Three New Indole-Containing Diketopiperazine Alkaloids from a Deep-Ocean Sediment Derived Fungus *Penicillium griseofulvum*

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Three new indole-containing diketopiperazine alkaloids, named variecolorins M – O (**1** – **3**), together with the eight known analogues **4** – **11**, were isolated from a deep-ocean sediment-derived fungus, *Penicillium griseofulvum*. Their structures were determined by analysis of the spectroscopic data. The 2,2-diphenyl-1-picrylhydrazinyl (DPPH) radical-scavenging activities and the cell-proliferation inhibitory activities of the three new compounds **1** – **3** were investigated.

Introduction. – Marine-derived fungi are widely recognized as emerging sources of active secondary metabolites, and much more attention has been paid to those obtained from extreme environments in recent years [1][2], such as from deep ocean, which possesses characteristics of high hydrostatic pressure, low temperature with the notable exception of hydrothermal vent, low nutrient concentration, and complete darkness [3]. In our continuous search for new bioactive natural products from the extremophilic/extreme-tolerant fungi, a deep-ocean sediment-derived fungus (depth 2481 m) identified as *Penicillium griseofulvum*, showed significant cytotoxic activity on the P388 cell line.

Investigation of the AcOEt extract of this fungus led to the isolation of three new indole-containing diketopiperazine alkaloids, variecolorins M – O[1]) (**1** – **3**), along with the eight known analogues variecolorin H (**4**) [4], neoechinulin A (**5**) [5], neoechinulin B (**6**) [6], isoechinulin B (**7**) [7], preechinulin (**8**) [8], tardioxopiperazine A (**9**) [9], echinulin (**10**) [9], and didehydroechinulin (**11**) [10] (*Fig. 1*). In this article, we describe the isolation, the structure elucidation, the radical-scavenging activity against 2,2-diphenyl-1-picrylhydrazinyl (DPPH), and the cytotoxic activities of the new compounds.

Results and Discussion. – 1. *Isolation and Structure Elucidation*. The AcOEt extract was concentrated and then separated by repeated column chromatography (silica gel, *Sephadex LH-20*) and semi-preparative HPLC to yield the three new indole-containing diketopiperazine alkaloids **1** – **3**, together with the eight known indole-containing diketopiperazine alkaloids **4** – **11**.

Variecolorin M (**1**) was obtained as a yellow amorphous powder. The molecular formula was determined as $C_{24}H_{29}N_3O_3$ by HR-ESI-MS (*m*/*z* 408.2278 ($[M+H]^+$)),

[1]) Arbitrary atom numbering; for systematic names, see *Exper. Part.*

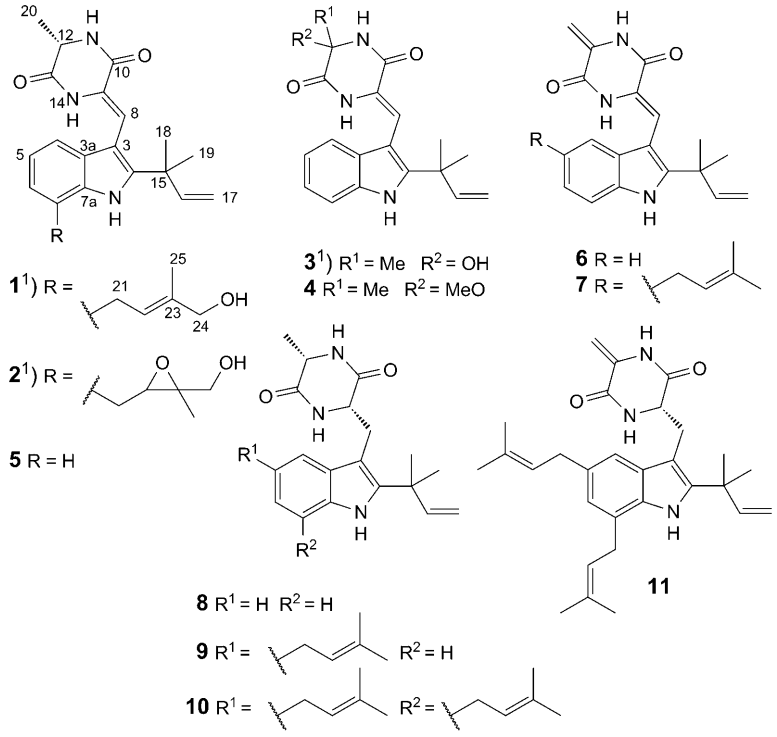

Fig. 1. *Compounds* **1**–**11**, *isolated from* Penicillium griseofulvum

indicating 12 degrees of unsaturation. UV Absorptions at λ_{max} 210, 271, and 337 suggested the presence of a conjugated indole moiety in **1** [5]. The IR spectrum of **1** showed the absorptions of OH (3357 cm^{-1}), amide or lactam (3191 and 1666 cm^{-1}), and aromatic functionalities (1613, 1427, and 1394 cm^{-1}). The ^{13}C-NMR signals at δ 159.8 and 166.3 confirmed the presence of two lactam C=O groups. The ^{1}H-NMR signals at δ 7.02 (*d*, J = 7.7 Hz), 6.95 (*dd*, J = 7.7, 6.6 Hz), and 6.88 (*d*, J = 6.6 Hz) (*Table 1*) indicated the presence of a 1,2,3-trisubstituted benzene moiety. The ^{13}C-NMR data together with the DEPT experiments established the presence of ten quaternary C-atoms and seven CH, three CH_2, and four Me groups (*Table 2*). Detailed analysis of the 1D-NMR data revealed that **1** was a dioxopiperazine alkaloid derivative containing an indole moiety and two isoprenyl units. The ^{1}H-NMR (*Table 1*) data of **1** was similar to that of variecolorin G [4], except for the absence of the Me(24) signal at δ 1.75 and the presence of two additional signals at δ 4.74 (OH–C(24)) and δ 3.86 (CH_2(24)). The difference was also observed in the ^{13}C-NMR spectrum with the replacement of the Me signal of variecolorin G at δ 25.6 (Me(24)) by an oxygenated CH_2 group at δ 66.3 (C(24)), indicating that **1** was the 24-hydroxy derivative of variecolorin G. The geometry of the C=C bond at C(8) was assigned as (*Z*), evidenced by the downfield chemical shift of H–C(8) (δ 6.87), which was caused by the deshielding effect of the C=O group. The absolute configuration of **1** was determined to be the same as that of

Table 1. *^{1}H-NMR Data* (600 MHz, (D_6)DMSO)[a]) *of Compounds* **1**–**3**[1]). δ in ppm, J in Hz.

	δ(H)		
	1	**2**	**3**
NH(1)	10.34 (*s*)	10.40 (*s*)	11.05 (*s*)
H–C(4)	7.02 (*d*, *J* = 7.7)	7.07 (*d*, *J* = 7.7)	7.30 (*d*, *J* = 7.7)
H–C(5)	6.95 (*dd*, *J* = 7.7, 6.6)	6.98 (*dd*, *J* = 7.7, 6.8)	6.99 (*dd*, *J* = 7.7, 6.6)
H–C(6)	6.88 (*d*, *J* = 6.6)	6.98 (*d*, *J* = 6.8)	7.07 (*dd*, *J* = 7.7, 6.6)
H–C(7)	–	–	7.40 (*d*, *J* = 7.7)
H–C(8)	6.87 (*s*)	6.87 (*s*)	6.93 (*s*)
NH(11)	8.34 (*d*, *J* = 2.2)	8.35 (*d*, *J* = 2.2)	8.82 (*s*)
H–C(12)	4.15 (*qd*, *J* = 6.6, 2.2)	4.15 (*qd*, *J* = 6.6, 2.2)	–
NH(14)	8.67 (*s*)	8.73 (*s*)	8.88 (*s*)
H–C(16)	6.14 (*dd*, *J* = 17.6, 9.9)	6.13 (*dd*, *J* = 16.5, 10.9)	6.08 (*dd*, *J* = 16.5, 9.9)
CH_2(17)	5.05 (*d*, *J* = 9.9), 5.02 (*d*, *J* = 17.6)	5.05 (*d*, *J* = 9.9), 5.02 (*d*, *J* = 16.5)	5.06 (*d*, *J* = 9.9), 5.04 (*d*, *J* = 16.5)
Me(18)	1.51 (*s*)	1.50 (*s*)	1.47 (*s*)
Me(19)	1.51 (*s*)	1.50 (*s*)	1.47(*s*)
Me(20)	1.36 (*d*, *J* = 6.6)	1.36 (*d*, *J* = 6.6)	1.50 (*s*)
CH_2(21)	3.69 (*d*, *J* = 6.6)	3.16 (*d*, *J* = 6.5), 3.26 (*d*, *J* = 11.0)	–
H–C(22)	5.64 (br. *t*, *J* = 6.6)	3.16 (*dd*, *J* = 6.5,11.0)	–
CH_2(24)	3.86 (*d*, *J* = 5.5)	3.41 (*d*, *J* = 6.0)	–
Me(25)	1.72 (*s*)	1.36 (*s*)	–
OH–C(12)	–	–	6.68 (*s*)
OH–C(24)	4.74 (br. *t*, *J* = 5.5)	4.87 (br. *t*, *J* = 6.0)	–

[a]) Recorded at 600 MHz for ^{1}H-NMR using TMS as internal standard.

Table 2. *^{13}C-NMR Data* (150 MHz, (D_6)DMSO) *of Compounds* **1**–**3**[1]). δ in ppm.

	δ(C)				δ(C)		
	1	**2**	**3**		**1**	**2**	**3**
C(2)	143.7 (*s*)	143.8 (*s*)	144.3 (*s*)	C(13)	166.3 (*s*)	166.4 (*s*)	165.8 (*s*)
C(3)	104.0 (*s*)	104.1 (*s*)	103.8 (*s*)	C(15)	39.3 (*s*)	39.5 (*s*)	39.4 (*s*)
C(3a)	126.0 (*s*)	126.1 (*s*)	126.3 (*s*)	C(16)	145.5 (*d*)	145.4 (*d*)	145.2 (*d*)
C(4)	116.6 (*d*)	117.2 (*d*)	119.4 (*d*)	C(17)	111.4 (*t*)	111.5 (*t*)	111.4 (*t*)
C(5)	119.7 (*d*)	119.7 (*d*)	119.4 (*d*)	C(18)	27.6 (*q*)	27.5 (*q*)	27.8 (*q*)
C(6)	121.3 (*d*)	120.0 (*d*)	120.8 (*d*)	C(19)	27.6 (*q*)	27.5 (*q*)	27.5 (*q*)
C(7)	124.3 (*s*)	121.5 (*s*)	111.7 (*d*)	C(20)	19.6 (*q*)	19.6 (*q*)	24.7 (*q*)
C(7a)	136.3 (*s*)	134.0 (*s*)	135.1 (*s*)	C(21)	28.3 (*t*)	29.6 (*t*)	–
C(8)	110.2 (*d*)	110.1 (*d*)	111.6 (*d*)	C(22)	121.3 (*d*)	59.6 (*d*)	–
C(9)	125.2 (*s*)	125.3 (*s*)	125.0 (*s*)	C(23)	133.8 (*s*)	61.4 (*s*)	–
C(10)	159.8 (*s*)	159.8 (*s*)	161.4 (*s*)	C(24)	66.3 (*t*)	65.4 (*t*)	–
C(12)	50.5 (*d*)	50.5 (*d*)	79.1 (*s*)	C(25)	13.7 (*q*)	14.3 (*q*)	–

variecolorin G [4] by comparison of their optical rotation ($[\alpha]_D^{25} = -36$ for **1** *vs.* -16 for variecolorin G). Thus, the structure of **1** was established (*Fig. 1*), and named variecolorin M.

Variecolorin N (**2**) was isolated as a yellow amorphous powder. The molecular formula was established as $C_{24}H_{29}N_3O_4$ on the basis of the HR-ESI-MS (*m*/*z* 424.2243

($[M+H]^+$)), indicating the presence of one more O-atom compared to **1**. IR Absorptions showed the presence of OH (3206 cm^{-1}) and C=O groups (1685 cm^{-1}) and of aromatic rings (1633, 1442, and 1195 cm^{-1}). Comparison of the 1D-NMR data of **1** and **2** (*Tables 1* and *2*) suggested that they had the same molecular scaffold with a different side chain involving C(21) to C(25). The major difference was the replacement of two olefinic C-atoms of **1** at δ 121.3 (C(22)) and 133.8 (C(23)) by two oxygenated C-atoms at δ 59.6 and 61.4, indicating the presence of an oxirane cycle in **2**, compatible with the chemical shifts and molecular formula. The structure was further confirmed by the ^{1}H,^{1}H-COSY and HMBC data (*Fig. 2*). The alanine moiety in **2** was determined as L ($[\alpha]_D^{25} = -58$) by comparison with the analogous optical rotation of **1** ($[\alpha]_D^{25} = -36$). But the configuration of the oxirane cycle could not be determined due to the lack of signals in the NOESY plot.

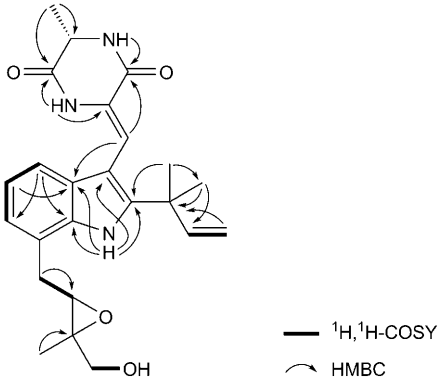

Fig. 2. *Key ^{1}H,^{1}H-COSY and HMBC data of compound* **2**

Variecolorin O (**3**) was separated as a yellow amorphous powder. The HR-ESI-MS indicated that the molecular formula was $C_{19}H_{21}N_3O_3$ (*m/z* 340.1659 ($[M+H]^+$)). The NMR data showed that the structure of **3** was very similar to that of variecolorin H (**4**) [4]. Careful comparison of the NMR data of **3** (*Tables 1* and *2*) with those of variecolorin H (**4**) [4] indicated that the major difference of the two compounds is due to the substitution at C(12). An additional OH signal (δ 6.68) was observed in the ^{1}H-NMR spectrum of **3**, with the absence of the MeO signal of **4** (δ 3.24), which suggested that the MeO group of **4** was replaced by an OH group in **3**. Thus, the structure of **3** was identified as shown in *Fig. 1*.

2. *Radical-Scavenging Activity.* The new compounds **1**–**3** were evaluated for their radical-scavenging activities against 2,2-diphenyl-1-picrylhydrazinyl (DPPH) [11]. Compounds **1**–**3** showed weak activities with IC_{50} values of 135, 120, and 91 μM, respectively (ascorbic acid as positive control, $IC_{50} = 26$ μM).

3. *Cytotoxic Activity.* The cytotoxic activities of the new compounds **1**–**3** were also tested on BEL-7402, HL-60, and A-549 cell lines by the MTT (=2-(4,5-dimethylthiazol-2-yl)-3,5-dimethyl-2*H*-tetrazolium bromide) [12] and SRB (=sulforhodamine B) methods [13]. None of them showed activities against these cell lines.

Indole-containing diketopiperazine alkaloids are widespread microbial products commonly found in cultures of marine fungi, such as *Aspergillus*, *Penicillium*, *Pestalotiopsis*, and *Chromocleista* [14]. This class of alkaloids is characteristic of a reversed isoprenic chain at the C(2), C(5), and/or C(7)-position of the indole moiety. This family of compounds attracted much attention of synthetic chemists due to their diverse biological activities in various pharmacological assay systems such as antinitration, anti-oxidant, or cytoprotective activities, *etc.* [15].

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Experimental Part

General. CC = Column chromatography. Semi-prep. HPLC; *ODS* column (*YMC-Pack ODS-A*, 10×250 mm, 5 μm); flow 4 ml/min; detection by UV (λ_{max} (log ε) in nm). Optical rotations: *Jasco-P-1020* digital polarimeter. IR Spectra: *Nicolet-Nexus-470* spectrophotometer; KBr discs; $\tilde{\nu}$ in cm^{-1}. ^{1}H- and ^{13}C-NMR, DEPT, and 2D-NMR Spectra: *Jeol-Eclipse-600* spectrometer; δ in ppm rel. to Me_4Si as internal standard and *J* in Hz. HR-ESI-MS: *Q-TOF-Ultima-Global-GAAo76* LC mass spectrometer; in *m*/*z*.

Fermentation and Extraction. The fungus was isolated from a deep-ocean-sediment sample collected at a depth of 2481 m. Working stocks were prepared on potato/dextrose-agar (PDA) slants stored at 4°. Spores growing on the PDA slant were inoculated into conical flasks (1000 ml) containing 300 ml of the culture medium (maltose (20 g), mannitol (20 g), glucose (10 g), monosodium glutamate (10 g), KH_2PO_4 (0.5 g), $MgSO_4$ (0.3 g), yeast-extract paste (3 g), and maize paste (1 g) dissolved in 1 l of seawater, pH 6.5) and cultured at 28° for 30 d. The fermented whole broth (60 l) was filtered through cheese cloth to separate into supernatant and mycelia. The former was extracted three times with AcOEt, while the latter was extracted three times with acetone and concentrated to afford an aq. soln. which was extracted three times with AcOEt. Both AcOEt solns. were combined and concentrated to give the crude extract (97.5 g).

Purification. The crude AcOEt extract was subjected to vacuum CC (silica gel, gradient petroleum ether/$CHCl_3$/MeOH): *Fractions 1–6. Fr. 3* was subjected to CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1), followed by CC (silica gel, petroleum ether/acetone 10:1) and prep. HPLC: **7** (11.5 mg), **8** (9.0 mg), **9** (10.6 mg), and **10** (8.5 mg). *Fr. 4* was subjected to CC (silica gel, gradient $CHCl_3$/MeOH) and prep. HPLC (60% MeOH/H_2O): **1** (2.3 mg), **4** (5.5 mg), **5** (6.0 mg), **6** (9.4 mg), and **11** (3.0 mg). *Fr. 5* was subjected to CC (*Sephadex LH-20*, MeOH) and further purified by prep. HPLC (65% MeOH/H_2O): **2** (5.5 mg) and **3** (3.0 mg).

Variecolorin M (= *(3Z,6S)-3-{[2-(1,1-Dimethylprop-2-en-1-yl)-7-[(2E)-4-hydroxy-3-methylbut-2-en-1-yl]-1H-indol-3-yl}methylidene}-6-methylpiperazine-2,5-dione*; **1**): Yellow amorphous powder. $[\alpha]_D^{25} = -36$ ($c = 0.1$ MeOH). UV (HPLC, mobile phase): 210 (3.97), 271 (3.51), 337 (3.68). IR (KBr): 3357, 3191, 2972, 2906, 1666, 1613, 1427, 1394, 731. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-ESI-MS: 408.2278 ($[M + H]^+$, $C_{24}H_{30}N_3O_3^+$; calc. 408.2287).

Variecolorin N (= *(3Z,6S)-3-{[2-(1,1-Dimethylprop-2-en-1-yl)-7-{[3-(hydroxymethyl)-3-methyloxiran-2-yl]methyl}-1H-indol-3-yl}methylidene}-6-methylpiperazine-2,5-dione*; **2**): Yellow amorphous powder. $[\alpha]_D^{25} = -58$ ($c = 0.1$ MeOH). UV (HPLC, mobile phase): 210 (4.30), 268 (3.77), 349 (3.96). IR (KBr): 3383, 3206, 2959, 2925, 2846, 1685, 1633, 1442, 1334, 1195, 1037, 783. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-ESI-MS: 424.2243 ($[M + H]^+$, $C_{24}H_{30}N_3O_4^+$; calc. 424.2236).

Variecolorin O (= *(6Z)-6-{[2-(1,1-Dimethylprop-2-en-1-yl)-1H-indol-3-yl]methylidene}-3-hydroxy-3-methylpiperazine-2,5-dione*; **3**): Yellow amorphous powder. $[\alpha]_D^{25} = -8.9$ ($c = 0.1$ MeOH). UV (HPLC, mobile phase): 210 (4.30), 337 (4.02). IR (KBr): 3206, 2923, 2846, 1670, 1645, 1412, 1334, 1175, 743. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. HR-ESI-MS: 340.1659 ($[M + H]^+$, $C_{19}H_{22}N_3O_3^+$; calc. 340.1661).

Biological Assays. The DPPH scavenging assay and the MTT and SRB methods were processed according to [11–13].

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