundantly but conidiogenesis was sparse and inconspicuous. Colonies on CzA were 2.8 cm in diameter after 7 days at 25°C, overlaid by white floccose pustule formed in patch, and wrinkled and buckled in radial pattern. The surface of colonies was white at first and turned light gray due to the development of abundant cleistothecium. Colonies on MEA at 25°C attained a diameter of 2.8 cm in 7 days, and were plane and zonate consisting predominantly of a layer of contiguous cleistothecia. Other characteristics were similar to those on CYA. Cleistothecia were warm gray to orange gray in color and 60~230 $\mu$m in length. Asci were 8-spored, born in single, and spherical to oval shape of 5.6~7.3 $\mu$m length and 4.3~6.2 $\mu$m width. Ascopores were lenticular, hyaline to pale yellow, smooth to finely roughen and ornamented with two longitudinal flanges, and 2.6~3.2×1.8~2.6 $\mu$m in size. Conidiospores were mostly born from surface hypae which were 300~500 $\mu$m in length and smooth-walled, but were born rarely from aerial mycelium (Fig. 2). Conidia were born in disordered chains, ellipsoidal, smooth and 2.5~3.2×2.1~2.6 $\mu$m in size (Fig. 2). Penicilli were mono- or bi-verticillate with metulae in verticals of 2~5. Phialides were ampulliform with narrow collular.

Fig. 2. Scanning electron micrograph of conidiospore.



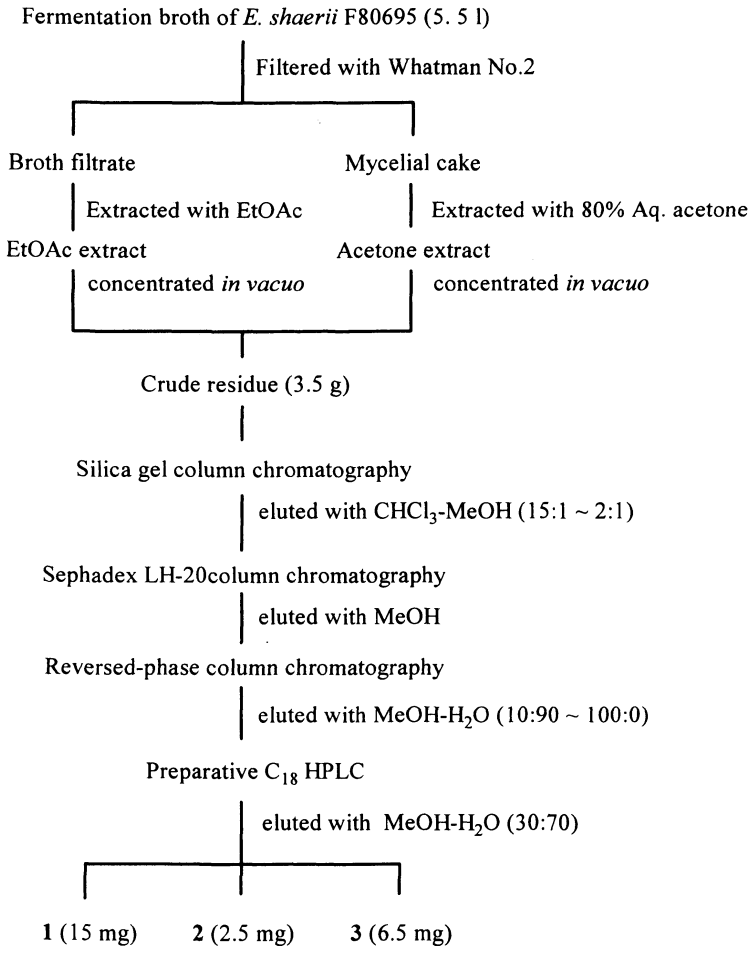
10 $\mu$m

From the above characteristics, the strain F80695 was identified as *Eupenicillium shaerii*. This strain has been deposited in the Korean Collection for Type Cultures as KCTC 0855BP.

Isolation

The isolation procedure for **1**, **2** and **3** is shown schematically in Fig. 3. About 6 liters of culture broth were filtered to separate the broth filtrate and the mycelium cake. The mycelial cake was extracted with 6 liters of 80% acetone. The acetone extracts were filtered and evaporated under reduced pressure to remove acetone. The resulting residue and the broth filtrate were extracted with EtOAc (6

Fig. 3. Isolation procedure of melanocins A, B and C.




liters) twice, and then the EtOAc extract was dried over anhydrous $Na_2SO_4$ and evaporated to dryness. The resulting residue (3.5 g) was chromatographed on a silica gel column (Merck silica gel 60) and eluted with the mixtures of $CHCl_3$-MeOH 15:1 to 1:1. The active fractions were collected and concentrated under reduced pressure to give a yellow oil. This oily substance was applied to a Sephadex LH-20 column and developed with MeOH as the eluting solvent. The active fractions were concentrated to give yellow powder (258.5 mg). The powder was rechromatographed with an eluting solvent of MeOH-$H_2O$ (70:30). The active fractions were concentrated and chromatographed using reverse phase HPLC (YMC, J' sphere, ODS-H80, 20×250 mm, YMC Co., Ltd.) with a solvent of MeOH-$H_2O$ (30:70) at flow rate of 6.0 ml/minute to give melanocin A (15.0 mg), B (2.5 mg) and C (6.5 mg).

## Biological Activities

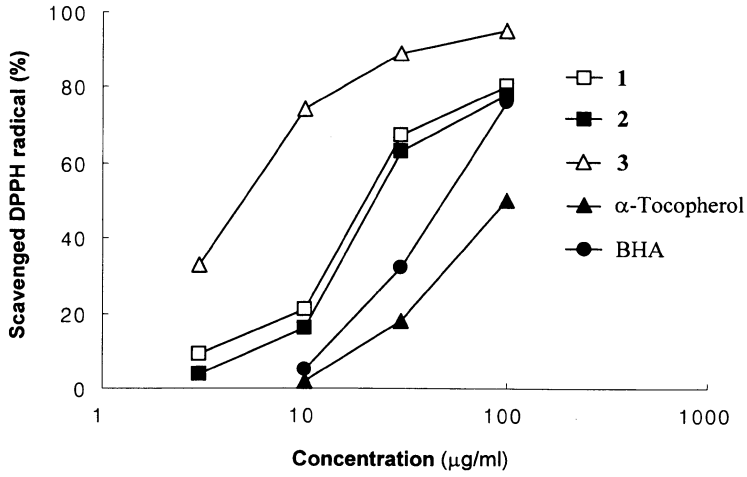
The inhibitory effects of the isolated compounds on mushroom tyrosinase and melanin formation in *S. bikiniensis* and B16 melanoma cells are shown in Table 1. Melanocin A (**1**), an isocyanide compound, inhibited strongly mushroom tyrosinase with an $IC_{50}$ of 9.0 nM. It was 3,300 and 4,200 times more active, respectively, than kojic acid and arbutin which are now being used as whitening agents in cosmetics. Compound **1** inhibited melanin synthesis in *S. bikiniensis* and strongly inhibited the melanogenesis of B16 melanoma cells with MIC of 0.9 $\mu$M. In comparison, the MIC by arbutin was 36.8 $\mu$M. The structurally related melanocins B (**2**) and C (**3**) which do not have isocyanide group showed no inhibitory effect against mushroom tyrosinase, *S. bikiniensis* and B16 melanoma cells. It has been proposed that the inhibitory activity of melanin synthesis inhibitors such as MR304A[10],

Table 1. Inhibitory effects of **1**~**3** and positive controls against mushroom tyrosinase and melanin formation in *Streptomyces bikiniensis* and B16 melanoma cells.

| Compound | *S. bikiniensis* NRRL-1049 | B16 Melanoma | Mushroom tyrosinase |
|---|---|---|---|
| | Inhibition zone (mm)[a] | MIC (μM) | $IC_{50}$ (μM) |
| **1** | 51 | 0.9 | 0.009 |
| **2** | 0 | >150 | >1000 |
| **3** | 0 | >150 | >1000 |
| Kojic acid | 0 | 106.3 | 31.0 |
| Hydroquinone | 25 | >150 | 9.1 |
| Arbutine | 0 | 36.8 | 38.0 |
| 4-hydroxyanisol | 30 | >150 | 120.0 |

[a] 30 μg compound / paper disk

Fig. 4. DPPH radical scavenging activity of **1**~**3** and standard antioxidants.




MR566A and B[11,12] is contributed by the isocyanide groups in their structures. Therefore, it is inferred that the potent inhibitory effect of **1** on tyrosinase and melanin synthesis is due to the isocyanide group, and the absence of melanin synthesis inhibitory activity of **2** and **3** is due to their absence of isocyanide group.

Melanocins A, B and C showed potent antioxidant activity. They scavenged DPPH radical (Fig. 4) and superoxide anion radical (Fig. 5). The $EC_{50}$ values of free radical scavenging effect are shown in Table 2. Compare with standard antioxidants, α-tocopherol and BHA, **1**~**3** showed higher activity. Melanocin C (**3**) showed the highest free radical scavenging activity among the tested compounds. The first two steps in the biosynthetic pathway of melanin are the hydroxylation of monophenol tyrosine to *o*-diphenol DOPA and the oxidation of DOPA to DOPA-quinone, both using molecular oxygen. Tyrosinase catalyzes these steps, and antioxidants may inhibit these oxidation steps. Although, **1**, **2** and **3** showed potent antioxidant effect, **2** and **3** inhibit neither tyrosinase activity nor melanin synthesis. Therefore, the potent inhibition activity of **1** against tyrosinase and melanin synthesis is not due to its antioxidant ability and can only be attributed to its isocyanide group in the structure.

Fig. 5. Superoxide anion radical scavenging activity of **1**~**3** and standard antioxidants.

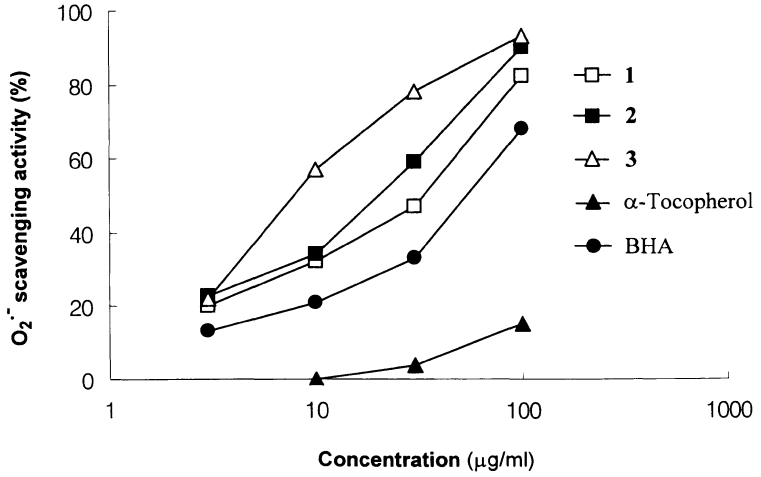



Table 2. DPPH and superoxide radical scavenging activity of **1**~**3** and standard antioxidants.

| Compound | $EC_{50}$ (μg/ml) | |
|---|---|---|
| | DPPH | Superoxide |
| 1 | 22.0 | 55.0 |
| 2 | 25.0 | 26.0 |
| 3 | 3.4 | 9.8 |
| α-Tocopherol | >100 | >100 |
| BHA | 63.0 | 65.5 |

Acknowledgments

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