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 ongoing 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 cytoprotective and nitrite inhibitory activities.

Compounds 1 and 2 were identified to be (+)-deoxyisoaustamide 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 (δH 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 unusual 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 Penicillium,1-5 and NOESY correlation between H-11 and H-17. In addition, comparison of its optical rotation value, [α]D25 + 140 (c 0.04, CHCl3) with the reported compound was in good agreement with this assignment.6 Although deoxydihydroisoaustamide (2) was synthesized in the course of total synthesis of (+)-deoxyisoaustamide,6 this is the first case to

Table 1. 1H and 13C NMR data for compounds 2 and 3

<table>
<thead>
<tr>
<th>Position</th>
<th>2</th>
<th>3</th>
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<tr>
<td></td>
<td>δH (mult., J in Hz)</td>
<td>δC</td>
</tr>
<tr>
<td>1</td>
<td>10.64 (s)</td>
<td>10.72 (s)</td>
</tr>
<tr>
<td>2</td>
<td>141.3</td>
<td>141.0</td>
</tr>
<tr>
<td>3</td>
<td>102.8</td>
<td>102.7</td>
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<tr>
<td>4</td>
<td>7.36 (d, 8.0)</td>
<td>118.1 7.32 (d, 7.2)</td>
</tr>
<tr>
<td>5</td>
<td>6.88 (ddd, 0.8, 7.2, 8.0)</td>
<td>117.9 6.89 (ddd, 1.2, 6.4, 7.2)</td>
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<tr>
<td>6</td>
<td>6.97 (ddd, 0.8, 7.2, 8.0)</td>
<td>120.1 6.94 (ddd, 1.2, 6.4, 7.2)</td>
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<td>7</td>
<td>7.27 (d, 8.0)</td>
<td>110.4 7.19 (d, 7.2)</td>
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<tr>
<td>8</td>
<td>134.5</td>
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<tr>
<td>9</td>
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<tr>
<td>10</td>
<td>3.44*</td>
<td>25.9 3.61 (d, 15.6)</td>
</tr>
<tr>
<td></td>
<td>3.24*</td>
<td>3.25 (dd, 6.4, 15.6)</td>
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<tr>
<td>11</td>
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<td>58.8 4.20 (d, 6.4)</td>
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<td>12</td>
<td>163.4</td>
<td>166.3</td>
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<tr>
<td>13</td>
<td>3.23 (m)</td>
<td>44.2 3.93 (m)</td>
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<td>2.95 (m)</td>
<td>2.68 (m)</td>
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<tr>
<td>15</td>
<td>1.44 (m)</td>
<td>20.3 1.81 (m)</td>
</tr>
<tr>
<td>16</td>
<td>0.87 (m)</td>
<td>1.49 (m)</td>
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<tr>
<td></td>
<td>0.07 (m)</td>
<td>28.5 3.89*</td>
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<tr>
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<td>3.78 (dd, 6.4, 10.8)</td>
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<td>18</td>
<td>165.6</td>
<td>162.0</td>
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<td>20</td>
<td>5.77 (s)</td>
<td>122.6 5.81 (s)</td>
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<td>22</td>
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<td>37.2</td>
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<td>1.51 (s)</td>
<td>27.2 1.52 (s)</td>
</tr>
<tr>
<td>24</td>
<td>1.32 (s)</td>
<td>32.0 1.32 (s)</td>
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†Spectra were recorded in dimethyl sulfoxide-d6. †400 MHz. ‡100 MHz.
*Overlapped signal. Assignments were confirmed by HSQC, HMBC, and COSY spectra.

Figure 1. Structures of compounds 1-3.
Compound 3 was isolated as a yellow solid, and its molecular formula was established to be C_{22}H_{35}NO_{5} by HRESITOFMS at m/z 394.1750 [M−H] (calcd for C_{22}H_{34}NO_{5}, 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, $\text{J} = 1.2, 6.4, 7.2$ Hz), 6.94 (1H, ddd, $\text{J} = 1.2, 6.4, 7.2$ Hz), 7.19 (1H, d, $\text{J} = 7.2$ 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. 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 downfield 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, C-8, and C-9, from H-4 to C-3 and C-8, and from H-7 to C-3 and C-8 (Figure 2). The close linkage between the indole and diketopiperazine 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/δ_C 47.5. 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. 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 $\alpha$, therefore the orientation of proton at C-11 of 3 could be assumed as $\alpha$ based on the biogenic considerations. In the NOESY spectrum, an NOE correlation observed between H-11 and H-16 indicated that the hydroxyl group at C-16 is $\beta$-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 $\beta$, which is opposite to that of the methoxy group of verpacamide D. 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. Thus the gross structure of compound 3 was identified as 16β-hydroxy-17β-methoxy-deoxydihydroisoaustamide (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. 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.

In vitro cytoprotective effects of the metabolites were

![Figure 2](image_url)
assessed using glutamate and r-BHP-induced 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 $^1$J$_{CH}$ = 140 Hz and $^2$J$_{CH}$ = 8 Hz, respectively. ESI-MS 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 x 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 sterile seawater. One mL of the diluted sample was transported to the laboratory, where it was kept frozen until scuba equipment off the shores of Jeju Island in February 2009. An unidentified sponge that was manually collected using scuba equipment off the shores of Jeju Island in February 2009


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$_3$CN in H$_2$O (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; [α]$_D^{25}$ +142 (c 0.02, CHCl$_3$); LRESIMS m/z 348 [M + H]$^+$.

Deoxydihydroisoaustamide (2): Yellow solid; [α]$_D^{25}$ +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 394.1750 [M + H]$^+$ (caled for C$_{12}$H$_{12}$N$_2$O$_3$, 394.1767).

16β-Hydroxy-17β-methoxy-deoxydihydroisoaustamide (3): Yellow solid; [α]$_D^{25}$ +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]$^+$ (caled for C$_{12}$H$_{12}$N$_2$O$_3$, 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).

Cytotoxicity 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 r-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 formazan 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$^6$ 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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References