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Pleofungins, Novel Inositol Phosphorylceramide Synthase Inhibitors, from *Phoma* sp. SANK 13899

I. Taxonomy, Fermentation, Isolation, and Biological Activities

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Abstract In the course of a screening for inositol phosphorylceramide (IPC) synthase inhibitors, the novel inhibitors pleofungins A, B, C, and D were found in a mycelial extract of a fungus, *Phoma* sp. SANK13899. Purification was performed by 50% methanol and ethyl acetate extraction, reversed phase open-column chromatography, and HPLC separations. Pleofungin A inhibited the IPC synthase of *Saccharomyces cerevisiae* and *Aspergillus fumigatus* at IC_{50} values of 16 and 1.0 ng/ml, respectively. The inhibitor also suppressed the growth of *Candida albicans*, *Cryptococcus neoformans*, and *A. fumigatus* at MIC values of 2.0, 0.3, and 0.5 μg/ml, respectively. These biological properties indicate that pleofungins belong to a novel class of IPC synthase inhibitors efficacious against *A. fumigatus*.

Keywords sphingolipid, inositol phosphoryceramide synthase, anti-fungal, *Aspergillus fumigatus*, pleofungin

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Introduction

Incidence of systemic fungal disease has increased during the last two decades and now fungal infections are recognized as one of the major life threatening diseases. However, the options of clinically available antifungal drugs are limited, and these drugs also have problems in their severe side effects and emergence of resistance in several pathogens. Fungicidal agents effective to *Aspergillus fumigatus* are of particular interest because of the rapid progression of this infection which is difficult to control, and generally fatal.

Inositol phosphorylceramide (IPC) synthase is recently reported as one of the novel targets for antifungal drugs because these lipids are not detectable in mammalian cells, in which sphingomyelin seems to be the counterpart of IPC [1]. IPC and its derivatives are the major species of sphingolipids and share 30% of the total phospholipids [2, 3] in yeast, and these sphingolipids and their precursors are known to regulate cell growth and death in the *Aspergillus* species [4] as well as in yeast [5~9]. Aureobasidin A [10], rustmicin [11, 12], and khafrefungin [13] have already been reported to inhibit IPC synthase potently, and also to suppress the growth of *Saccharomyces cerevisiae*, *Candida albicans*, and *Cryptococcus neoformans*. All these known IPC synthase inhibitors, however, lack fungicidal activity against *A. fumigatus*.

During the screening of IPC synthase inhibitors with an anti-*A. fumigatus* effect, we isolated pleofungins A, B, C, and D in the mycelial extract of a fungus *Phoma* sp. SANK13899. In this paper, we report the taxonomy and fermentation of the producing fungus, and also isolation and biological properties of pleofungins. The structure

elucidation of them is published in the following paper in this issue [22].

Materials and Methods

Taxonomic Studies

The producing fungus SANK13899 was isolated from a soil sample collected at Tokyo, Japan. For identification of the fungus, the following media were used; potato dextrose agar (PDA, Nissui, Tokyo), corn meal agar (CMA, Nissui, Tokyo) and Miura's medium (LCA) [14]. The observation of the colonies was made after a two-week incubation at 23°C. The color names used in this study were taken from Kornerup and Wanscher [15].

Fermentation and Purification

In order to acquire the pleofungins A and B, the strain *Phoma* sp. SANK13899 was inoculated from a slant culture into 500 ml of sterilized seed cultivation medium consisting of glycerol 3.0%, glucose 3.0%, soluble starch 2.0%, soybean meal 1.0%, gelatin 0.25%, yeast extract 0.25%, NH_4NO_3 0.25% and cultured in a 2-liter Erlenmeyer flask by shaking on a rotary shaker (210 rpm) at 23°C for 6 days. The culture (1.5 liters) was transferred to 30 liters of the fresh seed medium in a 60-liter tank-fermentor, and cultured at 23°C for 2 days. Then, 15 liters of the pre-culture was transferred to 300 liters of fermentation medium-1 consisting of glucose 8.0%, malt extract 2.0%, yeast extract 0.2%, tripton 1.0%, NH_4NO_3 0.1%, $NaNO_3$ 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1% in a 600-liter tank-fermentor and fermented at 23°C for 7 days. For the production of pleofungins C and D, the strain was pre-cultured in the same way as described above and 750 ml of the pre-culture was transferred into 15 liters of fermentation medium-2 consisting of glucose 10%, malt extract 2.0%, Pharmamedia 1.0%, GE90M 1.0%, NH_4NO_3 0.1%, $NaNO_3$ 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1% in a 30-liter jar-fermentor and fermented at 23°C for 7 days.

In order to purify the active compounds from the culture broth, Celite 545, Cosmosil 140 C18 OPN, YMC Pack ODS-AM, and Shodex Asahipak C8P 90 2F were purchased from Celite Corporation, NACALAI TESQUE, INC., YMC Co., Ltd., and Showa Denko K.K., respectively.

Analysis of IPC Synthase Activity

Crude IPC synthase of *A. fumigatus* was prepared as follows. The spores of *A. fumigatus* IAM 2034 (3.0×10^8 cells) were inoculated in 100 ml of 0.2% agar containing YPD medium (yeast extract 10%, polypeptone 20%, glucose 20%), and cultured at 26°C for 2 days to acquire stationary growing fungal cells. The culture (10 ml) was transferred to 100 ml of fresh YPD medium and further cultured at 26°C for 16 hours to bring the cells to an exponentially growing phase. The cells were harvested by a filtration with a filter paper (Whatman) and washed once with ice-cold lysis buffer (50 mM potassium phosphate pH 6.5, 10 mM EDTA, 5 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A). The wet mycelium (50 g) was resuspended in 400 ml of ice-cold lysis buffer and 250 ml of chilled glass beads (SIGMA), and stirred with Dyno-Mil 3 times at 2000 rpm for 30 seconds with 1 minute intervals. The cell lysate was passed through cheesecloth and the flow-throughs were centrifuged for 1 hour at 10,000×g to remove cell debris and glass beads. The resultant supernatant was concentrated by an ultrafiltration with a YM-30 membrane (Millipore). Finally, the concentrated material was resuspended in the lysis buffer containing 20% of glycerol and stored at −80°C.

For preparation of crude IPC synthase of *S. cerevisiae*, *S. cerevisiae* AHP250 strain was cultured with 1 liter of YPD medium at 30°C to OD_{660} of 4.0. The yeast cells were processed and their microsomes were acquired as previously described [16]. The microsomes were dissolved in the lysis buffer and stored at −80°C.

To measure the *A. fumigatus* IPC synthase activities *in vitro*, 30 μg of crude enzyme was mixed with liposomes containing 1.6 μM BODIPY-FL-C5-ceramide (Molecular Probes) and 89 μM phosphatidyl inositol (SIGMA) in 50 μl of reaction buffer (10 mM potassium phosphate pH 6.5, 2 mM dithiothreitol, 1.4 mM PMSF, 14 μg/ml leupeptin, 14 μg/ml pepstatin A), and incubated at 32°C for 4 hours. For evaluation of *S. cerevisiae* IPC synthase activity, 12.5 μg of *S. cerevisiae* microsomes was mixed with the same liposomes as described above in 50 μl of reaction buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and incubated at 30°C for 1 hour. To test the inhibitory activities of the samples, the final 2% of the samples were included in the reactions. After the incubation of the reaction mixtures, BODIPY-labeled IPC was separated by the addition of solvent mixture (MeOH : diethylether, 41 : 91), and their fluorescent values were measured. The specific activities of the crude IPC synthase in the *A. fumigatus* cell lysate and *S. cerevisiae* microsome were 0.26 and 2.4 (nM of BODIPY-IPC formed/sec/mg of protein), and Km values for BODIPY-C5-ceramide were 5.9 and 5.2 μM, respectively. The reactions were over 50% complete based on TLC analysis of BODIPY-labeled IPC conversion product.

Analysis of *de novo* Sphingolipid Biosynthesis

In order to assess the inhibitory effects of inhibitors on the biosynthesis of the sphingolipids in *A. fumigatus*, exponentially growing cells were prepared by a precultivation of the *A. fumigatus* spores (2.5×10^7 cells) for 12 hours at 26°C. The cells were further cultured with 5 μCi/ml of ^{3}H-dihydrosphingosine (ARC) and inhibitors for 6 hours at 30°C. Cell pellets were acquired by centrifugation for 3 minutes at 3000 rpm, and washed 3 times with ice-cold water. The pellets were suspended in 1 ml of solvent mixture (95% ethanol : distilled water : diethylether : pyridine : NH_4OH, 15 : 15 : 5 : 5 : 0.018), and the total lipids were extracted by heating for 30 minutes at 60°C [17]. The lipid extracts were dried under an N_2 gas flow and dissolved in 100 μl of dichloromethane : methanol (1 : 1, v/v). Total lipid solutions (20 μl) were spotted on the silica-gel thin-layer chromatography plates (20 cm Whatman LK5) and developed with dichrolomethane : methanol : 2 N acetic acid (6 : 4 : 1, v/v). Visualization and densitometric analysis of [^{3}H]-labeled lipids were performed with a BAS-2000 imaging analyzer (FUJI film).

For analysis of the IPC biosynthesis in *S. cerevisiae*, 1.0×10^7 cells/ml of exponentially growing *S. cerevisiae* YPH250 cells were prepared in a synthetic complete medium [0.67% Bacto-yeast nitrogen base without amino acids (Difco), 2.0% glucose; supplemented with amino acids], and further cultured with 10 μCi of ^{3}H-dihydrosphingosine (ARC) and inhibitors for 1.5 hours at 30°C. After the incubation, cell pellets were obtained by centrifugation at 3,000×*g* for 10 minutes and washed with 2 ml of ice-cold water. The total lipids were extracted from the pellets as described above. The lipid extracts were dried and dissolved in the same solvent as described above. Aliquots of the solutions were spotted on the silica-gel thin-layer chromatography plates (20 cm Whatman LK5) and developed with dichloromethane : methanol : 2 N NH_4OH (9 : 7 : 2, v/v). Visualizations and analyses were carried out in the same way as described in *A. fumigatus* [^{3}H]-labeled lipids. The respective sphingolipids were detected by comparing the TLC profiles of the wild type, *sur2Δ*, *csg2Δ*, and *ccc2Δ* strain.

For analysis of sphingomyelin biosynthesis, 1.0×10^6 cells of the A549 human lung carcinoma cell line were preincubated with inhibitors for 1 hour and further incubated for 24 hours after the addition of 10 μCi of [^{3}H]choline (NEN). Cells were scraped with a rubber policeman and washed once with ice cold PBS. The total lipids were extracted from the cell pellets as described above. Aliquots of the total lipids were developed on the silica-gel thin-layer chromatography with a solvent system, dichloromethane : methanol : 2 mM $MgCl_2$ (65 : 25 : 4, v/v), and analyzed as described above.

Antifungal Activities

Antifungal activities were measured following the National Committee for Clinical Laboratory Standard method [18]. For determination of MIC against *Candida* species and *Cryptococcus neoformans*, cells were diluted into 5.0×10^3 CFU/ml in RPMI medium. The cell suspensions (100 μl) were transfered onto 96-well microtiter plates. And then, 100 μl of diluted samples with RPMI medium were added to the cell suspensions on the microtiter plates. After the mixing of the samples and cell suspensions of *Candida* and *Cryptococcus*, the plates were incubated at 37°C for 24 and 48 hours, respectively. The growth conditions were determined by naked-eye observation by measuring the OD_{600}. For MICs against the *Aspergillus* species, the spore number was adjusted to 3.0×10^4 CFU/ml in RPMI medium. Cell suspensions (90 μl) were distributed onto 96-well microtiter plates and further added with 100 μl of diluted samples and 10 μl of Alamour Blue. Then, the plates were incubated at 26°C for 72 hours and the growth conditions were determined by measuring the OD_{595}.

For evaluation of minimal fungicidal concentrations (MFCs) against *Candida*, *Cryptococcus*, and *Aspergillus*, 1.5 μl of the mixtures were pipetted from 96-well microtiter plates after the MIC measurements and spread on SDA (Sabouraud Dextrose Agar) or PDA (Potato Dextrose Agar) plates. Then, the number of colonies was counted. The concentrations at which the colony numbers were less than 4 or 20 were defined as the MFCs against *Candida* and *Cryptococcus*, or *Aspergillus*, respectively.

Results

Taxonomy of Producing Organism

Colonies on the PDA plates reached 13～17 mm in diameter. The colony surface was tomentose, floccus at the center and colored Grey (3B1) to White. The reverse side of the colonies was Brown (6E5～4). Colonies on the CMA plates reached 15～17 mm in diameter. The colony surface was farinaceous and colored Greyish Green (27E5) to Dull Green (27E4). The reverse side of the colonies was Dark Green (27F4). Formation of pycnidia was observed after a one-month incubation on LCA. The pycnidia were 100～200 μm in diameter, globose to subglobose, immersed to subimmersed in the medium and colored brown to black (Fig. 1A). The conidiogenous cells were 7.5～9×1.5～2 μm in size, phialidic, cylindrical to ampulliform and hyaline. The conidia were

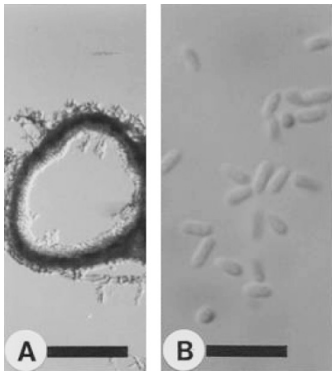

Fig. 1 Morphology of the strain SANK 13899.

A) Section of the pycnidium. Bar represents 100 μm. B) Conidia. Bar represents 10 μm.

3.5~5×1.5~2 μm in size, cylindrical, one-celled and hyaline (Fig. 1B).

The above characteristics of the strain SANK13899 indicate that this fungus belongs to the genus *Phoma* [19]. The strain has been deposited at the National Institute of Bioscience and Technology, Japan as *Phoma* sp. SANK13899 under the accession number of FERM BP-6851. In addition, this strain was clustered within the Pleosporales by means of the molecular phylogenetic analysis based on the 18S rDNA gene sequence (data not shown).

Isolation of Pleofungins A, B, C and D

Fermentation of SANK13899 strain was carried out as in described in materials and methods. The harvested broth (370 liters) was added with 18 g of Diatomaceous Earth (Celite 545) and which was then filtered to acquire supernatant and mycelium. The active material was extracted from the mycelium by adding 50% methanol (MeOH) followed by pH adjustment (pH 2.0) and filtration. A 310-liter aliquot of the filtrate was applied onto 30 liters of a reversed phase open column (Cosmosil 140 C18 OPN) which was equilibrated with 50% MeOH containing 0.05% of trifluoroacetic acid (TFA). After washing the column with 270 liters of 50% MeOH containing 0.05% TFA and 100 liters of 40% acetonitrile (MeCN) containing 0.05% TFA, the active compounds were eluted with 60% MeCN containing 0.05% TFA and the active fractions were pooled (30 liters). Active compounds were further extracted from the pooled fractions with 50 liters of ethyl acetate (EtOAc) after pH neutralization with NaOH. The extract was washed with saturated saline, dried over with anhydrous Na_2SO_4 and concentrated *in vacuo* to obtain 32.3 g of crude oily material. Further purification was performed by a primary HPLC (column, YMC Pack ODS-AM, 100 i.d.×500 mm; mobile phase, MeCN : 1.0% triethylamine-phosphate buffer (pH 6.0), 3 : 1; flow rate, 240 ml/minute; detection, UV at 210 nm). The eluate (26 liters) containing pleofungins A and B was mixed with 20 liters of water and EtOAc (1 : 1) to extract the active compounds. The EtOAc phase was washed, dried over and concentrated as described above to yield 7.8 g of oily material. A 326 mg aliquot of the material was dissolved in MeOH, and subjected to repeated secondary HPLC (column, Shodex Asahipak C8P 90 2F, 20 i.d.×250 mm; mobile phase, 60% MeCN containing 10 mM NH_4HCO_3; flow rate, 14 ml/minute; detection, UV at 210 nm). HPLC fractions possessing the aimed activity were combined and concentrated to acquire pure pleofungin A as a colorless powder (275 mg). In order to prepare pleofungin B, a 2.1 g aliquot of the oily material prepared by primary HPLC as mentioned above was dissolved in MeOH and applied onto 170 ml of a reversed phase open column (resin, Cosmosil 140 C18 OPN; mobile phase, 60 to 90% MeCN containing 0.05% TFA, linear gradient). Active fractions were pooled and concentrated by evaporation and lyophilization to give pale yellow powder (109 mg). Finally, the powder was purified by HPLC (column, Shodex Asahipak C8P 90 2F, 20 i.d.×250 mm; mobile phase, 60% MeCN containing 10 mM NH_4HCO_3; flow rate, 14 ml/minute; detection, UV at 210 nm). Pure pleofungin B (69.5 mg) was acquired as a colorless powder after concentration of the active fractions.

For purification of pleofungins C and D, the harvested broth (55 liters) was processed in the same manner as described above, and the mycelium was subjected to 50% MeOH extraction. After the filtration and pH neutralization of the extract, the active compounds were further extracted with EtOAc. Oily material (107 mg) was obtained from the EtOAc phase by washing and concentration as described above. This material was dissolved in MeOH and applied to a reversed phase open column (resin, 3 liters of Cosmosil 140 C18 OPN; mobile phase, 30% MeCN containing 0.05% TFA), and consequently 3.8 g of oily material was acquired from the combined active fractions. This material was further purified by repeating the preparative HPLC (column, Shodex Asahipak C8P 90 2F, 20 i.d.×250 mm; mobile phase, 60% MeCN containing 10 mM NH_4HCO_3; flow rate, 14 ml/minute; detection, UV at 210 nm). Finally, pure pleofungins C (19.5 mg) and D (6.3 mg) were acquired as colorless powders after integration and concentration of the active fractions.

Biological Properties of Pleofungin A

Inhibitory activities of pleofungin A on IPC synthase of *A. fumigatus* and *S. cerevisiae* were evaluated by comparing them to those of aureobasidin A. As shown in Fig. 2A and

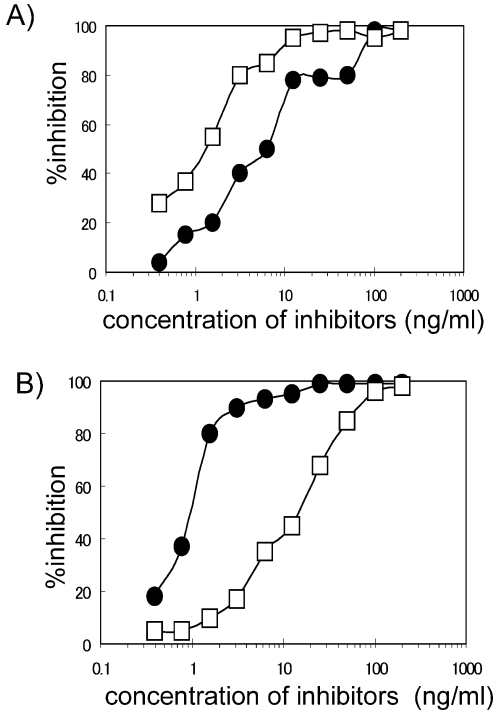

Fig. 2 Inhibition of IPC synthases by pleofungin A.

A) Inhibition of *Saccharomyces cerevisiae* IPC synthase, and B) *Aspergillus fumigatus* IPC synthase by pleofungin A. Closed circles and open squares represent the inhibitory activities of pleofungin A and aureobasidin A, respectively.

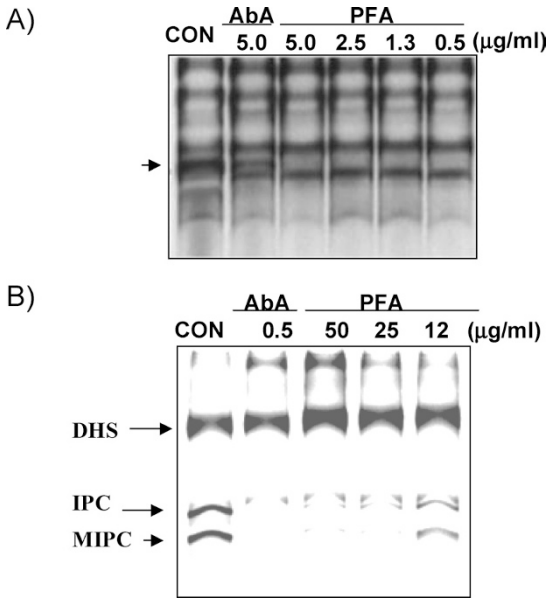

Fig. 3 Effects of pleofungin A on *de novo* sphingolipid biosynthesis.

A) Inhibition of dihydrosphingosine derived lipid biosynthesis in *A. fumigatus* by pleofungin A. An arrow indicates a dihydrosphingosine derived lipid whose biosynthesis is vulnerable to pleofungin A.

B) Inhibition of de novo IPC biosynthesis in *S. cerevisiae* by pleofungin A. *S. cerevisiae* were cultured with dihydrosphingosine and total lipids were analyzed as described in Materials and Methods. Abbreviations are as follows: PFA: pleofungin A, AbA: aureobasidin A, DHS: dihydrosphingosine, IPC: inositolphosphorylceramide, MIPC: mannosylinositolphosphorylceramide, CON: control (drug free).

B, pleofungin A strongly inhibited the *A. fumigatus* IPC synthase at an IC_{50} of 1.0 ng/ml, while aureobasidin A inhibited the enzyme at an IC_{50} of 16 ng/ml. However, pleofungin A was less effective against *S. cerevisiae* enzyme than aureobasidin A (IC_{50} of pleofungin A and aureobasidin A, 7.0 and 1.5 ng/ml, respectively). IPC synthase inhibitory effects and the anti-fungal activities of pleofungins B, C and D were much weaker than those of pleofungin A (IC_{50} of B, C, and D against *S. cerevisiae* IPC synthase, 80, 300, and 300 ng/ml, respectively; MICs, Table 2). Thus, only pleofungin A was used for further analyses.

In the next stage, the effects of pleofungin A on *de novo* sphingolipid biosynthesis in *A. fumigatus* and *S. cerevisiae* were examined. As shown in Fig. 3A, pleofungin A completely suppressed the biosynthesis of [^{3}H]dihydrosphingosine-derived lipids in *A. fumigatus* at the concentration of 0.5 μg/ml, but aureobasidin A barely inhibited the synthesis of the same lipid even at 5.0 μg/ml. In contrast, pleofungin A could not completely block the IPC biosynthesis in *S. cerevisiae* at less than 12 μg/ml, whereas aureobasidin A inhibited it at 0.5 μg/ml (Fig. 3B). These patterns on the inhibition of the sphingolipid biosynthesis by both inhibitors well reflected on their inhibitory activities against each enzyme. We next examined the effects of pleofungin A on the mammalian sphingolipid biosynthesis with using a culture cell line, A549, to confirm its selectivity for fungal enzymes over the corresponding mammalian counterpart. Pleofungin A showed no inhibition for *de novo* sphingomyelin biosynthesis in A549 cells at the concentrations up to 5.0 μg/ml, demonstrating high specificity to the fungal sphingolipid biosynthesis (Fig. 4).

Antifungal properties of pleofungins against pathogenic fungi are shown as values of MIC and MFC, as well as those of the known IPC synthase inhibitors, aureobasidin A and rustmicin (Table 1). Pleofungin A showed growth inhibitory effects on the various kinds of fungi, but preferentially inhibited the growth of *A. fumigatus* and *Cryptococcus neoformans*. As previously reported, aureobasidin A and rustmicin effectively inhibited the growth of *Candida* species and *Cryptococcus neoformans*, but not that of *A. fumigatus*. Pleofungin A and aureobasidin

Table 1 Antifungal spectrum of IPC synthase inhibitors

	Pleofungin A		Aureobasidin A		Rustmicin	
	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans ATCC90028	2.0	5.0	0.5	0.6	2.5	10
C. parapsilosis IFO1396	0.5	2.5	0.5	1.3	0.6	0.6
C. grabrata ATCC90030	0.3	1.3	0.5	0.6	0.3	0.3
C. tropicalis IAM4185	2.0	2.5	0.5	0.6	1.3	0.3
Aspergillus fumigatus IAM2034	0.5	1.3	>20	>20	>20	>20
Cryptococcus neoformans IAM4772	0.3	0.3	1.0	1.3	0.3	0.3
Saccharomyces cerevisiae YPH250	10	NT	0.5	NT	NT	NT

Minimum inhibitory concentration (MIC, μg/ml) and minimum fungicidal concentration (MFC, μg/ml) of inhibitors were measured as described in Materials and Methods. NT: not tested.

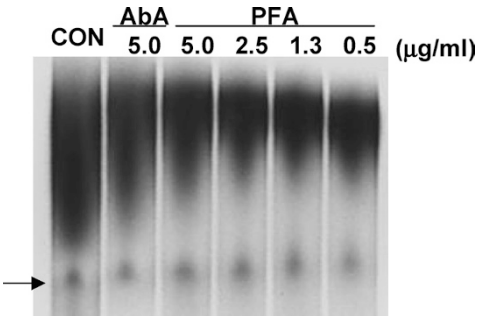

Fig. 4 Effect of pleofungin A on sphingomyelin biosynthesis in A549 cells.

A549 cells were labeled with choline and their total lipids were analyzed in the presence of pleofungin A and aureobasin A. An arrow indicates the choline labeled sphingomyelin. Abbreviations are as follows: PFA: pleofungin A, AbA: aureobasidin A, CON: control (drug free).

Table 2 Antifungal spectrum of pleofungins (MIC, μg/ml)

	B	C	D
Candida albicans ATCC90028	>65	8.0	16
C. parapsilosis IFO1396	>64	4.0	8.0
C. grabrata ATCC90030	1.0	1.0	2.0
C. tropicalis IAM4185	>64	8.0	>64
Aspergillus fumigatus IAM2034	>64	2.0	8.0
Cryptococcus neoformans IAM4772	1.0	0.3	0.3

A also inhibited the growth of *S. cerevisiae* at MIC of 10 and 0.5 μg/ml, respectively. These antifungal properties are consistent with their trends in their efficacies on IPC synthases and *de novo* sphingolipid biosynthesis. As in Table 1, almost all the MFC values of pleofungin A and the other inhibitors are close to the MIC values, suggesting that these IPC synthase inhibitors suppress the fungal growth through fungicidal effects.

Discussion

The novel IPC synthase inhibitors, pleofungins A, B, C, and D were isolated from the culture broth of *Phoma* sp. SANK13899. Pleofungin A more strongly inhibited IPC synthase of *A. fumigatus* than that of *S. cerevisiae* in our experimental conditions, however, aureobasidin A showed an opposing tendency in the effects on those enzymes to pleofungin A. Pleofungin A also showed stronger growth inhibition against *A. fumigatus* and *Cryptococcus neoformans* than against *Candida* species and *S. cerevisiae*. It was previously indicated that the homology between *Aspergillus* and yeast IPC synthase genes is very low [20, 21], regardless of the high homology among the enzymes of *Aspergillus* species, such as *A. fumigatus* and *A. nidulans*. Our data suggested that pleofungin A may recognize the structural differences between the *Aspergillus*- and yeast-type enzymes, and preferably interact with *Aspergillus*-type enzymes. It was reported that the major cause of the aureobasidin A resistance of *A. fumigatus* is the increased efflux *via* an unknown transporter. However, even in the presence of an efflux inhibitor, such as verapamil, which sensitize *A. fumigatus* to a certain extent, the MIC of aureobasidin A against *A. fumigatus* is still higher than that against *Candida albicans* [22]. Those data suggest that the selectivity of the antifungal effect of aureobasidin A is also partly attributable to its potency of inhibition against the enzymes. It should be an intriguing subject to elucidate the determinants of the affinities of the inhibitors to *A.*

fumigatus and *S. cerevisiae* IPC synthase, and generate more ideal inhibitors with a wider spectrum of antifungal effects than the known inhibitors.

It was also shown that pleofungin A has no inhibition against sphingomyelin biosynthesis in the A549 cell line, suggesting its selective action on fungal growth and low toxicity on animals. These data strongly support the possibility of the therapeutic effect of pleofungin A on *A. fumigatus* infected animals.

The lack of fungicidal action against *A. fumigatus* with previously reported inhibitors such as aureobasidin A, rustmicin, and khafrefungin, limits their potential for clinical development. Our discovery could facilitate the future research for the development of the IPC synthase inhibitors as a novel chemotherapeutic drug.

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