Novoamauromine and *ent*-Cycloechinulin: Two New Diketopiperazine Derivatives from *Aspergillus novofumigatus*

Kazuki Ishikawa,[a] Tomoo Hosoe,*[,a] Takeshi Itabashi,[a] Daigo Wakana,[a] Kayoko Takizawa,[b] Takashi Yaguchi,[b] and Ken-ichi Kawai[a]

[a] *Faculty of Pharmaceutical Sciences, Hoshi University; Ebara 2–4–41, Shinagawa-ku, Tokyo 142–8501, Japan: and* [b] *Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University; Inohana 1–8–1, Chuo-ku, Chiba 260–8673, Japan.* Received October 10, 2009; accepted February 26, 2010; published online March 4, 2010

Two new diketopiperazine metabolites, novoamauromine (1) and *ent*-cycloechinulin (2) have been isolated from *Aspergillus novofumigatus* CBS117520. The structures of 1 and 2 were established on the basis of spectroscopic and chemical investigation, including a detailed comparison of the spectroscopic and physico-chemical data of amauromine (3) and cycloechinulin (4).

Key words *Aspergillus novofumigatus*; novoamauromine; *ent*-cycloechinulin; diketopiperazine

The fungus *Aspergillus fumigatus* is known as an important human pathogen and a strain that produces many secondary metabolites. The fungus *Aspergillus novofumigatus* CBS117520 was isolated originally as *A. fumigatus* from Equadorian soil in 1965. In 2005, Hong *et al.* re-identified it as the new *Aspergillus* sp., closely related to *A. fumigatus*.[1] We have isolated two new diketopiperazines, novoamauromine (**1**) and *ent*-cycloechinulin (**2**), along with *epi*-aszonalenins A and C,[2] and helivolic acid,[3—5] from the methanolic extract of this fungus cultivated on rice using a thin layer chromatography (TLC) analysis-guided fractionation. This report describes the isolation, structure, and antifungal and cytotoxic activities of **1** and **2**.

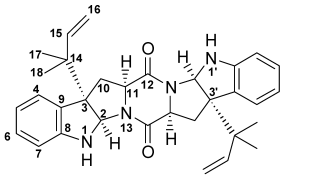
Solid-substrate fermentation cultures of *A. novofumigatus* CBS117520 grown on rice were extracted with MeOH, and the evaporated extract was suspended in water and extracted with ethyl acetate. The evaporated extract was partitioned with acetonitrile (ACN) and hexane to yield an ACN-soluble fraction. The fraction was extracted sequentially with hexane, benzene, chloroform, ethyl acetate, and MeOH. The benzene extract was chromatographed using a Sephadex LH-20 column, followed by medium pressure liquid column chromatography (MPLC) on silica gel. A positive color test with phosphomolybdic acid (5%)–ceric acid (trace) in 5% H_2SO_4 (orange or blue) on TLC led to the isolation of compounds. Further purification of these fractions by HPLC yielded compounds **1** (2 mg), **2** (5 mg), *epi*-aszonalenin A (217 mg), and C (85 mg), which are indole derivatives, and helivolic acid (162 mg), identified by comparison with the spectral data from reports in the literature.[2—5]

The molecular formula of **1** was determined as $C_{32}H_{36}N_4O_2$ by high resolution fast atom bombardment (HR-FAB) mass spectrometry. From the sixteen peaks observed in the ^{13}C-NMR spectrum, it was suggested that **1** was a dimer, which consisted of two identical partial C16 units. The presence of an amine NH group was deduced from broad absorption at 3420 cm^{-1} in the IR spectrum. The presence of a tertiary amide group was inferred from the ^{13}C-NMR spectrum (168 ppm) and broad infrared absorption at 1673 cm^{-1}. Two methyl groups at δ 1.10 (s) and δ 0.93 (s), vinyl groups at H_2-16 (δ 5.08 and δ 5.12) and H-15 (δ 5.92), CH_2–CH units at H_2-10 (δ 2.54 and δ 2.71) and H-11 (δ 4.08), and an *ortho*-disubstituted phenyl ring were deduced from ^{1}H–^{1}H correlation spectroscopy (COSY) and heteronuclear multiple quantum correlation (HMQC) data. The results of heteronuclear multiple bond correlation (HMBC) correlations of H-4 to C-3, and H-2 to C-8 and C-9, showed a dihydroindole unit incorporating the *ortho*-disubstituted phenyl ring. HMBC correlations of the methyl signals at δ 1.10 and δ 0.93, and vinyl resonance at H_2-16 showed a quaternary carbon signal (C-14) at δ 41.7, giving rise to the dimethylpropenyl unit. The HMBC correlations of methyl proton signals of the dimethylpropenyl unit at C-3 indicated that the dimethylpropenyl unit should be attached to the C-3 of the dihydroindole unit. The gross plate structure of **1** was identical to that of amauromine (**3**).[6,7] However, HPLC analysis showed that **1** is different from amauromine (**3**); the retention time (t_R) of **1** was 8.5 min and that of **3** was 7.5 min under the same conditions. These results indicated that **1** must be a diastereomer of amauromine (**3**) (Fig. 2).

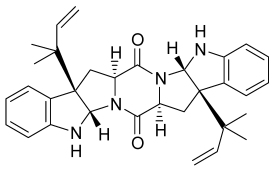
Nuclear Overhauser effect spectroscopy (NOESY) results also showed that the relative stereochemistry of **1** is different from that of amauromine (**3**) (Fig. 3). The signal for H-2 correlated with H-11 and two methyl proton signals of the dimethylpropenyl unit, and H-11 also correlated with the two methyl proton signals. Thus, H-2, H-11, and the dimethylpropenyl unit must be on the same face of the ring system. The absolute stereochemistry of amauromine (**3**) has been determined by comparing the circular dichroism (CD) spectral data of amauromine derivatives and related compounds by Takase *et al.*[7] The CD spectra of **1** showed a positive Cotton effect at 245 nm, almost identical to that of compound **5**. Therefore, compound **1** was assumed to have the same absolute configuration at C-2, C-3, and C-11 as **5**. Thus, the absolute stereochemistry of **1** was determined from the above results (Fig. 1).

The molecular formula of **2** was determined as $C_{20}H_{21}N_3O_3$ by high resolution electron ionization (HR-EI) mass spectrometry. The UV, IR, ^{1}H- and ^{13}C-NMR spectra, COSY, HMQC and HMBC data of **2** were identical to those of cycloechinulin (**4**), which was isolated from the fungus *Aspergillus ochraceus*.[8] However, the optical rotation in **2** showed that it was opposite that of **4**. Furthermore, the acid hydrolysis of **2**, followed by derivatization using 1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide (Marfey's reagent)[9,10] and HPLC comparison to alanine standards indicated that the

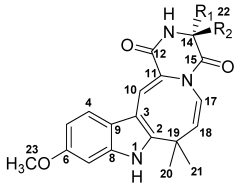
* To whom correspondence should be addressed. e-mail: hosoe@hoshi.ac.jp



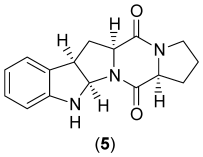
Novoamauromine (**1**)



Amauromine (**3**)



ent-Cycloechinulin (**2**) ; $R_1 = CH_3$, $R_2 = H$
Cycloechinulin (**4**) ; $R_1 = H$, $R_2 = CH_3$



(**5**)

Fig. 1. Structures of Compounds **1**—**5**

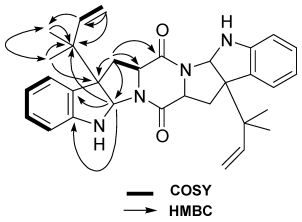


Fig. 2. Important COSY and HMBC Correlations of Novoamauromine (**1**)

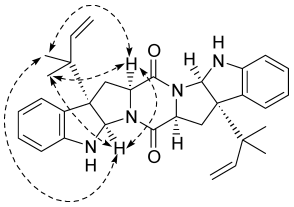


Fig. 3. Important NOESY Correlations of Novoamauromine (**1**)

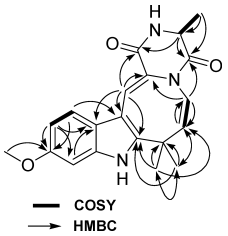


Fig. 4. Important COSY and HMBC Correlations of *ent*-Cycloechinulin (**2**)

alanyl residue obtained as the degradation product had D configuration. From the above results, it was determined that the C-14 position in **2** has *R* configuration, and **2** is an enantiomer of **4** (Fig. 1).

Antifungal activity was studied using the paper disk method, as described previously.[11] Novoamauromine (**1**) and *ent*-cycloechinulin (**2**) showed non-specific antifungal activities against *Aspergillus fumigatus*, *A. niger*, *Candida albicans*, and *Cryptococcus neoformans*, at 100 μg per disk. Compounds **1** and **2** were tested for cytotoxic activities against A549 human lung cancer cells, Hela human cervical cancer cells, and LNCap human prostate adenocarcinoma cells. Compound **1** inhibited the cell proliferation of A549, Hela, and LNCap with IC_{50} values of 97.4, 70.0, and 94.6 μM, respectively. Compound **2** did not inhibit cell proliferation at 100 μM.

We succeeded in isolating new amauromine and cycloechinulin analogs **1**—**2** from *A. novofumigatus*. Their stereochemistries are different from those of **3** and **4**, which were already isolated from other fungi. Furthermore, only the isolation of *epi*-aszonalenin A—C from *A. novofumigatus*[2] has been reported; therefore, this fungus appears to have a specific biosynthesis different from other cyclic dipeptide analogue-producing fungi. Recently, Yin *et al.* have reported the biosynthesis of an aszonalenin and its derivatives from *A. fumigatus* and *Neosartorya fischeri*, and described the stereo specific features as a *cis*-configuration between the two five-membered rings.[12—14] We hope that further investigations in the near future will reveal new information about the stereo specific biosynthesis.

Experimental

General Melting points were determined on a micro-melting point apparatus (Yanagimoto Ltd., Kyoto, Japan), and are uncorrected. FAB-MS and EI-MS data were measured using a JMS-MS700 and a JMS-MS600W spectrometer (JEOL Co., Ltd., Tokyo, Japan), respectively. UV and IR spectra were recorded on an Ultrospec 2100 pro UV-visible spectrophotometer (Amersham Biosciences Ltd., Tokyo, Japan) and a FT/IR-4100 instrument (JASCO Co. Ltd.), respectively. ^{1}H- and ^{13}C-NMR spectra were recorded using an AVANCE-400 spectrometer (400.13 MHz for ^{1}H, 100.61 MHz for ^{13}C, Bruker Biospin K. K., Kanagawa, Japan). Chemical shifts (δ) were measured in ppm using tetramethylsilane as an internal standard. Circular dichroism (CD) curves were determined on a J-820 spectropolarimeter (JASCO Co., Ltd.). Optical rotations were measured with a P-1020 photopolarimeter (JASCO Co., Ltd.). TLC was visualized by UV light at 254 nm, and/or by spraying with phosphomolybdic acid (5%)-ceric acid (trace) in 5% H_2SO_4 and then heating. Column chromatography was performed using a Sephadex LH-20 (GE Healthcare Bio-Science AB, Uppsala, Sweden). MPLC was performed using a Chemco Low-Prep 81-M-2 pump (Chemco Scientific Co., Ltd., Osaka, Japan) and an ULTRA PACK SI-40B column (300×26 mm, Yamazen Corp., Osaka, Japan). HPLC was performed using a Senshu SSC-3160 pump (flow rate 4 ml/min, Senshu Scientific Co., Ltd., Tokyo, Japan) and a YMC-Pack PEGASIL Silica 60-5 column (300×10 mm, YMC Co., Ltd., Kyoto, Japan), equipped with a YRD-883 RI detector (Shimamuratech Ltd., Tokyo, Japan). HPLC analytical conditions for novoamauromine (**1**) and amauromine (**3**) were as follows: column, a YMCPack PEGASIL Silica 60-5 column (300×10 mm, YMC Co., Ltd.); mobile phase, *n*-hexane–acetone (3 : 1); room temperature; flow rate, 4.0 ml/min; detector, a YRD-883 RI detector (Shimamuratech Ltd.). Amauromine (**3**) isolated from other fungus was used as a standard. HPLC analytical conditions for Marfey's method were as follows: column, Inertsil ODS-3, 4.6×250 mm (GL Sciences Inc., Tokyo, Japan); mobile phase, CH_3CN–0.1% trifluoroacetic acid (TFA) (50 : 50); flow rate, 1.0 ml/min; column oven temperature at 40 °C; detector, MD-2010 PLUS photodiode array (JASCO Co., Ltd.).

Isolation of Metabolites from *Aspergillus novofumigatus* CBS117520 Polished rice (Akitakomachi, 24 kg) was soaked in water for 30 min and then sterilized with an autoclave. *A. novofumigatus* CBS117520 was cultivated for 14 d in Roux flasks, each containing 140 g of moist rice. The cultivated rice was extracted with MeOH, and the extract was concentrated *in vacuo*. The residue was suspended in water and extracted with ethyl acetate. The EtOAc extract (52.3 g) was partitioned between hexane and acetonitrile to yield an acetonitrile-soluble mixture. The acetonitrile extract (29.4 g) was extracted sequentially with hexane (100 ml), benzene (100 ml), chloroform (100 ml), ethyl acetate, and MeOH (100 ml). The benzene extract (18 g) was chromatographed using a Sephadex LH-20 column [solvent system: *n*-hexane/$CHCl_3$ (1 : 4) (180 ml), $CHCl_3$/acetone (3 : 2) (220 ml), (1 : 4) (200 ml), acetone (200 ml), and MeOH (500 ml)] to yield five fractions. Fraction 2 [$CHCl_3$/acetone (3 : 2) eluate] was rechromatographed using

Table 1. ^{13}C- and ^{1}H-NMR Data of Novoamauromine (**1**) and *ent*-Cycloechinulin (**2**)

Carbon No.	Novoamauromine (**1**)			*ent*-Cycloechinulin (**2**)		
	^{13}C	^{1}H	J (Hz)	^{13}C	^{1}H	J (Hz)
1-NH					8.29 br s	
2	79.3	5.27 s		146.0		
3	62.5			105.3		
4	125.6	7.12 d	7.5	118.3	7.67 d	8.7
5	118.6	6.69 dt	0.7, 7.5	110.6	6.83 dt	2.1, 8.7
6	128.5	7.01 dt	0.7, 7.6	156.6		
7	109.6	6.31 d	7.6	95.0	6.84 d	2.1
8	148.5			134.2		
9	130.7			124.3		
10	34.8	2.54 dd	7.3, 13.8	115.3	7.60 s	
		2.71 dd	9.3, 13.8			
11	59.8	4.08 br t	7.3, 9.3	124.5		
12	168.3			165.1		
14	41.7			50.9	4.15 dq	2.3, 7.0
15	143.9	5.92 dd	10.8, 17.4	167.3		
16	114.6	5.08 dd	0.8, 17.4			
		5.12 dd	0.8, 10.8			
17	22.5	0.93 s		122.0	5.82 d	8.3
18	22.6	1.10 s		139.8	5.94 d	8.3
19				35.9		
20				27.1	1.67 s	
21				26.9	1.68 s	
22				18.3	1.54 d	7.0
23				55.6	3.81 s	

MPLC with a silica gel [*n*-hexane/acetone (2 : 1) to acetone] to give novoamauromine (**1**: 2 mg), *epi*-aszonalenin C (85 mg), helivolic acid (162 mg), *epi*-aszonalenin A (217 mg), and *ent*-cycloechinulin (**2**: 5 mg). The spectral data of *epi*-aszonalenin C, helivolic acid and *epi*-aszonalenin A were identical to those from reports in the literature.

Novoamauromine (1) Colorless amorphous solid; $[\alpha]_D$ +155° (c=0.425, $CHCl_3$); HR-FAB-MS obsd 509.2921, calcd for $C_{32}H_{37}N_4O_2$ $(M+H)^+$ 509.2917; UV λ_{max}^{MeOH} nm (log ε) 205 (4.7), 239 (4.1), 297 (3.6); IR ν_{max}^{KBr} cm^{-1} 3420, 1673; CD (c=2.26×10^{-5}, EtOH) $\Delta\varepsilon$ (nm) 87.6 (210), 25.1 (245), 10.5 (297). The ^{1}H- and ^{13}C-NMR signal assignments are summarized in Table 1.

***ent*-Cycloechinulin (2)** Yellow solid (mp 275—277°, from MeOH); $[\alpha]_D$ +47.6° (c=1.00, $CHCl_3$); HR-EI-MS obsd 351.1582, calcd for $C_{20}H_{21}N_3O_3$ (M^+) 351.1583; UV λ_{max}^{MeOH} nm (log ε) 215 (4.0), 268 (3.7), 302 (3.8), 377 (3.7); IR ν_{max}^{KBr} cm^{-1} 3341 (br), 1682, 1667; CD (c=8.55×10^{-5}, MeOH) $\Delta\varepsilon$ (nm) 2.5 (206), −5.6 (227), −2.3 (301), 3.4 (375). The ^{1}H- and ^{13}C-NMR signal assignments are summarized in Table 1.

Amino Acid Analysis of *ent*-Cycloechinulin (2) *ent*-Cycloechinulin (**2**: 1.0 mg) was dissolved in 100 μl of 6 M HCl and heated at 110 °C for 16 h. The resulting hydrolyzate was allowed to cool and then neutralized with $NaHCO_3$. Then, 200 μl of Marfey's reagent (PIERCE, IL, U.S.A.) and 40 μl of 1 M $NaHCO_3$ were added to this mixture, and the mixture was heated at 40 °C for 1 h. HCl (2 M, 20 μl) was added upon cooling to room temperature. The solution was then analyzed by reversed phase HPLC, as previously described. Coinjection with D- and L-alanine standards (t_R=5.0 and 4.6 min, respectively) indicated that the alanyl residue in **2** has D-configuration.

Antifungal Assay Using the Paper Disk Method Antifungal assay was performed using a previously reported method,[11] the paper disk method, against *A. niger* IFM 41398, *A. fumigatus* IFM 41362, *C. albicans* IFM 40009, and *C. neoformans* ATCC 90112 as test organisms. Novoamauromine (**1**) and *ent*-cycloechinulin (**2**) were applied to the paper disk (diameter: 8 mm) at 100 μg per disk and the disks were placed on assay plates. The test organisms were cultivated in potato dextrose agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 25 °C. After 48—72 h of incubation, the zones of inhibition (the diameter measured in millimeters) were recorded.

Cytotoxicity Assay Cytotoxicity assay was performed by a modified method of the previous paper.[11] Cells were seeded into 96-well microplates at 4000 cells per well, and allowed to attach for 4—6 h. A549 human lung cancer cells and Hela human cervical cancer cells were then incubated in Dulbecco's modified Eagle's medium (Invitrogen Co., Ltd., Carlsbad, CA, U.S.A.), and LNCap human prostate adenocarcinoma cells in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) until 80% confluency. Media were supplemented with the indicated concentrations of isolated compounds for 48—72 h. Cell proliferation was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) to count living cells by combining WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium) and 1-methoxy PMS (1-methoxy-5-methylphenazinium methysulfate). Briefly, 10 μl of Cell Counting Kit-8 solution was added to each well after the medium was removed, and the plates were incubated for 4 h. Cell number was determined by scanning with a Bio-Rad Model Q4 550 microplate reader at 450 nm.

Acknowledgements We are indebted to Prof. K. Takahashi, Meiji Pharmaceutical University, for the FAB-MS measurements. This work was supported by an "Open Research Center" Project from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by a Grant-in-Aid for Scientific Research (No. 20590017) from the Japan Society for the Promotion of Science. This study also was partly supported by the Cooperative Research Program of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University (09-2).

References

1) Hong S.-B., Go S.-J., Shin H.-D., Frisvad J. C., Samson R. A., *Mycologia*, **97**, 1316—1329 (2005).
2) Rank C., Phipps R. K., Harris P., Frisvad J. C., Gotfredsen C. H., Larsen T. O., *Tetrahedron Lett.*, **47**, 6099—6102 (2006).
3) Okada S., Iwasaki S., Tsuda K., Sano Y., Hata T., Udagawa S., Nakayama Y., Yamaguchi H., *Chem. Pharm. Bull.*, **12**, 121—124 (1964).
4) Fujimoto H., Negishi E., Yamaguchi K., Nishi N., Yamazaki M., *Chem. Pharm. Bull.*, **44**, 1843—1848 (1996).
5) Lee S.-Y., Kinoshita H., Ihara F., Igarashi Y., Nihira T., *J. Biosci. Bioeng.*, **105**, 476—480 (2008).
6) Takase S., Kawai Y., Uchida I., Tanaka H., Aoki H., *Tetrahedron Lett.*, **25**, 4673—4676 (1984).
7) Takase S., Kawai Y., Uchida I., Tanaka H., Aoki H., *Tetrahedron*, **41**, 3037—3048 (1985).
8) Guzman F. S., Gloer J. B., Wicklow D. T., Dowd P. F., *J. Nat. Prod.*, **55**, 931—939 (1992).
9) Marfey P., Ottesen M., *Carlsberg Res. Comm.*, **49**, 585—590 (1984).
10) Marfey P., *Carlsberg Res. Commun.*, **49**, 591—596 (1984).
11) Wakana D., Hosoe T., Wachi H., Itabashi T., Fukushima K., Yaguchi T., Kawai K., *J. Antibiot.*, **62**, 217—219 (2009).
12) Yin W.-B., Cheng J., Li S.-M., *Org. Biomol. Chem.*, **7**, 2202—2207 (2009).
13) Yin W.-B., Grundmann A., Cheng J., Li S.-M., *J. Biol. Chem.*, **284**, 100—109 (2009).
14) Ruan H.-L., Yin W. B., Wu J. Z., Li S.-M., *ChemBioChem*, **9**, 1044—1047 (2008).