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New Beauvericins, Potentiators of Antifungal Miconazole Activity, Produced by *Beauveria* sp. FKI-1366

I. Taxonomy, Fermentation, Isolation and Biological Properties

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Three new beauvericins, designated beauvericins D, E and F, were isolated along with known beauvericin and beauvericin A, from the culture of *Beauveria* sp. FKI-1366 by solvent extraction, ODS column chromatography and HPLC. These compounds potentiate miconazole activity against not only wild *Candida albicans* but also fluconazole resistant *C. albicans*. Beauvericins D and E decreased the IC_{50} value of miconazole against fluconazole resistant *C. albicans* from 1.3 μM to 0.25 and 0.31 μM, respectively.

Opportunistic infections caused by certain fungi, in particular problematic *Candida albicans*, have increased recently and become a public concern. Patients with compromised immune systems, *e.g.* patients receiving organ transplants and cancer chemotherapy, or those infected by human immunodeficiency virus (HIV), are particularly prone to such infections[1]. Azole derivatives, which inhibit fungal ergosterol biosynthesis by blockade of the cytochrome P-450 reaction involved in 14-α demethylation, are the most commonly used agents. However, new antifungal agents of a different mechanism of action have been sought extensively.

On the basis of new concept "antiinfective drugs"[2], we discovered fungal funicone-related compounds including a new compound named actofunicone of fungal origin as potentiators of antifungal miconazole activity[3]. The compounds showed no antimicrobial activity themselves, but reinforced miconazole activity against *C. albicans*. During the course of our continuous screening program, five beauvericins (Fig. 1) were isolated from the culture broth of fungal strain *Beauveria* sp. FKI-1366. Three were found to be new, designated beauvericins D, E and F[4], although two compounds were identified as beauvericin[5] and beauvericin A[6]. They all potentiated miconazole activity against *C. albicans*.

In this paper, we describe the taxonomy of the producing fungus, fermentation, isolation and miconazole-potentiating activity of these beauvericins.

Materials and Methods

General Experimental Procedures

The strain FKI-1366 was isolated from soil collected on Amamiooshima Island, Kagoshima, Japan and was used for production of beauvericins D, E and F. *C. albicans* ATCC64548 (wild type) and ATCC64550 (fluconazole resistant type) were purchased from ATCC (Virginia, USA). For determination of the amounts of beauvericins in the culture broths, the samples, dissolved in methanol, were analyzed on an HP 1100 system (Huwllet Packerd Inc., Germany) under the following conditions: column,

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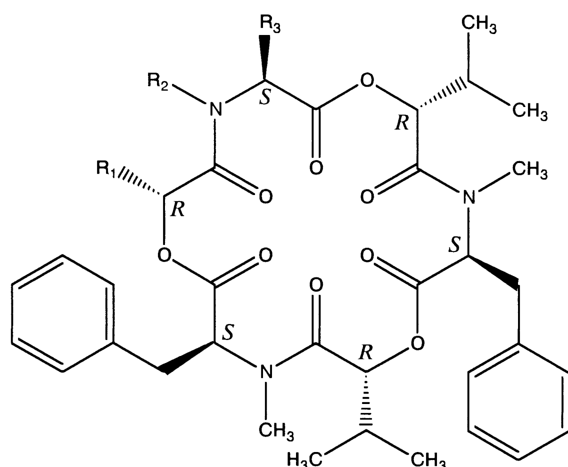
Fig. 1. Structures of beauvericin, beauvericins A, D, E and F.



Compound	R_1	R_2	R_3
Beauvericin	$CH(CH_3)_2$	CH_3	$CH_2C_6H_5$
Beauvericin A	$CH(CH_3)CH_2CH_3$	CH_3	$CH_2C_6H_5$
Beauvericin D	$CH(CH_3)_2$	H	$CH_2C_6H_5$
Beauvericin E	$CH(CH_3)_2$	H	$CH_2CH(CH_3)_2$
Beauvericin F	$CH_2CH(CH_3)_2$	CH_3	$CH_2C_6H_5$

Symmetry (2.1×150 mm, Waters Inc., Missouri, USA); flow rate, 0.2 ml/minute; mobile phase, a 20-minute linear gradient from 60% CH_3CN/0.05% H_3PO_4 to 100% CH_3CN/0.05% H_3PO_4; detection, UV at 210 nm.

Taxonomic Studies of the Producing Organism

For identification of the fungus, potato dextrose agar (PDA) (Difco, Maryland, USA), malt extract agar (MEA) (Difco), corn meal agar (CMA) (Difco) and Miura's medium (LcA) 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 (Vanox-S AH-2, Olympus, Tokyo, Japan) and a scanning electron microscope (JSM-5600, JEOL, Tokyo, Japan).

Assay for Miconazole-potentiating Activity

Wild and fluconazole resistant types of *C. albicans* were inoculated into a 50-ml test tube containing 10 ml of seed medium (potato extract containing peptone 0.5% and glucose 1%), and were grown for 24 hours on the rotary shaker. In Method A, the seed culture of wild *C. albicans* (0.1%, v/v) was transferred to the two different agar plates, GY agar (glucose 1%, yeast extract 0.5% and agar 0.8%) (Plate A) and GY agar plus miconazole (0.06 μM) (Plate B). The concentration (0.06 μM) of miconazole showed no effect on the growth of *C. albicans*. In Method B, the seed culture of fluconazole resistant *C. albicans* (0.1%, v/v) was transferred to the two different agar plates, GY agar (Plate C) and GY agar plus miconazole (0.1 μM) (Plate D). The concentration (0.1 μM) of miconazole showed no effect on the growth of fluconazole resistant *C. albicans*. Paper disks (8 mm, ADVANTEC, Tokyo, Japan) containing a sample were put on Plates A, B, C and D, which were incubated at 27°C for 24 hours. Samples showing inhibition zones selectively on Plate B in Method A and/or Plate D in Method B, were selected as potentiators of miconazole activity against *C. albicans*.

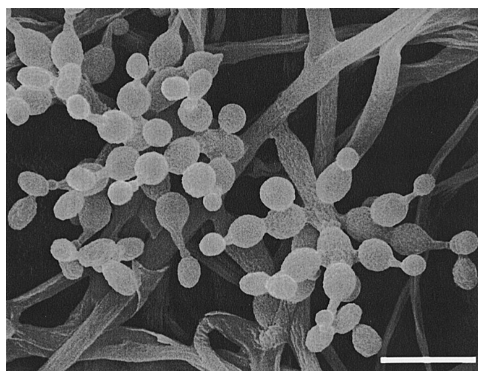
In Method C, the broth microdilution test using 96-well microplates (Corning, New York, USA) was performed according to the guidelines of NCCLS document M27-A[7]. Five colonies of fluconazole resistant *C. albicans* with diameters of 1 mm were suspended in sterile 0.85% saline to adjust to a 0.5 McFarland standard by spectrophotometric measurement. The seed of *C. albicans* was diluted 1,000 times with medium A (165 mM morpholinepropanesulfonic acid buffer (pH 7.0)), and the diluted seed (100 μl) containing serial concentration of miconazole (0~50 μM) in the absence or presence of a sample (1.0 μg/ml) to make the total volume 200 μl/well. *C. albicans* in microplates was incubated at 35°C for 24 hours, and the growth of *C. albicans* was measured at 630 nm with a microplate reader (model Elx 808, BIO-TEK Instruments, Vermont, USA). The IC_{50} values of miconazole against *C. albicans* in the absence or presence of a sample (1.0 μg/ml) were calculated.

Results

Taxonomy of the Producing Strain FKI-1366

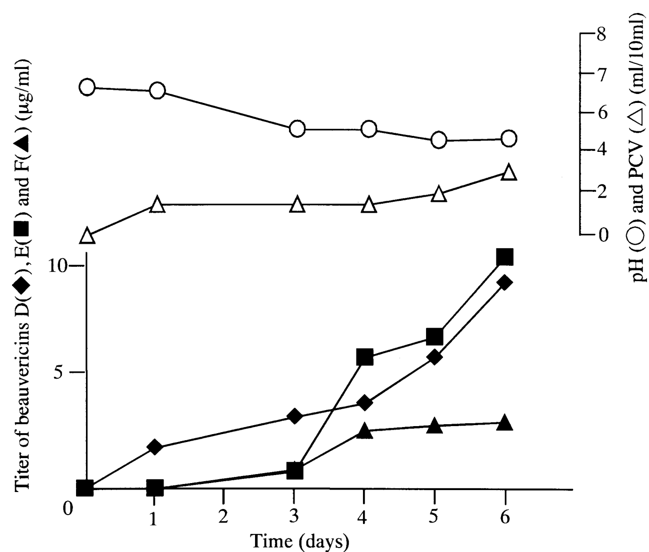
This strain grew abundantly to form white to brownish white colonies with abundant sporulation, attending a diameter of 40~70 mm after 14 days incubation at 25°C. Reverse of the colonies was brownish white to pale yellow. The colony was powdery to floccose. The conidiogenous apparatus were born directly from aerial hyphae or through short branch, and formed dense clusters. The proximal part of conidiogenous apparatus was swollen globose to flask shape, 3.3~5.6 μm in length, and 2.0~3.3 μm in width. In producing conidia, the tip of the conidiogenous apparatus elongated sympodually with a zig-zag shaped,

Fig. 2. Scanning electron micrograph of *Beauveria* sp. FKI-1366 grown on LcA.



Bar represents 5 μm.

Fig. 3. A typical time course of production of beauvericins D, E and F by *Beauveria* sp. FKI-1366.



The amounts of beauvericins D (◆), E (■) and F (▲) in culture broths were determined by HPLC as described in Materials and Methods.

PCV, packed cell volume (ml) from 10 ml of the whole culture broth after centrifugation at 3000 rpm.

5.0~12.0 μm in length. The conidia were hyaline in color, globose to subglobose and 1.8~3.0×1.8~2.6 μm in size, and their surface was smooth. The chlamydospores were formed clavate or irregular shape with branch. No teleomorph was observed in this strain. The scanning electron micrograph of FKI-1366 is shown in Fig. 2. From the above characteristics, the strain FKI-1366 was identified as a member of the genus *Beauveria*[8].

Fermentation

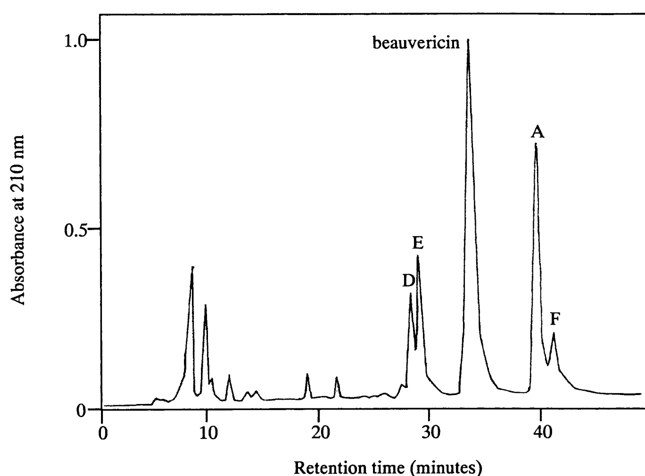
A slant culture of the strain FKI-1366 grown on LcA medium (glycerol 0.1%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02% and agar 1.5%, pH 6.0) was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (glucose 2.0%, 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 flask was shaken on a rotary shaker at 27°C for 3 days. The seed culture (100 ml) was transferred into a 7.5-liter jar fermenter (B. E. MARUBISHI, Japan) containing 2 liters of the production medium (glycerol 3.0%, oat meal 2.0%, dry yeast 1.0%, KH_2SO_4 1.0%, Na_2HPO_4 1.0% and $MgCl_2 \cdot 6H_2O$ 0.5%). The fermentation was carried out at 27°C for 6 days with an aeration of 2.5 liters/minute and an agitation of 200 rpm.

A typical time course of the fermentation is shown in Fig. 3. Beauvericin D was detected in the culture broth from day 1 after inoculation, and others were produced from day 3. The concentrations of beauvericins D, E and F on day 6 reached levels of 9.9, 11.1 and 3.3 μg/ml, respectively.

Isolation

The 6-day old culture broth (4 liters) was centrifuged to separate mycelium and supernatant. The mycelium was extracted with 4 liters of acetone. After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with 4 liters of ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield an oily material (4.5 g). The material was dissolved in a small volume of CH_3CN and applied on an ODS column (Senshu ODS-7515-12A, 225 g), and eluted stepwise with 60 : 40, 40 : 60, 20 : 80 and 0 : 100 (v/v) of H_2O - CH_3CN solvents (1000 ml each). The miconazole-potentiating activity was observed in the 20 : 80 fraction, which was concentrated to give a brown oily material (1.1 g). The material was purified by HPLC; ODS column (20×250 mm, Pegasil, Senshu Sci. Co. Tokyo, Japan), 85% CH_3CN, 6.0 ml/minute, and UV at 210 nm. Under the conditions, beauvericin D, beauvericin E, beauvericin, beauvericin A and beauvericin F were eluted

Fig. 4. A chromatographic profile of purification of beauvericins D, E and F by preparative HPLC.



Column, Senshu Pak PEGASIL ODS (20×250 mm); solvent, 50% CH_3CN; detection, UV at 320 nm; flow rate, 6.0 ml/minute; sample, 50 μg of active materials dissolved in 5 μl of MeOH.

as peaks with retention times of 28, 29, 33, 39 and 42 minutes, respectively (Fig. 4). Each peak was collected and concentrated to yield beauvericin D (15.9 mg), beauvericin E (28.9 mg), beauvericin (383.7 mg), beauvericin A (42.0 mg) and beauvericin F (6.9 mg) as coloreless needles or powder.

Biological Properties

Miconazole-potentiating Activity by Beauvericins

The miconazole-potentiating activity of five beauvericins was tested by Methods A, B and C. In Method A, all beauvericins themselves showed no inhibition against wild *C. albicans* at up to 50 μg/disk on Plate A. However, beauvericin D, beauvericin E, beauvericin F, beauvericin and beauvericin A showed clear inhibition zones (17, 18, 18, 20 and 21 mm i.d., respectively) at 50 μg/disk on Plate B (containing 0.06 μM miconazole) (Table 1). The potentiating activity by beauvericins was observed even at 1 μg/disk. In Method B, although they showed no inhibition against fluconazole resistant *C. albicans* at up to 50 μg/disk on Plate C, beauvericin D, beauvericin E, beauvericin F, beauvericin and beauvericin A showed clear inhibition zones (16, 20, 15, 22 and 16 mm i.d., respectively) at 50 μg/disk on Plate D (containing 0.1 μM miconazole)

Table 1. Effect of cyclodepsipeptides on miconazole activity against wild *Candida albicans*.

Compound	Concentration	Inhibition zone (mm)	
	µg/disk	Plate A	Plate B
Beauvericin D	1	-	14
	5	-	16
	50	-	17
Beauvericin E	1	-	16
	5	-	16
	50	-	18
Beauvericin F	1	-	15
	5	-	17
	50	-	18
Beauvericin	1	-	14
	5	-	16
	50	-	20
Beauvericin A	1	-	14
	5	-	20
	50	-	21
Enniatin A	1	15	14
	5	12	17
	50	20	28
Enniatin A1	1	17	14
	5	18	22
	50	19	27
Enniatin B	1	14	16
	5	15	25
	50	18	27
Enniatin B1	1	15	19
	5	17	24
	50	20	29
Enniatin E	1	15	16
	5	15	20
	50	15	25
Enniatin F	1	12	14
	5	13	19
	50	13	21
Bassianolide	1	-	-
	5	-	-
	50	17	16
Beauveriolide III	1	-	-
	5	-	-
	50	-	-

Paper disks (8 mm i.d.) containing three concentration of a compound were put on Plate A (wild *C. albicans* in GY agar) and Plate B (wild *C. albicans* in GY agar+0.06 μM miconazole), and incubated at 27°C. After 24 hours, the diameters of inhibition zones were measured (Method A).

Table 2. Potentiation of miconazole activity against fluconazole resistant *C. albicans* by beauvericins.

Compound	Concentration µg/disk	Inhibition zone (mm) Plate C	Plate D
Beauvericin D	1	-	-
	5	-	-
	50	-	16
Beauvericin E	1	-	-
	5	-	20
	50	-	20
Beauvericin F	1	-	-
	5	-	-
	50	-	15
Beauvericin	1	-	15
	5	-	17
	50	-	22
Beauvericin A	1	-	-
	5	-	-
	50	-	16

Paper disks (8 mm i.d.) containing three concentrations of a compound were put on Plate C (fluconazole resistant *C. albicans* in GY agar) and Plate D (fluconazole resistant *C. albicans* in GY agar + 0.1 mM miconazole), and incubated at 27°C. After 24 hours, the diameters of inhibition zones were measured (Method B).

Table 3. Potentiation of miconazole activity against fluconazole resistant *C. albicans* by beauvericins.

Addition	IC_{50} of miconazole (µM)	Ratio (control /+ drug)
No (control)	1.3	1
+ Beauvericin D	0.25	5.2
+ Beauvericin E	0.31	4.2
+ Beauvericin F	1.2	1.1
+ Beauvericin	0.48	2.7
+ Beauvericin A	0.19	6.8

The IC_{50} values of miconazole against the growth of fluconazole resisitant *C. albicans* in the absence (control) or presence of a compound (1.0 µg/ml) were measured using 96-well microplates (Method C).

(Table 2). These results indicated that beauvericins potentiate miconazole activity against wild and fluconazole resistant *C. albicans*.

Potentiation of miconazole activity by beauvericins was evaluated by Method C. In the absence of beauvericins, the IC_{50} value of miconazole against fluconazole resistant *C. albicans* was calculated to be 1.3 µM. In combination with beauvericin D, beauvericin E, beauvericin F, beauvericin

and beauvericin A (1.0 μg/ml each), the respective IC_{50} values of miconazole were decreased to 0.25, 0.31, 1.2, 0.48 and 0.19 μM (Table 3). Thus, it was confirmed that they potenitate miconazole activity against fluconazole resistant *C. albicans*.

Discussion

As demonstrated in this paper, beauvericins were found to show a new biological activity of potentitating miconazole-derived activity against pathogenic fungus *C. albicans*. The potentiating activity was effective on not only wild *C. albicans* but also fluconazole resistant strain. Beauvericins belonging to 18-membered cyclodepsipeptides have been reported to show a variety of biological activities. For example, beauvericin and enniatins possess ionophoric activity[9~11] and inhibitory activity against acyl-CoA: cholesterol acyltransferase (ACAT)[12,13]. Accordingly, enniatines were tested in Method A. Although inhibition zones caused by enniatins on Plate B were lager than those on Plate A (Table 1), the compounds inhibited the wild *C. albicans* growth on both Plate A and Plate B (with miconazole), indicating that enniatins are not ideal potentiators. Furthermore, other cyclodepsipeptides such as 13-membered beauveriolide III[14] and 24-membered bassianolide[15] did not potentiate the miconazole activity against wild *C. albicans*. Thus, miconazole potentiating activity might be very specific to the beauvericin-type structures among cyclodepsipeptides.

As shown in Tables 2 and 3, the potentiation of miconazole activity by beauvericins is also effective on fluconazole resistant *C. albicans*. Therefore, the target molecule of beauvericins in miconazole potentiating activity is of interest. Several mechanisms of azole resistance in *C. albicans* have been reported; 1) overexpression of transporters like ATP binding cassette (ABC) transporter and major facilitator superfamily (MSF) transporter[16], 2) mutation of cytochrom P-450 14-α demethylase (P-450 14DM)[17], 3) overexpression of P450 14DM[18], and 4) inactivation of $\Delta^{5,6}$ sterol desaturase[19]. It might be plausible that beauvericins inhibit one or some of these enzymes or proteins responsible for the resistant mechanism as a potential target of beauvericins in potentiating miconazole activity. Studies on the mechanism of action of beauvericin are now in progress.

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