Analysis of Beauvericin and Unusual Enniatins Co-Produced by *Fusarium oxysporum* FB1501 (KFCC 11363P)

SONG, HYUK-HWAN¹, JOONG-HOON AHN², YOONG HO LIM³, AND CHAN LEE¹*

¹Department of Food Science and Technology, BET Research Institute, Chung-Ang University, Ansan 456-756, Korea
²Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea

Received: December 14, 2005
Accepted: March 2, 2006

**Abstract** Beauvericins and enniatins are cyclodepsipeptides exhibiting various biological activities on animal systems, including humans. *Fusarium oxysporum* FB1501 (KFCC 11363P) that produces four different cyclodepsipeptides was isolated from soil in Korea and the structures of the four cyclodepsipeptides elucidated by HPLC, MS, IR, and NMR analyses. The molecular weights for compounds 1, 2, 3, and 4 were determined to be 654.5, 784.5, 668.6, and 682.5, respectively, on the basis of ESI-MS measurements. The IR spectra for all the compounds exhibited absorptions for ester bonds that were very similar to those for beauvericin and enniatins with ester and amide absorptions. The results of the NMR analysis (¹H, ¹³C, 135-DEPT, COSY, HMQC, and HMBC; in CDCl₃) revealed that compounds 1, 3, and 4 consisted of L-N-methyl valine (N-MeVal), N-α-hydroxyisovaleric acid (Hiv), and 2-hydroxy-3-methylpentanoic acid (Hmp) residues (compound 1: three N-MeVal residues, two Hiv residues, and one Hmp residue; compound 3: three N-MeVal residues, one Hiv, and two Hmp residues; compound 4: three N-MeVal residues and three Hmp residues). Therefore, the compounds were identified as enniatin H (compound 1), enniatin I (compound 3), and enniatin MK1688 (compound 4). Compound 2 was analyzed as beauvericin according to 1D and 2D NMR analyses. This study is the first report related to the co-production of beauvericin with other unusual enniatins, such as enniatin H, enniatin I, and enniatin MK1688, by *Fusarium oxysporum*.

**Key words:** Cyclodepsipeptide, beauvericin, enniatin H, enniatin I, enniatin MK1688, *Fusarium oxysporum*

*Fusarium* genera have been found to produce biologically active second metabolites [4, 13, 26, 28] including mycotoxins, such as trichotheccenes, fumonisins, fusaric acid, moniliformin, and fusaproliferin [7, 10, 14, 30]. They also produce beauvericin (BEA) and enniatins (ENs) that exhibit various biological activities and are well-known cyclic hexadepsipeptides showing ionophoric properties [12, 29]. Because of their ionophoric structures, BEA and ENs also have various biological effects on animal systems, including altering the ion transport across membranes, resulting in the disruption of the cationic selectivity of cell wall [16]. These basic mechanisms then lead to a large array of biological abilities, including antimicrobial, insecticidal, and a strong cytotoxicity towards several cell lines from invertebrates, rodents, farm animals, and humans [2, 3, 6, 8]. Consequently, these findings on the potential of BEA and ENs in biological systems have stimulated further examination of the significance of such cyclodepsipeptides.

BEA and ENs contain an alternating sequence of three N-methyl-l-α-amino acids [N-methyl-l-phenylalanine (N-MePhe); BEA, N-methyl-l-valine (N-MeVal), N-methyl-l-leucine (N-MeLeu), or N-methyl-l-isoleucine (N-MeIle); ENs] and three N-α-hydroxyisovaleric acids (Hiv) in their molecular structure. ENs have been reported as natural contaminants, and include enniatin A (EN A), enniatin A1 (EN A1), enniatin B (EN B), and enniatin B1 (EN B1) [9, 23, 29]. EN A, EN A1, EN B, and EN B1 are composed of three Hivs and three amino acid residues including N-MeLeu and/or N-MeVal, whereas EN C is formed with N-MeLeu and Hiv. In the case of novel EN D, EN E, and EN F, they consisted of both Hiv and N-MeLeu, with N-MeLeu or N-MeVal as the other amino acid residue in their structure [31]. Three ENs of the B series, designated as B2, B3, and B4, were previously characterized from a liquid culture of *F. acuminatum* and *F. compactum* by Visconti *et al.* [32], and EN G, EN H, EN I, and EN MK1688 were recently isolated from the insect pathogenic fungus *Verticillium hemipterigenum* BCC 1449 [15, 20, 25]. In addition, EN L, EN M1, EN M2, and EN N were the first enniatin groups

*Corresponding author
Phone: 82-31-670-3035; Fax: 82-31-676-8865;
E-mail: chanlee@cau.ac.kr
to reveal a hydroxyl group in the side chain of the 2-hydroxyarboxylic acid residues [33].

Accordingly, in this study, a *Fusarium* species co-producing several ionophoric cyclic hexadepsipeptides was isolated from soil in Korea and the strain identified as *F. oxysporum* based on morphological criteria and synthetic keys. To investigate the pattern of cyclic hexadepsipeptides produced from this isolate, the hexadepsipeptides were purified and their structures were elucidated using several instrumental analyses, including HPLC, MS, IR, and NMR.

**Materials and Methods**

**Fusarium Isolate**

*Fusarium* strain FB1501 (KFCC 11363P) isolated from soil in Korea was identified according to the morphological criteria and synthetic keys of Samson *et al.*[27] and Nelson *et al.*[24]. The *Fusarium* strain was cultivated in FDM (25 g of sucrose, 4.25 g of NaNO₃, 5 g of NaCl, 2.5 g of MgSO₄·7H₂O, 1.36 g of KH₂PO₄, 0.01 g of FeSO₄·7H₂O, and 0.0029 g of ZnSO₄·7H₂O per liter) as described by Madry *et al.*[19]. For submerged cultures, 100 ml of the medium in a 250-ml Erlenmeyer flask was inoculated with approximately 1×10⁵ spores and the culture incubated at 25°C with shaking at 120 rpm for 6 days.

**Purification of Cyclic Hexadepsipeptides**

The liquid culture of *F. oxysporum* FB1501 (KFCC 11363P) including the mycelium was extracted twice with a double volume of chloroform [1]. The bottom layer was then evaporated to dryness and the residue was resuspended in methanol (HPLC grade). Thereafter, the extract was applied to a high-performance liquid chromatography (HPLC) system for further purification steps. HPLC was applied to a high-performance liquid chromatography (HPLC) system (Karlsruhe, Germany) at a temperature of 298 K. The 1D-NMR (H NMR, 13C NMR, DEPT, COSY, and HMBC) were performed on a Bruker Avance 500 spectrometer system (Karlsruhe, Germany) and the 2D-NMR (COSY, HMQC, and HMBC) were collected in CDCl₃. The long-ranged coupling time for HMBC was 70 msec. Prior to a Fourier transformation, zero filling of 2 K and a sine squared bell window function were applied using XWIN-NMR (Bruker, Karlsruhe, Germany).

**Measurement of Picrate Transferring Activity**

The mobile phase (acetonitrile-water) was removed from the extract under reduced pressure, and the residue was resuspended in 20 ml of chloroform. One ml of the compound in a chloroform solution was then transferred to a test tube and the tube shaken vigorously after the addition of 1 ml of a picrate solution (picric acid 1 g, and 1 N KOH 100 ml, per liter). After centrifugation, the absorbance of the chloroform layer was determined, at 370 nm using chloroform as the reference for the UV spectrometer (Uvikon 933, Kontron, Italy)[1].

**Thin-Layer Chromatography (TLC)**

The presence of BEA and ENs was analyzed by TLC. BEA and ENs were spotted on a silica HP TLC plate (E. Merck, Darmstadt, Germany), which was then developed with a mixture of acetic acid, methanol, and water (100:5:1, v/v/v). After developing, the plate was air dried and the spots on the TLC plate were detected using iodine vapor.

**Structure Analysis**

Electrospray ionization (ESI) was performed using an LC-MSD Trap VL mass spectrometer (Agilent, U.S.A.). During the LC-MS-analysis, the LC effluent entered the mass spectrometer without splitting at a source voltage of 4.5 kV. The mass spectrometer was programmed to perform full scans between m/z 100–900 for BEA and ENs. The IR spectra were recorded with an FTIR-8400S infrared spectrophotometer (Shimadzu, Japan) in a KBr pellet. All the NMR measurements of BEA were performed on a Bruker Avance 400 spectrometer system (9.4 T, Karlsruhe, Germany) at a temperature of 298 K. The 1D-NMR (H NMR, 13C NMR, and DEPT-135) measurements of ENs were performed on a Bruker Avance 500 spectrometer system (Karlsruhe, Germany) and the 2D-NMR (COSY, HMQC, and HMBC) measurements performed on a Bruker DMX 600 spectrometer system (Karlsruhe, Germany). The NMR spectra of H NMR, 13C NMR, DEPT, COSY, HMQC, and HMBC were collected in CDCl₃. The long-ranged coupling time for HMBC was 70 msec. Prior to a Fourier transformation, zero filling of 2 K and a sine squared bell window function were applied using XWIN-NMR (Bruker, Karlsruhe, Germany).

**Results and Discussion**

**Identification of Fusarium strain FB1501 (KFCC 11363P)**

*Fusarium* strain FB1501 (KFCC 11363P) was identified as *F. oxysporum* according to the methods of Samson *et al.*
The colonies of the *Fusarium* strain were usually fast growing, brightly colored, and had a cottony aerial mycelium in a potato dextrose agar (PDA). The color of the thallus ranged from whitish to brown shades. In a morphological study, kidney-shaped microconidia formed from false heads were found in abundance, whereas the sickle-shaped macroconidia exhibited three thin-walls, clamydospores were observed singly or in pairs, and conidiophores showed branched monophialides (Fig. 1). Since these morphological characteristics exactly matched the criteria and synoptic keys for the *F. oxysporum* as described by Samson *et al.* [27] and Nelson *et al.* [24], the fungi was identified as *F. oxysporum* with the number FB1501 and maintained at the Korea Federation of Culture Collections (KFCC) with the number 11363P.

Purification of Cyclic Hexadepsipeptides

Several peaks were found in the HPLC chromatogram of the methanol extract after the first purification step. Four compounds were eluted with retention times of 16.5, 18.5, 21.5, and 28.5 min (peaks 1, 2, 3, and 4, respectively) and exhibited different picrate transferring activities (Fig. 2 and Table 1). Picrate is insoluble in chloroform and can only be transferred in the presence of ionophoric compounds. As a result, it was proved that the isolated compounds possessed an ability to carry picrate into a chloroform phase. Each fraction was then further purified in the second step and the purified compounds exhibited a single spot on a TLC plate after exposure to iodine gas (Fig. 3). The \( R_f \) value for compound 2 was 0.85, whereas all the other compounds showed the same \( R_f \) value of 0.76 on the TLC.

---

Fig. 1. Photograph of *Fusarium* strain FB1501 (KFCC 11363P) (×400).
A & B, microconidia; C & D, macroconidia; E, donidiophore; and F, clamydospore.
The molecular weights of compounds 1, 2, 3, and 4 were determined to be 654.5, 784.5, 668.6, and 682.5, respectively, on the basis of ESI-MS measurements (data not shown). Their IR spectra also showed absorptions indicating ester ($v_{1733-1739}$ cm$^{-1}$) and amide ($v_{1650-1655}$ cm$^{-1}$) bonds (data not shown), which were very similar to those of enniatins containing ester and amide bonds in their molecular structures.

**Compound 1.** The chemical shifts of the $^{13}$C and $^1$H NMR spectra (in CDCl$_3$) for compound 1 are summarized in Table 2. An analysis of the $^1$H-$^1$H COSY and $^{13}$C-$^1$H HMBC spectra revealed the three partial structures for compound 1 (Fig. 4). The $^{13}$C-$^1$H long-range couplings of $^2$J and $^3$J observed in the $^{13}$C-$^1$H HMBC experiments (Fig. 5) provided the following evidence. The signal at 8-H ($\delta_{5.271}$) assigned to the proton situated at the $\alpha$-position ($2$-H, $\delta_{4.569}$) attached to C-8, ($\delta_{74.526}$) showed vicinal coupling (COSY) to a multiple signal at 9-H ($\delta_{2.013}$) attached to C-9 ($\delta_{36.297}$). This methine (C-9), in turn, was connected to a methyl group ($12$-H, $\delta_{0.963}$ overlapping signal; C-12, $\delta_{14.803}$) and a methylene ($10$-H, $\delta_{1.438}$ and $\delta_{1.184}$ overlapping signal; C-10, $\delta_{25.600}$). The C-10 methylene was attached to a terminal methyl ($11$-H, $\delta_{0.916}$, overlapping signal; C-11, $\delta_{11.514}$) as indicated by the COSY cross-signal. Therefore, the 2-hydroxycarboxylic acid residue was 2-hydroxy-3-methylpentanoic acid (Hmp). The $^1$H and $^{13}$C NMR assignments of the three $\text{\textit{L}}$-N-MeVal residues for this compound could not be distinguished, owing to the very close signal overlap. However, the partial structure was confirmed by a 2D-NMR analysis (COSY and HMBC) as a set of signals. The important HMBC correlations for the $\text{\textit{N}}$-MeVal residues were 2-H ($\delta_{64.564}$) to C-3 ($\delta_{628.063}$) and one carbonyl signal at C-1 ($\delta_{170.541}$). The $\text{\textit{D}}$-$\alpha$-hydroxyisovaleric acid (Hiv) residues were also assigned as a set of signals.

Finally, the $^{13}$C NMR assignment of the carbonyl carbons, which only appeared as two signals at $\delta_{170.541}$ (C-1) and $\delta_{169.536}$ (C-7, 13), was achieved based on the HMBC correlations from H-6 ($\delta_{3.129}$) to C-7 and 13, not to C-1. Therefore, the C-7 and 13 signals were assigned to amide carbonyls (C-7 for Hmp and C-13 for Hiv), and the C-1 ($\delta_{170.541}$) signal assigned to ester carbonyl (C-1 for three N-MeVal). The results of the NMR analyses ($^1$H, $^{13}$C, 13DEPT, COSY, HMQC and HMBC; in CDCl$_3$) revealed that the compound was composed of three N-MeVal, two Hiv, and one Hmp residue, in association with the results of the molecular mass determination. Thus, the protons of the three N-MeVal residues and two Hiv residues appeared in the $^1$H NMR spectrum of compound 1 as superimposed signals in the up field. N-MeVal and Hiv were classified as the position of N-Methyl according to the results of the 2D-NMR spectra.

The result for the molecular weight (654.6) exactly matched those of enniatin BI [5], enniatin D [31], and enniatin H [25]. Furthermore all the present data were also consistent with the data for the EN H previously reported by Nilanonta et al. [25]. The structure of compound 1 was elucidated, as shown in Fig. 6.
Compound 2. The molecular weight of compound 2 was determined to be 784.5 on the basis of ESI-MS measurements (data not shown). The IR spectra showed absorptions for ester (ν 1,743 cm⁻¹) and amide (ν 1,649 cm⁻¹), which were very similar to those for beauvericin with ester and amide absorptions (data not shown). The chemical shifts of the ¹³C and ¹H NMR spectra (in CDCl₃) of compound 2 are summarized in Table 3. An analysis of the ¹H-¹H COSY and ¹³C-¹H HMBC spectra revealed the partial structure shown in Fig. 7. The ¹³C-¹H long-range couplings of J and J observed in the ¹³C-¹H HMBC experiments provided the following evidence (Fig. 8). The cross-peaks from 2-H (δ 5.52) to C-1 (δ 169.70), C-3 (δ 34.75), and C-10 (δ 121.61), from 3-H (δ 3.38) to C-1, C-2 (δ 87.16), C-4 (δ 136.58), C-5 (δ 128.83), and C-9 (δ 128.83), 12-H (δ 4.90) to C-11 (δ 169.95), C-13 (δ 29.75), C-14 (δ 29.75), and C-15 (δ 29.75), from 13-H (δ 2.00) to C-12 (δ 79.94), C-14, and C-15, from 15-H (δ 0.80) to C-12, C-13, and C-14, and from 14-H (δ 0.42) to C-12, C-13, and C-15, supported the partial structure. The NMR analyses (¹H, ¹³C, DEPT135, COSY, HMQC, and HMBC; in CDCl₃) revealed that the compound consisted of three N-MePhe and three Hiv residues, which was consistent with the measured molecular weight. Taken together, the structure of compound 2 was elucidated as shown in Fig. 9. All the present data agreed well with previously reported data for BEA [11, 12, 17, 21, 22].

Compound 3. The ¹H and ¹³C NMR spectra (Table 2) for compound 3 were similar to those for compound 1 (Enniatin H), where the chemical shifts of the protons and carbons in each residue were superimposed, yet with a different composition: three N-MeVal, one Hiv, and two Hmp. An analysis of the 2D-NMR spectra (COSY, HMQC, and HMBC) further confirmed the connectivity and assignment of each residue (Fig. 5). The ¹³C-¹H long-range couplings

<table>
<thead>
<tr>
<th>Table 2. NMR data for compounds 1, 3, and 4 in CDCl₃.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
</tr>
<tr>
<td>¹³C</td>
</tr>
<tr>
<td>N-MeVal</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>Hmp</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>Hiv</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

²H signals are overlapping.
³Assignments can be interchanged.
of $^3J$ and $^3J$ observed in the $^{13}C$-H HMBC experiments were also similar to those for compound 1 (EN H). The $^{13}C$ NMR assignment of the carbonyl carbons, which only appeared as two signals at $\delta 170.613$ (C-1) and $\delta 169.529$ (C-7, 13), was achieved based on the HMBC correlations from H-6 ($\delta 3.102$) to C-7 and 13, not to C-1. Therefore, the C-7 and 13 signals were assigned to amide carbonyls (C-7 for Hmp and C-13 for Hiv), and the C-1 ($\delta 170.541$) signal to ester carbonyls (C-1 for three N-MeVal). The data assigned here were very similar to the NMR and other data for compound 1. The molecular weight of compound 2 was determined to be 668.5 on the basis of ESI-MS measurements (data not shown). So far, three ENs, i.e., EN A1 [5], EN E [31], and EN I [25], have exhibited this molecular mass.

The NMR analysis ($^1H$, $^{13}C$, 135-DEPT, COSY, HMQC, and HMBC; in CDCl$_3$) revealed that the only possible structure for this compound was a cyclic depsipeptide structure containing three N-MeVal residues, one Hiv residue, and two Hmp residues, in accordance with the results of the molecular mass determination. These results were consistent with those previously reported for EN I. Thus, compound 3 was identified as EN I, in which Hmp was substituted with Hiv when compared with the structure of the EN H reported by Nilanonta et al. [25]. The structure of compound 3 was elucidated, as shown in Fig. 6.

**Compound 4.** The $^1H$ and $^{13}C$ NMR spectra (Table 2) for compound 4 exhibited similar results to the Hmp and N-MeVal residues in compounds 1 and 3. From the results of the molecular weight determination and data on the chemical shifts of the protons and carbons in compound 4, it was postulated that the compound was composed of three N-MeVal and three Hmp. An analysis of the 2D-NMR spectra (COSY, HMQC, and HMBC) further confirmed

---

**Fig. 4.** Partial structures of enniatins (A, N-MeVal; B, Hmp; and C, Hiv).

**Fig. 5.** HMBC and COSY correlations of enniatin H (compound 1).

**Fig. 6.** Chemical structure of enniatins. R1=CH$_2$CH$_3$, R2, R3=CH$_3$; compound 1 (Enniatin H); R1, R2=CH$_2$CH$_3$, R3=CH$_3$; compound 3 (Enniatin I); R1, R2, R3=CH$_3$; compound 4 (Enniatin MK1688).
the connectivity and assignment of each residue (A and B in Fig. 4). Thus, in the 'H NMR spectrum of compound 4, the protons of three Hmp residues appeared. The signal at 8-H (δ 5.286) assigned to the proton situated at the α-position (2-H, 4.599; attached to C-2, δ74.562) showed vicinal coupling (COSY) to a multiplet signal at the 9-H (δ2.011) attached to C-9 (δ36.418). This methane (C-9), in turn, was connected to a methyl group (12-H, δ0.962, overlapping signal; C-12, δ14.817) and a methylene (10-H, δ1.459 and 1.189 overlapping signal; C-10, δ25.594). The C-10 methylene was attached to a terminal methyl (11-H, δ0.914, overlapping signal; C-11, δ11.549) as indicated by the COSY cross-signal. Therefore, the hydroxycarboxylic acid residue was assigned to N-MeVal. The important HMBC correlations for N-MeVal residues are 2-H (δ4.599) to C-4 (δ20.497) and C-5 (δ19.571) and one carbonyl signal at C-1. The 13C NMR assignment of the carbonyl carbons, which only appeared as two signals at δ170.644 (C-1) and δ169.490 (C-7), was achieved based on the HMBC correlations from H-6 (δ3.105) to C-7 (δ169.529), not to C-1 (δ170.613). Therefore, the C-7 signal was assigned to amide carbonyls (C-7 for three Hmp), and the C-1 (δ170.541) signal to ester carbonyls (C-1 for three N-MeVal).

All the data assigned coincided with the results for the N-MeVal and Hmp residues in compounds 1 and 3. The molecular weight for compound 4 (682.6) was the same as those for EN B1 [5], EN F [31], and EN MK1688 [20, 25]. The NMR analysis and molecular weight determination revealed that the data for compound 4 were consistent with that previously reported for the EN MK1688 isolated from the insect pathogenic fungus Verticillium hemipterigenum with

Table 3. 1H and 13C NMR chemical shifts for compound 2.

<table>
<thead>
<tr>
<th>N-MePhe</th>
<th>1H Chemical shifts (ppm)</th>
<th>13C Chemical shifts (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 units</td>
<td>169.70</td>
<td>5.286 (1H, dd, J = 11.9, 4.9)</td>
</tr>
<tr>
<td></td>
<td>34.75</td>
<td>3.38 (2H, dd, J = 14.6, 4.9 Hz)</td>
</tr>
<tr>
<td></td>
<td>32.22</td>
<td>3.01 (1H, s)</td>
</tr>
<tr>
<td>Hiv</td>
<td>169.95</td>
<td>4.90 (1H, d, J = 8.5)</td>
</tr>
<tr>
<td>3 units</td>
<td></td>
<td>2.00 (1H, m)</td>
</tr>
<tr>
<td></td>
<td>18.75</td>
<td>0.42 (3H, d, J = 6.9 Hz)</td>
</tr>
<tr>
<td></td>
<td>17.38</td>
<td>0.80 (3H, d, J = 6.6 Hz)</td>
</tr>
</tbody>
</table>

Fig. 7. Partial structures of beauvericin (A: N-MePhe; B: Hiv).

Fig. 8. HMBC and COSY correlations with beauvericin (compound 2).

Beauvericin and Unusual Enneatins Co-produced by Fusarium oxysporum
Fig. 9. Chemical structure of beauvericin.

EN B, EN G, EN H, and EN I [25]. The structure of compound 4 was elucidated, as shown in Fig. 6.

Several researchers have already reported on the productions of EN A, EN A1, EN B, and EN B1 with BEA by Fusarium species [18, 23]. However, there have been no previous reports related to the co-production of BEA (0.17 g/l) with EN H (0.16 g/l), EN I (0.55 g/l), and EN MK1688 (0.81 g/l). Furthermore, no Fusarium species has ever been reported to produce a strain of BEA with EN H, EN I, and EN MK1688. Therefore, this study is the first report on the co-production of BEA with other unusual ENs, such as EN H, EN I, and EN MK1688, from F. oxysporum.

Acknowledgments

The authors would like to thank Dr. Jae-Hong Han for his invaluable assistance with the NMR analysis. This work was supported by a grant (No. R01-2005-000-10881-0) from the Basic Research Program of the Korea Science & Engineering Foundation.

REFERENCES

BENZFLUERIDE AND UNUSUAL ENNIATINS CO-PRODUCED BY Fusarium oxysporum


