FOUR CYCLODipePTIDES, ASNOVOlenINS A-B AND ASNOVOZINEs A-B, FROM ASPERGIllUS NOVOFUMIgATUs

Kazuki Ishikawa,* Daigo Wakana, Takeshi Itabashi, Hisashi Takeda, Takashi Yaguchi, Ken-ichi Kawai, and Tomoo Hosoe

Department of Organic Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo, 142-8501, Japan. Medical Mycology Research Center Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba City, Chiba, 260-8673, Japan. E-mail: hosoe@hoshi.ac.jp

Abstract – Four new cyclodipeptides, asnovolenins A (1) and B (2), and asnovozines A (3) and B (4), were isolated from the fungus Aspergillus novofigatus CBS 117520. The structures of 1-4 were determined by the detailed analysis of mainly 1D- and 2D-NMR and MS data. Compounds 1 and 2 are composed of epi-aszonalenin (5) and dihydroterrein (6), and they are 2'-epimers of each other. Compounds 3 and 4 consist of D-alanine and tryptophan attached to a 3-methyl-1-butene group. The stereochemistry of 1 and 2 was determined from ROESY spectra and the exciton chirality method from CD spectra, and that of 3 and 4 was determined from NOE or NOESY spectra using the modified Marfey’s method.

INTRODUCTION

The genus Aspergillus includes an extremely diverse array of filamentous ascomycetous fungi found ubiquitously around the world. One member in particular, A. fumigatus, is an important bioresource that produces diverse secondary metabolites exhibiting a variety of pharmacologic activities.1 In a previous study, we reported the isolation of secondary metabolites from methanol extracts of A. novofigatus CBS 117520 cultivated on rice medium. These metabolites included novobezomalvins A-C2 and asnovolins A-E3 as fibronectin expression regulators. The cyclodipeptides novoamauromine and ent-cycloechinulin were also isolated from same extract of A. novofigatus.4 We therefore hypothesized that further investigation of methanol extracts of A. novofigatus would lead to the isolation of other novel compounds. In this report, we describe the isolation and structure elucidation of the novel
cyclo-dipeptides asnovolenins A (1) and B (2) and asnovozines A (3) and B (4) using detailed analysis of 1D- and 2D-NMR, MS, and CD spectral data and Marfey’s method.\(^5\)

![Chemical structures of 1-6 isolated from *A. novofumigatus*, and the benzoate derivatives (7 and 8) of asnovolenins A and B](image)

**RESULTS AND DISCUSSION**

Methanol extracts of *Aspergillus novofumigatus* CBS 117520 cultured on rice medium were separated and purified by HPLC to obtain asnovolenins A (1) and B (2) and asnovozines A (3) and B (4), along with *epi*-aszonalenin C (5)\(^6\) and dihydroterrein (6).\(^7\)

Compound 1 was obtained as a colorless amorphous solid, and the molecular formula was determined as C\(_{31}\)H\(_{33}\)N\(_3\)O\(_5\) (17 degrees of unsaturation) by high-resolution chemical ionization mass spectrometry (HRCIMS). The IR bands (1699 and 1636 cm\(^{-1}\)) indicated the presence of amide groups.

The \(^1\)H-NMR spectrum of 1 exhibited signals for three methyl protons (\(\delta_H\) 1.38, 1.15, and 1.02), two methylene proton signals (\(\delta_H\) 3.08 and 2.74, \(\delta_H\) 2.79 and 2.54), two oxygenated methine protons (\(\delta_H\) 4.43 and 3.99), three nitrogenous methine protons (\(\delta_H\) 5.96, 4.39, and 4.05), two sp\(^2\) methine protons (\(\delta_H\) 5.91 and 5.81), one exomethylene group (\(\delta_H\) 5.09 and 5.06), and eight aromatic protons constituting two 1,2-disubstituted benzene moieties. The \(^13\)C-NMR spectrum of 1 showed the presence of two amide carbonyl carbons (\(\delta_C\) 170.6 and 167.8) and an \(\alpha,\beta\)-unsaturated ketone carbon (\(\delta_C\) 203.1), 16 sp\(^2\) carbons including 12 aromatic sp\(^2\) carbons, 3 methyl carbons (\(\delta_C\) 24.0, 22.9, and 15.7), 5 sp\(^3\) methine carbons (\(\delta_C\) 85.7, 80.0, 77.4, 58.2, and 52.6) at the adjacent heteroatom positions, 2 sp\(^3\) methylene carbons (\(\delta_C\) 35.6 and 33.8), and 2 sp\(^3\) quaternary carbons (\(\delta_C\) 61.0 and 41.9) (Table 1). \(^1\)H-\(^1\)H COSY and HMBC
Table 1. NMR spectroscopic data for asnovolenins A (1) and B (2)

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_c^a$</th>
<th>$\delta_H^b$ ($J$ in Hz)</th>
<th>$\delta_c^a$</th>
<th>$\delta_H^b$ ($J$ in Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>85.7</td>
<td>5.96 s</td>
<td>81.7</td>
<td>6.10 s</td>
</tr>
<tr>
<td>3</td>
<td>61.0</td>
<td></td>
<td>61.9</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>124.8</td>
<td>7.27 m</td>
<td>124.3</td>
<td>7.27 m</td>
</tr>
<tr>
<td>5</td>
<td>118.4</td>
<td>6.71 m</td>
<td>118.3</td>
<td>6.67 dd (7.5, 7.3)</td>
</tr>
<tr>
<td>6</td>
<td>128.1</td>
<td>7.08 dd (7.5, 7.5)</td>
<td>128.5</td>
<td>7.09 dd (7.7, 7.3)</td>
</tr>
<tr>
<td>7</td>
<td>109.9</td>
<td>6.69 d (8.3)</td>
<td>108.2</td>
<td>6.55 d (7.7)</td>
</tr>
<tr>
<td>8</td>
<td>146.7</td>
<td></td>
<td>148.2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>133.5</td>
<td></td>
<td>132.7</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>33.8</td>
<td>2.79 m</td>
<td>34.2</td>
<td>2.74 dd (14.3, 6.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.54 dd (13.6, 8.5)</td>
<td>2.56 m</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>58.2</td>
<td>4.05 dd (8.5, 6.4)</td>
<td>58.4</td>
<td>4.00 dd (8.5, 6.4)</td>
</tr>
<tr>
<td>12</td>
<td>170.6</td>
<td></td>
<td>170.9</td>
<td></td>
</tr>
<tr>
<td>13-NH</td>
<td></td>
<td>9.19 brs</td>
<td>9.23</td>
<td>brs</td>
</tr>
<tr>
<td>14</td>
<td>136.5</td>
<td></td>
<td>136.8</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>122.0</td>
<td>6.96 d (6.8)</td>
<td>122.0</td>
<td>7.00 d (6.7)</td>
</tr>
<tr>
<td>16</td>
<td>133.4</td>
<td>7.33 dd (6.8, 6.6)</td>
<td>133.5</td>
<td>7.33 dd (6.7, 6.7)</td>
</tr>
<tr>
<td>17</td>
<td>125.1</td>
<td>7.19 dd (7.6, 6.6)</td>
<td>124.8</td>
<td>7.17 dd (7.5, 6.7)</td>
</tr>
<tr>
<td>18</td>
<td>130.9</td>
<td>7.93 d (7.6)</td>
<td>131.0</td>
<td>7.97 d (7.5)</td>
</tr>
<tr>
<td>19</td>
<td>125.7</td>
<td></td>
<td>125.5</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>167.8</td>
<td></td>
<td>167.5</td>
<td></td>
</tr>
<tr>
<td>21-N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>41.9</td>
<td></td>
<td>41.7</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>144.3</td>
<td>5.91 dd (17.1, 10.6)</td>
<td>144.5</td>
<td>5.94 dd (17.5, 11.1)</td>
</tr>
<tr>
<td>24</td>
<td>114.4</td>
<td>5.09 d (17.1)</td>
<td>114.2</td>
<td>5.08 d (17.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.06 d (10.6)</td>
<td>5.04 d (11.1)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>22.9</td>
<td>1.15 s</td>
<td>23.2</td>
<td>1.16 s</td>
</tr>
<tr>
<td>26</td>
<td>24.0</td>
<td>1.02 s</td>
<td>24.3</td>
<td>1.03 s</td>
</tr>
<tr>
<td>1'</td>
<td>15.7</td>
<td>1.38 d (6.3)</td>
<td>16.9</td>
<td>1.36 d (6.4)</td>
</tr>
<tr>
<td>2'</td>
<td>52.6</td>
<td>4.39 m</td>
<td>49.9</td>
<td>4.08 m</td>
</tr>
<tr>
<td>3'</td>
<td>35.6</td>
<td>3.08 dd (14.0, 10.3)</td>
<td>34.3</td>
<td>2.92 dd (17.3, 9.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.74 m</td>
<td>2.60 m</td>
<td></td>
</tr>
<tr>
<td>4'</td>
<td>175.8</td>
<td></td>
<td>176.4</td>
<td></td>
</tr>
<tr>
<td>5'</td>
<td>128.4</td>
<td>5.81 s</td>
<td>127.4</td>
<td>5.79 s</td>
</tr>
<tr>
<td>6'</td>
<td>203.1</td>
<td></td>
<td>203.6</td>
<td></td>
</tr>
<tr>
<td>7'</td>
<td>80.0</td>
<td>3.99 brs</td>
<td>81.2</td>
<td>4.08 brs</td>
</tr>
<tr>
<td>8'</td>
<td>77.4</td>
<td>4.43 brs</td>
<td>77.9</td>
<td>4.30 brs</td>
</tr>
</tbody>
</table>

$^a$Recorded at 100 MHz in CDCl$_3$. $^b$Recorded at 400 MHz in CDCl$_3$. 
correlations of 1 are shown in Figure 2. $^1$H-$^1$H COSY correlations indicated six sequences (H-4 to H-7, H2-10 to H-11, H-15 to H-18, H-23 to H2-24, H3-1’ to H2-3’, and H-7’ to H-8’), as shown by bold lines in Figure 1. HMBC correlations of H-4 ($\delta_H$ 7.27) with C-8 ($\delta_C$ 146.7), H-7 ($\delta_H$ 6.69) with C-9 ($\delta_C$ 133.5), H-17 ($\delta_H$ 7.19) with C-19 ($\delta_C$ 125.7), and H-18 ($\delta_H$ 7.93) with C-14 ($\delta_C$ 136.5) indicated the existence of two benzene rings. The presence of a 3-methyl-1-butene group was revealed from HMBC correlations of H3-25 ($\delta_H$ 1.15) and H3-26 ($\delta_H$ 1.02) with C-23 ($\delta_C$ 144.3) and C-22 ($\delta_C$ 41.9). HMBC correlations of H-2 ($\delta_H$ 5.96) with C-3 ($\delta_C$ 61.0) and C-10 ($\delta_C$ 33.8) indicated the existence of a hexahydropyrrolo[2,3-b]indole unit. Moreover, it was revealed that the 3-methyl-1-butene group was attached at the C-3 position of a hexahydropyrrolo[2,3-b]indole unit, based on HMBC correlation of H3-25 with C-3. HMBC correlations of H-10 ($\delta_H$ 2.54) with C-12 ($\delta_C$ 170.6) and H-18 with C-20 ($\delta_C$ 167.8) indicated that two amide bonds were located between a hexahydropyrrolo[2,3-b]indole unit and a benzene ring. The linkage of N-1 between the sequence of C-1’ to C-3’ was revealed from the HMBC correlation of H-2 with C-2’. HMBC correlations of H-5’ ($\delta_H$ 5.81) with C-6’ ($\delta_C$ 203.1), C-7’ ($\delta_C$ 80.0), and C-8’ ($\delta_C$ 77.4) indicated the presence of a 4,5-dihydroxy-2-cyclopenten-1-one group. The planar structure of 1 was deduced from the combination of C-3’ ($\delta_C$ 35.6) with C-4’ ($\delta_C$ 175.8) in which the dihydroterrein$^8$ residue is attached to the N-1 of the aszonalenin$^7$ residue.

![Figure 2](image-url)  

Figure 2. Key 2D-NMR spectra correlations for 1 and 2

Compound 2 was isolated as a colorless amorphous solid, and the molecular formula was determined as C$_{31}$H$_{33}$N$_3$O$_5$ (17 degrees of unsaturation), the same as 1, by high-resolution electrospray ionization mass spectrometry (HRESIMS). Furthermore, $^1$H- and $^{13}$C-NMR spectra were very similar to those of 1, except for chemical shifts at the C-2’ position (2: $\delta_H$ 4.08 and $\delta_C$ 49.9; 1: $\delta_H$ 4.39 and $\delta_C$ 52.6). Therefore, the planar structure of 2 was determined to be the same as that of 1. However, the difference in retention time
on preparative HPLC indicated that 1 (tR = 8.2 min) and 2 (tR = 8.7 min) are diastereomers at the C-2’ position. The relative structure of the aszonalenin residue in 1 and 2 was established from analysis of rotating-frame Overhauser enhancement and exchange spectroscopy (ROESY) spectra (Figure 2). ROESY correlations of H3-25/H-2 and H-11 in 1 and H3-25/H3-26 and H-2/H-11 in 2 indicated the relative configuration of the aszonalenin residue in 1 and 2 was same as that of epi-aszonalenis C (5). A previous study indicated that the stereochemistry of C-2, C-3, and C-11 depends on the cotton effect at 250 nm, and the same positive cotton effect at 250 nm was observed in each CD spectrum of 1, 2, and 5. On the other hand, 6 showed no positive cotton effect at 250 nm on the CD spectrum (Figure 3).

Therefore, the absolute configuration of the aszonalenin residues in 1 and 2 was determined to be 2R, 3R, and 11S (Figure 1).

Scheme 1. Esterification of 1 and 2 to 7 and 8
On the other hand, the small coupling constants at H-7’ and H-8’ in the $^1$H-NMR spectrum indicated that the relative stereochemistry of the hydroxy groups at C-7’ and C-8’ in 1 and 2 are *trans*. In order to determine the absolute configuration of C-7’ and C-8’ of the dihydroterrein residue, 1 and 2 were esterified to 7 and 8 using 4-(dimethylamino)bezoyl (DMAB) chloride in dichloromethane (Scheme 1). Positive cotton effects (325 nm) and negative cotton effects (300 nm) in CD spectra of the DMAB derivatives of 7 and 8 (Figure 4) indicated the absolute configurations of C-7’ and C-8’ as 7’*R* and 8’*S* based on exciton chirality analysis. From these results, the absolute configurations of 1 and 2, except for C-2’, were determined, as shown in Figure 1.

![Figure 4. CD spectra of 1, 2, 7 and 8](image)

Compound 3 was isolated as a colorless amorphous solid. The molecular formula of 3 was determined as C$_{20}$H$_{25}$N$_3$O$_3$ based on high-resolution electron ionization mass spectrometry (HREIMS). IR bands (3382, 1676, and 1631 cm$^{-1}$) and $^{13}$C-NMR signals ($\delta$ C 168.9 and 168.6) revealed the presence of two amide bonds. Moreover, $^1$H-NMR spectra of 3 exhibited signals for three methyl groups ($\delta$H 1.45 × 2 and 1.19), a methoxy group ($\delta$H 3.73), three methine protons ($\delta$H 6.14, 3.82 and 3.17), methylene and exomethylene protons ($\delta$H 3.20 and 3.08; 5.06 and 5.02), and three aromatic protons constituting a 1,2,4-trisubstituted benzene moiety (Table 2). In addition, $^1$H-$^1$H COSY spectra indicated four substructures of 3: the sequence of H-4 to H-5, H$_2$-10 to H-11, H-14 to H$_3$-17, and H-1’ to H-2’, as shown by bold lines in Figure 5. HMBC correlations from H-2’ to C-3’, C-4’ and C-5’, and between H$_3$-4’ and H$_3$-5’ to C-2’ established the presence of a 3-methyl-1-butene group (Figure 1).
### Table 2. NMR spectroscopic data for asnovozines A (3) and B (4)

<table>
<thead>
<tr>
<th>Position</th>
<th>δc&lt;sup&gt;a&lt;/sup&gt;</th>
<th>δH&lt;sub&gt;b&lt;/sub&gt; (J in Hz)</th>
<th>δc&lt;sup&gt;a&lt;/sup&gt;</th>
<th>δH&lt;sub&gt;b&lt;/sub&gt; (J in Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-NH</td>
<td>10.40 brs</td>
<td></td>
<td>10.87 brs</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>139.8</td>
<td></td>
<td>110.4</td>
<td>6.88 s</td>
</tr>
<tr>
<td>3</td>
<td>104.4</td>
<td></td>
<td>103.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>118.6</td>
<td>7.27 d (8.6)</td>
<td>119.6</td>
<td>7.08 d (8.6)</td>
</tr>
<tr>
<td>5</td>
<td>108.1</td>
<td>6.59 dd (8.6, 1.6)</td>
<td>109.2</td>
<td>6.68 dd (8.6, 2.4)</td>
</tr>
<tr>
<td>6</td>
<td>155.1</td>
<td></td>
<td>155.3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>94.1</td>
<td>6.82 d (1.6)</td>
<td>95.2</td>
<td>6.93 d (2.4)</td>
</tr>
<tr>
<td>8</td>
<td>135.4</td>
<td></td>
<td>135.9</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>123.3</td>
<td></td>
<td>120.3</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>29.4</td>
<td>3.20 m</td>
<td>142.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.08 dd (14.5, 8.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>48.6</td>
<td>3.17 m</td>
<td>124.6</td>
<td></td>
</tr>
<tr>
<td>12-NH</td>
<td>7.79 brs</td>
<td></td>
<td>8.56 s</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>168.9</td>
<td></td>
<td>156.0</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>56.8</td>
<td>3.82 m</td>
<td>50.6</td>
<td>4.15 q (7.0)</td>
</tr>
<tr>
<td>15-NH</td>
<td>8.13 brs</td>
<td></td>
<td>8.30 brs</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>168.6</td>
<td></td>
<td>166.5</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>17.1</td>
<td>1.19 d (7.0)</td>
<td>19.7</td>
<td>1.37 d (7.0)</td>
</tr>
<tr>
<td>18</td>
<td>55.1</td>
<td>3.73 s</td>
<td>55.3</td>
<td>3.76 s</td>
</tr>
<tr>
<td>1'</td>
<td>110.8</td>
<td>5.06 dd (16.9, 9.6)</td>
<td>111.6</td>
<td>5.03 dd (10.6, 1.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.02 dd (10.5, 9.6)</td>
<td></td>
<td>5.01 dd (17.6, 1.2)</td>
</tr>
<tr>
<td>2'</td>
<td>146.6</td>
<td>6.14 dd (16.9, 10.5)</td>
<td>145.3</td>
<td>6.05 dd (17.6, 10.6)</td>
</tr>
<tr>
<td>3'</td>
<td>39.1</td>
<td></td>
<td>39.0</td>
<td></td>
</tr>
<tr>
<td>4'</td>
<td>27.9</td>
<td>1.45 s</td>
<td>27.6</td>
<td>1.45 s</td>
</tr>
<tr>
<td>5'</td>
<td>27.9</td>
<td>1.45 s</td>
<td>27.6</td>
<td>1.45 s</td>
</tr>
</tbody>
</table>

<sup>a</sup>Recorded at 100 MHz in DMSO-<sup>d</sup><sub>6</sub>. <sup>b</sup>Recorded at 400 MHz in DMSO-<sup>d</sup><sub>6</sub>.

Furthermore, HMBC correlations from H-4 to C-6, C-8, and C-9; H-5 to C-7 and C-9; H-7 to C-5, C-6, C-8, and C-9; H<sub>2</sub>-10 (δ<sub>H</sub> 3.20 and 3.08) to C-2, C-3, C-9, and C-16; H-11 to C-16, and H<sub>3</sub>-18 to C-6 revealed the presence of a 6-methoxytryptophan group. In addition, HMBC correlations from H-2', H<sub>3</sub>-4', and H<sub>3</sub>-5' of a 3-methyl-1-butene group to the C-2 position of the 6-methoxytryptophan group indicated the linkage of a 3-methyl-1-butene group and a 6-methoxytryptophan group. Moreover, an alanine residue was revealed by HMBC correlations from H-14 and H<sub>3</sub>-17 to C-13. HMBC correlations from H-11 to C-13, C-16 and H-14 to C-13, C-16 indicated the presence of a diketopiperazine ring consisting of 6-methoxytryptophan and alanine residues. The relative structure of 3 was determined by NOE spectra.
and the modified Marfey’s method. The key NOE correlation of H-14 to H-10 ($\delta_H 3.08$) was observed; therefore, the stereochemistry of the diketopiperazine ring was anti (Figure 5). Moreover, acid hydrolysis of 3 followed by derivatization using 1-fluoro-2,4-dinitrophenyl-L-alanine-amide (Marfey’s reagent) and comparison of HPLC retention time with those of D/L-alanine derivative standards revealed that the alanine residue of 3 was the D-form. Thus, the stereochemistry of C-11 and C-14 were determined as $S$ and $R$ configuration, respectively, and the absolute configuration of 3 was established.

Compound 4 was isolated as a colorless amorphous solid, and the molecular formula was determined as $C_{20}H_{23}N_{3}O_{3}$ based on HRESIMS. The molecular formula of 4 had two fewer hydrogen atoms compared with that of 3. Moreover, $^1H$- and $^{13}C$-NMR spectra of 4 were very similar to those of 3, except for the absence of H-2-10 and H-11 signals and the appearance of an H-2 signal ($\delta_H 6.88$). Therefore, it was thought that the planar structure of 4 was formed by dehydration of C-10/11 and attachment to the 3-methyl-1-butene group at the C-10 position of 3. HMBC correlations from H-2 of the 6-methoxyindole ring and H-2’, H-3-4’, and H-3-5’ of the 3-methyl-1-butene group to the C-10 position indicated that the 3-methyl-1-butene group was attached to the C-10 position (Figure 1). Detailed analysis of NOESY spectrum indicated that key NOESY correlations of NH-12 ($\delta_H 8.56$ [s])/H-2 ($\delta_H 6.88$ [s]) and H-4 revealed that C10/11 was in the $Z$ form (Figure 5). The stereochemistry of the C-14 position was determined as 14$R$ using Marfey’s method, similar to 3.

Antifungal and cytotoxic activity of 1 and 2 was studied using the paper disk method, as described previously. Compounds 1 and 2 showed non-specific antifungal activity against *A. fumigatus*, *A. niger*, *Candida albicans*, and *Cryptococcus neoformans* at 100 $\mu$g per disk. Neither 1 nor 2 exhibited cytotoxic activity against A549 human lung cancer cells, HeLa human cervical cancer cells, and LNCap human prostate adenocarcinoma cells.

In a previous study, we reported several novel compounds along with known compounds such as helvolic acid and terrein. In this study, we isolated four novel cyclodipeptides and determined their
structures using a spectroscopic analysis and chemical approach. Asnovolenins A (1) and B (2) were found to consist of epi-aszonalein C (5) and dihydroterrein residues. The epi-aszonalein C (5) was found to be a cyclodipeptide consisting of two amino acids, L-tryptophan and anthranilic acid, and was isolated only from *A. novofumigatus*. By contrast, aszonalenins typically consist of D-tryptophan and anthranilic acid, and there are numerous reports of their isolation from a variety of fungi, such as *Aspergillus* sp. and *Neosartorya fischeri*. Asnovozines A (3) and B (4) were found to consist of two amino acids, D-alanine and L-tryptophan, and our results suggested that 3 is an intermediate of ent-cycloechinulin. Asnovozine B (4) had a 3-methyl-1-butene group attached at the C-10 position. To our knowledge, there are no reported examples of a 3-methyl-1-butene group attached at the C-10 position. Interestingly, a novoamauromine and an ent-cycloechinulin isolated only from *A. novofumigatus* were also epimers. These results suggest that *A. novofumigatus* could be characterized based on differences in the stereochemistry of compounds isolated from the fungus.

None of the compounds isolated in this study exhibited antifungal activity. Previous studies reported that aszonalenins act as substance P inhibitors for the human neuropeptide-1 receptor and that terrein is a melanogenesis inhibitor. These results suggest that 1 and 2, which include epi-aszonalenin C and dihydroterrein residues, would exhibit similar bioactivity.

**EXPERIMENTAL**

EI and CIMS data were collected using a JMS-MS600W spectrometer (JEOL Co., Ltd., Tokyo, Japan), and ESIMS data were collected using a JMS-T100LP spectrometer (JEOL Co. Ltd.). UV and IR spectra were recorded using an Ultrospec 2100 pro UV-visible spectrophotometer (Amersham Biosciences Ltd., Buckinghamshire, UK) and FT/IR-4100 instrument (JASCO Co. Ltd., Tokyo, Japan), respectively. 1H- and 13C-NMR spectra were recorded using an AVANCE-400 spectrometer (400.13 MHz for 1H, 100.61 MHz for 13C, Bruker Biospin, Billerica, MA). Chemical shifts (δ) were measured in ppm using tetramethylsilane as an internal standard. CD curves were determined on a J-820 spectropolarimeter (JASCO Co., Ltd.). Optical rotation was measured using a P-1020 photopolarimeter (JASCO Co., Ltd.). TLC plates were visualized under UV light at 254 nm and/or by spraying with phosphomolybdic acid (5%)-ceric acid (trace) in 5% H2SO4 and then heating. Column chromatography was performed using a Sephadex LH-20 column (GE Healthcare Bio-Science AB, Uppsala, Sweden). Middle-pressure liquid chromatography (MPLC) was performed using a Chemco Low-Prep 81-M-2 pump (Chemco Scientific Co., Ltd., Osaka, Japan) and ULTRA PACK SI-40B column (300 × 26 mm, Yamazen Corp., Osaka, Japan). Preparative HPLC was performed using a Senshu SSC-3160 pump (flow rate 4 mL/min, Senshu Scientific Co., Ltd., Tokyo, Japan) and Inertsil ODS-P column (250 × 10 mm, GL Sciences Inc., Tokyo, Japan) on a system equipped with a YRD-883 RI detector (Shimamuratech Ltd., Tokyo, Japan). Samples
were examined by Marfey’s method using PDA-HPLC with PU-980 and PU-1580 pumps (flow rate 1 mL/min; JASCO Co., Ltd.) and Inertsil ODS-3 column (5 μm, 4.6 mm × 250 mm; GL Science Inc., Tokyo, Japan) maintained at a temperature of 40 °C using a CO-2065 Plus column oven (JASCO Co.) and equipped with a MD-2010 Plus photodiode array detector (JASCO Co.).

**Fermentation and Isolation:** Polished rice (Akitakomachi, 24 kg) was soaked in water for 30 min and then sterilized in an autoclave. *Aspergillus novofumigatus* CBS 117520 was cultivated on sterilized rice (140 g) at 30 °C for 21 days in Roux flasks. Cultivated rice was then extracted with MeOH, and the MeOH extract was evaporated at 40 °C under reduced pressure. The resulting residue was suspended in water and extracted with EtOAc. The concentrated EtOAc extract (52 g) was partitioned between MeCN and n-hexane to yield an MeCN extract. The MeCN extract (29.4 g) was sequentially extracted by a solid-liquid separation method using n-hexane, benzene, CHCl₃, EtOAc, and MeOH. Then, n-hexane extract, benzene extract, CHCl₃ extract, EtOAc extract, and MeOH extract was obtained by removing organic solvent, respectively. The benzene extract was chromatographed on a Sephadex LH-20 column (solvent system: n-hexane/CHCl₃ [1:4], 200 mL; CHCl₃/acetone [3:2], 200 mL; CHCl₃/acetone [1:4], 200 mL; acetone, 200 mL; and then MeOH, 500 mL). Fraction 3 (CHCl₃/acetone [1:4] elute) was chromatographed by MPLC on a silica gel column (CHCl₃/MeOH [10:1]) followed by HPLC on a silica gel column (CHCl₃/MeOH [20:1]) to isolate asnovolenins A (1: 44.9 mg) and B (2: 72.0 mg) and asnovozine A (3: 1.0 mg). The other known compounds, *epi-*aszonalenin A (217 mg) and C (5: 85 mg), terrein, and dihydroterrein were isolated from the benzene extract, and identified by comparison with spectral data in the literature.⁷,⁸ The CHCl₃ extract was chromatographed on a Sephadex LH-20 column (solvent system: n-hexane/CHCl₃ [1:4], 200 mL; CHCl₃/acetone [3:2], 200 mL; CHCl₃/acetone [1:4], 200 mL; acetone, 200 mL; and then MeOH, 500 mL). Fraction 3 (CHCl₃/acetone [1:4] elute) was chromatographed by MPLC on a silica gel column (CHCl₃/MeOH [15:1]) followed by HPLC on a silica gel column (CHCl₃/MeOH [35:1] to [6:1]) to isolate asnovozine B (4: 3.1 mg).

**Asnovolenin A (1):** Colorless amorphous solid; [α]D<sub>18</sub> +211.7 (c 0.29, MeOH); UV (MeOH) λ<sub>max</sub> (logε) 216 (4.9), 252 (4.5), 300 (4.0) nm; IR (KBr) ν<sub>max</sub> 3447, 1699, 1636 cm⁻¹; CD (c 3.80×10⁻⁵, MeOH) Δε (λ<sub>max</sub>) −6.8 (208), 5.5 (217), −7.9 (230), 50.7 (252), −5.3 (300), 2.1 (322), −0.7 (345) nm. HRCIMS obsd. 528.2519 [M+H]<sup>+</sup> (calcd. 528.2498 for C₃₁H₃₄N₃O₅); The <sup>1</sup>H- and <sup>13</sup>C-NMR signal assignments are summarized in Table 1.

**Asnovolenin B (2):** Colorless amorphous solid; [α]D<sub>20</sub> +218.8 (c 0.25, MeOH); UV (MeOH) λ<sub>max</sub> (logε) 215 (4.5), 249 (4.1), 300 (3.6) nm; IR (KBr) ν<sub>max</sub> 3430, 1700, 1636 cm⁻¹; CD (c 3.80×10⁻⁵, MeOH) Δε (λ<sub>max</sub>) −3.3 (204), 6.7 (216), −5.6 (231), 6.7 (216), 50.7 (252), −4.8 (302), 1.0 (321), −2.5 (343) nm; HRCIMS obsd. 528.2512 [M+H]<sup>+</sup> (calcd. 528.2498 for C₃₁H₃₄N₃O₅); The <sup>1</sup>H- and <sup>13</sup>C-NMR signal assignments are summarized in Table 1.
Asnovolenin A di-4-(dimethylamino)benzoate (7): Compound 1 (6.2 mg, 0.012 mmol), 4-(dimethylamino)benzyl chloride (32.9 mg, 0.18 mmol) and DCM (1 mL) were mixed and stirred at 50 °C for 13 h. The reaction mixture was extracted with CHCl₃ and water. The extract was evaporated and residue purified by HPLC (benzene/acetonitrile [10:1]) to afford 3.0 mg of 7. Colorless amorphous solid; UV (MeOH) λ max (logε) 208 (4.8), 215 (4.8), 251 (4.4), 317 (4.8) nm; CD (c 2.44×10⁻⁵, MeOH) Δε (λ max) 12.2 (214), 15.5 (217), −5.5 (231), 51.9 (253), −26.8 (300), 61.8 (325) nm; ESIMS obsd. 844.0 [M+Na]⁺; ¹H-NMR (CDCl₃) δ : 1.03 (3H, s, H-26), 1.15 (3H, s, H-25), 1.33 (3H, d, J=6.9 Hz, H-3’), 2.60 (2H, m, H-3’), 2.93 (1H, dd, J=14.4, 4.8 Hz, H-10), 3.03 (6H, s, N-(CH₃)₂ × 2), 3.04 (6H, s, N-(CH₃)₂ × 2), 3.16 (1H, dd, J=14.4, 9.5 Hz, H-10), 4.14 (1H, dd, J=9.5, 4.8 Hz, H-11), 4.53 (1H, m, H-2’), 5.09 (1H, dd, J=10.9, 1.0 Hz, H-24), 5.10 (1H, J=17.2, 1.0 Hz, H-24), 5.56 (1H, d, J=3.0 Hz, H-7’), 5.94 (1H, dd, J=17.2, 10.9 Hz, H-23), 5.97 (1H, s, H-2), 6.30 (1H, dd, J=3.0, 0.8 Hz, H-8’), 6.51 (1H, d, J=8.2 Hz, H-4), 6.57 (2H, d, J=9.2 Hz), 6.58 (2H, d, J=9.2 Hz), 6.65 (1H, dd, J=7.6, 7.4 Hz, H-6), 6.86-6.92 (2H, m, H-7, H-15), 7.22-7.28 (2H, m, H-5, H-17), 7.42 (1H, dd, J=7.8, 7.7, 1.5 Hz, H-16), 7.76 (2H, d, J=9.2 Hz), 7.80 (1H, s, NH), 7.85 (2H, d, J=9.2 Hz), 8.03 (1H, dd, J=7.9, 1.5 Hz, H-18).

Asnovolenin B di-4-(dimethylamino)benzoate (8): Compound 2 (7.1 mg, 0.013 mmol), 4-(dimethylamino)benzoyl chloride (32.9 mg, 0.18 mmol) and DCM (1 mL) were mixed and stirred at 50 °C for 13 h. The reaction mixture was extracted with CHCl₃ and water. The extract was evaporated and residue purified by HPLC (benzene/acetonitrile [9:1]) to afford 1.0 mg of 8. Colorless amorphous solid; UV (MeOH) λ max (logε): 207 (4.7), 215 (4.7), 251 (4.2), 317 (4.7) nm; CD (c 2.44×10⁻⁵, MeOH) Δε (λ max) 8.1 (212), 12.0 (217), −5.0 (232), 46.3 (252), −19.2 (300), 47.8 (325) nm; ESIMS obsd. 844.0 [M+Na]⁺; ¹H-NMR (CDCl₃) δ : 1.03 (3H, s, H-26), 1.15 (3H, s, H-25), 1.33 (3H, d, J=6.9 Hz, H-3’), 2.59 (1H, dd, J=13.9, 9.7 Hz, H-10), 2.66 (1H, dd, J=17.7, 11.3 Hz, H-3’), 2.87 (1H, dd, J=17.7, 8.1 Hz, H-3’), 2.90 (1H, dd, J=13.9, 4.4 Hz, H-10), 3.04 (6H, s, N-(CH₃)₂ × 2), 3.04 (6H, s, N-(CH₃)₂ × 2), 4.14 (1H, dd, J=9.7, 4.4 Hz, H-11), 4.28 (1H, m, H-2’), 5.09 (1H, dd, J=17.6, 1.1 Hz, H-24), 5.10 (1H, dd, J=10.5, 1.1 Hz, H-24), 5.26 (1H, d, J=3.0 Hz, H-7’), 5.99 (1H, dd, J=17.6, 10.5 Hz, H-23), 6.08-6.13 (2H, m, H-2, H-8’), 6.31 (1H, d, J=0.7 Hz, H-5’), 6.49 (1H, d, J=8.0 Hz, H-4), 6.61 (4H, m), 6.71 (1H, dd, J=7.5, 7.3 Hz, H-6), 6.88 (1H, d, J=8.1 Hz, H-15), 7.02 (1H, t, J=7.5 Hz, H-17), 7.20-7.32 (2H, m, H-5, H-7), 7.44 (1H, ddd, J=8.1, 7.5, 1.5 Hz, H-16), 7.87 (2H, d, J=9.0 Hz), 7.87 (2H, d, J=9.0 Hz), 7.90 (1H, s, NH), 8.05 (1H, dd, J=7.5, 1.5 Hz, H-18).

Asnovozine A (3): Colorless amorphous solid; [α]D²⁶ −17.6 (c 0.05, MeOH); UV (MeOH) λ max (logε) 207 (4.3), 226 (4.4), 298 (3.6) nm; IR (KBr) ν max 3382, 1676, 1631 cm⁻¹; CD (c 5.63×10⁻⁵, MeOH) Δε (λ max) −7.4 (219), 1.9 (239) nm; HREIMS obsd. 355.1880 [M]+ (calcld. for C₂₀H₂₅N₃O₃ 355.1896); The ¹H- and ¹³C-NMR signal assignments are summarized in Table 2.
Asnovozine B (4): Colorless amorphous solid; [α] D27 +51.8 (c 0.16, MeOH); UV (MeOH) λ max (log ε): 223 (4.4), 301 (3.9), 344 (3.9) nm; IR (KBr) ν max 3216, 1673, 1629 cm−1; CD (c 8.50×10−5, MeOH) Δε (λ max ) 1.7 (211), 2.4 (221), −1.4 (241), −0.8 (262), −0.9 (300), 0.6 (346) nm; HRESIMS obsd. 376.16304 [M+Na] + (calcd. 376.16371 for C20H23N3O3Na); The 1H- and 13C-NMR signal assignments are summarized in Table 2.

Amino acid analysis of 3 and 4: Compounds 3 and 4 (each 1.0 mg) were dissolved in 100 μL of 6 M HCl and heated at 110 °C for 12 h. The resulting hydrolysates were allowed to cool and then neutralized with NaHCO3. Then, 200 μL of Marfey’s reagent (PIERCE, IL, USA) and 40 μL of 1 M NaHCO3 were added, and the mixture were heated at 40 °C for 1 h. Upon cooling to room temperature, 20 μL of 2 M HCl was added, respectively. The solution was then analyzed by reversed-phase PDA-HPLC (Analysis condition; Flow rate was 1 mL/min, and the column oven temperature was maintained at 30 °C. Mobile phase was 60% MeCN, and compounds were detected at a UV wavelength of 340 nm.), as previously described.10 Comparison with t R value for D- and L-alanine standards indicated that the alanyl residue in 3 and 4 has a D-configuration, respectively.

Antifungal assay using the paper disk method: Antifungal assay was performed according to the paper disk method using A. niger IFM 41398, A. fumigatus IFM 41362, C. albicans IFM 40009, and C. neoformans ATCC 90112 as test organisms. Asnovolenins A and B (1 and 2) were applied to the paper disk (diameter: 8 mm) at 100 μg per disk, and the disks were then placed on the 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, zones of inhibition (diameter measured in millimeters) were recorded.

ACKNOWLEDGEMENTS
We thank Dr. H. Kasai and Dr. M. Ikegami of Hoshi University for technical assistance. This work was supported in part by “Open Research Center” Project funds 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 was also supported by the Cooperative Research Program of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University.

REFERENCES


