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

DAISUKE MATSUDA[d], ICHIJI NAMATAME[a,c], HIROSHI TOMODA[a,b,*], SUSUMU KOBAYASHI[d], RAINER ZOCHER[e], HORST KLEINKAUF[e] and SATOSHI ŌMURA[a,b,*]

[a] Kitasato Institute for Life Sciences and Graduate School of Infection Control Sciences, Kitasato University, Shirokane, Minato-ku, Tokyo 108-8641, Japan
[b] The Kitasato Institute, Shirokane, Minato-ku, Tokyo 108-8642, Japan
[c] Japan Society for the Promotion of Science, Koujimachi, Chiyoda-ku, Tokyo 102-8471, Japan
[d] Faculty Graduate School of Pharmaceutical Sciences, Science University of Tokyo, Hunagawaramachi, Ichigaya, Shinjyuku-ku, Tokyo 162-0826, Japan
[e] Max Volmer Institut für Biochemie, Technische Universität Berlin, 29, Franklinstr, Berlin 10587, Germany

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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 atherosclerogenesis[1]. During the screening for inhibitiors of lipid droplet synthesis with this assay system, beauveriolides I and III were isolated from the culture broth of *Beauveria* sp. FO-6979[2~4]. They inhibited cholesteryl ester synthesis in macrophages specifically, resulting in a reduction of the number and the size of cytosolic lipid droplets[2]. Recently, we succeeded in the selective production of beauveriolide I or III by fermentation of the producing strain in the amino acid-supplemented media[5]. 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 Fa[6].

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 (10^8 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%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 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 slica gel column

* Corresponding author: tomoda@lisci.kitasato-u.ac.jp or omura-s@kitasato.or.jp

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 C_{18} (Waters), 2.1×150 mm; solvent, 20-minute isocratic with 45% CH_3CN in 0.05% H_3PO_4 and then 20-minute linear gradient from 45% CH_3CN in 0.05% H_3PO_4 to 80% CH_3CN in 0.05% H_3PO_4; 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 Fa[6]) 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 method[3].

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

Morphological assay for lipid droplet formation and biochemical assay for [^{14}C]cholesteryl ester (CE) and [^{14}C]triacylglycerol (TG) syntheses from [^{14}C]oleic acid in mouse peritoneal macrophages were carried out according to the established methods[1].

Other Biological Assays

Antimicrobial activity was tested using paper disks (i.d. 6 mm, ADVANTEC). Bacteria were grown on Müeller-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 μl) was transferred into a 50-ml test tube containing 10 ml of the seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4\cdot7H_2O$ 0.05%, Polypepton 0.5%, KH_2PO_4 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 μg/ml), V (51.8 μg/ml), VIII (15.5 μg/ml) and IX (28.7 μg/ml) was increased about two to four folds when compared with the control fermentation, but the production of beauveriolide I (25→5.6 μg/ml) was inhibited dramatically.

Table 1. Production of beauveriolides by amino acid addition.

	Beauveriolides production (μg/ml)					
		Amino acid addition				
Beauveriolide	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 μg/ml) and VI (45.5 μg/ml) was increased three to five folds, but the production of beauveriolide III was decreased (50.0→26.0 μg/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→47.6 μg/ml).

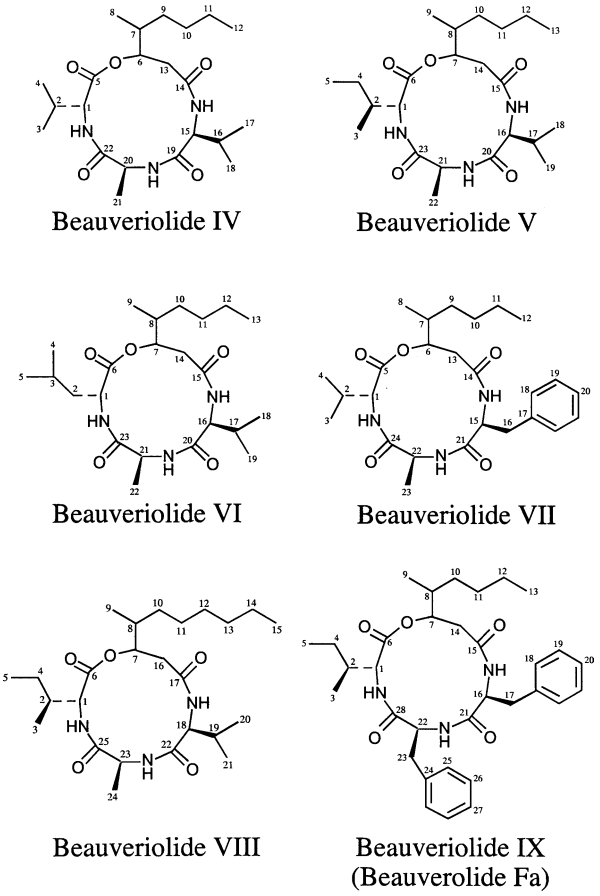
By L-Phe-added fermentation, the production of beauveriolides I (66.0 μg/ml), III (79.0 μg/ml), VII (24.0 μg/ml) and IX (19.0 μg/ml) was increased 1.6 to 2.6 folds. By L-Ala-added fermentation, the production of beauveriolides I (30.0 μg/ml), III (60.0 μg/ml), VI (19.7 μg/ml), VII (25.2 μg/ml) was slightly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

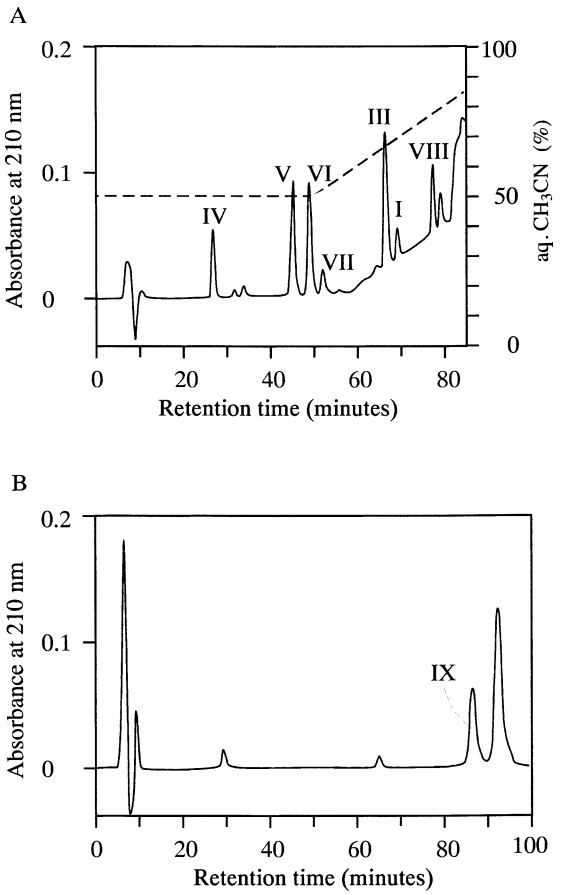
The yellow powder (1.8 g) from L-Val-added fermentation was used for isolation of beauveriolides IV, V, VI and VII. This powder was dissolved in $CHCl_3$ and was applied on a silica gel (200 g) column that was previously equilibrated with $CHCl_3$. The materials were eluted stepwise with $CHCl_3$ - CH_3OH 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), 20×250 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. Similiarly, 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 gladient 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 Fa[6].

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 H_3-3 (δ 0.89) to C-1 and C-4 (δ 26.68) and from H_2-4 (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 H_3-20 (δ 0.97) and H_3-21 (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 H_3-24 (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 H_3-9 (δ 0.92) to C-7 (δ 77.58), C-8 (δ 36.56) and C-10 (δ 31.57), from H_2-11 (δ 1.62, 2.07) to C-10, from H_2-12 (δ 1.26) to C-13 (δ 23.35) and C-14 (δ 30.41), from H_2-13 (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 H_3-15 (δ 0.87) to C-13 and from H_2-16 (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 metylene 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.

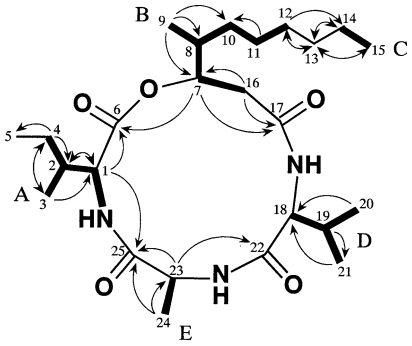
	Beauveriolide					
	IV	V	VI	VII	VIII	IX
Appearance	white powder	white powder	white powder	white powder	white powder	white powder
Molecular formula	$C_{22}H_{39}N_3O_5$	$C_{23}H_{41}N_3O_5$	$C_{23}H_{41}N_3O_5$	$C_{26}H_{39}N_3O_5$	$C_{25}H_{45}N_3O_5$	$C_{33}H_{45}N_3O_5$
Molecular weight	425	439	439	473	467	563
FAB-MS (*m/z*)						
Positive	426 $[M+H]^+$	440 $[M+H]^+$	440 $[M+H]^+$	474 $[M+H]^+$	468 $[M+H]^+$	564 $[M+H]^+$
	448 $[M+Na]^+$	462 $[M+Na]^+$	462 $[M+Na]^+$	496 $[M+Na]^+$	490 $[M+Na]^+$	586 $[M+Na]^+$
HRFAB-MS (*m/z*) (positive)						
MF+Na or MF+H	$C_{22}H_{39}N_3O_5Na$	$C_{23}H_{41}N_3O_5Na$	$C_{23}H_{42}N_3O_5$	$C_{26}H_{40}N_3O_5$	$C_{25}H_{46}N_3O_5$	$C_{33}H_{46}N_3O_5$
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
$[\alpha]_D^{25}$ ($CHCl_3$-CH_3OH, 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 $\lambda_{max}^{CH_3OH}$nm (ε)	209 (18,216)	208 (24,066)	209 (15,027)	209 (28,044)	207 (21,365)	206 (14,801)
IR υ_{max}^{KBr} (cm^{-1})	3300, 1724, 1684	3323, 1724, 1682	3292, 1724, 1682	3302, 1726, 1687	3298, 1722, 1686	3303, 1726, 1678
	1639, 1535	1639, 1537	1639, 1537	1639, 1537	1639, 1537	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_3CN, MeOH, $CHCl_3$ EtOAc	EtOH, CH_3CN, MeOH, $CHCl_3$ EtOAc	EtOH, CH_3CN, MeOH, $CHCl_3$ EtOAc	EtOH, CH_3CN, MeOH, $CHCl_3$ EtOAc	EtOH, CH_3CN, MeOH, $CHCl_3$ EtOAc	EtOH, CH_3CN, MeOH, $CHCl_3$ EtOAc
Insoluble	H_2O, *n*-Hexane	H_2O, *n*-Hexane	H_2O, *n*-Hexane	H_2O, *n*-Hexane	H_2O, *n*-Hexane	H_2O, *n*-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.

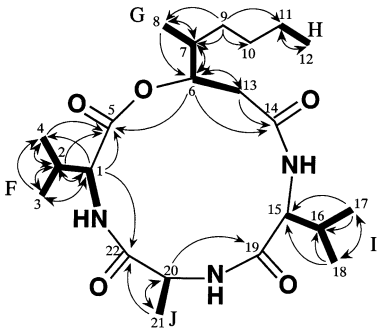
Structure of Beauveriolide IV

The ^{13}C NMR spectrum ($CDCl_3$ - CD_3OD, 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 ($CDCl_3$ - CD_3OD, 1 : 1) displayed 36 proton signals, whereas additional three proton signals (δ 6.21, δ 6.32 and δ 7.46) were observed in $CDCl_3$ (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-^{13}C 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-^{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 4.18) 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 H_3-3 (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 H_3-17 (δ 0.96) to C-15 (δ 62.96), C-16 (δ 28.65), and C-18 (δ 19.40) and from H_3-18 (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 H_3-21 (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.



Beauveriolide VIII



Beauveriolide IV

^{1}H-^{1}H COSY: ——, HMBC: H⟶C.

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 ^{1}H-^{1}H COSY and the ^{1}H-^{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 beauverolide M[7].

Biological Properties

Inhibition of Macrophage Foam Cell Formation by Beauveriolides

In the assay system, mouse peritoneal macrophages accummulated 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 μg/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 μg/ml) in our assays.

Table 3. ^{1}H 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]	^{1}H chemical shifts (ppm)[b]	Carbon No.	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)	Carbon No.	^{13}C chemical shifts (ppm)[a]	^{1}H 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				

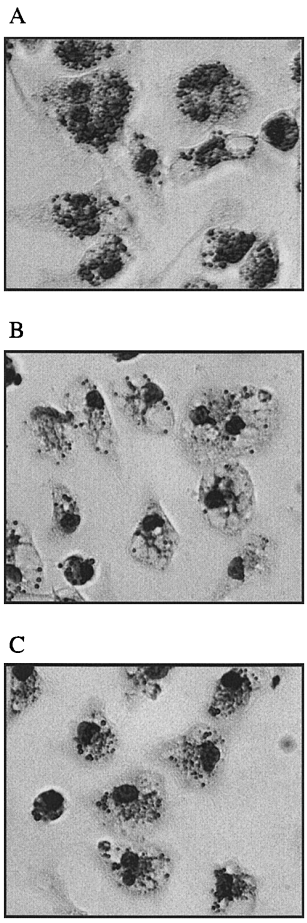
a) Chemical shifts are shown with reference to CD_3OD as 49.0 ppm.
b) Chemical shifts are shown with reference to CD_3OD as 3.30 ppm.
*) The signals were observed as the same chemical shifts.
**) The signals may be reversed.
***) The signals were observed as the same chemical shifts.

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

	Beauveriolide VII		Beauveriolide VIII		
Carbon No.	^{13}C chemical shifts (ppm)[a]	^{1}H chemical shifts (ppm)[b]	Carbon No.	^{13}C chemical shifts (ppm)[a]	^{1}H 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.

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

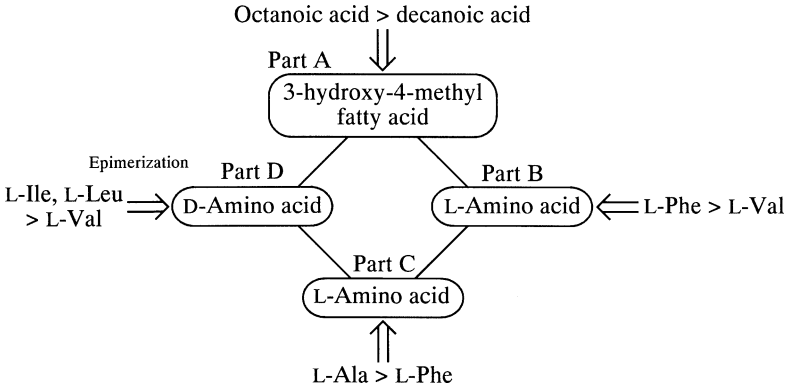


Macrophage monolayers (5.0×10^5 cells in 0.25 ml medium) grown in a tissue culture chamber (LAB-TEK 8-chamber, Nunc) were incubated with 10 μl of liposomes for 16 hours in the absence (A) or in the presence of 10 μM beauveriolide VII (B) or 3 μM beauveriolide IX (C), respectively. Fixation and staining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

Discussion

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 III[5]. Furthermore, selective production of beauveriolide I or III was achieved by fermentation in such media supplemented with L-Leu or L-Ile, respectively[5]. 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-ribosormal cyclodepsipeptide synthetases (*ex.* enniatin synthetases) contain amino acid epimerase domains[8~10]. 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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