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Structure Elucidation of a New Cyclic Hexadepsipeptide from *Beauveria felina*

HYUN SIK KIM[a,*], MIN HWAN JUNG[a], SANGDOO AHN[a], CHANG WOO LEE[b], SANG NYUN KIM[b] and JONG HOA OK[a]

[a] Analytical R&D Center, LG Chemical Ltd.
Research Park, 104-1 Moonji-dong, Yuseong-gu,
Daejeon 305-380, Korea
[b] LG Household & Personal Care R&D Center,
LG Household & Health Care Ltd.
84 Jang-dong, Yuseong-gu,
Daejeon 305-343, Korea

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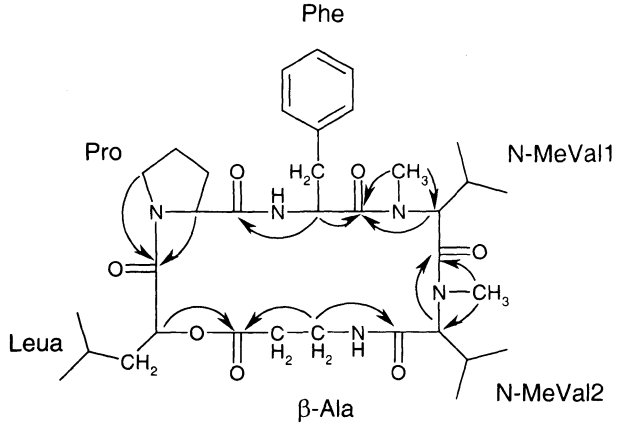
In the course of our screening for new insecticidal compounds from microbial metabolites, a novel destruxin derivative, [Phe^3, *N*-MeVal^5] destruxin B (**1**, Figure 1), was isolated from the fermentation broth of a *Beauveria* sp. Spectroscopic analyses revealed that compound **1** was a new cyclic hexadepsipeptide belonging to the destruxin family that has shown a variety of biological activities including insecticidal, phytotoxic, nematicidal, and immunosupressant activities[1~5]. In this paper, we report the fermentation, isolation, and structure determination of [Phe^3, *N*-MeVal^5] destruxin B (**1**).

Beauveria felina KCTC 6542 was cultured with shaking for 7 days at 27°C in medium consisting of Malt extract (2%) and Bacto peptone (0.5%). The fermentation broth (3 liters) was centrifuged and filtered, and the mycelial cake was extracted with methanol (300 ml×3) and concentrated *in vacuo* to a syrup (4 g) under reduced pressure. The aqueous syrup solution (300 ml), prepared by dissolving the syrup in water, was partitioned with ethyl acetate (300 ml×2), and then the organic phase layer was concentrated *in vacuo* to dryness (1 g). The residue was fractionated on a silica gel column (Silica gel 60, Merck, 40 mm×300 mm) using sequential elution with chloroform (1 liter), ethyl acetate (1 liter) and methanol (1 liter), for the first purification step. Each eluting solution was concentrated *in vacuo* to dryness. The ethyl acetate fraction was observed to have an activity against the larvae of the lepidopteran insect. The fraction was chromatographed on a silica gel column (Silica gel 60, Merck, 40 mm×200 mm) using hexane - acetone (1 : 1) as an eluent for the second purification step. The eight fractions eluted from the column were collected and subjected to TLC [silica gel 60 F254 HPTLC plates, Merck, developing solvent: hexane - acetone (1 : 1), detection reagent: anisaldehyde-sulfuric acid solution]. The fraction containing a spot with a Rf value of 0.5 on silica gel TLC was collected and concentrated *in vacuo* to afford pale yellow syrup. This was further purified by semi-preparative HPLC [mobile phase: linear gradient, 40~53% B in 22 minutes; A (25% methanol in water), B (100% acetonitrile), flow rate : 3 ml/minute, column temp : 30°C, detection : UV at 213 nm] using an ODS column (ZORBAX 300 SB-C18 9.4 mm i.d.×250 mm) to give a pure compound of [Phe^3, *N*-MeVal^5] destruxin B (2 mg) (retention time: ~12 minutes).

Positive-ion electrospray ionization (ESI) and atmospheric pressure chemical ionization (ApCI) mass spectra were obtained using a LCQ (Finnigan) ion trap mass spectrometer. High-resolution mass spectra were performed on a VG AutoSpec (Micromass) equipped with an ESI source. In CID experiments, protonated molecular ions were excited to produce sufficient fragments by collisions with neutral nitrogen molecules. The collisional energy was optimized by adjusting the electric voltage between cone and octapole ion guide inside the LCQ. For the sequence analysis of the peptide, we performed the collision induced dissociation (CID) experiments with the desalted **1** using an onGuard-H (Dionex). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DMX-600 spectrometer at 600.13 and 150.92 MHz, respectively; chemical shifts

Fig. 1. Structure of [Phe^3, *N*-MeVal^5] destruxin B, **1**, and long-range ^{1}H-^{13}C correlation diagram (HMBC).



* Corresponding author: hskimd@lgchem.co.kr

were referenced to internal TMS. The HMQC spectra were recorded by using a pulse sequence with a BIRD pulse 0.5 s before each scan to suppress the signal originating from protons not directly bound to ^{13}C; interpulse delays were adjusted for an average $^{1}J_{CH}$ of 145 Hz. The HMBC spectra were recorded by using gradient pulses and no decoupling during the acquisition; interpulse delays were optimized on long range couplings for an average $^{n}J_{CH}$ of 10 Hz.

The molecular formula was established as $C_{35}H_{53}N_5O_7$ by HRESIMS. In the spectrum, the mass of the sodiated molecular ion was 678.3857 Da within 1.4 mDa deviation from the theoretically calculated value. Based on the extensive NMR and MS experimental data (see Tables 1 and 2) the structure of **1** was determined to be a cyclic hexadepsipeptide consisting of leucic acid (Leua), proline (Pro), phenyalanine (Phe), *N*-methyl-valine (*N*-MeVal), and *β*-alanine (*β*-Ala) amino acids.

The CID experiments showed 3 distinct series of ring-opening fragments as shown in Figure 2. With the analysis of fragmentation patterns arising from successive losses of the common constitutive amino acid residues from each ring-opened hexadepsipeptide, we could identify the amino acid composition and sequence of compound **1**. Additionally we found 3 series of B, A, and D type fragments from the summarized list of abundant ions produced by ESI and ApCI methods shown in Table 1. We annotated each fragment with the previously described nomenclature.[6] Predominant ring openings near to *N*-MeVal give rise to the series of acylium ($^{4\text{-}5}B_i$, $^{5\text{-}6}B_i$) and immonium ions ($^{4\text{-}5}A_i$, $^{5\text{-}6}A_i$) with relatively small peaks of the 6-1 ring opening fragment. In addition to the sequence information, internal $^{6\text{-}1}D_2$ (D+) was observed as a typical destruxin-specific fragment[7] at *m/z* 194. By comparing fragmentation patterns and sequence with those of the reported destruxins[6], we could find common structural parts. **1** and several destruxins have Pro, *N*-MeVal, *β*-Ala (at the same position; 2nd, 4th, and 6th, respectively) and Leua. Compound **1**, however, has Phe and *N*-MeVal at the 3rd and 5th amino acids which have not been found in destruxins at those positions[8~12]. To supply the pathway of each fragmentation in CID experiments, we have analyzed $(MS)^n$ spectra of major fragments of **1** and found the typical acylium and immonium ions in the pathways. Absolute sequence information, *cyclo*(-Leua -Pro -Phe -*N*-MeVal(1) -*N*-MeVal(2) -*β*-Ala-), could be fully obtained from 2 series of successive amino acid losses, $^{4\text{-}5}B_5 \rightarrow {}^{4\text{-}5}B_4 \rightarrow {}^{4\text{-}5}B_3$, $^{6\text{-}1}B_5 \rightarrow {}^{6\text{-}1}B_4 \rightarrow {}^{6\text{-}1}B_3 \rightarrow {}^{6\text{-}1}B_2$, and their immonium ions ($^{6\text{-}1}A_5$, $^{6\text{-}1}A_4$, $^{6\text{-}1}A_3$, $^{6\text{-}1}A_2$, $^{4\text{-}5}A_4$).

The ^{13}C-NMR and DEPT spectra showed **1** to have eight methyl, seven methylene, thirteen methine and seven quaternary carbons (five amino carbonyl, one ester carbonyl and one aromatic). To ascertain the proton and carbon connectivities, homo- and heteronuclear 2D-COSY experiments were performed, which revealed the presence of the following seven partial structural elements: $(CH_3)_2$–CH–CH_2–, –CH–CH_2–CH_2–CH_2–, –CH_2–CH–NH–, –C_6H_5, $(CH_3)_2$–CH–, $(CH_3)_2$–CH–, and –CH_2–CH_2–NH–. The long-range ^{1}H-^{13}C correlations observed in the HMBC spectrum clearly revealed that those partial structures come from Leua , Pro, Phe, Phe, *N*-MeVal(1), *N*-MeVal(2), and *β*-Ala, respectively. The relative locations of amino acids in the cyclic depsipeptide were also determined by the HMBC experiment, with evaluation of the ^{2}J or ^{3}J (C,H) couplings between an α position proton and ester carbonyl carbons at different amino acids. For example, the proton at δ_H 4.09 (H-α, Pro) showed couplings to carbonyl carbons at δ_C

Table 1. List of observed fragments generated by the in-source CID experiment on a LCQ MS spectrometer with ESI and ApCI methods.

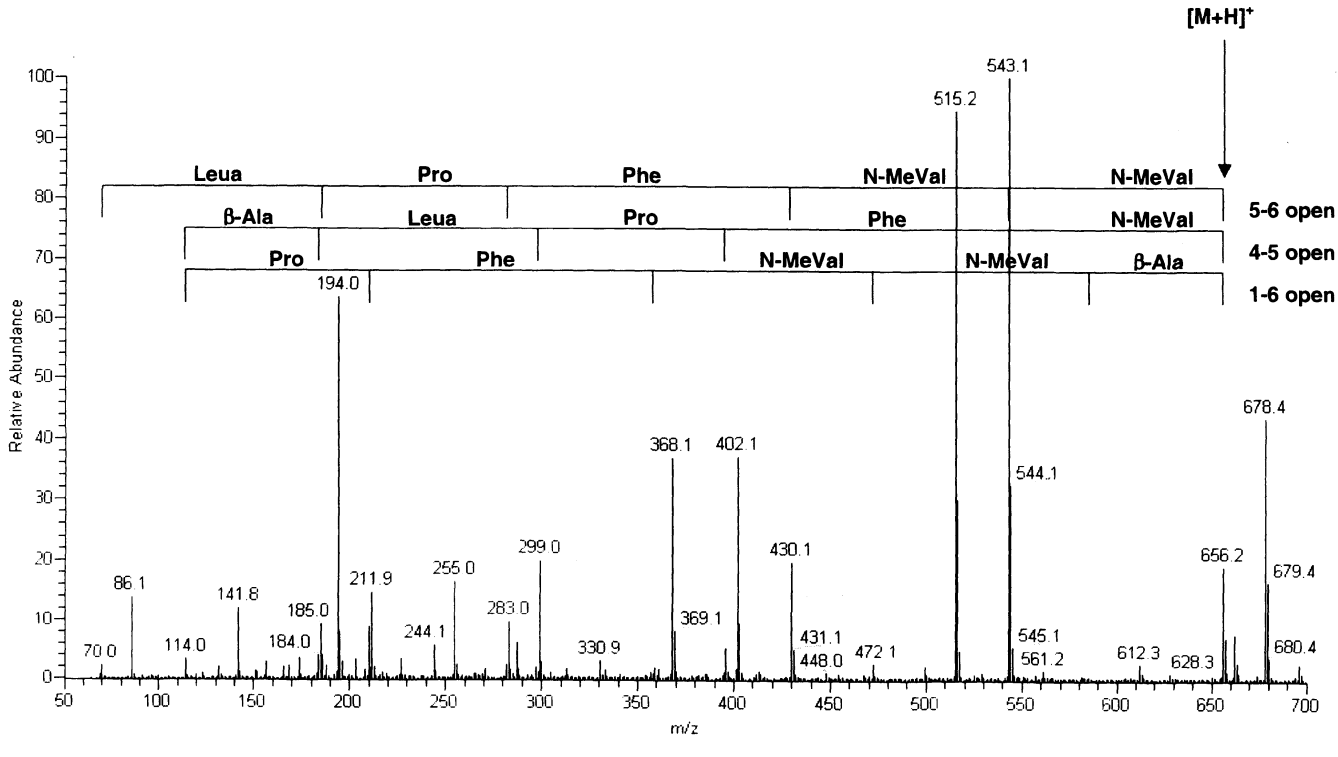
Ring Open	Fragments	Mass [u]	Relative Intensity	
			ESI	ApCI
4-5	$^{4\text{-}5}B_1$	114.1	3	7
	$^{4\text{-}5}B_2$	185.1	9	5
	$^{4\text{-}5}B_3$	299.2	20	8
	$^{4\text{-}5}B_4$	396.2	5	3
	$^{4\text{-}5}B_5$	543.3	100	100
	$^{4\text{-}5}A_1$	86.1	14	8
	$^{4\text{-}5}A_2$	157.1	3	1
	$^{4\text{-}5}A_3$	271.2	2	1
	$^{4\text{-}5}A_4$	368.3	37	16
	$^{4\text{-}5}A_5$	515.3	95	69
5-6	$^{5\text{-}6}B_1$	72.0	0.4	0.1
	$^{5\text{-}6}B_2$	186.1	4	2
	$^{5\text{-}6}B_3$	283.2	10	3
	$^{5\text{-}6}B_4$	430.2	20	14
	$^{5\text{-}6}B_5$	543.3	100	100
	$^{5\text{-}6}A_1$	44.1	-	-
	$^{5\text{-}6}A_2$	158.1	0.4	0.1
	$^{5\text{-}6}A_3$	255.2	16	5
	$^{5\text{-}6}A_4$	402.2	37	20
	$^{5\text{-}6}A_5$	515.3	95	69
6-1	$^{6\text{-}1}B_1$	115.1	1	1
	$^{6\text{-}1}B_2$	212.1	15	10
	$^{6\text{-}1}B_3$	359.2	2	2
	$^{6\text{-}1}B_4$	472.3	3	4
	$^{6\text{-}1}B_5$	585.4	1	0.2
	$^{6\text{-}1}A_1$	87.1	1	0.4
	$^{6\text{-}1}A_2$	184.1	4	5
	$^{6\text{-}1}A_3$	331.2	3	0.4
	$^{6\text{-}1}A_4$	444.3	1	0.2
	$^{6\text{-}1}A_5$	557.4	1	0.3
	$^{6\text{-}1}D_2$	194.1	64	36

Table 2. ^{1}H and ^{13}C NMR data for **1** in $CDCl_3$ (298 K).

AA	δ_C	δ_H (mult, J in Hz)	AA	δ_C	δ_H (mult, J in Hz)
Leua CO	169.9	–	NMeVal 1 CO	169.7	–
α	73.3	5.34 (d,11.1)	α	57.5	5.13 (d, 8.3)
β	38.7	1.97 (m), 1.26 (m)	β	27.6	2.43 (m)
γ	24.7	1.97 (m)	γ -Me	18.8[b]	0.88 (d, 6)
δ -Me	20.5[a]	1.00 (d, 6)	γ -Me	20.2[b]	0.88 (d, 6)
δ -Me	23.3[a]	1.00 (d, 6)	NMe	29.6	3.14 (s)
Pro CO	172.0	–	NMeVal 2 CO	168.6	–
α	60.9	4.09 (d, 8.3)	α	66.5	4.29 (d,10.5)
β	32.2	2.22 (m), 2.13 (m)	β	27.7	2.43 (m)
γ	22.0	1.77 (m), 1.33 (m)	γ -Me	19.5[c]	0.93 (d, 6)[d]
δ	47.1	3.50 (m)	γ -Me	19.7[c]	0.88 (d, 6)[d]
			NMe	29.0	2.97 (s)
Phe CO	173.6	–			
α	53.8	4.66 (m)	β -Ala CO	174.0	–
β	35.0	3.00 (m)	α	35.3	2.62 (m), 2.46 (m)
γ $-C_1$	136.3	–	β	35.5	4.16 (m), 3.16 (m)
C_2, C_6	128.7	7.22	NH	–	7.41 (d, 9.8)
C_3, C_5	128.7	7.27			
C_4	127.2	7.22			
NH	–	8.14 (d, 6.8)			

a), b), c), d) The assignments may be interchanged.

Fig. 2. In-source CID mass spectrum of **1**.



172.0 (Pro) and 169.9 (Leua), which implied the connection between *Leua* and *Pro*. Similar correlations, as shown in Figure 1, between the carbon and proton signals unambiguously indicated the locations of the six amino acids and the cyclic sequence, which were completely in accord with the MS experimental results.

Further investigations about biological activities, especially insecticidal, of compound **1** are now under way.

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