MR566A and MR566B, New Melanin Synthesis Inhibitors Produced by *Trichoderma harzianum*

I. Taxonomy, Fermentation, Isolation and Biological Activities

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New melanin synthesis inhibitors (MR566A and B) and six related known isocyanocyclopentenes were isolated from the fermentation broth of *Trichoderma harzianum*. The IC_{50} values of MR566A and B against mushroom tyrosinase were 1.72 and 47 μM, respectively. They inhibited melanin biosynthesis in B16 melanoma cells with MIC values of 0.1 and 2.2 μM, respectively. Also isolated from the same culture extract of *T. harzianum* was a new oxazole (MR93B), which showed no inhibitory activity against mushroom tyrosinase at a concentration of 1,000 μg/ml.

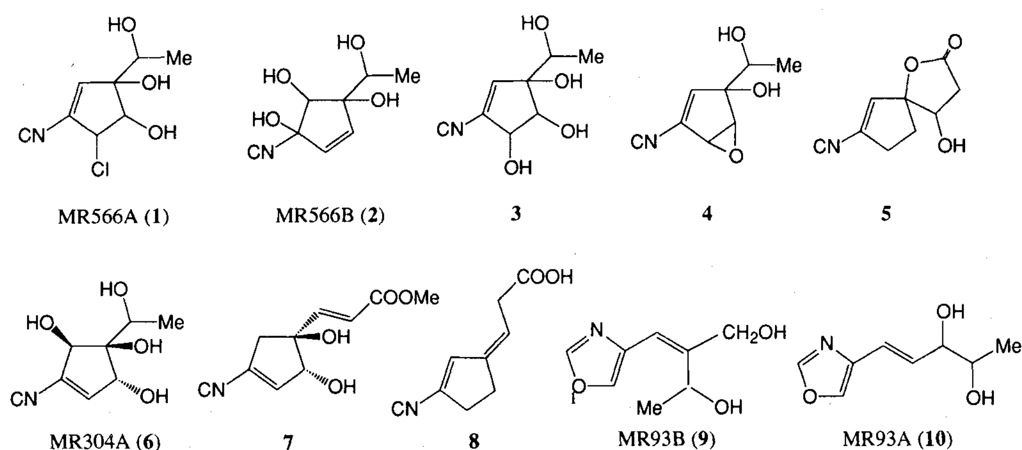
In our continuing search for new melanin synthesis inhibitors, we have reported a new isocyanocyclopentene, MR304A (**6**),[1] and a new oxazole, MR93A (**10**),[2] which were produced by *Trichoderma harzianum* KCTC 0114BP. A further study led to the discovery of other novel isocyanide compounds, MR566A (**1**) and B (**2**), and a novel oxazole, MR93B (**9**), from the culture broth of *Trichoderma harzianum* MR566 which had been isolated from a soil sample, together with six known isocyanide compounds (**3**~**8**) (Fig. 1).[3] In this paper we describe taxonomical studies of the producing strain, fermentation, isolation and biological activities of **1** and **2**.

Materials and Methods

Microorganism

The producing microorganism, strain MR566, was isolated from soil collected in Taejon, Korea. The strains were examined mainly according to RIFAI[4] and DOMSCH *et al.*[5] For the evaluation of cultural characteristics, the strain was incubated in OA (oatmeal agar, Difco) and MEA (malt extract 1%, peptone 0.1%, glucose 2.0%, agar 2.0%) for 5 days at 28°C. The morphological characteristics were determined using an optical microscope (Nikon Microphot FXA, Japan) and a scanning electron microscope (Philips SEM 515, Netherland). The restriction fragment length polymorphism (RFLP) patterns of 5.8S rDNA of the strain was analyzed by a previously described method.[6~8]

Fig. 1. Structures of MR566A (1), B (2), MR93B (9) and related compounds.



Fermentation

A slant culture of the KCTC 0187BP grown on PDA (Difco Co.) was used to inoculate a 500 ml conical flask containing 100 ml of the medium containing yeast extract 0.3%, malt extract 0.3%, Bacto-tryptone 0.5% and glucose 1%. The flask was placed on a rotary shaker at 200 rpm, at 25°C. After 3 days, 100 ml of the seed culture was inoculated into 5 liter of fermenter containing 3 liters of the medium described above. The fermentation was agitated at 150 rpm with aeration at 25°C for 4 days prior to being harvested.

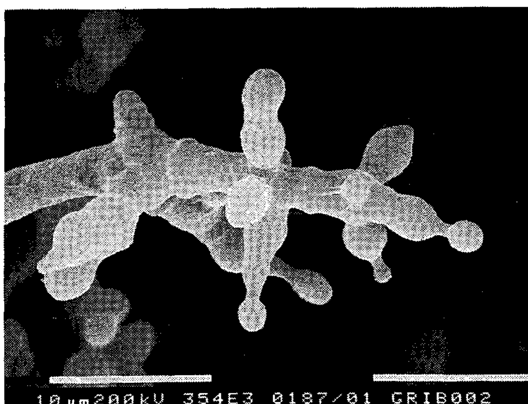
Isolation of **6**, **7**, and **8**

From the culture broth of *T. harzianum* KCTC0123, **6**, **7**, and **8** were purified by Diaion HP-20 column chromatography, EtOAc extraction, and Sephadex LH-20 column chromatography, and finally by repetitive HPLC on ODS under same conditions as shown in Scheme 1.

Biological Activities

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[9] and inhibition of melanin formation in B16 melanoma cells.[10] The reaction mixture for the mushroom tyrosinase activity determination consisted of 15 μl of sample solution, 150 μl of 0.1 M phosphate buffer (pH 6.5), 25 μl of 1.5 mM L-tyrosine and 7 μl 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 (Bio-Rad 3550, CA, USA).

Fig. 2. Scanning electron micrograph of conidiophore. ×3100, bar represents 10 μm.



Results and Discussion

Taxonomic Studies of the Producing Strain

Colonies on OA and MEA were 9 cm in diameter after culturing at 20°C for 5 days. The colony surface was yellow but turning dark green. The reverse side of the colony was colorless. As shown in Fig. 2, morphological observation was carried out using a scanning electron microscope. Smooth hyphae had developed on the OA medium. The hyphae with hyaline and smooth walls were 3～5 μm in diameter. Chlamydospores were hyaline, smooth-walled and globose. Conidiophores with complicated dendroid branching systems were long and slender. Phialide were ampulliform to subglobose, not crowded and regularly disposed. Conidia were subglobose to short-ovoid (length : width ratio = 1 : 3). From the characteristics mentioned above, the fungal strain MR-566 was identified as *Trichoderma harzianum* and has been deposited in the Korean Collection for Type Cultures as KCTC 0187BP. The identification of the producing strain was confirmed by comparison of RFLP patterns of *Trichoderma harzianum* strains (data not shown). The 7 strains of *T. harzianum* which were examined could be divided into two groups. According to FUJIMORI *et al.*,[11] there were no strains that produced both isocyanide antibiotics and dark brown pigments, and *T. harzianum*

Table 1. Pigment and inhibitor production patterns of *Trichoderma harzianum*.

Strain[1]	Pigment	Tyrosinase inhibitory activity (%)[2]
T. harzianum 1		
KCTC[3] 0114 (MR93)	-	100
KCTC 0123 (MR304)	-	100
KCTC 6504 (IMI[4] 288012)	-	100
KCTC 0187 (MR566)	-	100
T. harzianum 2		
KCTC 6426 (ATCC[5] 8678)	+	25
KCTC 6043 (ATCC 24274)	+	32
KCTC 6385 (ATCC 18647)	+	24

[1] Cultured on YMB for 5 days in 250 ml △-flask

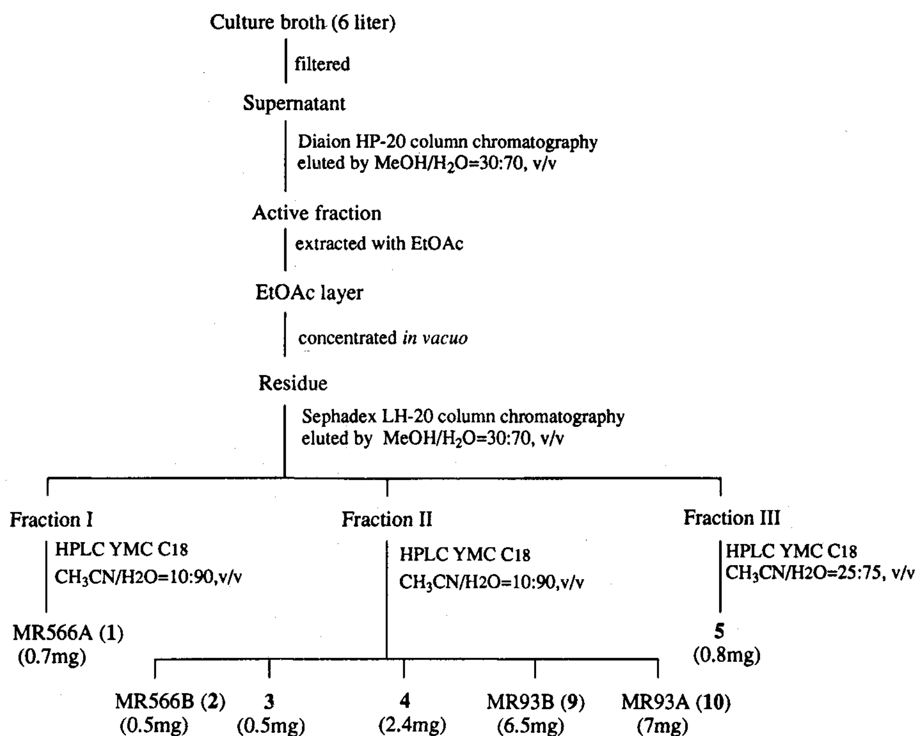
[2] 2% (V/V) in reaction mixture

[3] Korean Collection for Type Cultures

[4] International Mycological Institute (U.K)

[5] American Type Culture Collection

Scheme 1. Isolation procedure of **1**, **2**, **3**, **4**, **5**, **9** and **10** from *T. harzianum* MR566.



could be divided into types 1 and 2 by their production of isocyanides and pigments. In these experiments (Table 1), there were no strains that exhibited both tyrosinase inhibitory activities and these pigments. To the best of our knowledge, about 14 isocyanocyclopentenes have been isolated from *Trichoderma* sp.[1,12] Also 9 of those were purified as mushroom tyrosinase inhibitors in our experiments.

Isolation and Purification

The procedure for isolation is shown in Scheme 1. The fermentation broth (6 liters, pH 7.5) was filtered with Whatman No. 2 filter paper. The filtrate was applied to a Diaion HP-20 column (6 i.d. × 60 cm) eluting with water (6 liters), MeOH - H_2O (3 : 7) (6 liters) and MeOH - H_2O (7 : 3) (6 liters). The activity was found in the MeOH - H_2O (3 : 7) fraction, which was concentrated by evaporation and extracted with ethyl acetate. The organic layer was evaporated and applied to a column of Sephadex LH-20 (2 i.d. × 80 cm), and then developed with MeOH - H_2O (3 : 7) to give three active fractions (Fraction I, II and III). Fraction I was concentrated *in vacuo* and chromatographed on a HPLC (column, YMC-Pack ODS-AM, 4.6 × 205 mm, 10 μm; mobile phase, acetonitrile - water (1 : 9); flow rate, 0.7 ml/minute; detection, UV at 220 nm). MR566A (**1**) (0.7 mg) was obtained as a brown powder. Fraction II was concentrated *in vacuo* and chromatographed on HPLC to give three known compounds (**3**, **4**, and **10**) and two novel compounds, MR566B (**2**) and MR93B (**9**). Fraction III was also concentrated and chromatographed on YMC C_{18} HPLC column. **5** was obtained by elution with CH_3CN - H_2O (25 : 75). **3** and **5** have been purified from *T. hamatum* as rhodium complexes by BOYD *et al.*[13] This is the first report on complete isolations of **3** and **5**.

Biological Activities

The inhibitory effects of the isolated isocyanide compounds and known melanogenesis inhibitors on mushroom tyrosinase and melanin formation in *S. bikiniensis* and B16 melanoma cells are shown in Table 2. MR566A (**1**) strongly inhibited mushroom tyrosinase with an IC_{50} value of 1.72 μM in comparison with kojic acid. All eight isolated isocyanide compounds (**1**~**8**) inhibited mushroom tyrosinase with IC_{50} values of 0.0014~47 μM. **8** showed the strongest inhibitory activity against mushroom tyrosinase with an IC_{50} value of 0.0014 μM, which was 3,300 times more active than that of **6** and 2,200 times more active than that of kojic acid which is now being used as a whitening agent in cosmetics. To determine the active part of isocyanocyclopentenes (**1**~**8**), the inhibitory activities of related compounds

Table 2. Inhibitory effects on mushroom tyrosinase and melanin formation in *Streptomyces bikinienesis* and B16 melanoma cells.

Compound	*S. bikiniensis* NRRL-1049			B16 Melanoma	Mushroom tyrosinase
	Inhibition zone (mm)			MIC (μM)	IC_{50} (μM)
	10 μg	20 μg	30 μg		
1	25	30	38	0.10	1.72
2	10	16	20	2.20	47
3	50	55	60	0.32	3.6
4	50	55	60	0.48	4.9
5	15	25	35	0.34	0.089
6	15	20	25	5.4	47
7	10	12	15	0.24	1.72
8	45	55	63	0.25	0.0014
9	0	0	0	>600	>6000
10	0	0	0	>600	>6000
Kojic acid	0	0	0	106	31
Hydroquinone	10	19	20	–	9.1
Arbutin	–	–	–	36.8	38
p-Methoxyphenol	15	25	35	–	120

–, Not determined.

were compared (Table 3). Cyclopentene compounds without an isocyano group inhibited mushroom tyrosinase with IC_{50} values above 8 mM. Cyclohexyl isocyanide inhibited it with IC_{50} of 0.41 μM. From these results it could be proposed that isocyano group plays a more important role in the inhibitory activity of mushroom tyrosinase than does the cyclopentene ring. The structure-activity relationship of isocyanocyclopentenes with synthetic compounds will be reported in a separate paper.

1~**8** inhibited melanin synthesis in *S. bikiniensis* and also inhibited strongly the melanogenesis of B16 melanoma cells with MIC of 0.1~5.4 μM in comparison with arbutin (36.8 μM). But there was no relative correlation between the mushroom tyrosinase inhibitory activities and melanogenesis inhibitory activities of B16 melanoma cells. Compounds **1** and **2** exhibited strong cytotoxicity against B16 melanoma cells with IC_{50} values of 9.8 μM and 54.3 μM, respectively. Two oxazoles (**9** and **10**) showed no inhibitory activities against mushroom tyrosinase at a concentration of 1 mg/ml (6 mM) and did not exhibit melanogenesis inhibitory activities on *S. bikiniensis* or B16 melanoma cells, either. According to NAKAGAWA *et al.*,[14,15] MR93A (melanoxadin, **10**) and melanoxazale exhibited inhibitory activities against mushroom tyrosinase with IC_{50} values of 9.8 and 4.2 μg/ml, respectively. In our experiments, we first purified **9** and **10** as mushroom tyrosinase inhibitors, but after repetitive purification of those compounds they did not exhibit any more inhibitory activities. From these results, it can be proposed that the inhibitory activities of oxazoles (**9** and **10**) have been originated from small amounts of impurities, such as isocyanides, which showed strong tyrosinase inhibitory activities.

Table 3. Inhibitory activities (IC_{50}) of cyclopentene and related compounds on mushroom tyrosinase.

Compound	IC_{50}
Cyclopentene[a]	100 mM
2-Cyclopenten-1-one[a]	60 mM
1,3-Cycolopentanedione[a]	8 mM
2-Cyclopenten-1-ly acetic acid[a]	10 mM
Cyclohexyl isocyanide[a]	0.41 μM
Cyclohexane[b]	> 100 mM
Cyclohexanone[b]	> 150 mM

[a] Merck [b] Junsei Chemical

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