New Beauveriolides Produced by Amino Acid-supplemented Fermentation of *Beauveria* sp. FO-6979

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Five new beauveriolides were isolated from the acetone extracts of *Beauveria* sp. FO-6979 mycelia fermented in amino acid-supplemented media. The structures were elucidated by spectroscopic studies including NMR experiments and chemical degradation. All the beauveriolides are cyclodepsipeptides consisting of one 3-hydroxy-4-methyl fatty acid, two L-amino acids and one D-amino acid in common. Beauveriolide VII with the structure of cyclo-[3-hydroxy-4-methyloctanoyl-L-phenylalanyl-L-alanyl-D-valyl] inhibited lipid droplet formation and cholesteryl ester synthesis in macrophages, but the other beauveriolides showed only slight or almost no effect on lipid droplet formation.

We established the assay system of lipid droplet synthesis in intact macrophages as a model of foam cell formation, the early event of atherosclerogenesis¹⁾. During the screening for inhibitors of lipid droplet synthesis with this assay system, beauveriolides I and III were isolated from the culture broth of *Beauveria* sp. FO-6979²⁻⁴⁾. They inhibited cholesteryl ester synthesis in macrophages specifically, resulting in a reduction of the number and the size of cytosolic lipid droplets²⁾. Recently, we succeeded in the selective production of beauveriolide I or III by fermentation of the producing strain in the amino acid-supplemented media⁵⁾. During experiments analyzing such fermentation broths by LC/UV, several peaks having UV spectra similar to those of beauveriolides increased or appeared newly, suggesting the presence of beauveriolide analogs. As a result, six analogs named beauveriolides IV to IX were obtained; beauveriolides IV to XIII were new compounds, but beauveriolide IX was identified as

beauverolide Fa⁶⁾.

In this paper, production, isolation, structure elucidation and biological activities of new beauveriolides are described.

Materials and Methods

General Experimental Procedures

Beauveria sp. FO-6979 was used for production of beauveriolides. The strain was stored as a spore suspension (10⁸ spores/ml) in 50% glycerol at -80°C. The suspension was prepared by recovering spores from the 14-day culture of the strain on the YpSs agar medium (soluble starch 1.5%, yeast extract 0.4%, K₂HPO₄ 0.1%, MgSO₄·7H₂O 0.05%, and agar 2.0%, pH 6.0) by washing its surface with 5 ml of sterilized water.

Kieselgel 60 (E. Merck) was used for silica gel column

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chromatography. HPLC was carried out using the RANIN systems (Model SD-200).

Quantitative Analysis of Beauveriolides by HPLC

For determination of the amounts of beauveriolides, the samples dissolved in methanol were analyzed by LC/UV (Series 1100 system, Hewlett Packard) under the following conditions: column, Symmetry C₁₈ (Waters), 2.1×150 mm; solvent, 20-minute isocratic with 45% CH₃CN in 0.05% H₃PO₄ and then 20-minute linear gradient from 45% CH₃CN in 0.05% H₃PO₄ to 80% CH₃CN in 0.05% H₃PO₄; flow rate, 0.2 ml/minute; detection, UV at 210 nm. Under the conditions, beauveriolides I, III, IV, V, VI, VII, VIII and IX (identical with beauverolide Fa⁶⁾) were eluted as peaks with retention times of 27.0, 25.6, 11.1, 15.8, 17.5, 18.5, 32.1, and 35.9 minutes, respectively.

Spectroscopic Studies

UV spectra were recorded on a BECKMAN spectrophotometer (Model DU640). IR spectra were recorded on a Horiba infrared spectrometer (Model FT-210). Melting point was measured with a Yanaco micromelting point apparatus. Optical rotations were obtained with a JASCO digital polarimeter (Model DIP-370). FAB-MS spectra were recorded on a JEOL mass spectrometer (Model JMS-DX300), and HRFAB-MS spectra were recorded on a JEOL mass spectrometer (Model JMS-AX505 HA). The various NMR spectra were obtained on a Varian spectrometer (Model XL-400).

Amino Acid Analysis of Hydrolysates of Beauveriolides by HPLC

The absolute stereochemistry of amino acid constituents in beauveriolides obtained by amino acid-supplemented fermentation was determined by HPLC using a chiral column (Sumichiral OA-5000, 4.6×150 mm, Sumika Chemical Analysis Service, Ltd.) according to the established method³⁾.

Assay for Lipid Droplet Formation and Syntheses of [¹⁴C]Cholesteryl Ester and [¹⁴C]Triacylglycerol from [¹⁴C]Oleic Acid in Mouse Macrophages

Morphological assay for lipid droplet formation and biochemical assay for [¹⁴C]cholesteryl ester (CE) and [¹⁴C]triacylglycerol (TG) syntheses from [¹⁴C]oleic acid in mouse peritoneal macrophages were carried out according to the established methods¹⁾.

Other Biological Assays

Antimicrobial activity was tested using paper disks (i.d.

6 mm, ADVANTEC). Bacteria were grown on Müller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after a 24-hour incubation at 37°C for bacteria and after a 48-hour incubation at 27°C for fungi and yeasts.

Results

Fermentation in Amino Acid-supplemented Media

The spore suspension (2.0×10^7 spores in 200 μ l) was transferred into a 50-ml test tube containing 10 ml of the seed medium (glucose 2.0%, yeast extract 0.2%, MgSO₄·7H₂O 0.05%, Polypepton 0.5%, KH₂PO₄ 0.1% and agar 0.1%, pH 6.0). The tubes were shaken on a reciprocal shaker for 3 days at 27°C. The seed culture (1 ml) was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the same seed medium. The flasks were shaken on a rotary shaker at 27°C for 3 days. Two hundred ml of the seed culture was transferred into a 30-liter jar fermenter containing 20 liters of the production medium (glucose 1.0%, tryptone 0.5%, yeast extract 0.3%, malt extract 0.3% and agar 0.1%, pH 6.0). On day 1, an amino acid (0.1% L-Leu, 0.1% L-Ile, 0.1% L-Phe, 0.1% L-Ala or 0.3% L-Val) was added to the culture. The production of beauveriolides was determined by HPLC (Table 1).

In case of L-Ile-added fermentation, beauveriolides I, III, V, VIII and IX were detected in the culture broth on day 3 after inoculation, and their concentrations reached a maximum on day 5. Beauveriolides IV, VI and VII were not detected. The production of beauveriolides III (172 μ g/ml), V (51.8 μ g/ml), VIII (15.5 μ g/ml) and IX (28.7 μ g/ml) was increased about two to four folds when compared with the control fermentation, but the production of beauveriolide I (25→5.6 μ g/ml) was inhibited dramatically.

Table 1. Production of beauveriolides by amino acid addition.

Beauveriolide	Beauveriolides production (μ g/ml)					
	Amino acid addition					
	Control	+L-Ile	+L-Leu	+L-Phe	+L-Ala	+L-Val
I	25.0	5.6	127	66.0	30.0	6.8
III	50.0	172	26	79.0	60.0	7.9
IV	4.8	-	-	-	-	47.6
V	13.6	51.8	-	-	-	20.3
VI	13.0	-	45.5	-	19.7	25.7
VII	18.9	-	-	24.0	25.2	44.5
VIII	7.2	15.5	-	-	16.8	-
IX	11.9	28.7	-	19.0	-	-

In case of L-Leu-added fermentation, the production of beauveriolides I (127 $\mu\text{g/ml}$) and VI (45.5 $\mu\text{g/ml}$) was increased three to five folds, but the production of beauveriolide III was decreased (50.0 $\rightarrow$ 26.0 $\mu\text{g/ml}$). Beauveriolides IV, V, VII, VIII and IX were not produced in this medium.

In case of L-Val-added fermentation, the production pattern changed significantly; both beauveriolides I and III were decreased severely, while beauveriolides IV to VII were increased. Especially, production of beauveriolides IV enhanced about 10 folds (4.8 $\rightarrow$ 47.6 $\mu\text{g/ml}$).

By L-Phe-added fermentation, the production of beauveriolides I (66.0 $\mu\text{g/ml}$), III (79.0 $\mu\text{g/ml}$), VII (24.0 $\mu\text{g/ml}$) and IX (19.0 $\mu\text{g/ml}$) was increased 1.6 to 2.6 folds. By L-Ala-added fermentation, the production of beauveriolides I (30.0 $\mu\text{g/ml}$), III (60.0 $\mu\text{g/ml}$), VI (19.7 $\mu\text{g/ml}$), VII (25.2 $\mu\text{g/ml}$) was slightly increased. Beauveriolide VIII (16.8 $\mu\text{g/ml}$) was increased 2.3 folds.

Isolation

The 5-day old whole broth (20 liters) fermented in the medium supplemented with each amino acid (0.3% L-Val and 0.1% L-Ile) was centrifuged to collect the mycelium. The mycelial part was treated with 20 liters of acetone. The extracts were filtered and concentrated to give brown powder. The powder was then washed with hexane (3 liters) and was centrifuged to give yellow powder (1.8 g from L-Val- and 2.8 g from L-Ile-added fermentation).

The yellow powder (1.8 g) from L-Val-added fermentation was used for isolation of beauveriolides IV, V, VI and VII. This powder was dissolved in CHCl_3 and was applied on a silica gel (200 g) column that was previously equilibrated with CHCl_3 . The materials were eluted stepwise with CHCl_3 - CH_3OH solutions (1.1 liters each, 100:0, 100:1, 100:2, 100:3 and 0:100), and each 50 ml of the elution was collected. The fractions mainly including beauveriolides IV, V, VI and VII (100:2, 9th~19th) were pooled and concentrated *in vacuo* to give Fr. 1 (425 mg) as white powder. Then, they were subjected to preparative HPLC under the following conditions: CAPCELLPAK C_{18} UG120 column (SHISEIDO), 20 $\times$ 250 mm; UV at 210 nm; 6.0 ml/minute; a 50-minute isocratic solvent with 50% aq CH_3CN and then a 35-minute linear gradient from 50% aq CH_3CN to 85% aq CH_3CN . Beauveriolides IV, V, VI and VII were eluted as peaks with retention times of 27.0, 45.0, 49.0 and 52.0 minutes, respectively (Fig. 2A). The fractions of these peaks were concentrated *in vacuo* to dryness to give pure beauveriolides IV (86 mg), V (36 mg), VI (46 mg) and VII (80 mg) as white powder.

Fig. 1. Structures of beauveriolides IV to IX.

The yellow powder (2.8 g) from L-Ile-added fermentation was used for isolation of beauveriolides V, VIII and IX. Similarly, this powder was separated by silica gel column chromatography, and the fractions mainly including beauveriolides V and VIII (CHCl_3 - CH_3OH 100:2, 35th~37th) and beauveriolide IX (100:1, 7th) were pooled and concentrated *in vacuo* to give Fr. 2 (245 mg) and Fr. 3 (14 mg) as white powder, respectively. Finally, Fr. 2 was purified by the same HPLC conditions. Beauveriolide V and VIII were eluted as peaks with respective retention times of 45.0 and 77.0 minutes (Fig. 2A). The fractions of the peaks were concentrated *in vacuo* to dryness to give pure beauveriolides V (93 mg) and VIII (28 mg) as white powder. On the other hand, Fr. 3 was purified by HPLC using isocratic 58% aq CH_3CN as solvent, and beauveriolide IX was eluted as a peak with a retention time of 86.0 minutes (Fig. 2B). The fraction of the peak was concentrated *in vacuo* to dryness to give pure beauveriolides IX (4.2 mg) as white powder.

Fig. 2. Chromatographic profile of isolation of beauveriolides IV to IX by preparative HPLC.

Column, CAPCELLPAK C_{18} UG120 (20×250 mm); UV at 210 nm; 6.0 ml/minute. A, solvent 50% aq CH_3CN (0~50 minutes), and then linear gradient from 50% aq CH_3CN to 85% aq CH_3CN (50~85 minutes) for beauveriolides IV to VIII.

B, solvent 58% aq CH_3CN for beauveriolide IX.

Physico-chemical Properties of Beauveriolides IV to IX

Physico-chemical properties of beauveriolides IV to IX are summarized in Table 2. All these beauveriolides showed the end absorption (206~209 nm) in UV spectra. From the amino acid analysis, all the beauveriolides consisted of two L-amino acids and one D-amino acid. These data suggested that beauveriolides IV to IX are structurally related to beauveriolides I and III. Beauveriolide IX was identified as beauverolide Fa⁶⁾.

Structure of Beauveriolide VIII

The ^{13}C NMR spectrum ($CDCl_3$ - CD_3OD , 1:1) showed

25 resolved peaks (Table 4), which were classified into seven methyl, seven methylene, three sp^3 methine, three *N*-methine, one *O*-methine, and four carbonyl carbons by analyses of the DEPT spectra. The 1H NMR spectrum ($CDCl_3$ - CD_3OD , 1:1) displayed 43 proton signals, whereas additional two proton signals (δ 7.18 and 7.78) were observed in $CDCl_3$ (Table 4), suggesting the presence of two NH protons. These results supported the molecular formula. The connectivity of proton and carbon atoms was confirmed by the 1H - ^{13}C HMQC spectrum (Table 4). Analysis of the 1H - 1H COSY revealed the five partial structures A to E (Fig. 3). From amino acid analyses, beauveriolide VIII contained L-Val, L-Ala and D-*allo*-Ile (Table 2). In addition to these findings, observation of the 1H - ^{13}C long-range in the 1H - ^{13}C HMBC experiments (Fig. 3) provided structural evidence as follows; 1) The long-range couplings from H-1 (δ 4.26) to C-2 (δ 37.80) and C-6 (δ 170.35), from H-2 (δ 1.76) to C-1 (δ 60.39), from H₃-3 (δ 0.89) to C-1 and C-4 (δ 26.68) and from H₂-4 (δ 1.13) to C-2, C-3 (δ 15.08) and C-5 (δ 19.42) confirmed the presence of the D-*allo*-Ile moiety containing the partial structure A. 2) The cross peaks from H-19 (δ 2.25) to C-21 (δ 11.29) and from H₃-20 (δ 0.97) and H₃-21 (δ 0.92) to C-18 (δ 62.84) showed the presence of the L-Val moiety containing the partial structure D. 3) The cross peaks from H-23 (δ 3.92) to C-25 (δ 172.97), from H₃-24 (δ 1.37) to C-23 (δ 50.41) and C-25 showed the presence of the L-Ala moiety containing the partial structure E. And 4) the cross peaks from H-7 (δ 4.94) to C-17 (δ 173.38), from H₃-9 (δ 0.92) to C-7 (δ 77.58), C-8 (δ 36.56) and C-10 (δ 31.57), from H₂-11 (δ 1.62, 2.07) to C-10, from H₂-12 (δ 1.26) to C-13 (δ 23.35) and C-14 (δ 30.41), from H₂-13 (δ 1.28) to C-12 (δ 32.64), C-14 and C-15 (δ 14.30), from H-14 (δ 1.26) to C-13, from H₃-15 (δ 0.87) to C-13 and from H₂-16 (δ 2.43, 2.60) to C-7 and C-17 showed that the partial structure B was linked to the partial structure C *via* a methylene unit. Therefore, the part containing the partial structures B to C gave a 3-hydroxy-4-methyldecanoic acid moiety.

The sequence of the four constituents was determined as shown in Fig. 3. The long-range couplings from H-1 to C-25 and the chemical shift of C-1 indicated that L-Ala moiety is attached to the D-*allo*-Ile *via* an amide bond. The cross peaks from H-23 to C-22 (δ 173.06) and the chemical shift of C-23 indicated that the L-Ala is attached to L-Val *via* an amide bond. The cross peaks from H-7 to C-6 and the chemical shift of C-7 indicated that the 3-hydroxy-4-methyl-decanoyl moiety is attached to D-*allo*-Ile *via* an ester bond. Finally, the cyclic structure was elucidated. The chemical shifts of C-17 and C-18 indicated that they are

Table 2. Physico-chemical properties of beauveriolides IV to IX.

	Beauveriolide					
	IV	V	VI	VII	VIII	IX
Appearance	white powder	white powder	white powder	white powder	white powder	white powder
Molecular formula	C ₂₂ H ₃₉ N ₃ O ₅	C ₂₃ H ₄₁ N ₃ O ₅	C ₂₃ H ₄₁ N ₃ O ₅	C ₂₆ H ₃₉ N ₃ O ₅	C ₂₅ H ₄₅ N ₃ O ₅	C ₃₃ H ₄₅ N ₃ O ₅
Molecular weight	425	439	439	473	467	563
FAB-MS (<i>m/z</i>)						
Positive	426 [M+H] ⁺ 448 [M+Na] ⁺	440 [M+H] ⁺ 462 [M+Na] ⁺	440 [M+H] ⁺ 462 [M+Na] ⁺	474 [M+H] ⁺ 496 [M+Na] ⁺	468 [M+H] ⁺ 490 [M+Na] ⁺	564 [M+H] ⁺ 586 [M+Na] ⁺
HRFAB-MS (<i>m/z</i>) (positive)						
MF+Na or MF+H	C ₂₂ H ₃₉ N ₃ O ₅ Na	C ₂₃ H ₄₁ N ₃ O ₅ Na	C ₂₃ H ₄₂ N ₃ O ₅	C ₂₆ H ₄₀ N ₃ O ₅	C ₂₅ H ₄₆ N ₃ O ₅	C ₃₃ H ₄₆ N ₃ O ₅
Calcd:	448.2787	462.2943	440.3124	474.2968	468.3437	564.3437
Found:	448.2787	462.2944	440.3124	474.2968	468.3437	564.3437
Amino acid analysis	L-Ala, L-Val D-Val	L-Ala, L-Val D-allo-Ile	L-Ala, L-Val D-Leu	L-Ala, L-Phe D-Val	L-Ala, L-Val D-allo-Ile	L-Phe, L-Phe D-allo-Ile
[α] _D ²⁵ (CHCl ₃ -CH ₃ OH, 4:1)	-44° (c 0.41)	-37° (c 0.50)	-65° (c 0.47)	-9.0° (c 0.20)	-45° (c 0.30)	ND
UV λ _{max} ^{CH₃OH} (ε)	209 (18,216)	208 (24,066)	209 (15,027)	209 (28,044)	207 (21,365)	206 (14,801)
IR ν _{max} ^{KBr} (cm ⁻¹)	3300, 1724, 1684 1639, 1535	3323, 1724, 1682 1639, 1537	3292, 1724, 1682 1639, 1537	3302, 1726, 1687 1639, 1537	3298, 1722, 1686 1639, 1537	3303, 1726, 1678 1639, 1539
Melting point	244-246 °C	240-242 °C	243-246 °C	249-251 °C	244-246 °C	231-233 °C
Solubility						
Soluble	EtOH, CH ₃ CN, MeOH, CHCl ₃ , EtOAc	EtOH, CH ₃ CN, MeOH, CHCl ₃ , EtOAc	EtOH, CH ₃ CN, MeOH, CHCl ₃ , EtOAc	EtOH, CH ₃ CN, MeOH, CHCl ₃ , EtOAc	EtOH, CH ₃ CN, MeOH, CHCl ₃ , EtOAc	EtOH, CH ₃ CN, MeOH, CHCl ₃ , EtOAc
Insoluble	H ₂ O, <i>n</i> -Hexane	H ₂ O, <i>n</i> -Hexane	H ₂ O, <i>n</i> -Hexane	H ₂ O, <i>n</i> -Hexane	H ₂ O, <i>n</i> -Hexane	H ₂ O, <i>n</i> -Hexane

linked to an amide nitrogen. Furthermore, considering the degree of unsaturation and molecular formula, L-Val should lie between L-Ala and 3-hydroxy-4-methyldecanoyl moieties. Taken together, the structure of beauveriolide VIII was elucidated as shown in Fig. 1.

Structure of Beauveriolide IV

The ¹³C NMR spectrum (CDCl₃-CD₃OD, 1:1) showed 22 resolved peaks (Table 3), which were classified into seven methyl, four methylene, three *sp*³ methine, three *N*-methine, one *O*-methine, and four carbonyl carbons by analyses of the DEPT spectra. The ¹H NMR spectrum (CDCl₃-CD₃OD, 1:1) displayed 36 proton signals, whereas additional three proton signals (δ 6.21, δ 6.32 and δ 7.46) were observed in CDCl₃ (Table 3), suggesting the presence of three NH protons. These results supported the molecular formula. The connectivity of proton and carbon atoms was confirmed by the ¹H-¹³C HMQC spectrum (Table 3). Analysis of the ¹H-¹H COSY revealed the five

partial structures F to J (Fig. 3). From amino acid analyses, beauveriolide IV contained L-Val, L-Ala and D-Val (Table 2). In addition to these findings, observation of the ¹H-¹³C long-range in the ¹H-¹³C HMBC experiments (Fig. 3) provided structural evidence as follows; 1) The long-range couplings from H-1 (δ 4.18) to C-2 (δ 31.27) C-3 (δ 19.35), C-4 (δ 19.54), and C-5 (δ 169.97), from H-2 (δ 1.93) to C-1 (δ 61.22), C-3, C-4 and C-5, from H₃-3 (δ 0.98) to C-1, C-2 and C-4 and from H-4 (δ 0.98) to C-2 and C-5 confirmed the presence of the D-Val moiety containing the partial structure F. 2) The cross peaks from H₃-17 (δ 0.96) to C-15 (δ 62.96), C-16 (δ 28.65), and C-18 (δ 19.40) and from H₃-18 (δ 0.96) to C-15, C-16, and C-17 (δ 19.30) showed the presence of the L-Val moiety containing the partial structure I. 3) The cross peaks from H-20 (δ 3.98) to C-21 (δ 15.44), from H₃-21 (δ 1.37) to C-20 (δ 49.99) and C-22 (δ 172.47) showed the presence of the L-Ala moiety containing the partial structure J. And 4) the cross peaks from H-6 (δ 4.93) to C-7 (δ 36.20), C-13 (δ 36.32) and C-14 (δ 172.93), from H-7 (δ 2.09) to C-6 (δ

Fig. 3. NMR experiments of beauveriolide VIII and IV.

77.27) and C-8 (δ 15.84), from H_3 -8 (δ 0.91) to C-6 and C-7, from H_2 -9 (δ 1.04, 1.46) to C-7, C-8, C-10 (δ 30.06) and C-11 (δ 23.44), from H_2 -11 (δ 1.27) to C-12 (δ 14.21), from H_3 -12 (δ 0.87) to C-11 and from H_2 -13 (δ 2.45, 2.56) to C-6 and C-14 showed that the partial structure G was linked to the partial structure H via two methylene units. Therefore, the part containing the partial structures G and H gave a 3-hydroxy-4-methyloctanoic acid moiety.

The sequence of the four constituents was determined as shown in Fig. 3. The long-range couplings from H-1 to C-22 and the chemical shift of C-1 indicated that L-Ala moiety is attached to the D-Val via an amide bond. The cross peaks from H-20 to C-19 (δ 172.77) and the chemical shift of C-20 indicated that the L-Ala is attached to L-Val via an amide bond. The cross peaks from H-6 to C-5 and the chemical shift of C-6 indicated that the 3-hydroxy-4-methyloctanoyl moiety is attached to D-Val via an ester bond. Finally, the cyclic structure was elucidated. The chemical shifts of C-14 and C-15 indicated that they are linked to an amide nitrogen. Furthermore, considering the

degree of unsaturation and molecular formula, L-Val should lie between L-Ala and 3-hydroxy-4-methyloctanoyl moieties. Taken together, the structure of beauveriolide IV was elucidated as shown in Fig. 1.

Structures of Beauveriolides V, VI and VII

From amino acid analyses, beauveriolide V contained L-Val, L-Ala and D-*allo*-Ile, beauveriolide VI did L-Val, L-Ala and D-Leu, and beauveriolide VII did L-Phe, L-Ala and D-Val (Table 2). From analyses of the ^1H - ^1H COSY and the ^1H - ^{13}C HMBC experiments, beauveriolides V, VI and VII contained 3-hydroxy-4-methyloctanoyl moieties. Similarly, the structures of beauveriolides V, VI and VII were elucidated as shown in Fig. 1. The planar structure of beauveriolide VI was identical with that of beauveriolide M⁷⁾.

Biological Properties

Inhibition of Macrophage Foam Cell Formation by Beauveriolides

In the assay system, mouse peritoneal macrophages accumulated massive amounts of lipid droplets in the cytosols, which were observed microscopically after oil red O staining (Fig. 4A). However, beauveriolides VII and IX caused a dose-dependent reduction in the size and number of lipid droplets in macrophages as shown in Fig. 4B and 4C. No cytotoxic effect was observed even at the highest dose (25 μM), indicating that both beauveriolides inhibit the lipid droplet formation specifically. Furthermore, beauveriolides VII and IX inhibited [^{14}C]CE synthesis from [^{14}C]oleic acid by macrophages with IC_{50} values of 21 and 4.4 μM , respectively, and they showed almost no effect on [^{14}C]TG synthesis. However, beauveriolides IV, V, VI and VIII showed only slight effect on lipid droplet formation and [^{14}C]CE synthesis even at the highest dose (25 μM).

Other Biological Activities

No antimicrobial activity of beauveriolides IV to IX was observed at a concentration of 10 $\mu\text{g}/\text{disk}$ (2 mM) against the following microorganisms; *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces cerevisiae*, *Pyricularia oryzae*, *Mucor racemosus* and *Aspergillus niger*.

None of these beauveriolides showed cytotoxic, nematocidal or insecticidal activity at 0.2 mM (100 $\mu\text{g}/\text{ml}$) in our assays.

Table 3. ^1H and ^{13}C NMR chemical shifts of beauveriolides IV to VI.

Beauveriolide IV			Beauveriolide V			Beauveriolide VI		
Carbon No.	^{13}C chemical shifts (ppm) ^a	^1H chemical shifts (ppm) ^b	Carbon No.	^{13}C chemical shifts (ppm) ^a	^1H chemical shifts (ppm)	Carbon No.	^{13}C chemical shifts (ppm) ^a	^1H chemical shifts (ppm) ^b
1-NH		(6.21 ppm in CDCl_3)**	1-NH		6.98 (1H, d, $J=9.5$ Hz)	1-NH		
C-1	61.22	4.18 (1H, d, $J=10.0$ Hz)	C-1	59.54	4.28 (1H, t, $J=9.5$ Hz)	C-1	53.51	4.61 (1H, t, $J=9.5$ Hz)
C-2	31.27	1.93 (1H, m)	C-2	37.01	1.75 (1H, m)	C-2	41.93	1.56 (2H, m)
C-3	19.35	0.98* (3H, d, $J=6.5$ Hz)	C-3	13.61	0.85 (3H, d, $J=7.0$ Hz)	C-3	25.72	1.57 (1H, m)
C-4	19.54	0.98* (3H, d, $J=6.5$ Hz)	C-4	29.76	1.15 (2H, m)	C-4	19.44	0.91 (3H, d, $J=6.5$ Hz)
C-5	169.97		C-5	14.32	0.88 (3H, t, $J=7.0$ Hz)	C-5	19.65	0.96 (3H, d, $J=6.5$ Hz)
C-6	77.27	4.93 (1H, m)	C-6	172.29		C-6	170.71	
C-7	36.20	2.09 (1H, m)	C-7	76.81	4.94 (1H, ddd, $J=4.0, 4.5, 10.5$ Hz)	C-7	77.34	4.93 (3H, m)
C-8	15.84	0.91 (3H, d, $J=7.0$ Hz)	C-8	35.81	2.06 (1H, m)	C-8	36.43	2.10 (3H, m)
C-9	31.08	1.04 (1H, m)	C-9	18.90	0.97 (3H, d, $J=6.0$ Hz)	C-9	15.91	0.89 (3H, d, $J=6.0$ Hz)
		1.46 (1H, m)	C-10	30.52	1.06 (1H, m)	C-10	31.54	1.47 (2H, m)
C-10	30.06	1.24 (1H, m)			1.44 (1H, m)	C-11	30.38	1.26 (2H, m)
		1.27 (1H, m)	C-11	25.91	1.10 (1H, m)	C-12	30.27	1.26 (2H, m)
C-11	23.44	1.27 (2H, m)			1.42 (1H, m)	C-13	14.29	0.89 (3H, t, $J=7.0$ Hz)
C-12	14.21	0.87 (3H, t, $J=7.0$ Hz)	C-12	29.64	1.25 (2H, m)	C-14	36.58	2.47 (1H, dd, $J=10.0, 14.0$ Hz)
C-13	36.32	2.45 (1H, t, $J=10.0, 14.0$ Hz)	C-13	18.67	0.89 (3H, t, $J=7.0$ Hz)			2.54 (1H, dd, $J=4.5, 14.0$ Hz)
		2.56 (1H, t, $J=4.5, 13.5$ Hz)	C-14	35.69	2.44 (1H, dd, $J=10.5, 14.0$ Hz)	C-15	173.20	
C-14	172.93				2.59 (1H, dd, $J=4.5, 13.5$ Hz)	16-NH		
15-NH		(6.32 ppm in CDCl_3)**	C-15	172.64		C-16	63.12	3.61 (1H, d, $J=7.0$ Hz)
C-15	62.96	3.60 (1H, d, $J=9.0$ Hz)	16-NH			C-17	29.01	2.07 (1H, m)
C-16	28.65	2.09 (1H, m)	C-16	62.10	3.65 (1H, d, $J=9.0$ Hz)	C-18	22.59	0.93 (3H, m)
C-17	19.30	0.96*** (3H, d, $J=10.0$ Hz)	C-17	28.18	2.06 (1H, m)	C-19	22.64	0.93 (3H, m)
C-18	19.40	0.96*** (3H, d, $J=10.0$ Hz)	C-18	15.14	0.93 (3H, m)	C-20	173.15	
C-19	172.77		C-19	10.53	0.91 (3H, m)	21-NH		
20-NH		(7.46 ppm in CDCl_3)**	C-20	169.55		C-21	49.85	3.97 (1H, m)
C-20	49.99	3.98 (1H, m)	21-NH			C-22	15.56	1.35 (3H, d, $J=7.0$ Hz)
C-21	15.44	1.37 (3H, d, $J=7.0$ Hz)	C-21	49.62	3.93 (1H, m)	C-23	172.59	
C-22	172.47		C-22	14.71	1.38 (3H, d, $J=7.0$ Hz)			
			C-23	172.14				

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.

**) The signals may be reversed.

***) The signals were observed as the same chemical shifts.

Table 4. ^1H and ^{13}C NMR chemical shifts of beauveriolides VII and VIII.

Beauveriolide VII			Beauveriolide VIII		
Carbon No.	^{13}C chemical shifts (ppm) ^a	^1H chemical shifts (ppm) ^b	Carbon No.	^{13}C chemical shifts (ppm) ^a	^1H chemical shifts (ppm)
1-NH		6.66 (1H, d, $J=8.0$ Hz)	1-NH		6.97 (1H, d, $J=9.5$ Hz)
C-1	60.66	4.09 (1H, dd, $J=7.8, 8.0$ Hz)	C-1	60.39	4.26 (1H, t, $J=10.0$ Hz)
C-2	30.98	1.77 (1H, m)	C-2	37.80	1.76 (1H, m)
C-3	18.73	0.81 (3H, d, $J=6.5$ Hz)	C-3	15.08	0.89 (3H, d, $J=7.0$ Hz)
C-4	18.98	0.80 (3H, d, $J=6.5$ Hz)	C-4	26.68	1.13 (2H, m)
C-5	169.46		C-5	19.42	0.91 (3H, t, $J=7.0$ Hz)
C-6	76.64	4.85 (1H, ddd, $J=4.5, 4.8, 10.0$ Hz)	C-6	170.35	
C-7	35.73	1.96 (1H, m)	C-7	77.58	4.94 (1H, ddd, $J=4.0, 4.5, 10.0$ Hz)
C-8	15.59	0.77 (3H, d, $J=7.0$ Hz)	C-8	36.56	2.06 (1H, m)
C-9	30.71	0.91 (1H, m)	C-9	15.87	0.92 (3H, d, $J=6.0$ Hz)
		1.31 (1H, m)	C-10	31.57	1.048 (1H, m)
C-10	29.60	1.05 (1H, m)			1.46 (1H, m)
		1.20 (1H, m)	C-11	28.93	1.62 (1H, m)
C-11	23.00	1.12 (1H, m)			2.07 (1H, m)
		1.20 (1H, m)	C-12	32.64	1.26 (2H, m)
C-12	13.94	0.75 (3H, t, $J=6.5$ Hz)	C-13	23.35	1.28 (2H, m)
C-13	36.08	2.32 (1H, dd, $J=10.0, 14.0$ Hz)	C-14	30.41	1.26 (2H, m)
		2.38 (1H, dd, $J=4.5, 14.0$ Hz)	C-15	14.30	0.87 (3H, m)
C-14	172.26		C-16	36.47	2.43 (1H, dd, $J=10.0, 13.5$ Hz)
15-NH					2.60 (1H, dd, $J=4.5, 13.5$ Hz)
C-15	57.21	4.09 (1H, dd, $J=7.8, 8.7$ Hz)	C-17	173.38	
C-16	35.75	2.86 (1H, dd, $J=7.8, 13.5$ Hz)	18-NH		(7.18 ppm in CDCl_3)**
		2.96 (1H, dd, $J=8.7, 13.5$ Hz)	C-18	62.84	3.62 (1H, m)
C-17	136.62		C-19	28.22	2.25 (1H, m)
C-18	129.19	7.05 (2H, d, $J=9.6$ Hz)	C-20	19.64	0.97 (3H, d, $J=6.5$ Hz)
C-19	128.69	7.15 (2H, dd, $J=9.6, 9.6$ Hz)	C-21	11.29	0.92 (3H, m)
C-20	127.09	7.09 (1H, dd, $J=9.6, 9.6$ Hz)	C-22	173.06	
C-21	171.64		23-NH		(7.78 ppm in CDCl_3)**
22-NH			C-23	50.41	3.92 (1H, m)
C-22	49.61	3.76 (1H, q, $J=7.0$ Hz)	C-24	15.47	1.37 (3H, d, $J=7.0$ Hz)
C-23	15.01	1.15 (3H, d, $J=7.0$ Hz)	C-25	172.97	
C-24	171.64				

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 may be reversed.

Discussion

Fig. 4. Inhibition of lipid droplet formation in macrophages by beauveriolides VII and IX.

Macrophage monolayers (5.0×10^5 cells in 0.25 ml medium) grown in a tissue culture chamber (LAB-TEK 8-chamber, Nunc) were incubated with $10 \mu\text{l}$ of liposomes for 16 hours in the absence (A) or in the presence of $10 \mu\text{M}$ beauveriolide VII (B) or $3 \mu\text{M}$ beauveriolide IX (C), respectively. Fixation and staining with oil red O and hematoxylin were then performed. Original magnification, $\times 200$.

From our studies on improvement of the beauveriolide production by fermentation of *Beauveria* sp. FO-6979, we found that tryptone-containing media, such as the control medium described in this paper, give a high production of beauveriolides I and III⁵). Furthermore, selective production of beauveriolide I or III was achieved by fermentation in such media supplemented with L-Leu or L-Ile, respectively⁵). At the same time, we noticed that different beauveriolides having similar UV spectra were produced in various amino acid-supplemented fermentation broths, resulting in isolation of eight beauveriolides (I and III to IX). All beauveriolides are cyclodepsipeptides composed of four constituents; two L-amino acids, one D-amino acid and one 3-hydroxy-4-methyl fatty acid. A number of amino acids were supplemented to the fermentation medium to see the change of these beauveriolide production. L-Ile, L-Leu, L-Phe, L-Ala and L-Val yielded a big change in the production (Table 1), but other amino acids such as D-amino acids, L-Met, L-Lys, L-Gln, L-Ser, L-Pro, L-Trp, L-His, L-Arg and L-Asp gave almost no change (data not shown). From analysis of beauveriolide production, specific molecules appear incorporated into each part of beauveriolides (Fig. 5). 3-Hydroxy-4-methyloctanoic acid is almost exclusively incorporated into the part A under the fermentation conditions. Only beauveriolide VIII has longer 3-hydroxy-4-methyldecanoic acid in this part. Regarding amino acid parts, L-Phe and L-Ala are preferentially incorporated, and L-Val and L-Phe are slightly into the parts B and C, respectively. Interestingly, D-amino acids are not directly incorporated into the part D, whereas the corresponding L-amino acids are found to be incorporated efficiently there. L-Ile-supplemented fermentation enhanced the production of D-allo-Ile-

Fig. 5. Selective incorporation of precursors into the four parts A to D of beauveriolide.

containing beauveriolides III, V, VIII and IX, and L-Leu enhanced the production of D-Leu-containing beauveriolides I and VI. L-Ile and L-Leu are good substrates for the part D. L-Val was also found to be a substrate for this part, to increase the production of D-Val-containing beauveriolides IV and VII. These findings suggested that putative beauveriolide synthetase has the substrate specificity in part by part as shown in Fig. 5, and the enzyme can epimerize L-Leu, L-Ile and L-Val to corresponding D-amino acids for incorporation as constituents into the part D. KLEINKAUF and VON DÖHREN reported, in fact, that non-ribosomal cyclodepsipeptide synthetases (*ex. enniatin synthetases*) contain amino acid epimerase domains⁸⁻¹⁰. Therefore, it is plausible that a homologous synthetase catalyzes the beauveriolide biosynthesis in this fungal strain.

Among eight beauveriolides isolated from *Beauveria* sp. FO-6979, beauveriolides I and III are the most potent inhibitors of lipid droplet formation in mouse macrophages. As described in this paper, beauveriolide VII and IX also inhibit the formation, but their potency seems much weaker than that of beauveriolides I and III. The other beauveriolides showed only slight effect on the formation. These results indicated that the presence of L-Phe and D-Leu/D-*allo*-Ile in the molecules is important for eliciting inhibitory activity against lipid droplet formation in macrophages.

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