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

II. Physico-chemical Properties and Structural Elucidation

CHOONG HWAN LEE, HIROYUKI KOSHINO†, MYUNG CHUL CHUNG, HO JAE LEE, JONG KI HONG††, JONG SHIN YOO†† and YUNG HEE KHO*

Korea Research Institute of Bioscience & Biotechnology,
Yusong P.O. Box 115, Taejon 305-333, Korea
†The Institute of Physical and Chemical Research (RIKEN),
Wako-Shi, Saitama 351-01, Japan
††Korea Basic Science Institute,
Yusong P.O. Box 41, Taejon 305-333, Korea

(Received for publication December 6, 1996)

New melanin synthesis inhibitors (MR566A and B) and six related known isocyanocyclopentenes were isolated from the fermentation broth of *Trichoderma harzianum*, and their structures were elucidated by spectroscopic methods. The structures of novel isocyanides, MR566A (**1**) and B (**2**), were elucidated as 1-(3-chloro-1,2-dihydroxy-4-isocyano-4-cyclopenten-1-yl)ethanol, 1-(1,2,3-trihydroxy-3-isocyano-4-cyclopenten-1-yl)ethanol, respectively. The structure of novel oxazole, MR93B (**9**), was elucidated as 4-[(1*Z*)-3-hydroxy-2-hydroxymethyl-1-propen-1-yl]oxazole.

MR566A (**1**) and B (**2**) were isolated as new melanin biosynthesis inhibitors from the culture broth of *Trichoderma harzianum* which had been isolated from a soil sample, together with a new oxazole, MR93B (**9**) and six known isocyanide compounds[1]. The eight isocyanide compounds (**1**~**8**) showed melanogenesis inhibitory activities in *Streptomyces bikinienesis* and B16 melanoma cells. In the preceding paper[1] we described the taxonomy of the producing strain, fermentation, isolation and the biological activities of these compounds. In what follows, we present the elucidation of the structures of **1**, **2** and **9** with relative stereochemistry of **7** and NMR assignments of **3** and **5**. Compounds **3** and **5** have been purified from *T. hamatum* as rhodium complexes by BOYD *et al.*[2]. However there were no complete NMR assignments of intact compounds.

Results and Discussion

Structural Determination of MR566A (1)

The physico-chemical properties of MR566A (**1**) are summarized in Table 1. The observation of characteristic absorption at 2121 cm^{-1} in the IR spectrum of **1** indicated the presence of an isocyano group[3]. From the observation of CI-MS, the molecular weight of **1** could be assigned as 203. The molecular formula of **1** was

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

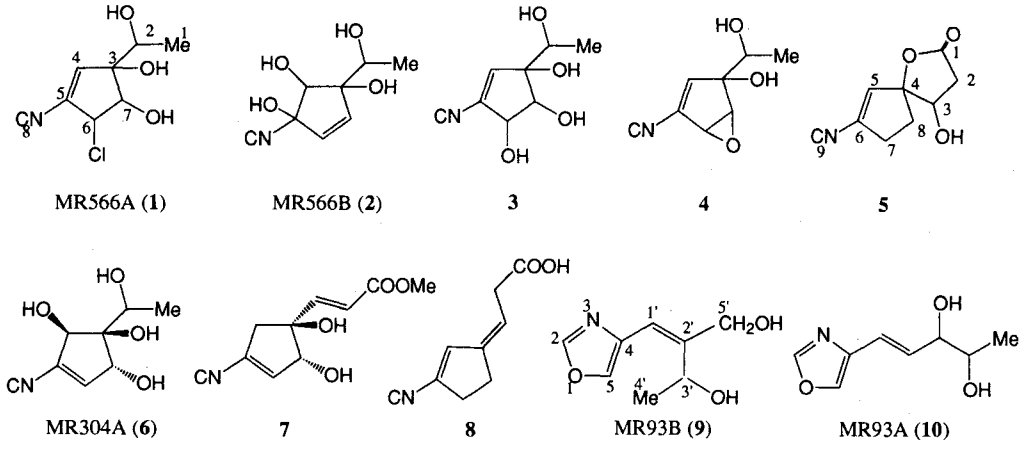


Table 1. Physico-chemical properties of **1**, **2** and **9**.

	1	**2**	**9**
Appearance	Brown powder	Brown powder	Colorless oil
$[\alpha]_D^{25}$	10° (*c* 0.1, MeOH)	70° (*c* 0.2, MeOH)	- 4.0° (*c* 0.1, MeOH)
Molecular formula	$C_8H_{10}ClNO_3$	$C_8H_{11}NO_4$	$C_8H_{11}NO_3$
HRCI-MS *m/z* $(M+H)^+$	204.0437 (calcd 204.0427)		170.0817 (calcd 170.0812)
IR (KBr) cm^{-1}	3361, 2121, 1051, 759	3413, 2138, 1335, 1159, 970	3361, 2929, 1681, 1517, 1400, 1068, 617
UV (MeOH)λ_{max} nm (ε)	End	End	230 (15138)

Table 2. ^1H NMR data for MR566A (**1**), MR566B (**2**), **3**, **4** and **5** in CD_3OD.

Position	**1** (600 MH)	**2** (300 MHz)	**3** (300 MHz)	**4** (300 MHz)	**5**[a] (500MHz)
1	1.18 (3H, d, 6.6)	1.33 (3H, d, 6.3)	1.17 (3H, d, 6.0)	1.19 (3H, d, 6.3)	
2	3.73 (1H,q, 6.6)	3.26 (1H, q, 6.3)	3.70 (1H, q, 6.0)	3.70 (1H, q, 6.3)	2.66 (1H, dd, 18.1, 2.8) 3.16 (1H, dd, 18.1, 5.9)
3					4.5 (1H, dd, 5.9, 2.8)
4	6.18 (1H, d, 1.2)	4.12 (1H, dd, 1.2, 1.2)	6.0 (1H, d, 1.2)	5.90 (1H, dd, 2.4, 2.4)	
5					6.23 (1H, s)
6	4.72 (1H, dd, 4.5, 1.2)	6.08 (1H, dd, 6.0, 1.2)	4.49 (1H, dd, 4.5, 1.2)	3.90 (1H, dd, 2.7, 2.7)	
7	4.25 (1H, d, 4.5)	6.39 (1H, dd, 6.0, 1.2)	3.89 (1H, d, 4.5)	3.74 (1H, dd, 2.7, 2.4)	2.84 (1H, m) 2.74 (1H, m)
8					2.37 (1H, ddd, 4.5, 8.6, 14.4) 2.23 (1H, ddd, 4.5, 8.6, 14.4)

[a] Sample was dissolved in D_2O

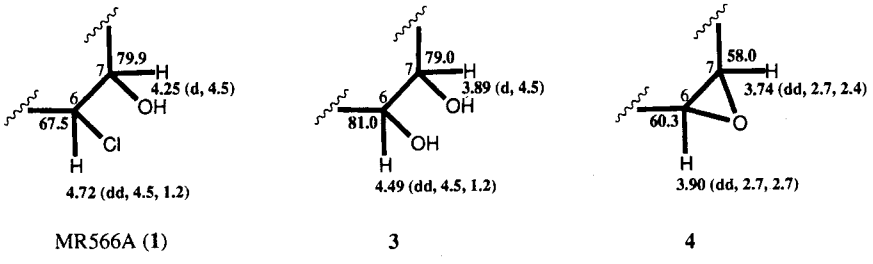
Table 3. ^{13}C NMR data for MR566A (**1**), MR566B (**2**), **3**, **4** and **5** in CD_3OD.

Position	**1** (500 MHz)	**2** (500 MHz)	**3** (500 MHz)	**4** (300 MHz)	**5**[a] (500 MHz)
1	17.7	16.8	17.9	18.8	179.3
2	71.4	75.5	71.6	72.5	38.7
3	83.2	85.1	81.9	85.1	74.5
4	137.2	87.9	134.1	138.4	101.9
5	137.1	93.0	-[b]	135.5	128.5
6	67.5	129.9	81.0	60.3	-[b]
7	79.9	142.2	79.0	58.0	33.6
8	170.9	164.6	169.7	169.0	33.5
9					169.1

[a] Sample was dissolved in D_2O

[b] not detected

Fig. 2. NMR data and partial structures of MR566A (**1**), **3** and **4**.



MR566A (**1**) **3** **4**

Fig. 3. Long-range C-H correlations observed in HMBC spectrum of MR566A (**1**).

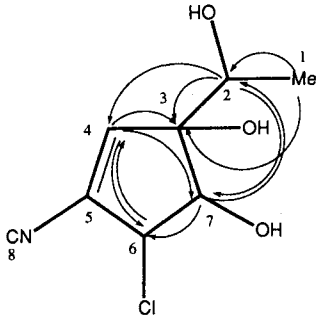
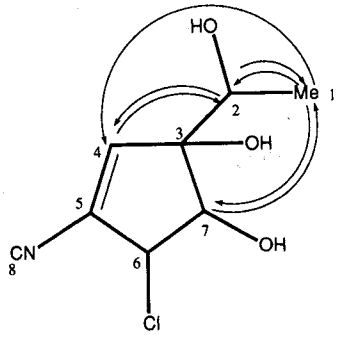


Fig. 4. NOE data of MR566A (**1**).



confirmed as $C_8H_{10}ClNO_3$ by the high resolution MS data [calcd.: 204.0427 for $(M+H)^+$, found: 204.0437 in the positive ion mode and calcd.: 203.0349 for M^-, found: 203.0346 in the negative ion mode]. The ^1H and ^{13}C NMR spectral data for **1** are shown in Tables 2 and 3. One tri-substituted double bond, two oxygenated methines, one chlorinated methine, one oxygenated quaternary carbon, one methyl, and one isocyano carbon at 171.0 ppm were found from the ^1H and ^{13}C NMR spectra of **1**. These data suggested that **1** was an analogue of the trichoviridin type isocyanide antibiotic[4,5]. The presence of a chlorine atom was confirmed by the HR-MS data and the detection of the M+2 peak of the isotope having approximately one-third of the intensity of the molecular ion peak, together with absorption at 795 cm^{-1} in the IR spectrum. The position of the chlorine atom was determined to be at C-6 based on its chemical shift. In the ^{13}C NMR data of **1**, the C-6 signal was observed at 67.5 ppm which was upfield compared with that of **3** with hydroxyl group and was lower than that of **4** with epoxy (Fig. 2). The ^1H NMR spectral data of **1** was similar to those of **3** and **4**, but the C-6 methine signal appeared down field compared with those of **3** and **4**. These data indicated that **1** was a chlorinated analogue of **3**. Finally the planar structure of **1** was determined by a HMBC experiment and its data are summarized in Fig. 3. NOE data (Fig. 4) also supported the proposed structure. Based on this spectral evidence, the structure of **1** was elucidated to be 1-(3-chloro-1,2-dihydroxy-4-isocyano-4-cyclopenten-1-yl)-ethanol. According to the best of our knowledge, this is the first report of isocyanide compound with a chlorine atom from *Trichoderma* sp.

Structural Determination of MR566B (**2**)

The physico-chemical properties of **2** are summarized in Table 1. No parent ion for **2** was detected in several MS experiments. The molecular formula of **2** was determined to be $C_8H_{11}NO_4$ by CI-MS data of dehydrated ion peak at *m/z* 168 $(M+H-H_2O)^+$ and ^1H and ^{13}C NMR data (Tables 2 and 3). In the ^{13}C NMR spectrum of **2**, two olefinic methines, two oxygenated methines, one methyl, and three quaternary carbons were detected. In the IR spectrum of **2**, absorption of isocyano group was observed at 2138 cm^{-1}. The carbon signal of isocyanide was observed at 164.6 ppm which is shifted upfield compared to those of α,β-unsaturated isocyano carbons (Table 3). Two sp^3 quaternary carbons were assigned to be oxygenated from chemical shifts at 85.1 ppm (C-3) and 93.0 ppm (C-5). We speculated that the extremely low chemical shift of C-5 was caused by

the attachment of the isocyano group and oxygen. All of the oxygens of the four oxygenated carbons were assigned to the carbons with a hydroxyl group. The possibility of the presence of an epoxide group was disproved by the ^{13}C chemical shift and the values of $^{1}J_{CH}$ for C-2 and C-4, 148 Hz and 158 Hz, respectively[6,7]. The positions of the double bond and oxygenated carbons together with all NMR assignments were determined by C-H long-range correlations observed in the HMBC spectrum (Fig. 5). Based on these spectral data, the structure of **2** was determined to be 1-(1,2,3-trihydroxy-3-isocyano-4-cyclopenten-1-yl)ethanol. Presence of the tertiary alcohol at C-5 with an isocyano group might be the reason for the difficulty in detecting the parent ion in several MS spectra.

Relative Stereochemistry of 7

Compound **7** has been isolated from *T. hamatum* by BALDWIN *et al.*[8]. However, there have been no reports on the stereochemisty of **7**. The relative stereochemistry of **7** was determined by NOE (Fig. 6). From the observed NOE data between H-8 and H-5β, the relative stereochemistry of H-8 and H-5β could be determined to *syn*. From the strong NOE between C-3 methine proton and H-5α, the relative configuration between C-3 and H-5α could be proposed.

Structural Determination of MR93B (9)

The physico-chemical properties of MR93B (**9**) are also summarized in Table 1. Compound **9** was purified as a colorless oil. The HRCI-MS gave a $(M+H)^+$ peak at *m/z* 170.0817 for $C_8H_{11}NO_3$ (calcd 170.0812). In the UV spectrum of **9** only a single maximum was observed at 230 nm (ε 15138). The ^{1}H, ^{13}C and HMBC NMR data of **9** are shown in Table 4. The ^{1}H NMR spectrum exhibited three singlet signals for H-2, H-5 and H-1', one doublet for methyl protons H-4', one doublet of doublets for methylene protons for H-5', and a quartet for methine proton H-3'. The ^{13}C NMR, C-H COSY and DEPT spectra exhibited signals for four CH carbons, one CH_2 group, one CH_3 group, and two quaternary carbons. By comparison of spectral data of **9** to those of MR93A

Fig. 5. Long-range C-H correlations observed in HMBC spectrum of MR566B (**2**).

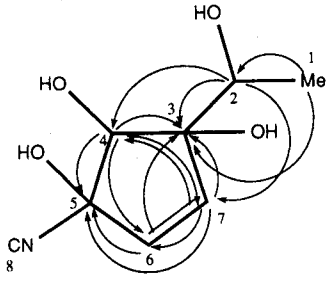


Fig. 6. NOE data with percentage of enhancement of **7**.

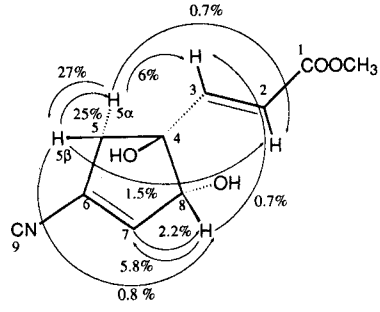


Table 4. ^{1}H, ^{13}C and HMBC NMR data for MR93B (**9**) in $CDCl_3$.

Position	^{1}H(ppm)	^{13}C(ppm)	HMBC[a]
2	7.91 (1H, s)	150.7 (dd, 230.7, 7.9)	C-5
4		137.2 (s)	
5	7.66 (1H, s)	137.0 (d, 208.1)	C-4
1'	6.34 (1H, s)	112.9 (d, 149.5)	C-4, C-2', C-3', C-5'
2'		146.8 (s)	
3'	4.81 (1H, q, 6.75)	66.8 (d, 159.9)	
4'	1.44 (3H, d, 6.75)	21.9 (q, 126.4)	C-2', C-3'
5'	4.30 (2H, dd, 13.7, 48.3)	65.6 (t, 159.9)	C-1', C-2'

[a] Carbon resonances that were long-range correlated with protons.

Table 5. ^{13}C NMR chemical shift and $^{1}J_{CH}$ of oxazole ($CDCl_3$).

	^{13}C (ppm)			$^{1}J_{CH}$ (Hz)		
	C-2	C-4	C-5	C(2)-H(2)	C(4)-H(4)	C(5)-H(5)
Oxazole*	150.6	124.5	138.1	230	194	210
9	150.7		137.0	230.7		208.1
10	151.3		137.8	230.5		206.0

* Reference (ADAMCZESKI *et al.*)[12]

(**10**) and melanoxazal[9,10], it was speculated that **9** was analogue of monosubstituted oxazole, **10**. The ^{13}C NMR chemical shift of C-5′ (65.6 ppm) exhibited that C-5′ of **9** was a hydroxyl methyl instead of an aldehyde (192 ppm) of melanoxazal. From these data the side chain was determined to be 3-hydroxy-2-hydroxymethyl-1-propenyl group. The position of side chain on the monosubstituted oxazole was established as C-4 by chemical shifts of ^{13}C NMR and HMBC data. To confirm the substitution pattern of the oxazole, the $^{1}J_{CH}$ values were measured. From the obtained $^{1}J_{CH}$ values, $^{1}J_{C_2H_2}=230.7$ Hz and $^{1}J_{C_5H_5}=208.1$ Hz, the position of the side chain was assigned to C-4[9,11] (Table 5). An *Z*-orientation of the 1′,2′-double bond was established from NOE between 1′-H and 5′-H (data not shown). Based on the above mentioned spectral data, the structure of **9** was determined to be 4-[(1*Z*)-3-hydroxy-2-hydroxymethyl-1-propen-1-yl]oxazole.

Experimental

General Procedure

NMR spectra were recorded on a Bruker AMX-500 and JEOL JNM-A600 spectrometers in CD_3OD, D_2O or $CDCl_3$ solutions. HRCI-MS were obtained on JMS-SX102A double focusing mass spectrometer, using methane as a reagent gas. The instrument was calibrated for both negative and positive ion mode using perfluorokerosene as a reference compound. Samples were introduced into the ion source using direct insertion probe. The source temperature was set at 120°C. ESI-MS were obtained on VQ Quattro 4000. Infrared spectra (KBr pellet) and ultraviolet spectra were taken on a Laser Precision Analytical IFX-65s and Shimadzu UV-260 spectrophotometer, respectively. Optical rotation was recorded on a Schmidt+Haensch POLARTRONIC polarimeter.

1-(1,4,5-Trihydroxy-3-isocyanocyclopenten-2-enyl)-ethanol (**3**): White powder; UV (MeOH) λ_{max} nm (ε) end; $C_8H_{11}NO_4$; ESI-MS *m/z* 184 $(M-H)^-$.

2-Hydroxy-4-isocyano-α-methyl-6-oxabicyclo[3.1.0]-hex-3-ene-2-methanol (**4**): Brown powder; UV (MeOH) λ_{max} nm (ε) 218 (5172); $C_8H_9NO_3$; $[\alpha]_D$ −82°, *c* 0.1.

4-Hydroxy-8-isocyano-1-oxaspiro[4.4]cyclonon-8-en-2-one (**5**): Brown powder; UV (MeOH) λ_{max} nm (ε) 229 (4453); $C_9H_9NO_3$; ESI-MS *m/z* 202 $(M+Na)^+$.

Methyl-3-(1,5-dihydroxy-3-isocyanocyclopent-3-enyl)prop-2-enoate (**7**): Brown powder; UV (MeOH) λ_{max} nm (ε) end; $C_{10}H_{11}NO_4$; ESI-MS *m/z* 232 $(M+Na)^+$.

References

1) LEE, C. H.; M. C. CHUNG, H. J. LEE, K. S. BAE & Y. H. KHO: MR566A and MR566B, New melanin synthesis inhibitors produced by *Trichoderma harzianum*. I. Taxonomy, fermentation, isolation and biological activities. J. Antibiotics 50: 469～473, 1997
2) BOYD, R. K.; A. J. MCALEESS, A. TAYOR & J. A. WALTER: Isolation of new isocyanide metabolites of *Trichoderma hamatum* as their (η^5-pentamethylcyclopentadienyl)- or (η^5-ethyltetramethylcyclopentadienyl)-di-μ-thiocyanato-rhodium complexes. J. Chem. Soc. Perkin Trans. 1: 1461～1465, 1991
3) BREWER, D.; E. J. GABE, A. E. HANSON & A. TALOR: Isonitrile acids from cultures of the fungus *Trichoderma hamatum*. J. Chem. Soc. Chem. Comm.: 1061～1062, 1979
4) TURNER, W. D. & D. C. ALDRIDGE: Fungal metabolites II. p. 474, Academic Press, London, 1983
5) LEE, C. H.; H. KOSHINO, M. C. CHUNG, H. J. LEE & Y. H. KHO: MR304A, a new melanin synthesis inhibitor produced by *Trichoderma harzianum*. J. Antibiotics 47: 1168～1170, 1995
6) KOBAYASHI, H.; R. SUNAGA, K. FURIHATA, N. MORISAKA & S. IWASAKI: Isolation and structures of an antifungal antibiotic, fusarielin A, and related compounds produced by a *Fusarium* sp. J. Antibiotics 48: 42～52, 1995
7) SEKI-ASANO, M.; Y. TSUCHIDA, K. HANADA & K. MIZOUE: Structures of new 18-membered macrolides FD-891 and FD-892. J. Antibiotics 47: 1234～1241, 1994
8) BALDWIN, J. E.; R. M. ADLINGTON, J. CHONDROGIANNI, M. S. EDENBOROUGH, J. W. KEEPING & C. B. ZIEGLER: Structure and synthesis of new cyclopentenyl isonitriles from *Trichoderma hamatum* (Bon.) Bain. aggr. HLX 1379. J. Chem. Soc. Chem. Commun.: 816～817, 1985
9) LEE, C. H.; M. C. CHUNG, H. J. LEE, Y. H. KHO & H. KOSHINO: MR93A, a new oxazole from *Trichoderma harzianum*. KCTC 0114BP. J. Nat. Prod. 58: 1605～1607, 1995
10) TAKAHASHI, S.; R. HASHIMOTO, K. HAMANO, T. SUZUKI & A. NAKAGAWA: Melanoxazal, new melanin biosynthesis inhibitor discovered by using the larval haemolymph of the silk worm, *Bombyx mori*. J. Antibiotics 49: 513～518, 1995
11) ADAMCZESKI, M.; E. QUINOA & P. CREWS: Unusal anthelminthic oxazoles from a marine sponge. J. Am. Chem. Soc. 110: 1598～1602, 1988