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

SONG, HYUK-HWAN[1], JOONG-HOON AHN[2], YOONG HO LIM[2], AND CHAN LEE[1]*

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

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Abstract Beauvericins and enniatins are cyclohexadepsipeptides exhibiting various biological activities on animal systems, including humans. *Fusarium oxysporum* FB1501 (KFCC 11363P) that produces four different cyclohexadepsipeptides was isolated from soil in Korea and the structures of the four cyclohexadepsipeptides 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 (1,733–1,743 cm^{-1}) and amide (1,649–1,655 cm^{-1}) bonds that were very similar to those for beauvericin and enniatins with ester and amide absorptions. The results of the NMR analysis (^{1}H, ^{13}C, 135-DEPT, COSY, HMQC, and HMBC; in $CDCl_3$) revealed that compounds 1, 3, and 4 consisted of L-*N*-methyl valine (*N*-MeVal), D-α-hydroxyisovaleic 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 trichothecenes, 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-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 D-α-hydroxyisovaleic 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*-MeIle 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*-MeIle 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-hydroxycarboxylic 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 synoptic 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 synoptic 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 $NaNO_3$, 5 g of NaCl, 2.5 g of $MgSO_4 \cdot 7H_2O$, 1.36 g of KH_2PO_4, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 0.0029 g of $ZnSO_4 \cdot 7H_2O$ 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 1×10^5 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 for the purification of BEA and ENs. The extract in methanol was filtered through an MF3 filter (pore size, 0.5 μm; Advantec, MFS. Inc., Pleasanton, CA, U.S.A.), before HPLC purification. HPLC-grade water and organic solvents purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.) were used throughout the procedure. The purification of the cyclodepsipeptides was performed as described by Moretti *et al.* [22] with minor modifications. The purification of BEA and ENs was carried out in two steps. A GROM-sil pack ODS preparative column (1.0×25 cm) (Alltech Grom GmbH, Germany) was used for the first purification, where the mobile phase was an acetonitrile-water solution (65:35, v/v) at a flow rate of 4 ml/min for 50 min. The picrate transferring activity of each peak was measured at 370 nm. After pooling the peaks exhibiting picrate transferring activity, the compounds were further purified with a Shiseido pack C18 column (0.5×30 cm) (Shiseido Co., Japan) using HPLC. The second HPLC was performed for 45 min at a constant flow rate (1 ml/min) with a mixture of acetonitrile and water (70:30, v/v) as the eluent. The peaks were detected at 210 nm.

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 by 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 (^{1}H NMR, ^{13}C 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 ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC, and HMBC were collected in $CDCl_3$. 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.*

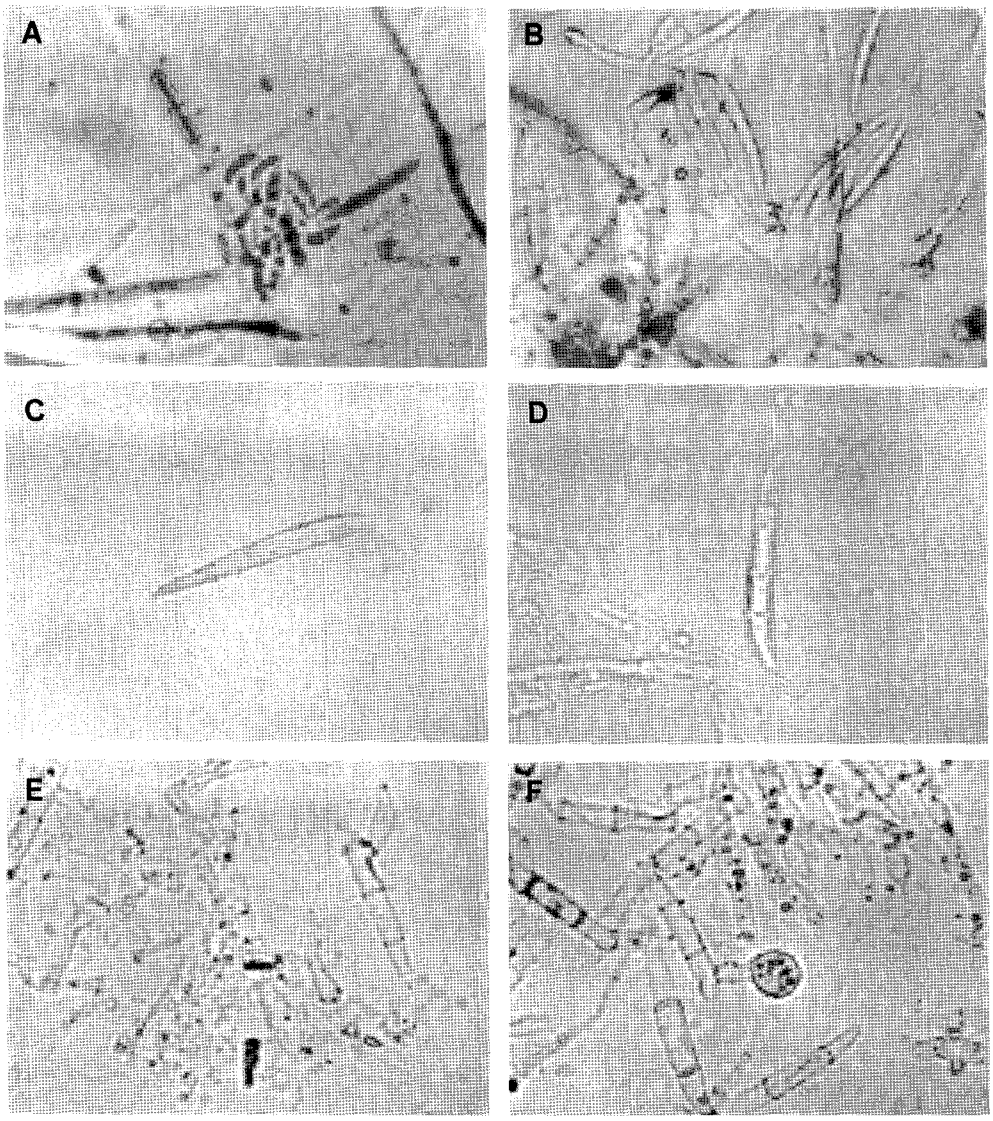

Fig. 1. Photograph of *Fusarium* strain FB1501 (KFCC 11363P) (×400).
A & **B**, microconidia; **C** & **D**, macroconidia; **E**, donidiophore; and **F**, chlamydospore.

[27], and Nelson *et al.* [24]. 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.

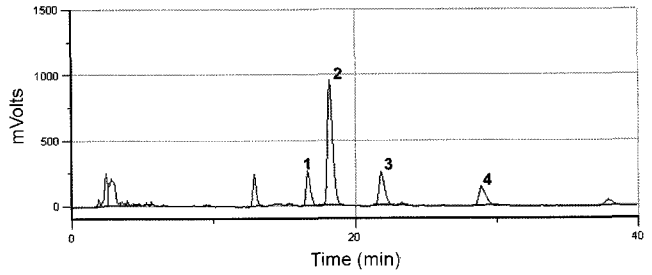

Fig. 2. HPLC chromatogram of *F. oxysporum* FB1501 (KFCC 11363P) extract.

Structural Elucidations of Cyclodepsipeptides

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 (ν 1,733–1,739 cm^{-1}) and amide (ν 1,650–1,655 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 (δ5.271) assigned to the proton situated at the α-position (2-H, δ4.569); attached to C-8, (δ74.526) showed vicinal coupling (COSY) to a multiple signal at 9-H (δ2.013) attached to C-9 (δ36.297). This methine (C-9), in turn, was connected to a methyl group (12-H, δ0.963 overlapping signal; C-12, δ14.803) and a methylene (10-H, δ1.438 and 1.184 overlapping signal; C-10, δ25.600). The C-10 methylene was attached to a terminal methyl (11-H, δ0.916, overlapping signal; C-11, δ11.514) 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 L-*N*-methyl valine (*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

Table 1. Picrate transferring activity of compounds in chloroform extracts.

Compounds No.	Concentration (mg)	O.D. (370 nm)
Blank ($CHCl_3$)	0	0.0381
1	0.1	0.4115
2	0.1	0.4460
3	0.1	0.4919
4	0.1	0.6181

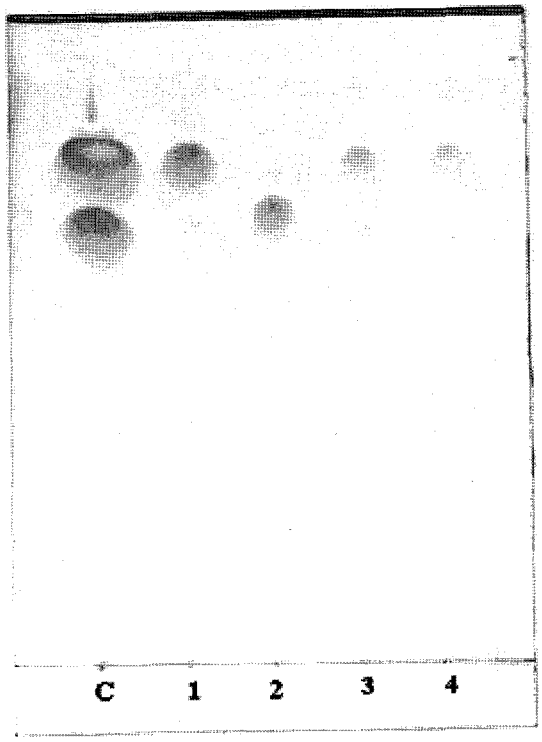

Fig. 3. TLC of the compounds 1, 2, 3, and 4 after purification (C: Extract from *F. oxysporum* FB1501).

HMBC correlations for the *N*-MeVal residues were 2-H (δ4.564) to C-3 (δ28.063) and one carbonyl signal at C-1 (δ170.541). The D-α-hydroxyisovaleic 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 δ170.541 (C-1) and δ169.536 (C-7, 13), was achieved based on the HMBC correlations from H-6 (δ3.129) 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 (170.541) signal assigned to ester carbonyl (C-1 for three *N*-MeVal). The results of the NMR analyses (^{1}H, ^{13}C, 135-DEPT, 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 B1 [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.

Table 2. NMR data for compounds 1, 3, and 4 in $CDCl_3$.

Position	Compound 1 (Enniatin H)		Compound 3 (Enniatin I)		Compound 4 (Enniatin MK1688)	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
N-MeVal	3 units		3 units		3 units	
1	170.541		170.613		170.644	
2	63.030, 63.429	4.564 (1H, m)	63.319	4.569 (1H, m)	63.311	4.599 (1H, d, J=8.75 Hz)
3	28.063, 28.116	2.293 (1H, m)[a]	28.030, 28.119	2.289 (1H, m)[a]	28.068	2.281 (1H, m)
4	20.555	1.067 (3H, m)	20.568	1.065 (3H, m)	20.497	1.060 (3H, d, J=6.11 Hz)
5	19.481, 19.579, 19.691	0.916 (3H, m)	19.510, 19.600, 19.715	0.919 (3H, m)	19.571	0.914 (3H, q, J=14.8, 7.18 Hz)
6	33.218	3.129, 3.128, 3.146 (3H, s)	32.999, 33.169	3.102, 3.117, 3.133 (3H, s)	32.921	3.105 (1H, s)
Hmp	1 units		2 unit		3 units	
7	169.536		169.529		169.490	
8	74.526	5.271 (1H, d, J=6.75 Hz)	74.454, 74.600	5.269 (1H, t, J=7.74 Hz)	74.562	5.286 (1H, d, J=6.02 Hz)
9	36.297	2.013 (1H, m)	36.352	2.012 (1H, m)	36.418	2.011 (1H, m)
10	25.600	1.184 (1H, m), 1.438 (1H, m)	25.594	1.162 (1H, m), 1.459 (1H, m)	25.549	1.189 (1H, m), 1.459 (1H, m)
11	11.514	0.916 (3H, m)	11.549	0.919 (3H, m)	11.564	0.914 (3H, t, J=7.18 Hz)
12	14.803	0.963 (3H, m)[a]	14.800	0.962 (3H, m)	14.817	0.962 (3H, d, J=6.11 Hz)
Hiv	2 unit		1 units			
13	169.536		169.529			
14	75.853, 76.022	5.144 (1H, m)	75.942	5.149 (1H, d, J=8.09 Hz)		
15	29.881, 30.158	2.293 (1H, m)[a]	30.192	2.289 (1H, m)[a]		
16	18.722[b]	0.963 (3H, d, J=6.70 Hz)[a]	18.710[b]	0.962 (3H, d, J=9.32 Hz)[a]		
17	18.881[b]	0.992 (3H, d, J=6.52 Hz)[a]	18.881[b]	0.988 (3H, d, J=6.83 Hz)[a]		

[a]^{1}H signals are overlapping.
[b]Assignments can be interchanged.

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 ^{13}C and ^{1}H NMR spectra (in $CDCl_3$) of compound 2 are summarized in Table 3. An analysis of the ^{1}H-^{1}H COSY and ^{13}C-^{1}H HMBC spectra revealed the partial structure shown in Fig. 7. The ^{13}C-^{1}H long-range couplings of ^{2}J and ^{3}J observed in the ^{13}C-^{1}H HMBC experiments provided the following evidence (Fig. 8). The cross-peaks from 2-H (δ5.52) to C-1 (δ169.70), C-3 (δ34.75), and C-10 (δ32.22), from 3-H_2 (δ3.38) to C-1, C-2 (δ57.16), C-4 (δ136.58), C-5 (δ128.83), and C-9 (δ128.83), 12-H (δ4.90) to C-11 (δ169.95), C-13 (δ29.75), C-14 (δ18.75), and C-15 (δ17.38), from 13-H (δ2.00) to C-12 (δ79.4), C-14, and C-15, from 15-H_3 (δ0.80) to C-12, C-13, and C-14, and from 14-H_3 (δ0.42) to C-12, C-13, and C-15, supported the partial structure. The NMR analyses (^{1}H, ^{13}C, DEPT135, COSY, HMQC, and HMBC; in $CDCl_3$) 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 ^{1}H and ^{13}C NMR spectra (Table 2) for compound 3 were similar to those for compound 1 (EN 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 ^{13}C-^{1}H long-range couplings

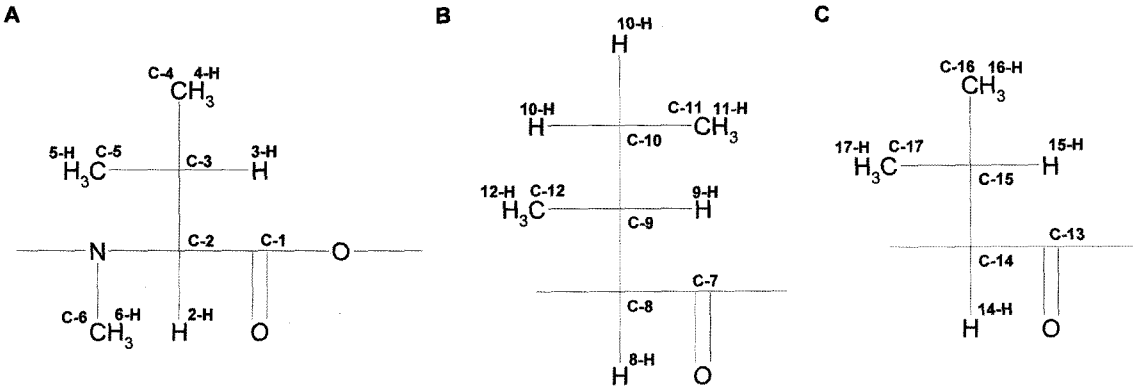

Fig. 4. Partial structures of enniatins (**A**, *N*-MeVal; **B**, Hmp; and **C**, Hiv).

of ^{2}J and ^{3}J observed in the ^{13}C-^{1}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 δ170.613 (C-1) and δ169.529 (C-7, 13), was achieved based on the HMBC correlations from H-6 (δ3.102) 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 (δ170.541) 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 (^{1}H, ^{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 ^{1}H 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

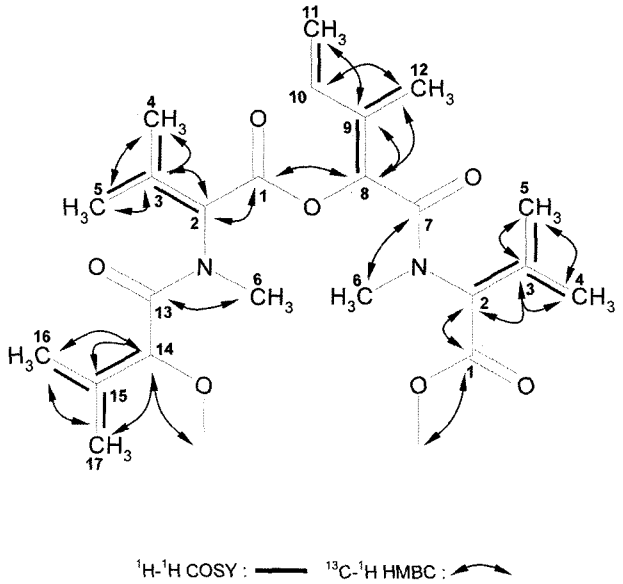

Fig. 5. HMBC and COSY correlations of enniatin H (compound 1).

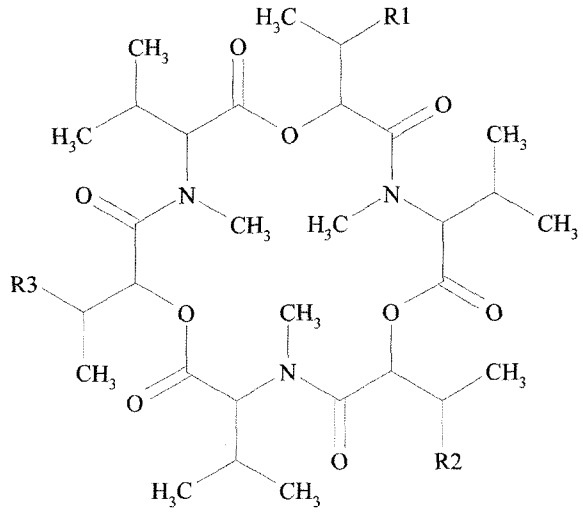

Fig. 6. Chemical structure of enniatins.
R1=CH_2CH_3, R2, R3=CH_3: compound 1 (Enniatin H); R1, R2=CH_2CH_3, R3=CH_3: compound 3 (Enniatin I); R1, R2, R3=CH_2CH_3: compound 4 (Enniatin MK1688).

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

		^{13}C Chemical shifts (ppm)	^{1}H Chemical shifts (ppm)
N-MePhe	1	169.70	
3 units	2	57.16	5.52 (1H, dd, J = 11.9, 4.9)
	3	34.75	3.38 (2H, dd, J = 14.6, 4.9 Hz)
	4	136.58	
	5	128.83	7.18-7.35 (1H, m)
	6	128.57	7.18-7.35 (1H, m)
	7	126.82	7.18-7.35 (1H, m)
	8	128.57	7.18-7.35 (1H, m)
	9	128.83	7.18-7.35 (1H, m)
	10	32.22	3.01 (1H, s)
Hiv	11	169.95	
3 units	12	75.60	4.90 (1H, d, J = 8.5)
	13	29.75	2.00 (1H, m)
	14	18.75	0.42 (3H, d, J = 6.9 Hz)
	15	17.38	0.80 (3H, d, J = 6.6 Hz)

the connectivity and assignment of each residue (**A** and **B** in Fig. 4). Thus, in the ^{1}H NMR spectrum of compound 4, the protons of three Hmp residues appeared. The signal at 8-H (δ5.286) 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, 11.549) 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 ^{13}C 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

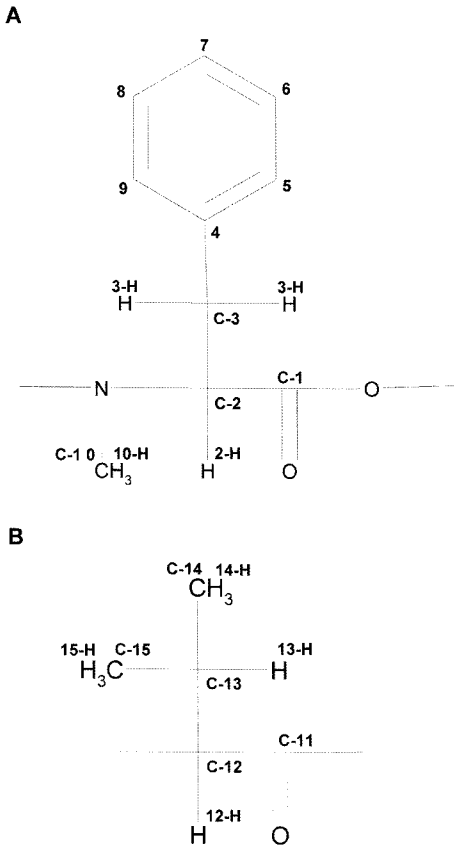

Fig. 7. Partial structures of beauvericin (**A**: *N*-MePhe; **B**: Hiv).

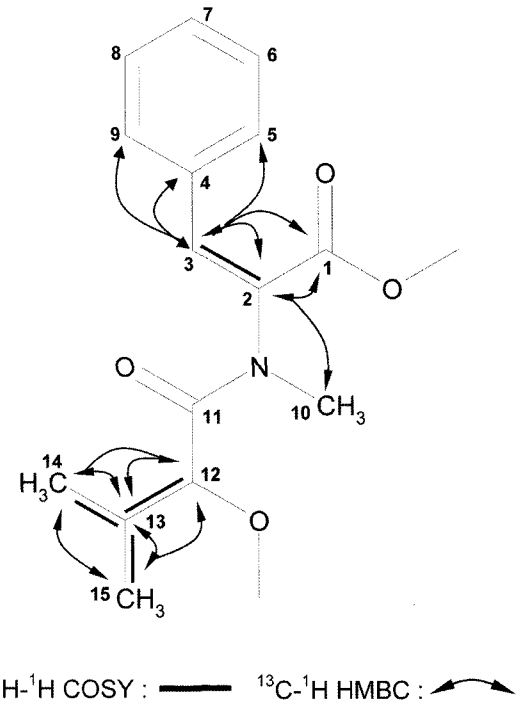

Fig. 8. HMBC and COSY correlations with beauvericin (compound 2).

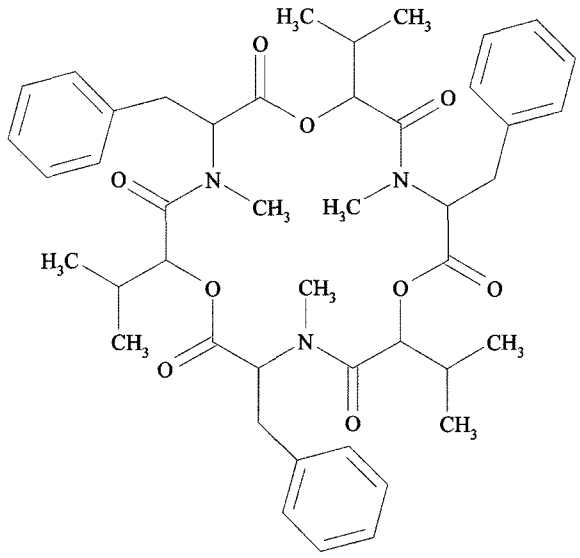

Fig. 9. Chemical structure of beauvericin.

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*.

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