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Structure Elucidation of Fungal Beauveriolide III, a Novel Inhibitor of Lipid Droplet Formation in Mouse Macrophages

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The structure of fungal beauveriolide III, an inhibitor of lipid droplet formation in mouse macrophages, was elucidated to be cyclo-[(3*S*,4*S*)-3-hydroxy-4-methyloctanoyl-L-phenylalanyl-L-alanyl-D-allo-isoleucyl] by spectral analyses and chemical degradation.

During our screen for microbial-based inhibitors of macrophage-derived foam cell formation, a novel cyclodepsipeptide named beauveriolide III was isolated along with a known compound beauveriolide I[1] from the fermentation broth of *Beauveria* sp. FO-6979[2] (Fig. 1). It was suggested that they are structurally related[2]. In this report, the structure elucidation of beauveriolide III is described.

Materials and Methods

Materials

Beauveriolides I and III were isolated from the culture broth of *Beauveria* sp. FO-6979 as described in the preceding paper[2].

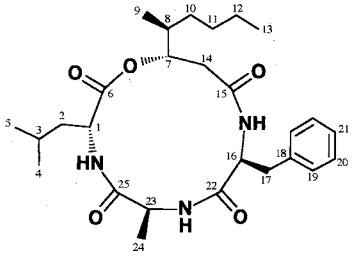
General Experimental Procedures

UV spectra were recorded on a Shimadzu UV-200S spectrophotometer. IR spectra were recorded on a Horiba FT-210 infrared spectrometer. Melting point was measured with a Yanaco micromelting point apparatus. Optical rotations were obtained with a JASCO DIP-370 digital polarimeter. FAB-MS spectra were recorded on a JMS-DX300 mass spectrometer, and HRFAB-MS spectra were recorded on a JMS-AX505 HA mass spectrometer. The various NMR spectra were obtained on a Varian XL-400 spectrometer.

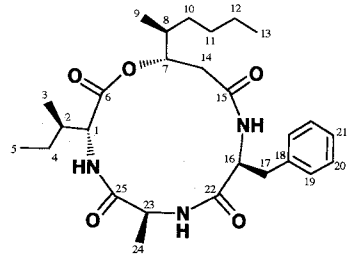
Acid Hydrolysis

Beauveriolide III (7.0 mg) was degraded in a gas phase of 6 M HCl (990 μl) and phenol (10 μl) at 150°C for 4 hours using the PICO·TAG work station (Waters). The degradation products were dissolved in acidic H_2O (pH 3, 2.0 ml) and extracted with diethyl ether (2.0 ml × 3 times). To determine the amino acid constituents, the aqueous layer (4.0 μl) was analyzed by HPLC (SUMICHIRAL OA-5000, 4.6 × 150 mm, UV at 254 nm, 1.0 ml/minute). HPLC was carried out using Shimadzu (LC-7A) Systems. Using 1 mM aq $CuSO_4$ as a solvent

Fig. 1. Structures of beauveriolides I and III.



Beauveriolide I

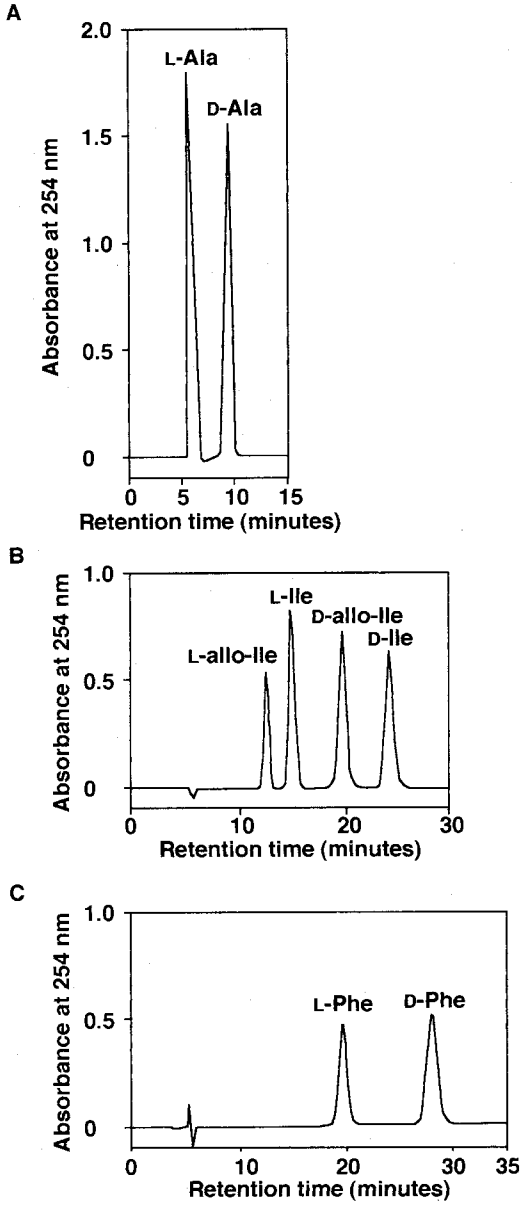


Beauveriolide III

Fig. 2. Separation of alanine, phenylalanine, isoleucine by HPLC column, SUMICHIRAL OA-5000 (4.6×150 mm); UV at 254 nm; 1 ml/minute.

A, solvent 1 mM aq $CuSO_4$ for L- and D-alanine. B, solvent 15% methanol in 2 mM aq $CuSO_4$ for L-, D-allo-isoleucine, L- and D-isoleucine. C, solvent 30% methanol in 2 mM aq $CuSO_4$ for L- and D-phenylalanine.

Each amino acid (5 μg) was injected.



for HPLC, L- and D-alanine were eluted as peaks with retention times of 6.0 and 10.0 minutes, respectively. Using 15% methanol in 2 mM aq $CuSO_4$, L-, D-allo-isoleucine, L- and D-isoleucine were eluted as peaks with retention times of 13.0, 20.0, 15.0 and 24.5, respectively. Using 30% methanol in 2 mM aq $CuSO_4$, L- and D-phenylalanine were eluted as peaks with retention times of 20.0 and 28.0, respectively (Fig. 2). To determine the stereochemistry of the 3-hydroxy-4-methyl-octanoic acid, the aqueous layer (700 μl) was also subjected to a Toyopak SP colunm (Tosoh) to adsorb amino acids. The pass-through fraction containing 3-hydroxy-4-methyl-octanoic acid was concentrated *in vacuo* and the residue (1.9 mg) was methylated with (trimethylsilyl)diazomethane (2.0 M solution, 100 μl) (Aldrich) in benzene (1 ml) and methanol (100 μl) at room temperature for 3 days. After evaporation, 3-hydroxy-4-methyloctanoate methyl ester (2.1 mg) was obtained.

Results

Physico-chemical Properties of Beauveriolide III

Physico-chemical properties of beauveriolide III are summarized in Table 1. The data of beauveriolide I is also shown for comparative purpose. Beauveriolides showed the same molecular weight (*m/z* 487) and the same UV maxima at 215 nm. The IR spectrum of beauveriolide III suggested the presence of amide group (3300 cm^{-1}) and four carbonyl groups (1726, 1686, 1639, and 1539 cm^{-1}). These data suggested that beauveriolide III is structurally related to beauveriolide I.

Structure of Beauveriolide III

The molecular formula of beauveriolide III was determined to be $C_{27}H_{41}N_3O_5$ on the basis of HREI-MS measurement (*m/z*, found 488.3127, calcd 488.3124 for $C_{27}H_{42}N_3O_5$ $[M+H]^+$). The ^{13}C NMR spectrum ($CDCl_3$-CD_3OD, 4:1) showed 25 resolved peaks corresponding to 27 carbons (Table 2), which were classified into five methyl, six methylene, two methine, three amino methine, one oxy methine, five sp^2 methine, one sp^2 quaternary, and four carbonyl carbons by analysis of the DEPT spectra. The ^1H NMR spectrum in $CDCl_3$-CD_3OD displayed 38 proton signals (Table 2), whereas ^1H NMR spectrum in $CDCl_3$ gave additional three proton signals (δ 7.41, 6.28 and 6.25), suggesting the presence of three NH protons. These results supported the molecular formula. The connectivity of proton and carbon atoms was confirmed by the HMQC spectrum (Table 2). Analyses of ^1H-^1H COSY spectrum revealed the presence of the six partial structures I to VI (Fig. 3). Preliminary analyses of the hydrolysates of beauveriolide III (6 M HCl containing 1% phenol at 115°C for 20 hours) by TLC using ninhydrin reagents suggested that the compound contained amino acids. On the basis of these findings, ^{13}C-^1H long-range coupl-

Table 1. Physico-chemical properties of beauveriolides I and III.

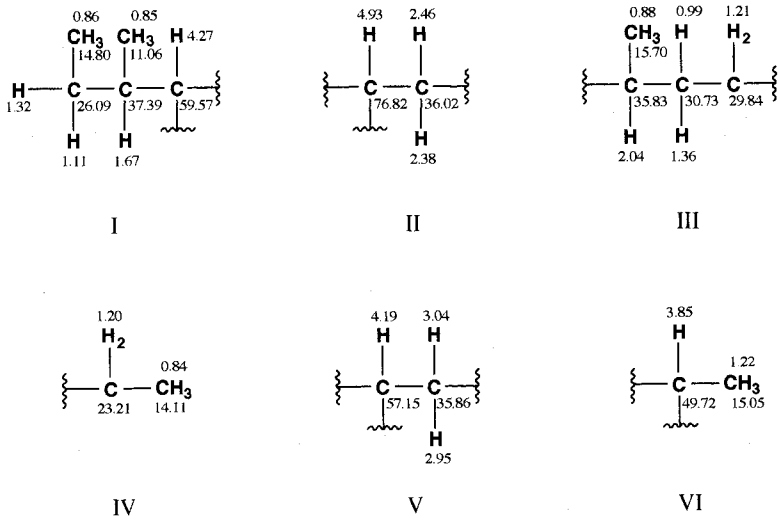
	Beauveriolide I	Beauveriolide III
Appearance	white powder	white powder
Molecular formula	$C_{27}H_{41}N_3O_5$	$C_{27}H_{41}N_3O_5$
Molecular weight	487	487
FAB-MS (*m/z*)		
Positive	488 $[M+H]^+$	488 $[M+H]^+$
	510 $[M+Na]^+$	510 $[M+Na]^+$
HRFAB-MS (*m/z*) (positive)		
MF+H	$C_{27}H_{42}N_3O_5$	$C_{27}H_{42}N_3O_5$
Calcd:	488.3124	488.3124
Found:	488.3112	488.3127
$[\alpha]_D^{25}$ ($CHCl_3$-CH_3OH, 4:1)	- 21 ° (*c* 0.44)	- 38 ° (*c* 0.40)
UV $\lambda_{max}^{CH_3OH}$ nm (ε)	215 (27,300)	215 (15,800)
IR υ_{max}^{KBr} (cm^{-1})	3298, 1724, 1684	3300, 1726, 1686
	1641, 1535	1639, 1539
Melting point	248-250 °C	246-248 °C
Solubility		
Soluble	EtOH, CH_3CN,	EtOH, CH_3CN,
	CH_3OH, $CHCl_3$	CH_3OH, $CHCl_3$
	EtOAc	EtOAc
Insoluble	H_2O, *n*-Hexane	H_2O, *n*-Hexane

Table 2. ^1H and ^{13}C NMR chemical shifts of beauveriolides I and III.

	Beauveriolide I		Beauveriolide III	
Carbon No.	^{13}C chemical shifts (ppm)[a]	^1H chemical shifts (ppm)[b]	^{13}C chemical shifts (ppm)[a]	^1H chemical shifts (ppm)[b]
1-NH				
C-1	53.49	4.60 (1H, t, *J*=7.0 Hz)	59.57	4.27 (1H, d, *J*=10.0 Hz)
C-2	41.91	1.53 (2H, m)	37.39	1.67 (1H, m)
C-3	25.71	1.52 (1H, m)	11.06	0.85 (3H, d, *J*=7.0 Hz)
C-4	22.67	0.93 (3H, d, *J*=6.0 Hz)	26.09	1.32 (1H, m)
				1.11 (1H, m)
C-5	22.57	0.91 (3H, d, *J*=6.0 Hz)	14.80	0.86 (3H, t, *J*=7.0 Hz)
C-6	170.59		169.70	
C-7	77.30	4.94 (1H, ddd, *J*=10.0, 4.5, 4.0 Hz)	76.82	4.93 (1H, ddd, *J*=10.0, 4.5, 4.0 Hz)
C-8	36.56	2.05 (1H, m)	35.83	2.04 (1H, m)
C-9	15.87	0.89 (3H, d, *J*=7.0 Hz)	15.70	0.88 (3H, d, *J*=7.0 Hz)
C-10	31.44	1.40 (1H, m)	30.73	1.36 (1H, m)
		1.04 (1H, m)		0.99 (1H, m)
C-11	30.32	1.23 (2H, m)	29.84	1.21 (2H, m)
C-12	23.65	1.27 (2H, m)	23.21	1.20 (2H, m)
C-13	14.30	0.88 (3H, t, *J*=7.0 Hz)	14.11	0.84 (3H, t, *J*=7.0 Hz)
C-14	36.69	2.53 (1H, dd, *J*=14.0, 4.5 Hz)	36.02	2.46 (1H, dd, *J*=14.0, 4.5 Hz)
		2.41 (1H, dd, *J*=14.0, 10.5 Hz)		2.38 (1H, dd, *J*=14.0, 10.0 Hz)
C-15	173.14		172.46	
16-NH				
C-16	57.68	4.25 (1H, dd, *J*=9.0, 7.5 Hz)	57.15	4.19 (1H, dd, *J*=8.5, 7.5 Hz)
C-17	36.51	3.04 (1H, dd, *J*=13.5, 7.5 Hz)	35.86	3.04 (1H, dd, *J*=13.5, 7.5 Hz)
		2.96 (1H, dd, *J*=13.5, 9.0 Hz)		2.95 (1H, dd, *J*=13.5, 8.5 Hz)
C-18	137.42		136.65	
C-19	129.85	7.15 (2H, ddd, *J*=8.0, 1.5, 1.0 Hz)	129.29	7.14 (2H, ddd, *J*=8.0, 1.5, 1.0 Hz)
C-20	129.20	7.23 (2H, ddd, *J*=8.0, 7.0, 1.0 H)	128.77	7.23 (2H, ddd, *J*=8.0, 7.0, 1.0 Hz)
C-21	127.59	7.18 (1H, dd, *J*=7.0, 1.5 Hz)	127.18	7.18 (1H, dd, *J*=7.0, 1.5 Hz)
C-22	172.54*		171.75	
23-NH				
C-23	50.33	3.80 (1H, q, *J*=7.0 Hz)	49.72	3.85 (1H, q, *J*=7.0 Hz)
C-24	15.33	1.20 (3H, d, *J*=7.0 Hz)	15.05	1.22 (3H, d, *J*=7.0 Hz)
C-25	172.54*		171.82	

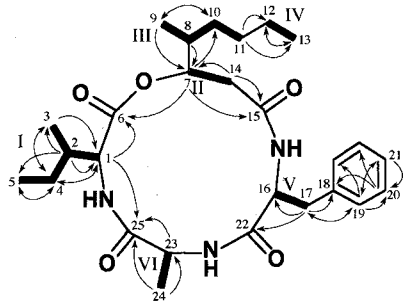
a) Chemical shifts are shown with reference to CD_3OD as 49.0 ppm. b) Chemical shifts are shown with reference to CD_3OD as 3.30 ppm. *) The signals were observed as the same chemical shifts.

Fig. 3. Partial structures I to VI of beauveriolide III.



ings of ^{2}J and ^{3}J were measured in the HMBC spectrum (Fig. 4), providing the following structural evidence; Regarding the amino acid components in the structure, 1) the cross peaks from H_3-24 (δ 1.22) to C-23 (δ 49.72) and C-25 (δ 171.82), and from H-23 (δ 3.85) to C-25 showed the presence of an alanine moiety containing the partial structure VI. 2) The long-range couplings from H-1 (δ 4.27) to C-4 (δ 26.09), from H-2 (δ 1.67) to C-1 (δ 59.57), C-3 (δ 11.06) and C-5 (δ 14.80), from H_3-3 (δ 0.85) to C-1 and C-4, from H_2-4 (δ 1.32, 1.11) to C-1, C-3 and C-5, and from H_3-5 (δ 0.86) to C-4 showed the presence of an isoleucine moiety containing the partial structure I. 3) The long-range couplings from H_2-14 (δ 2.46, 2.38) to C-7 (δ 76.82) and C-15 (δ 172.46), from H-7 (δ 4.93) to C-10 (δ 30.73) and C-15, from H-8 (δ 2.04) to C-7, from H-9 (δ 0.88) to C-7 and C-10, and from H_2-10 (δ 1.36, 0.99) to C-9 (δ 15.70) showed that the partial structure II was attached to the partial structure III. Furthermore, the long-range couplings from H_2-11 (δ 1.21) to C-12 (δ 23.21) and C-13 (δ 14.11), from H_2-12 (δ 1.20) to C-13, and from H_3-13 (δ 0.84) to C-12 showed that the partial structure III was attached to the partial structure IV. Therefore, the sequence II-III-IV was indicated to give a 2-hydroxy-3-methyl-octanoyl moiety. 4) The long-range couplings from H-17 (δ 3.04, 2.95) to C-16 (δ 57.15), C-18 (δ 136.65), C-19 (δ 129.29) and C-22 (δ 171.75), from H-19 (δ 7.14) to C-17 (δ 35.86), C-19, C-20 (δ 128.77) and C-21 (δ 127.18), tfrom H-20 (δ 7.23) o C-18 and C-20, and from H-21 (δ 7.18) to C-19 and C-20 showed the presence of a phenylalanine moiety containing the partial structure V.

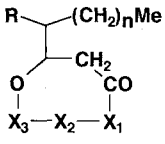
Fig. 4. NMR experiments of beauveriolide III.



^{1}H-^{1}H COSY: ▬, HMBC: H ⟶ C

The sequence of these components was determined as follows; 1) The long-range couplings from H-1 to C-25 and the chemical shift of C-1 (δ 59.57) suggested the alanyl-isoleucinyl sequence *via* amide ester bond. 2) The long-range couplings from H-1 and H-7 to C-6 (δ 169.70) and the chemical shift of C-7 (δ 76.82) suggested that the isoleucine residue was attached to 2-hydroxy-3-methyl-octanoylic acid moiety *via* ester bond. Finally, the cyclic structure was suggested because 1) the two carbons C-16 (δ 57.15) and C-23 (δ 49.72) should be bound to an amide nitrogen from the ^{13}C chemical shifts, and 2) the ^{13}C chemical shifts of C-15 (δ 172.46) and C-22 (δ 171.75) are assignable for an amide ester carbon. Thus, the phenylalanine moiety should lie between the alanine and 2-hydroxy-3-methyl-octanoyl moieties. This cyclic structure also satisfied the degree of unsaturation, the molecular formula and biosynthesis. From the structure

Table 3. The structures of beauveriolide type cyclodepsipeptides.



Compound	n	R	Amino acid X_1	Amino acid X_2	Amino acid X_3	Ref.
Beauveriolide III	3	Me	L-Phe	L-Ala	D-*allo*-Ile	
Beauverolide La	5	Me	L-Phe	L-Ala	D-*allo*-Ile	3
Beauveriolide I	3	Me	L-Phe	L-Ala	D-Leu	1
Beauveriolide II	5	Me	L-Phe	L-Ala	D-Leu	1
Beauverolide A	5	Me	L-Phe	L-Val	D-Val	4
Beauverolide D	3	Me	L-Phe	L-Val	D-Val	4
Beauverolide B	5	Me	L-Val	L-Phe	D-Ile	4
Beauverolide E	3	Me	L-Val	L-Phe	D-Ile	4
Beauverolide C	5	Me	L-Phe	L-Phe	D-Ile	4
Beauverolide F	3	Me	L-Phe	L-Phe	D-Ile	4, 5
Beauverolide H	4	H	L-Phe	L-Ala	D-Leu	6
Beauverolide I	6	H	L-Phe	L-Ala	D-Leu	6
Beauverolide Ba*)	5	Me	L-Val	L-Phe	D-*allo*-Ile	5, 7
Beauverolide Ea	3	Me	L-Val	L-Phe	D-*allo*-Ile	4
Beauverolide Ca	5	Me	L-Phe	L-Phe	D-*allo*-Ile	4, 7
Beauverolide Fa	3	Me	L-Phe	L-Phe	D-*allo*-Ile	4
Beauverolide Ja	3	Me	L-Trp	L-Phe	D-*allo*-Ile	7
Beauverolide Ka	5	Me	L-Trp	L-Phe	D-*allo*-Ile	7

*) Also named beauverilide A

as described above, beauveriolide III consists of three amino acids and a hydroxylic acid.

To determine their absolute configuration, beauveriolide III (7.0 mg) was hydrolyzed and the hydrolysate was extracted with diethyl ether. The aqueous layer was analyzed by HPLC using a chiral column. In comparison with peaks of the authentic amino acids (Fig. 2), the main amino acids comprising beauveriolide III were determined to be L-alanine, D-allo-isoleucine and L-phenylalanine, which were detected almost in equimolar quantities. Regarding the stereochemistry of the 3-hydroxy-4-methyl-octanoic acid, the aqueous layer was subjected to a Toyopak SP colunm to adsorb amino acids. The pass-through fraction containing 3-hydroxy-4-methyl-octanoic acid was concentrated *in vacuo* and the residue was treated with diazomethane to give 3-hydroxy-4-methyl-octanoic acid methyl ester (2.1 mg). After the structure was confirmed by NMR, the optical rotation ($[\alpha]_D^{25}$ $-29°$, *c* 0.2) was compared with that ($[\alpha]_D^{25}$ $-27°$, *c* 0.3) prepared from beauveriolide I, which was almost identical with the previously reported values[1]. Therefore, the stereochemistry was concluded

to be (3*S*,4*S*)-3-hydroxy-4-methyloctanoic acid.

Taken together, the structure of beauveriolide III was elucidated as shown in Fig. 1.

Discussion

The structure of beauveriolide III including the absolute configurations was elucidated cyclo-[(3*S*,4*S*)-3-hydroxy-4-methyloctanoyl-L-phenylalanyl-L-alanyl-D-allo-isoleucyl] by spectral analyses and degradation experiments. This type of cyclodepsipeptides, such as beauveriolide, beauverolide and beauverilide, where the common cyclic skeleton is comprised of an hydroxylic acid and three amino acids, are summarized in Table 3. They were reported to be produced by *Beauveria* sp.

This family shows the structural characteristies as follows; 1) Regarding the part of hydroxylic acid, methylation occured at the R position for fatty acids of an odd-numbered straight carbon chain (octanoic acid or decanoic acid), while no methylation occured for those of an even-numbered carbon. 2) Hydrophobic amino acids were occupied at the X_1, X_2 and X_3 positions. Interestingly, L-amino acids were introduced at the X_1 and X_2 positions, but D-amino acids were at the X_3 position. In this sense, the biosynthetic sequence for this type of cyclodepsipeptides might be interesting. Furthermore, the biological activity of this series of cyclodepsipeptides has not been clearly defined. Therefore, it will be interesting to test their inhibitory activity of lipid droplet formation in macrophages.

Acknowledgments

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