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Beauveriolides, Specific Inhibitors of Lipid Droplet Formation in Mouse Macrophages, Produced by *Beauveria* sp. FO-6979

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Beauveria sp. FO-6979, a soil isolate, was found to produce inhibitors of lipid droplet formation in mouse peritoneal macrophages. A new compound beauveriolide III was isolated along with a known compound beauveriolide I from the fermentation broth of the producing strain by solvent extraction, ODS column chromatography, silica gel column chromatography and preparative HPLC. Beauveriolides I and III caused a reduction in the number and size of cytosolic lipid droplets in macrophages at 10 μM without any cytotoxic effect on macrophages.

In the early stage of atherosclerosis, macrophages penetrate into the intima, efficiently take up modified low density lipoprotein (LDL), store cholesterol and fatty acids in the form of cholesteryl esters (CE) and triacylglycerides (TG), respectively, in the cytosolic lipid droplets, and are converted into foam cells, leading to the development of atherosclerosis in the arterial wall. Therefore, inhibitors of the macrophage-derived foam cell formation would be expected to retard the progression of atherosclerosis[1~4].

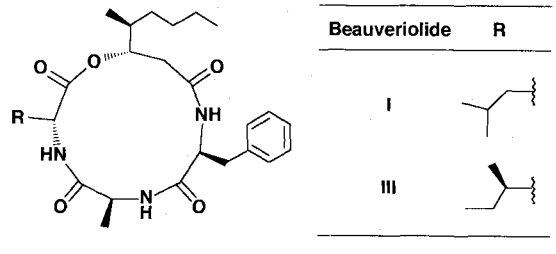
NISHIKAWA *et al.* reported a foam cell formation model using primary mouse peritoneal macrophages cultured in the presence of lipid liposomes containing acidic phospholipids[5]. In this cell assay, it is possible to observe the macrophage-derived foam cell formation, that is, a process from ligand (lipoproteins or liposomes) incorporation *via* scavenger receptors to lipid droplet formation in a cytosolic fraction of macrophages. We have developed a more optimized assay system for the primary screen of microbial inhibitors, where massive amounts of cytosolic neutral lipid droplets in macrophages cultured in a 96-well microplate were visualized by oil red O staining[5]. With this screen system, two structurally related compounds were isolated from the culture broth of a fungal strain FO-6979 as inhibitors of the lipid droplet formation. One was identified as beauveriolide I previously reported as an insecticidal agent[6], but the other was found to be a new compound named beauveriolide III[7] (Fig. 1). They belong to cyclodepsipeptide family like beauverilide A[8], beauverolides[9~11], beauvericin[12], enniatins[12] and bassianolide[13]. In this paper, the taxonomy of the producing strain, fermentation, isolation and biological properties of beauveriolides are described.

Materials and Methods

General Experimental Procedures

Fungal strain FO-6979 isolated from a soil sample was used for production of beauveriolides I and III. Kieselgel 60 (E. Merck) and SSC-ODS-7515-12 (Senshu Sci. Co.) were used for silica gel and octadecyl silyl (ODS) column chromatography, respectively. HPLC was carried out using RANIN (MODEL SD-200) or Shimadzu (LC-7A)

Fig. 1. Structures of beauveriolides I and III.



Systems. For determination of the amounts of beauveriolides, the samples dissolved in methanol were analyzed by HPLC as follows: column, Senshu pak Pegasil ODS, 6×250 mm; solvent, 55% CH_3CN; flow rate, 1.5 ml/minute; detection, UV at 215 nm. Under these conditions, beauveriolides I and III were eluted as peaks with retention times of 22 and 20 minutes, respectively.

Taxonomic Studies

For identification of the fungus, potato dextrose agar (PDA) (Difco), malt extract agar and corn meal agar (CMA) (Difco) were used. Morphological properties were examined after incubation at 25°C for 14 days on these agar media. Morphological observation was done under a light microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (JEOL JSM-5600).

Assay for Lipid Droplet Formation in Mouse Macrophages

Female ICR mice (25~30 g) were obtained from the Japan SLC Inc. Mouse peritoneal macrophages were prepared as described previously[5]. Peritoneal cells were harvested from unstimulated mice using Hank's buffered salt solution (HBSS) and then suspended at 2×10^6 cells/ml in GIT medium. Aliquots (0.125 ml) were dispensed into a 96-well plastic microplate (Corning Co.) and incubated in a humidified CO_2 (5% v/v) atmosphere at 37°C for 2 hours, after which each plate was washed three times with 0.125 ml HBSS to remove the unattached cells. The medium was then replaced immediately with 0.125 ml Dulbecco's modified Eagle medium containing 8% (v/v) lipoprotein-deficient serum[14], penicillin (100 units/ml) and streptomycin (100 μg/ml). After a 2-hour preincubation, 1.25 μl of samples (methanol solution), and 5.0 μl of liposomes (phosphatidylcholine 1.0 μmol, phosphatidylserine 1.0 μmol, dicetylphosphate 0.20 μmol and cholesterol 1.5 μmol suspended in 1.0 ml of 0.3 M glucose) prepared as described previously[15], were added to each culture. After a 14-hour incubation, the cells were washed three times with PBS and then fixed by soaking in 10% formalin. Nuclei and intracellular neutral lipid droplets were then stained with hematoxylin and oil red O, respectively, and the stained cells were examined by a light microscopy.

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.

Nematocidal and insecticidal assays were carried out using *Caenorhabditis elegans* and *Artemia salina*, respectively, as respective test organisms. Samples were dissolved in 250 μl of an assay medium (egg lecithin 0.01%, $NaHCO_3$ 7.5 mM, KCl 7.5 mM, $CaCl_2 \cdot 2H_2O$ 7.5 mM, and $MgSO_4 \cdot 7H_2O$ 7.5 mM) per well of 96-well plates. Then both *C. elegans* and *A. salina* were inoculated into each well and incubated at 27°C for 2 days. Movement of the organisms and their morphological changes were observed under microscope (Olympus Vanox-S AH-2) at 24 and 48 hours after inoculation as signs of toxicity.

Results

Taxonomy of the Producing Organism

The fungal strain FO-6979 grew moderately on PDM, malt extract agar and CMA to form white to brownish white colonies with a diameter of 30~54 mm. Reverse of the colonies was brownish white to pale yellow. The colony was powdery to floccose. The conidiogenous cells were born directly from vegetative hyphae or through short branches. They were variable in shape, ventricose to filamentous, 2.5~3.7 μm in length, and 2.0~3.3 μm in width. In producing conidia, the tip of the conidiogenous cells elongated sympodually, 12~21 μm in length. The conidia were hyaline in color, globose to ellipsoidal and $1.8 \sim 2.5 \times 2.5 \sim 3.3$ μm in size, and their surface was smooth. No teleomorph was observed in this strain. The scanning electron micrograph of FO-6979 is shown in Fig. 2. From the above characteristics, the strain FO-6979 was considered to belong to a member of the genus *Beauveria*[16]. The strain was deposited in National Institute of Bioscience and Technology, Japan, as *Beauveria* sp. FO-6979 with the accession number FERM P-16716.

Fermentation

A slant culture of the strain FO-6979 grown on YpSs agar (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) was used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of a seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flasks were

Fig. 2. Scanning electron micrograph of *Beauveria* sp. FO-6979 grown on corn meal agar.

Bar represents 10 μm.

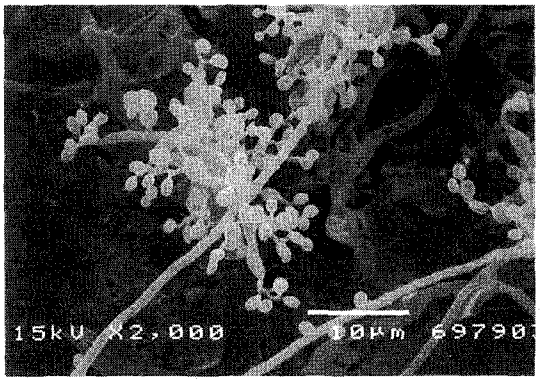
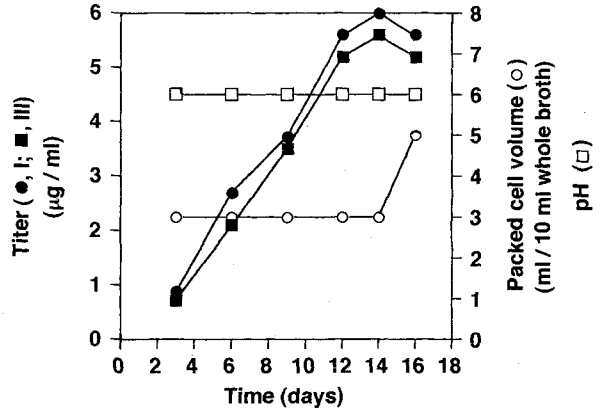


Fig. 3. A typical time course of beauveriolides production by *Beauveria* sp. FO-6979.

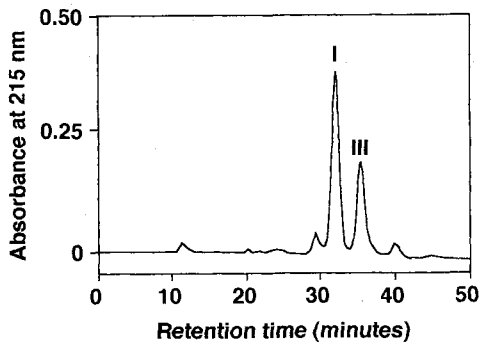


shaken on a rotary shaker for 4 days at 27°C. 2 ml of the seed culture was transferred into 200 ml of the production medium (sucrose 2.0%, glucose 1.0%, corn steep powder 0.5%, meat extract 0.5%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3%, agar 0.1%, and trace element containing in g/liter: $FeSO_4 \cdot 7H_2O$ 1.0, $MnCl_2 \cdot 4H_2O$ 1.0, $ZnSO_4 \cdot 7H_2O$ 1.0, $CuSO_4 \cdot 5H_2O$ 1.0 and $CoCl_2 \cdot 2H_2O$ 1.0 (1 ml), pH 6.0) in a 1,000-ml flask. The fermentation was carried out at 27°C under a stationary condition. A typical time course of the fermentation is shown in Fig. 3. Both beauveriolides I and III were detected in the culture broth on day 3 after inoculation, and their concentrations reached a maximum (6.0 and 5.6 μg/ml, respectively) on day 14.

Isolation

To the 14-day old whole broth (10 liters) was added acetone (10 liters). After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with 10 liters of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a brownish oil (9.4 g). The material suspended in 40% aq acetonitrile was subjected to an ODS column (470 g). The materials were eluted stepwise with aq acetonitrile solutions (500 ml, 40, 50, 60, 70, 80, 90 and 100%), and each 40 ml of the elution was collected. The 36th~49th fractions were pooled, concentrated and extracted with ethyl acetate to give a yellow powder (605 mg). Then, the residue was dissolved in chloroform-methanol solution (100:1, v/v) and applied on a silica gel column (30 g) previously equilibrated with chloroform-methanol solution (100:1, v/v). The materials were eluted stepwise with chloroform-methanol solutions (100 ml, 100:1, 100:2, 100:3, 100:4, 100:5, 100:10, and 0:100), and each 10 ml of the elution was collected. The active (18th~24th) fractions were pooled and concentrated *in vacuo* to give a white powder (168 mg). The resulting active powder was purified by preparative HPLC using a prepacked silica gel column (Senshu Pak PEGASIL Silica 120-5, 20×250 mm; hexane-2-propanol solution (100:3); UV at 215 nm; 6.0 ml/minute). Under the condition, beauveriolides I and III were eluted with retention times of 31.0 and 35.0 minutes, respectively (Fig. 4), each of which was concentrated *in vacuo* to dryness to give pure beauveriolides I (9.3 mg) and III (5.4 mg) as white

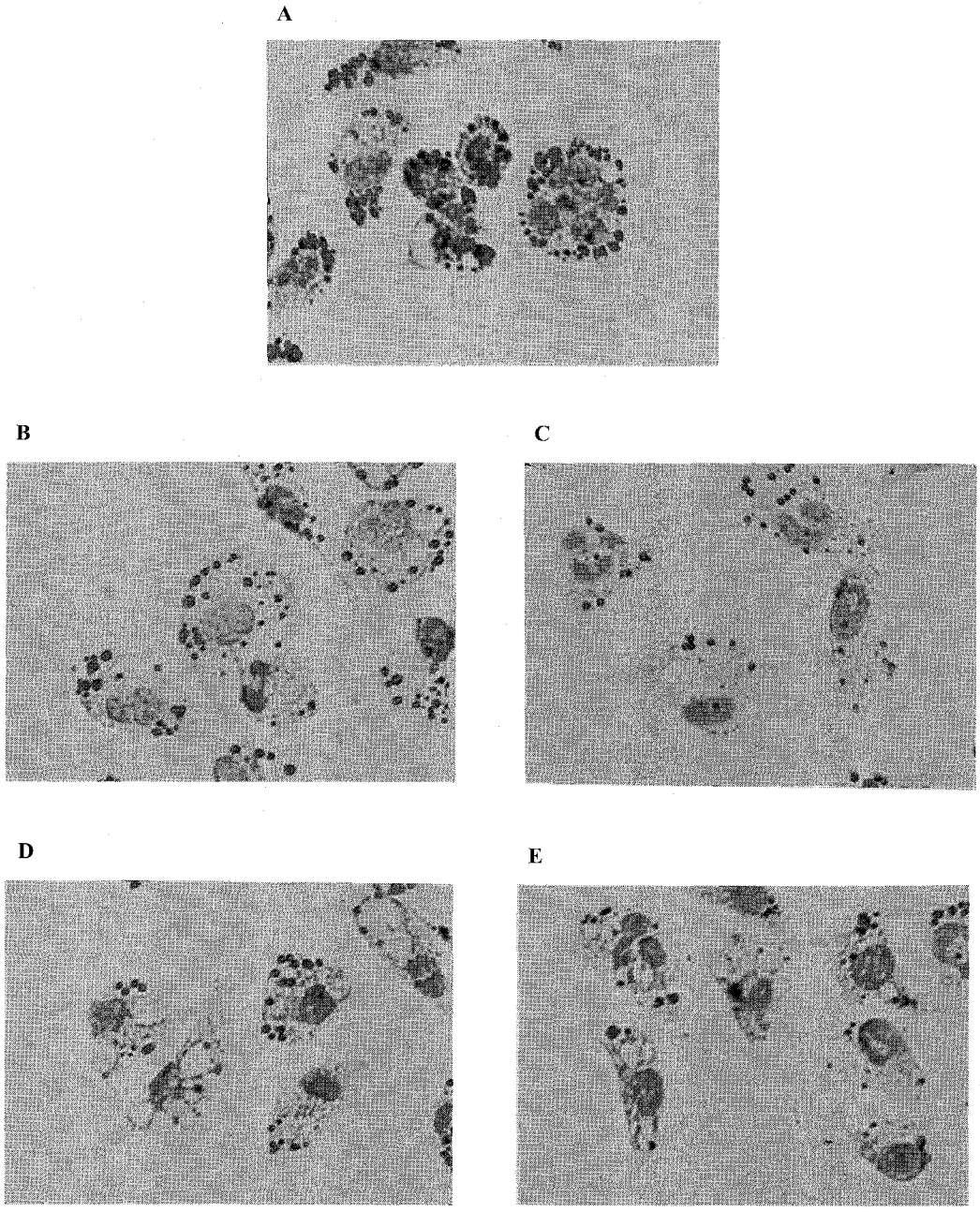
Fig. 4. A chromatographic profile of isolation of beauveriolides I and III by preparative HPLC.



Column, Senshu Pak Pegasil Silica 120-5, 20×250 mm; hexane-2-propanol (100:3); UV at 215 nm; 6.0 ml/minute; sample, 100 μg of active materials (obtained through silica gel column chromatography) dissolved in 50 μl CH_3CN was injected.

Fig. 5. Inhibition of lipid droplet formation in macrophages by beauveriolides I and III.

Macrophage monolayers (5×10^5 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 3 or 10 μM beauveriolide I (B and C) or III (D and E), respectively.



Fixation and staining with oil red O and hematoxylin were then performed as described in the "Materials and methods". Original magnification, ×200.

powders. Bassianolide (23.5 mg) was also purified from the fractions (1st~17th) of the silica gel column by preparative HPLC using an ODS column.

Biological Properties

Inhibition of Lipid Droplet Formation by Beauveriolides

Under the culture conditions, mouse peritoneal macrophages accumulated massive amounts of lipid droplets in the cytosols, which were observed microscopi-

cally after oil red O staining (Fig. 5A). In the presence of beauveriolides I or III, however, the drug caused a dose-dependent reduction in the size and number of lipid droplets in macrophages (Fig. 5B, 5C, 5D and 5E). No cytotoxic effect was observed even at the highest dose (20 μM), indicating that both beauveriolides inhibits the lipid droplet formation specifically. Under the same conditions, bassianolide showed only a cytotoxic effect on macrophages, and beauvericin caused a reduction of lipid droplets with severe morphological changes to macrophages.

Other Biological Activities

No antimicrobial activity of beauveriolides I and III was observed at a concentration of 2.1 mM (1.0 mg/ml, 10 μg/disk) against the following microorganism; *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces cerevisiae*, *Pyricularia oryzae*, *Mucor racemosus* and *Aspergillus niger*.

Both beauveriolides did not show any nematocidal or insecticidal activity in our assay at 0.2 mM (100 μg/ml). However, bassianolide showed potent insecticidal activity at concentrations of more than 0.1 μM (0.1 μg/ml).

Discussion

Much attention has been paid to the molecular mechanism of intracellular cholesterol metabolism as potential target for pharmaceutical intervention. Several types of compounds affecting the metabolism have been discovered; for example, cholesterol derivatives like pregnenolone[17], which inhibit cholesterol departure from the lysosome, and a number of inhibitors of acyl-CoA: cholesterol acyltransferase (ACAT) catalyzing cholesterol esterification in the endoplasmic reticulum. Preglenolone and some ACAT inhibitors like 58-035[18,19] were demonstrated to block the cholesteryl ester (CE) synthesis in macrophages, and were believed to reduce the cytosolic lipid droplets.

In the present paper, beauveriolides I and III, members of the cyclodepsipeptide family, were found to reduce the cytosolic lipid droplets without any significant toxic effect on mouse peritoneal macrophages at least up to 20 μM. Many cyclodepsipeptides were isolated as ionophore antibiotics, antifungal or insecticidal antibiotics[6,13]. We have reported previously that beauvericin and enniatins inhibit ACAT activity *in vitro* and that beauvericin inhibited the CE synthesis in J774 cells[12]. However, beauvericin showed a reduction in lipid droplets in parallel with morphological changes of macrophages, suggesting that the drug is not specific for inhibition of lipid droplet formation in this cell system. Beauveriolide I was originally reported to show insecticidial activity when injected directly to *Spodoptera litura* at a dose of 10 μg/body[6]. This suggested that beauveriolide I is a very weak insecticidal agent, which was also supported by no insecticidal activity in our assay. On the other hand, bassianolide, a well known insecticidal cyclodesipeptide[13], showed potent insecticidal activity against *A. salina* in our assay as well, and exhibited severe cytotoxic effects on macrophages in this assay.

Together, among the cyclodepsipeptides tested, beauveriolides I and III are the most specific inhibitors of mouse macrophage-derived foam cell formation. Recent our preliminary experiments showed that the beauveriolides inhibited the CE synthesis specifically. Therefore, the inhibition site in the foam cell formation process is under investigation.

Acknowledgments

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