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A New Deoxyisoaustamide Derivative from the Marine-derived Fungus *Penicillium* sp. JF-72

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Marine-derived fungi are currently considered as the rich sources of active secondary metabolites, and there is an increasing amount of research on their chemistry and biological effects. *Penicillium* species are known to produce numerous alkaloids, in which some diketopiperazine-type alkaloids have been reported so far.[1-5] In the course of our on-going research on the secondary metabolites of marine-derived fungi, we have investigated the chemistry of the methylethylketone extract of the *Penicillium* sp. JF-72, and the study resulted in the isolation of three indole-containing diketopiperazine alkaloids (**1-3**), including a new natural compound (**2**) and a new metabolite (**3**). Some biological effects of the metabolites were evaluated, including cyto-protective and nitrite inhibitory activities.

Compounds **1** and **2** were identified to be (+)-deoxyiso-austamide and deoxydihydroisoaustamide, respectively by comparison of their NMR and MS data with the reported values (Figure 1).[6] It is notable that the chemical shift value of H-16β in compound **2** was far shifted to up-field region (δ_H 0.07) (Table 1). By comparison of the NMR data of the diketopiperazine moiety of compound **2** with those of a reported diketopiperazine alkaloid, carneamide C, this un-usual up-field chemical shift was determined to be due to the magnetic anisotropy of the coplanar C-18 carbonyl group.[7] The absolute configuration of compound **2** was determined based on the biogenetic considerations of diketopiperazine alkaloids isolated from the fungal genus *Penecillium*,[1-5] and NOESY correlation between H-11 and H-17. In addition,

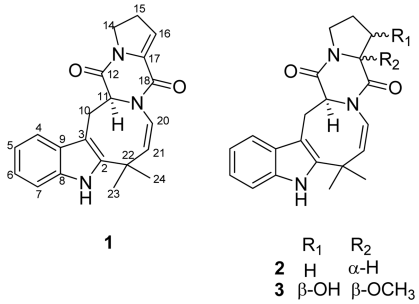

Figure 1. Structures of compounds **1-3**.

comparison of its optical rotation value, $[\alpha]_D^{25}$ + 140 (*c* 0.04, $CHCl_3$) with the reported compound was in good agreement with this assignment.[6] Although deoxydihydro-isoaustamide (**2**) was synthesized in the course of total synthesis of (+)-deoxyisoaustamide,[6] this is the first case to

Table 1. ^{1}H and ^{13}C NMR data for compounds **2** and **3**

Position	**2**		**3**	
	δ_H[a,b] (mult., *J* in Hz)	δ_C[a,c]	δ_H[a,b] (mult., *J* in Hz)	δ_C[a,c]
1	10.64 (s)		10.72 (s)	
2		141.3		141.0
3		102.8		102.7
4	7.36 (d, 8.0)	118.1	7.32 (d, 7.2)	117.3
5	6.88 (ddd, 0.8, 7.2, 8.0)	117.9	6.89 (ddd, 1.2, 6.4, 7.2)	118.5
6	6.97 (ddd, 0.8, 7.2, 8.0)	120.1	6.94 (ddd, 1.2, 6.4, 7.2)	120.4
7	7.27 (d, 8.0)	110.4	7.19 (d, 7.2)	110.3
8		134.5		134.8
9		128.0		128.2
10	3.44* 3.24*	25.9	3.61 (d, 15.6) 3.25 (dd, 6.4, 15.6)	25.9
11	4.26 (d, 6.4)	58.8	4.20 (d, 6.4)	58.5
12		163.4		166.3
14	3.23 (m) 2.95 (m)	44.2	3.93 (m) 2.68 (m)	42.6
15	1.44 (m) 0.87 (m)	20.3	1.81 (m) 1.49 (m)	28.7
16	1.62 (m) 0.07 (m)	28.5	3.89[d]	74.1
17	3.78 (dd, 6.4, 10.8)	57.7		93.8
18		165.6		162.0
20	5.77 (s)	122.6	5.81 (s)	121.7
21	5.77 (s)	140.3	5.81 (s)	140.5
22		36.9		37.2
23	1.51 (s)	27.2	1.52 (s)	28.2
24	1.32 (s)	32.0	1.32 (s)	32.2
17-OCH_3			1.44 (s)	47.5

[a]Spectra were recorded in dimethyl sulfoxide-d_6. [b]400 MHz. [c]100 MHz. [d]Overlapped signal. Assignments were confirmed by HSQC, HMBC, and COSY spectra.

report as a secondary metabolite.

Compound **3** was isolated as a yellow solid, and its molecular formula was established to be $C_{22}H_{25}N_3O_4$ by HRESITOFMS at *m/z* 394.1750 $[M-H]^-$ (calcd for $C_{22}H_{24}N_3O_4$, 394.1767). The ^{1}H NMR spectrum of **3** showed signals for an NH proton at δ_H 10.72 (1H, s), an 1,2-disubstituted aromatic ring [δ_H 6.89 (1H, ddd, J = 1.2, 6.4, 7.2 Hz), 6.94 (1H, ddd, J = 1.2, 6.4, 7.2 Hz), 7.19 (1H, d, J = 7.2 Hz), and 7.32 (1H, d, 7.2 Hz)], suggesting that compound **1** possessed an indole structural moiety (Table 1). The ^{1}H NMR spectrum further exhibited signals for two germinal methyl groups [δ_H 1.32 (3H, s) and 1.52 (3H, s)], two olefinic protons at δ_H 5.81 (2H, each s), and an oxymethine group at δ_H 3.89. The ^{13}C NMR and DEPT spectra revealed the presence of 22 carbon signals, including eight quaternary carbons, eight methine, three methylene, and three methyl groups. The ^{13}C NMR spectrum contained signals for two amide carbonyls at δ_C 166.3 (C-12) and 162.0 (C-18), an amino acid α-methine (δ_C 58.5, C-11), an oxymethine group (δ_C 74.1, C-16), and an oxygenated-quaternary carbon at (δ_C 93.8, C-17), suggesting the presence of a diketopiperazine ring (Table 1). Comparison of ^{13}C NMR data of **3** with those of **2**, and in combination with analyses of HSQC, HMBC, and COSY spectra indicated that the diketopiperazine ring was oxygenated at C-16 and C-17 (Figure 2). The down-field shifted ^{13}C NMR chemical shift value of C-17 (δ_C 93.8) indicated that this carbon was attached to a nitrogen atom. The position of the indole moiety of **3** was assigned based on the HMBC correlations from H-1 to C-2, C-3, C8, and C-9, from H-4 to C-3 and C-8, and from H-7 to C-8 and C-9 (Figure 2). The close linkage between the indole and diketo-piperazine moieties *via* a methylene bridge and an isoprenyl unit were deduced based on the HMBC correlations of H-10 with C-2, C-3, C-9, C-11, and C-12, of H-20 with C-18, C-21, and C-22, and of H-21 to C-2, C-23, and C-24, respectively. The ^{13}C NMR spectrum of **3** further showed the presence of a methoxy group at δ_C 47.5, which was attached to the signal at δ_H 1.44 (3H, s), as deduced by the HSQC spectrum. This methoxy group was then located at C-17 by the observation of the HMBC correlation between δ_H 1.44/ C-17. A noticeable point here is that the proton chemical shift value of the methoxy group was far shifted to up-field (δ_H 1.44), whereas the carbon chemical shift value slightly up-field shifted (δ_C 47.5). The ^{1}H and ^{13}C NMR data of the diketopiperazine moiety of **3** were found to be similar with those of the similar partial structure, verpacamide D, except for the chemical shift value of the methoxy group and C-17.[8] This difference might be due to the different configurations of the methoxy groups of these two compounds, so an NOESY experiment was carried out to determine the relative configuration of **3**. As all the diketopiperazine alkaloids isolated from the fungi *Penecillium* showed the configuration at C-11 to be *S*, therefore the orientation of proton at C-11 of **3** could be assumed as α based on the biogenetic considera-tions.[1-5] In the NOESY spectrum, an NOE correlation observed between H-11 and H-16 indicated that the hydroxyl group at C-16 is β-oriented (Figure 2). Furthermore, H-11 and H-16 did not give any NOE correlation with the methoxy group, suggesting that the orientation of the methoxy group at C-17 is β, which is opposite to that of the methoxy group of verpacamide D.[8] The observation of unusual upfield-shifted proton chemical shift of the methoxy group could be rationalized by the presence of coplanar C-18 carbonyl group. Molecular models of compound **3** indicated that the methoxy protons are positioned at shielding zone of the carbonyl group, thereby resonated at relatively upfield region compared to usual methoxy protons. Analogous patterns of chemical shifts for the H-16β of compound **2** and the reported diketopiperazine alkaloid, carneamide C were also observed.[7] Thus the gross structure of compound **3** was identified as 16β-hydroxy-17β-methoxy-deoxydihydroiso-austamide (Figure 1).

Diketopiperazine alkaloids, an important class of fungal metabolites, were biosynthesized by condensation of two amino acids, such as tryptophane, proline, histidine, and phenylalanine.[3] Noticeably, the structures of metabolites **1-3**, the products of mixed biogenetic origins derived from the condensation of tryptophane, proline and isoprene units, were found to be unusual based on the evaluation of the structural diversity of diketopiperazine alkaloids reported previously.[9]

In vitro cytoprotective effects of the metabolites were

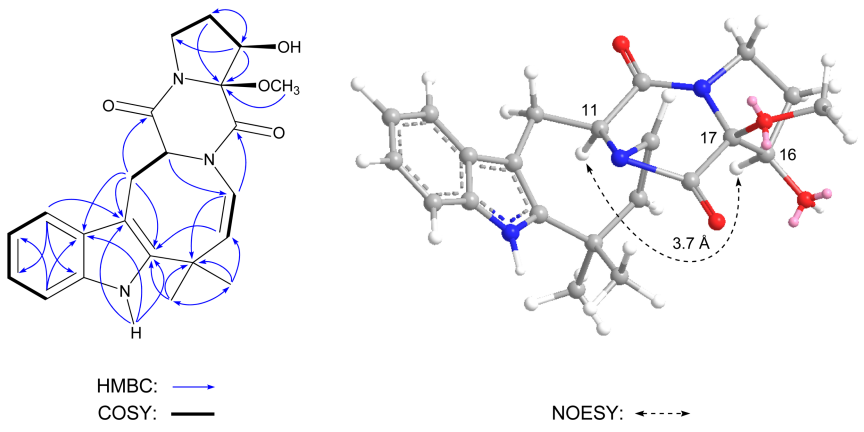

Figure 2. Key HMBC, COSY, and NOESY correlations of compound **3**.

assessed using glutamate and *t*-BHP-indcuced cytotoxicity in HT-22 and RIN-m5F cells, respectively. The inhibitory effects of the metabolites on nitrite production were also evaluated in LPS-stimulated RAW264.7 and BV2 cells. As the result, all the metabolites showed no significant effects on both cytoprotection and nitrite inhibition.

Experimental

General Procedures. Optical rotations were recorded on a Perkin Elmer 341 digital polarimeter. NMR spectra (1D and 2D) were recorded in dimethyl sulfoxide-d_6 using a JEOL JNM ECP-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C), and chemical shifts were referenced relative to the corresponding residual solvents signals (δ 2.50/39.5). HSQC and HMBC experiments were optimized for $^1J_{CH}$ = 140 Hz and $^nJ_{CH}$ = 8 Hz, respectively. ESIMS data were obtained using a Q-TOF micro LC-MS/MS instrument (Waters) at Korea University, Seoul, Korea. Solvents for extractions and flash column chromatography were reagent grade and used without further purification. Solvents used for HPLC were analytical grade. Flash column chromatography was carried out using YMC octadecyl-functionalized silica gel (C_{18}). HPLC separations were performed on Agilent semiprep-C_{18} column (21.2 × 150 mm; 5 μm particle size; 5 mL/min). Compounds were detected by UV absorption at 210 nm.

Fungal Material and Fermentation. *Penicillium* sp. JF-72 (deposited at the College of Medical and Life Sciences fungal strain repository, Silla University) was isolated from an unidentified sponge that was manually collected using scuba equipment off the shores of Jeju Island in February 2009. The sample was stored in a sterile plastic bag and transported to the laboratory, where it was kept frozen until further processing. The sample was diluted 10-fold using sterile seawater. One mL of the diluted sample was processed utilizing the spread plate method in potato dextrose agar (PDA) medium containing 3% NaCl. The plate was incubated at 25 °C for 14 days. After purifying the isolates several times, the final pure cultures were selected and preserved at -70 °C. A GenBank search with the 28S rRNA gene of JF-72 indicated *Penicillium expansum* DAOM 215350 (JN938952), *P. fuscoglaucum* CBS 261.29 (JQ434691), and *P. solitum* (JN642222 as the closest matches, showing a sequence identity of 100%. Therefore, the marine-derived fungal strain JF-72 was identified as a *Penicillium* sp.

Extraction and Isolation. The fungal strain was cultured on 100 petri plates (90-mm), each containing 20 mL of PDA with 3% NaCl. Plates were individually inoculated with 2 mL seed cultures of the fungal strain and incubated at 25 °C for a period of 10 days. Extraction of the combined agar media with methylethylketone (2 L) provided an organic phase, which was then concentrated *in vacuo* to yield 766.5 mg of an extract. The dried extract was subjected to C_{18} flash column chromatography (5 × 26 cm), eluting with a stepwise gradient of 20%, 40%, 60%, 80%, and 100% (v/v) MeOH in H_2O (500 mL each). A portion (90.3 mg) of the fraction which eluted at 80% MeOH (178.2 mg) was subjected to semi-preparative reversed-phase HPLC eluting with a gradient from 30-60% CH_3CN in H_2O (0.1% formic acid) over 70 min to yield compounds **3** (4.5 mg, t_R = 19.2 min), **2** (5.8 mg, t_R = 20.9 min), and **1** (4.0 mg, t_R = 22.3 min).

(+)-Deoxyisoaustamide (1): Yellow solid; $[\alpha]_D^{25}$ +142 (*c* 0.02, $CHCl_3$); LRESIMS *m/z* 348 $[M + H]^+$.

Deoxydihydroisoaustamide (2): Yellow solid; $[\alpha]_D^{25}$ +140 (*c* 0.04, $CHCl_3$); ^{1}H NMR (dimethyl sulfoxide-d_6, 400 MHz) and ^{13}C NMR data (dimethyl sulfoxide-d_6, 100 MHz), see Table 1; HRESIMS *m/z* 350.1863 $[M + H]^+$ (calcd for $C_{21}H_{24}N_3O_3$, 350.1869).

16β-Hydroxy-17β-methoxy-deoxydihydroisoaustamide (3): Yellow solid; $[\alpha]_D^{25}$ +196 (*c* 0.11, $CHCl_3$); ^{1}H NMR (dimethyl sulfoxide-d_6, 400 MHz) and ^{13}C NMR data (dimethyl sulfoxide-d_6, 100 MHz), see Table 1; HRESIMS *m/z* 394.1750 $[M–H]^-$ (calcd for $C_{22}H_{24}N_3O_4$, 394.1767).

Cell Culture. RAW264.7 macrophages, BV2 microglia, HT22 and RIN-m5F cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air at 5 × 10^5 cells/mL in DMEM medium and RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 mg/mL), and l-glutamine (2 mM).

Cytoprotective Assay. HT22 cells were pre-treated for 12 h with indicated concentrations of compounds, and then incubated for 12 h with glutamate (5 mM). In addition, RIN-m5F cells were pre-treated for 12 h with indicated concentrations of compounds, and then incubated for 12 h with *t*-BHP (50 μM). The effects of various experimental modulations on cell viability were evaluated by determining mitochondrial reductase function with an assay based on the reduction of tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into fomazan crystals. The synthesis of formazan is proportional to the number of functional mitochondria in living cells. For the determination of cell viability, 50 mg/mL of MTT was added to 1 mL of cell suspension (1 × 10^5 cells/mL in 96-well plates) for 4 h. The synthesized formation was dissolved in acidic 2-propanol and the optical density was measured at 590 nm. The optical density of the formazan formed in control (untreated) cells was considered as 100% viability.

Nitrite Production Determination. RAW264.7 macrophages and BV2 microglia were pretreated for 12 h with indicated concentrations of compounds, and treated 24 h with LPS (1 μg/mL). The nitrite concentration in the medium was measured as an indicator of NO production as per the Griess reaction. The nitrite present in the conditioned media was determined spectrophotometrically using the Griess reaction. An aliquot (100 μL) of each supernatant was mixed with an equal volume of Griess reagent (0.1% [w/v] *N*-(1-naphthyl)-ethylenediamine and 1% [w/v] sulfanilamide in 5% [v/v] phosphoric acid) for 10 min at room temperature. The absorbance of the final reactant at 525 nm was measured using an ELISA plate reader, and the nitrite concentration was determined using a standard curve of sodium nitrite prepared in DMEM without phenol red.

Supporting Information. The NMR spectra of compounds **2** and **3** and HRESIMS of compound **3**.

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