MR304A, a New Melanin Synthesis Inhibitor Produced by *Trichoderma harzianum*

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In the screening for new melanin synthesis inhibitors, we found a novel isonitrile inhibitor, MR304A from the culture broth of *Trichoderma harzianum*. There were some isonitrile antibiotics that were reported to have been isolated from *Trichoderma* sp.[1~4] and trichoviridin, one of these isonitriles, showed a melanin biosynthesis inhibitory activity[5].

In this paper we described the fermentation, isolation, physico-chemical properties, structure determination and biological activities of MR304A.

The producing microorganism, strain MR304, was isolated from a soil collected in Taejon, Korea. The fungus was identified as *Trichoderma harzianum* on the basis of its cultural properties and has been deposited with Korean Collection for Type Cultures, KCTC 0123BP.

A slant culture of the strain MR-304 grown on PDA (Difco Co.) was used to inoculate a 500 ml conical flask containing 100 ml of the seed medium containing glucose 2.5% and pharmamedia (Southern Cotton Oil Company) 2.5%. The flask was shaken on a rotary shaker for 42 hours at 25°C. 5 ml of the seed culture was transferred to 100 ml of the production medium containing glucose 2%, lactose 1%, peptone 0.3%, NaCl 0.5%, corn steep liquor 0.3%, KNO_3 0.23% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 6.0 in a 500 ml conical flask. The fermentation was carried out at 25°C for 7 days using a rotary shaker.

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[6] and inhibition of melanin formation in B16 melanoma cells[7]. A large scale fermentation was carried out in 5 liter jar fermenter. The seed culture prepared in 500 ml conical flasks was poured into a 5 liter jar fermenter containing 3 liters of the production medium described above. The fermentation was run at 25°C for 7 days with stirring at 250 rpm and aeration.

The fermentation broth (3 liters, pH 7.7) was filtered by Whatman No. 2 filter paper. The filtrate was applied to a Diaion HP-20 column (5 i.d. × 45 cm) eluting with water (3 liters), MeOH - H_2O (3 : 7) (3 liters) and MeOH - H_2O (7 : 3) (3 liters). The activity was found in the MeOH - H_2O (7 : 3) fraction, which was concentrated by evaporation and extracted with n-butyl alcohol. The organic layer was evaporated and applied to a column of Sephadex LH-20 (2.5 i.d. × 90 cm), and then developed with MeOH - H_2O (3 : 7). The active fractions were concentrated and chromatographed on a HPLC (column, PLRP-S, 7.5 × 300 mm, 8 μm, Phenomenex; mobile phase, acetonitrile - water (1 : 9); flow rate, 1.5 ml/minute; detection, UV at 220 nm). Final purification of MR304A was performed using preparative 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). MR304A (1.5 mg) was obtained as a brown powder.

Table 1. Physico-chemical properties of MR304A.

Appearance	Brown powder
$[\alpha]_D^{25}$	−36° (*c* 0.075, CH_3OH)
Molecular fomular	$C_8H_{11}NO_4$
ESI-MS[a]	
$(M+Na)^+$	208
$(M-H)^-$	184
HRCI-MS $(M+H)^+$	$C_8H_{12}NO_4$
Calcd:	186.0766
Found:	186.0755
UV (MeOH) λ_{max} nm (ε)	209 (4,204), 268 (726)
IR (KBr) cm^{-1}	3361, 2121, 1295, 1025

[a] Electrospray ionization MS.

Table 2. ^1H and ^{13}C NMR chemical shifts and coupling constants for MR304A.

Carbon No.	^{13}C Chemical shifts in ppm[a] (150 MHz)	^1H Chemical shifts in ppm[a] (600 MHz)
1	17.29 (q, J=125.7 Hz)	1.25 (3H, d, J=6.5 Hz)
2	71.07 (ddq, J=143.9, 5.0, 5.0 Hz)	4.02 (1H, q, J=6.5 Hz)
3	80.21 (d, J=3.3 Hz)	
4	75.99 (ddd, J=143.9, 8.3, 4.1 Hz)	4.82 (1H, dd, J=1.4, 1.4 Hz)
5	133.10 (br s)	
6	132.46 (d, J=173.7 Hz)	6.09 (1H, dd, J=3.1, 1.4 Hz)
7	80.00 (dd, J=150.5, 3.3 Hz)	4.35 (1H, dd, J=3.1, 1.4 Hz)
8	169.10 (s)	

[a] Sample was dissolved in CD_3OD.

The physico-chemical properties of MR304A are summarized in Table 1. The observation of characteristic absorption at 2121 cm^{-1} in the IR spectrum (Laser Precision Analytical, IFX-65s) indicated the presence of an isonitrile group[2]. The MR304A did not show clear molecular ion peak in the EI- and FAB-MS but did show in the electrospray ionization MS (VQ Quattro 4000, Manchester, U.K.) and CI-MS (JMS-HX 110A/110A, JEOL, Japan). Molecular formular of MR304A is determined as $C_8H_{11}NO_4$ by high resolution CI-MS data m/z 186.0755 $(M+H)^+$. The ^1H and ^{13}C NMR spectral data for MR304A are shown in Table 2. The ^{13}C NMR data indicated that the presence of one tri-substituted double bond, three oxygenated methines, one oxygenated quaternary carbon, one methyl and one isonitrile carbon at 169.10 ppm. These data and unsaturation number of four suggested that MR304A was an analogue of the trichoviridin type isonitrile antibiotics[8]. PFG (pulsed field gradient) DQF-COSY[9] and PFG-HMQC[10] data indicated that the protons of the methyl group at 1.25 ppm (d, $J=6.5$ Hz) are coupled to the methine at 4.02 ppm (q, $J=6.5$ Hz) and 71.07 ppm in ^{13}C NMR. These data also suggested two remaining oxymethine groups at 4.35 ppm (dd, $J=3.1$ and 1.4 Hz) and 4.82 ppm (dd, 1.4 and 1.4 Hz) that are located on the allylic position of the double bond at 6.09 ppm (dd, 3.1 and 1.4 Hz) in ^1H NMR. To confirm the planar structure and NMR assignments for MR304A, PFG-HMBC[11,12], NOE difference spectra were measured and the data obtained are summarized in Figs. 1 and 2. Based on the these spectral data the constitution for MR304A was determined to be 1-(1,2,5-trihydroxy-3-isocyanopent-3-enyl)-ethanol. MR304A is an isomer of 1-(1,4,5-trihydroxy-3-isocyanopent-2-enyl)ethanol which has been isolated from *T. hamatum*[1]. The relative stereochemistry of MR304A was suggested by the NOE data and vicinal C-H coupling constant values ($^3J_{CH}$)[13]. The coupling constant value $^3J_{C2,H4}=5.0$ Hz and NOE between H-2 and H-4 suggested that the dihedral angle between C-2 and H-4 is about 30° with syn stereochemistry. On the contrary, the coupling constant value between C-2 and H-7 was $^3J_{C2,H7}=ca.$ 0 Hz and the dihedral angle between C-2 and H-7 must be about 90°. Therefore the relative stereochemistry of C-2 and OH-7 could be determined to syn. Stereochemistry of C-2 is probably as shown in Fig. 3. From the values of the coupling constant ($^3J_{C7,H2}=ca.$ 0 Hz) and the observed NOE data between the methyl protons and H-7 the sterically favored anti conformation between OH-3 and H-2 is proposed. To confirm the relative stereochemistry of MR304A, some chemical studies or X-ray analysis may be necessary.

Inhibitory effects of MR304A and known melanogenesis inhibitors on mushroom tyrosinase and melanin

Fig. 1. Long-range C-H correlations observed in PFG-HMBC spectrum of MR304A.

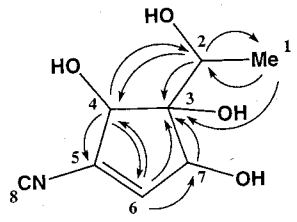


Fig. 2. NOE data with percentage of increase.

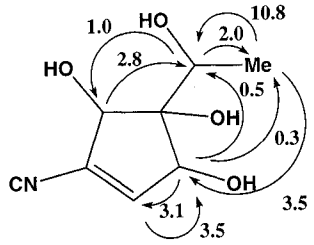
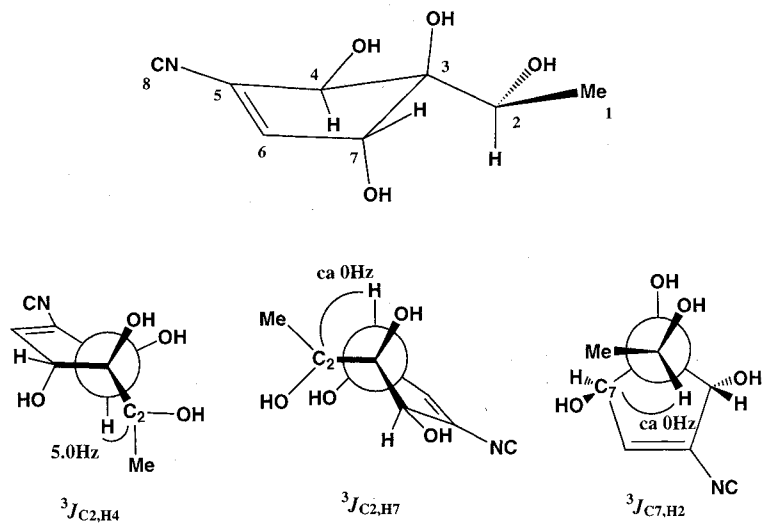


Fig. 3. Structure of MR304A with proposed relative stereochemistry and $^3J_{C,H}$ values.



formation in *S. bikiniensis* and B16 melanoma cells are shown in Table 3. MR304A inhibited mushroom tyrosinase strongly with IC_{50} value of 7.5 μg/ml in comparison with kojic acid and also inhibited melanin synthesis in *S. bikiniensis*. As shown in Fig. 4 the inhibition of MR304A against mushroom tyrosinase is noncompetitive with tyrosine, the *Ki* value of it being 4×10^{-5} M. MR 304A inhibited the melanogenesis of B16 melanoma cells with no inhibitory effect on cell growth at a concentration of 1 μg/ml.

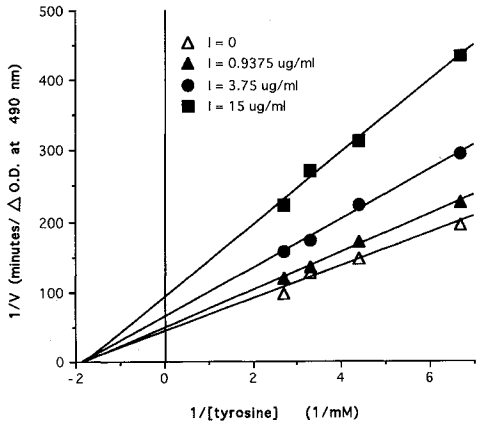
MR304A was shown no antimicrobial activity at 1 mg/ml concentration against *Escherichia coli* AB 11513, *Salmonella typhimurium* TV 119, *Staphylococcus aureus* IFO 12732 and *Candida albicans* IFO 1594 in paper disc agar diffusion method.

Table 3. Inhibitory effects on mushroom tyrosinase and melanin formation of *Streptomyces bikiniensis* and B16 melanoma cells.

Compound	*S. bikiniensis* NRRL B-1049[a]	B16 Melanoma	Mushroom tyrosinase
	Inhibition zone (cm)	IC_{50} (μg/ml)	IC_{50} (μg/ml)
MR-304A	2.5	1.0	7.5
Kojic acid	0	15.0	10.0
Hydroquinone	2.5	—[b]	1.0
Arbutin	—[b]	10.0	15.0
p-Methoxy-phenol	3.0	—[b]	15.0

[a] Compound 30 μg/ paper disc.
[b] Not determined.

Fig. 4. Lineweaver-Burk plot of mushroom tyrosinase by MR304.



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