VOL. 56 NO. 12, DEC. 2003 THE JOURNAL OF ANTIBIOTICS pp. 1000 — 1003

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

II. Physico-chemical Properties and Structure Elucidation

JONG-PYUNG KIM, BYOUNG-KWON KIM, BONG-SIK YUN, IN-JA RYOO, IN-KYOUNG LEE, WON-GON KIM, YU-RYANG PYUN[a] and ICK-DONG YOO*

Korea Research Institute of Bioscience and Biotechnology, Yuseong, Daejeon 305-600, Korea
[a] Department of Biotechnology & Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea

(Received for publication July 25, 2003)

New melanin synthesis inhibitors, melanocins A, B and C, were isolated from the fermentation broth and extract of mycelium of *Eupenicillium shearii* F80695. The structures of melanocins were established by spectroscopic methods. They are formamide compounds. In particular, melanocin A has an isocyanide group.

In the screening program for melanin biosynthesis inhibitors, we discovered novel substances designated melanocins A (**1**), B (**2**) and C (**3**) from the culture broth and mycelial cake of *Eupenicillium shearii* F80695. In the preceding paper[1] we described the taxonomy, fermentation, isolation, and biological properties of **1**~**3**. This article describes the physico-chemical properties and structural elucidations of **1**~**3** by spectroscopic studies including various two-dimensional NMR experiments.

Results and Discussion

Physico-chemical Properties

The physico-chemical properties of melanocins (**1**~**3**) are summarized in Table 1. Compounds **1**~**3** were readily soluble in DMSO and MeOH, but insoluble in $CHCl_3$ and H_2O. Compound **1** was obtained as yellow powder with the melting point of 212°C. Compounds **2** and **3** were obtained as dark brown powders. The UV absorption maxima of **1**~**3** were 220 and 336 nm (**1**), 253 and 341 nm (**2**) and 241 and 341 nm (**3**), which exhibited characteristic bathochromic shifts in alkaline solution typical for phenols. On the basis of high-resolution FAB-MS and NMR spectral analyses, the molecular formula of **1** was determined to be $C_{18}H_{14}N_2O_5$ based on the negative ion mode of FAB-MS [found *m/z* 337.0821 $(M-H)^-$, calcd. 337.0824 for $C_{18}H_{13}N_2O_5$]. The molecular formulas of **2** and **3** were established as $C_{17}H_{15}NO_6$ [found *m/z* 330.0982 $(M+H)^+$, calcd. 330.0987 for $C_{17}H_{16}NO_6$] and $C_{18}H_{14}N_2O_6$ [found *m/z* 355.0933 $(M+H)^+$, calcd. 355.0930 for $C_{18}H_{14}N_2O_6$], respectively. The IR spectra of **1**~**3** indicated the presence of hydroxyl groups [3458 cm^{-1} (**1**), 3447 cm^{-1} (**2**) and 3463 cm^{-1} (**3**)] and carbonyl groups [1690 cm^{-1} (**1**), 1695 cm^{-1} (**2**) and 1705 cm^{-1} (**3**)]. The characteristic intense IR absorption of **1** at 2110 cm^{-1} indicated the presence of isocyanide group[2].

Structure Elucidation

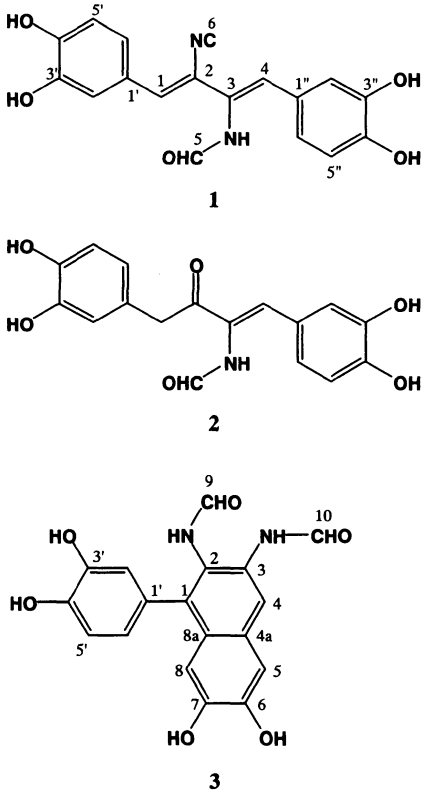
The structures of melanocins A (**1**), B (**2**) and C (**3**) were mainly deduced from various NMR spectral analyses including ^1H NMR, ^{13}C NMR, DEPT, ^1H-^1H COSY, PFG (pulsed field gradient)-HMQC and PFG-HMBC experiments (Fig. 1). The ^1H and ^{13}C NMR spectral data of **1**~**3** are shown in Table 2.

* Corresponding author: idyoo@kribb.re.kr

Table 1. Physico-chemical properties of melanocins A (**1**), B (**2**) and C (**3**).

	1	**2**	**3**
Appearance	Yellow powder	Dark brown powder	Dark brown powder
MP	212 °C	223 °C	190~200 °C (dec.)
Molecular weight	338	329	354
Molecular formula	$C_{18}H_{14}N_2O_5$	$C_{17}H_{15}NO_6$	$C_{18}H_{14}N_2O_6$
FAB-MS (*m/z*)	339 $(M+H)^+$	330 $(M+H)^+$	355 $(M+H)^+$
HRFAB-MS (*m/z*)			
Found :	337.0821 $(M-H)^-$	330.0980 $(M+H)^+$	355.0933 $(M+H)^+$
Calcd. :	337.0824 for $C_{18}H_{13}N_2O_5$	330.0978 for $C_{17}H_{16}NO_6$	355.0930 for $C_{18}H_{15}N_2O_6$
UV λ_{max} nm (ε) in MeOH	220 (32,300), 336 (13,100)	253 (31,400), 341 (8,300)	241 (42,650), 341 (5,150)
IR ν_{max} cm^{-1} (KBr)	1690, 2110, 2843, 2930, 3458	1695, 2361, 2925, 2930, 3447	1705, 2110, 2853, 2924, 3463
TLC (Rf value)	0.86	0.76	0.39

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



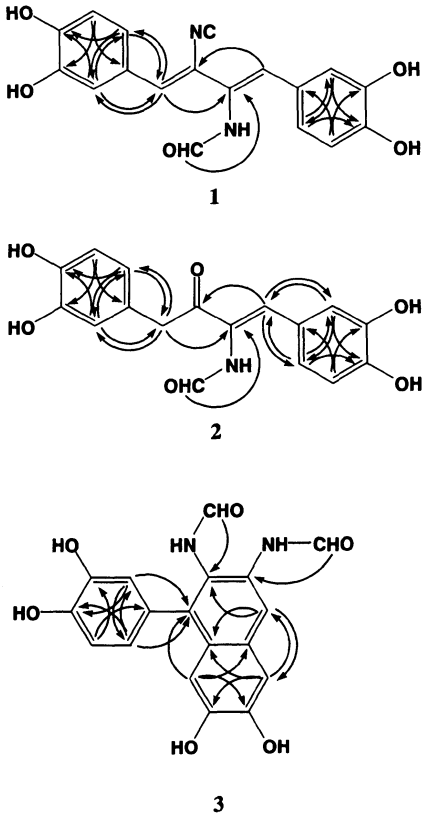
Melanocin A (**1**)

The ^{13}C NMR spectrum of **1** demonstrates 18 signals which were assigned to nine methines and nine quaternary carbons by DEPT experiments. The ^1H NMR spectrum of **1** recorded in CD_3OD indicated the presence of two 1,3,4-trisubstituted benzene rings [δ_H 6.76 (d), 6.91 (d) and 7.08 (s), and δ_H 6.6.81 (d), 7.11 (d) and 7.36 (s)], according to the coupling patterns. In addition, two olefinic protons at δ_H 6.65 and 6.82 and one aldehyde proton at δ_H 8.30 were observed. The aldehyde proton at δ_H 8.30 (H5) was considered as an aldehyde proton of formamido group by its upfield shift. The connectivity of proton and carbon atoms was established by PFG-HMBC experiment, and the data are summarized in Fig. 2. In the PFG-HMBC spectrum of **1**, the olefinic proton at δ_H 6.65 (H1) was correlated to the aromatic carbons at δ_C 117.2 (C2′) and 124.2 (C6′), and the quaternary carbon at δ_C 126.3 (C3). The other olefinic proton at δ_H 6.82 (H4) was correlated to the aromatic carbons at δ_C 116.8 (C2″) and 123.2 (C6″), and the quaternary carbon at δ_C 120.0 (C2). These HMBC data demonstrate the connectivity of the benzene rings and the butadiene moiety. The aldehyde proton of formamido moiety showed long-range coupling to the quaternary carbon C3. The unusually large chemical shift of δ_C 171.7 (C6) can be explained by the isocyanide group of which the presence had been expected from IR spectrum. Melanocin A is a formamide compound with an isocyanide group.

Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for melanocins A (**1**), B (**2**) and C (**3**) in CD_3OD.

	1		2			3	
Position	^{13}C (δ)	^{1}H (δ)	^{13}C (δ)	^{1}H (δ)	Position	^{13}C (δ)	^{1}H (δ)
1	127.8	6.65 (1H, s)	45.0	3.90 (2H, s)	1	130.2	
2	120.0		198.2		2	130.1	
3	126.3		129.5		3	122.7	
4	127.1	6.82 (1H, s)	137.4	7.37 (1H, s)	4	119.6	8.26 (1H, s)
5	163.4	8.30 (1H, s)	164.0	8.25 (1H, s)	4a	137.9	
6	171.7				5	110.5	7.10 (1H, s)
1'	126.5		127.8		6	148.5	
2'	117.2	7.36 (1H, s)	117.5	6.69 (1H, s)	7	147.7	
3'	148.5		146.2		8	110.0	6.81 (1H, s)
4'	146.4		145.2		8a	128.0	
5'	116.4	6.81 (1H, d, J=8.4 Hz)	116.4	6.70 (1H, d, J=8.2 Hz)	9	162.3	8.33 (1H, s)
6'	124.2	7.11 (1H, d, J=8.4 Hz)	121.8	6.55 (1H, d, J=8.2 Hz)	10	163.9	8.05 (1H, s)
1"	127.7		126.2		1'	130.0	
2	116.8	7.08 (1H, s)	118.0	7.18 (1H, s)	2'	118.2	6.70 (1H, s)
3"	147.4		146.5		3'	146.3	
4"	146.3		149.6		4'	146.0	
5"	116.4	6.76 (1H, d, J=8.4 Hz)	116.0	6.80 (1H, d, J=8.4 Hz)	5'	116.3	6.88 (1H, d, J=8.3 Hz)
6"	123.2	7.91 (1H, d, J=8.4 Hz)	125.3	7.00 (1H, d, J=8.4 Hz)	6'	122.5	6.58 (1H, d, J=8.3 Hz)

Fig. 2. ^{13}C-^{1}H long-range correlations for **1**, **2** and **3** observed in PFG-HMPC spectra.



Related isocyanide with two aromatic rings, darlucin A which has 1,2-diisocyanide groups, has been reported from a fungus *Sphaerellopsis filum*[3] as an antibiotic.

Melanocin B (**2**)

The ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1**. The ^{13}C NMR spectrum of **2** showed 17 signals, which were assigned to nine methines and eight quaternary carbons by DEPT experiments. Instead of highly upfield shifted isocyanide carbon, a new carbonyl carbon was observed at δ_C 198.2. The ^{1}H NMR signals of **2** (CD_3OD) were similar to those of **1** for two 1,3,4-trisubstituted benzene rings [δ_H 7.37 (d), 7.00 (d) and 7.18 (s), and δ_H 6.69 (d), 6.55 (d) and 6.70 (s)] and aldehyde proton of formamido group ([δ_H 8.25 (s)]. However, instead of the two olefinic protons of **1**, one olefinic proton [δ_H 7.37 (s)] and one methylene protons [3.90 (s)] were observed in ^{1}H NMR of **2**. In the PFG-HMBC spectrum of **2**, the olefinic proton at H1 was correlated to the aromatic carbons of C2′ and C6′, and the quaternary carbon C3. The other olefinic proton at δ_H 6.82 (H4) was correlated to the aromatic carbons of C2″ and C6″, and the quaternary carbon C2. The aldehyde proton of formamide moiety showed long-range coupling to the quaternary carbon at δ_C 126.3 (C3). It was revealed that the isocyanide group of 2-isocyano-3-

foramido-1,3-butadiene back bone in **1** is changed to a carbonyl group of 3-formido-3-butene-2-one back bone in **2**.

Melanocin C (**3**)

The ^{1}H NMR spectrum of **3** in CD_3OD exhibited the signals of one 1,3,4-trisubstituted benzene rings [δ_H 6.58 (d), 6.88 (d) and 6.70 (s)], three singlet methines at δ_H 8.26 (H4), 7.10 (H5) and 6.81 (H8), and two upfield shifted aldehyde protons (δ_H 8.05 and 8.33) of formamido groups. In the ^{13}C NMR spectrum of **3**, 18 signals were observed, which were assigned to eight methines and ten quaternary carbons by DEPT experiments. The carbons observed at δ_C 162.3 and 163.9 were assigned to the carbonyl carbons of two formamide groups. The two oxygenated aromatic sp^2 carbons [δ_C 146.0 (C3′) and 146.3 (C4′)] and three aromatic methine carbons [δ_C 118.2 (C2′), 116.3 (C5′) and 122.5 (C6′)] and the quaternary carbon at δ_C 130.0 (C1′) was assigned to form an *ortho*-dihydroxybenzene ring, which was confirmed by the ^{1}H-^{13}C long-range correlations from the aromatic methine protons at δ_H 6.70 and 6.58 to the aromatic carbons in the HMBC data. The long-range correlations between aromatic methine protons and aromatic carbons, from H8 to C1, C4a and C6, from H4 to C2, C5 and C8a, from H5 to C4, C7 and C8a, confirmed the naphthalene backbone. The aldehyde protons of formamido moieties showed long-range couplings from δ_H 8.33 to C2 and from δ_H 8.05 to C3. The long-range couplings from the aromatic protons H2′ and H6′ to C1 confirmed the connectivity of *ortho*-dihydroxybenzene ring and naphthalene ring. Compound **3** which is structurally related to **1** and **2** was revealed to be an 1,2-diformamido phenolic compound.

Experimental

General

NMR spectra were recorded on a JEOL JNM-A600 spectrometer for ^{1}H NMR at 600 MHz and ^{13}C NMR at 150 MHz in CD_3OD. Chemical shifts are expressed in δ values (ppm) with TMS as an internal standard. Standard techniques were used to obtain the ^{1}H-^{1}H COSY, PFG-HMQC and PFG-HMBC spectra. The PFG-HMQC and PFG-HMBC experiments were optimized for $^{1}J_{CH}$=145 Hz and $^{2\sim3}J_{CH}$=8.3 Hz, respectively. HRFAB-MS spectra were measured on a JEOL JMS HX-110 mass spectrometer with matrix of triethanolamine. IR and UV spectra were recorded on a Laser Precision Analect RFX-65S FT-IR and Shimazu UV-260 spectrometer, respectively. The samples for IR measurements were prepared as KBr tablets. Thin layer chromatography was performed using Silica gel 60 F_{254} precoated glass plates [Merck, No. 5715 (0.25 mm)]. Preparative HPLC was carried out using a Waters HPLC equipped with 510 pump, 991 phodiode array detector and a reversed-phase column (J'sphere ODS-H-80, YMC Co. Ltd, 20 mm i.d.×250 mm).

Acknowledgments

We are thankful to Mr. ESUMI YASUAKI (RIKEN, Japan) for negative FAB MS. This work was supported by Bioproducts and Biotechnology Research grants (to I. D. YOO) and National Research Laboratory Program (to I. D. YOO) from the Korean Ministry of Science and Technology.

References

1) KIM, J. P.; B. K. KIM, B. S. YUN, I. J. RYOO, C. H. LEE, I. K. LEE, W. G. KIM, S. LEE, Y. R. PYUN & I. D. YOO: Melanocins A, B and C, new melanin synthesis inhibitors produced by *Eupenicillium shearii*. I. Taxonomy, fermentation, isolation and biological properties. J. Antibiotics 56: 993~999, 2003
2) BREWER, D.; E. J. GABE, A. E. HANSON & A. TALOR: Isonitrile acids from cultures of the fungus *Tricoderma hamatum*. J. C. S. Comm: 1061~1062, 1979
3) ZAEF, S.; M. HOBFELD, H. ANKE, R. VELTEN & W. STEGLICH: Darlucins A and B, new isocyanide antiobiotics from *Sphaerellopsis filum* (*Darluca filum*). J. Antibiotics 48: 36~41, 1995