HISPIDOSPERMIDIN, A NOVEL PHOSPHOLIPASE C INHIBITOR PRODUCED BY *Chaetosphaeronema hispidulum* (Cda) Moesz NR 7127

I. SCREENING, TAXONOMY, AND FERMENTATION

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A novel phospholipase C inhibitor, hispidospermidin, was discovered from a fungal culture broth. The producing fungus, NR 7127, formed abundant pycnidia on banana leaf agar under near UV light. The ostiolate pycnidia were dark colored with a short beak possessing numerous protruding setae. The conidiogeneous cells were phialidic. The conidia were hyaline, 1 septate, smooth and spindle-shaped. From these distinctive characteristics, this strain was identified as *Chaetosphaeronema hispidulum* (Cda) Moesz of the Coelomycetes. Hispidospermidin was produced in a 50-liter jar fermentor containing 2% glucose, 2% potato starch, 2% Toast soya, 0.5% yeast extract, 0.25% NaCl, 0.0005% $ZnSO_4 \cdot 7H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.0005% $MnSO_4 \cdot 4H_2O$, 0.32% $CaCO_3$, and 0.3% Nissan disfoam CA-115. Fermentation was conducted at 27°C at an aeration rate of 30 liters/minute and agitated at 500 rpm for 95 hours. Maximum production yield of hispidospermidin was observed after 72 hours. Hispidospermidin inhibited rat brain phospholipase C at 16 μM of IC_{50}. This is the first recorded discovery of a secondary metabolite from the genus *Chaetosphaeronema*.

Phospholipase C (PLC) is a key enzyme for phosphoinositol metabolism, one of the signal transduction systems involved in various kinds of cellular responses by hormones, peptide growth factors, neurotransmitters, and the other regulatory ligands.[1~3] Therefore, PLC inhibitors would be expected to be cell growth inhibitors.[4] In our microbial screening program for new inhibitors of PLC, we discovered a novel inhibitor, hispidospermidin, in the fermentation broth of *Chaetosphaeronema hispidulum* (Cda) Moesz NR 7127. In this report, we describe a screening method for novel PLC inhibitors, taxonomy of the producing strain, and production of hispidospermidin. We also discuss the production of novel secondary metabolites from other fungal strains belonging to this genus.

Materials and Methods

Microorganisms

The producing organism, NR 7127, came from a soil sample collected in Antequela, Spain, on November 8, 1986. The strain was separated from the soil using the Percell density gradient modified from the method described by MARTIN N. J. and R. N. MACDONALD,[5] and then isolated on malt extract agar containing 30 mg/liter Rose Bengal. *Chaetosphaeronema hispidulum* IMI 261026b was purchased from International Mycological Institute, Egham, Surrey, United Kingdom. *C. hispidulum* CBS 216.75 and CBS 826.88, and *Chaetosphaeronema narasimhani* CBS 335.65 were purchased from the Centraalbureau voor Schimmelcultures, Baarn, the Netherlands. A herbarium specimen, *C. hispidulum* Herb-IMI 182342 was kindly loaned to us by Dr. SUTTON of the International Mycological Institute.

Taxonomy

Taxonomic work was done according to SUTTON.[6] Because NR 7127 had originally produced no

fruiting bodies or spores on any agar media tested, we tried to induce and stimulate its sporulation by using plant materials and light irradiation of various wavelengths.

Screening Method

The substrate suspension for the PLC inhibitory assay was prepared from [^{3}H] phosphatidyl inositol (PI) (NET-862, New England Nuclear), specific activity: 800 dpm/nmol, mixed with unlabeled PI (Sigma, P-5766). After removal of the solvent ($CHCl_3$) under N_2, this PI was brought into suspension (500 μM PI) in Tris-acetate buffer (200 mM, pH 5.5, 2 mM $CaCl_2$) by sonication for 20 minutes. The assay mixture (0.1 ml) contained 200 mM Tris-acetate buffer pH 5.5, 2 mM calcium chloride, 250 μM PI (sonicated [^{3}H] PI, approximately 20,000 dpm/assay), and 50 μg protein/ml of partially-purified rat brain PLC. This amount of enzyme led to approximately 30% hydrolysis of the substrate in 20 minutes at 30°C. In the standard screening assay, 20 μl of inhibitor or microbial culture broth diluted with 15 mM Tris-HCl buffer (pH 7.5), was mixed with the substrate suspension (50 μl). As a control for 100% inhibition of PLC, 20 μl of 25 mM EDTA was added instead of microbial broth. The reaction was started with the addition of 30 μl enzyme (*ca.* 5 μg protein). After 20 minutes incubation at 30°C, the reaction was stopped by addition of 1.5 ml chloroform-methanol-conc HCl (100:100:0.6) followed by addition of 1.45 ml of 1 N HCl containing 5 mM EGTA. After vortex-mixing, the sample was left for 20 minutes at room temperature and then mixed again. The sample was centrifuged for 5 minutes at 3,000 rpm, and a 200 μl portion of the upper phase was pippetted out into a scintillation vial. The radioactivity was measured by a liquid scintillation counter (Aloka).

Fermentation

A portion of the stock culture (0.1 ml) was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a medium consisting of 2% glucose, 2% potato starch, 2% Toast soya, 0.5% yeast extract, 0.25% NaCl, 0.0005% $ZnSO_4 \cdot 7H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.0005% $MnSO_4 \cdot 4H_2O$, 0.32% $CaCO_3$, and 0.3% Nissan disfoam CA-115. The pH of the medium was adjusted to 7.0 before the addition of calcium bicarbonate. This seed culture was shaken on a rotary shaker at 190 rpm at 27°C for 4 days. Six 500-ml flasks containing 100 ml of the same medium were each inoculated with 2 ml of the resultant seed culture and incubated under the same conditions for 3 days. Six hundred milliliters of the second seed culture was added to a 50-liter jar fermentor containing 30 liters of the same medium and 0.3% disfoam. The fermentation was conducted at 27°C at an aeration rate of 30 liters/minute and agitated at 500 rpm for 95 hours. The pH and dissolved oxygen were monitored throughout the fermentation. The activity against PLC was checked by withdrawing a small amount of broth every 24 hours after the first 48 hours of fermentation. Packed cell volume expressing organism growth was determined by centrifuging 10 ml of cultured broth in a conical tube at 3,500 rpm for 5 minutes.

Analysis of Hispidospermidin Production by Thin Layer Chromatography

NR 7127 and the other related strains were cultured and broth filtrates were examined for hispidospermidin by TLC. The seed culture of each strain was shaken on a rotary shaker at 220 rpm at 27°C for 3 days. Two ml of the resultant culture was transferred into a 500-ml flask containing the same medium mentioned earlier and incubated under the same conditions for 5 days. The culture filtrate adjusted to pH 11 was extracted with 100 ml of dichloromethane. The extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The dried residue was dissolved in 1 ml methanol. Ten μl of each extract was developed on two 20 × 20 cm TLC plates (Merck, Kieselgel 60 F_{254}) with dichloromethane-methanol-25% aqueous ammonia (8:2:0.1). The compounds on the TLC were detected by iodine and Ninhydrin reactions. Their Rf values were compared with that of the authentic hispidospermidin, purified from NR 7127, which was 0.19.

Results and Discussion

Taxonomy of the Producing Fungus

Since the fungal strain NR 7127 had formed no conidiogenous structures on any agar media tested,

we had difficulty with identification. After various attempts to induce it to form fruiting bodies, NR 7127 finally showed the following characteristics.

Cultural Characteristics

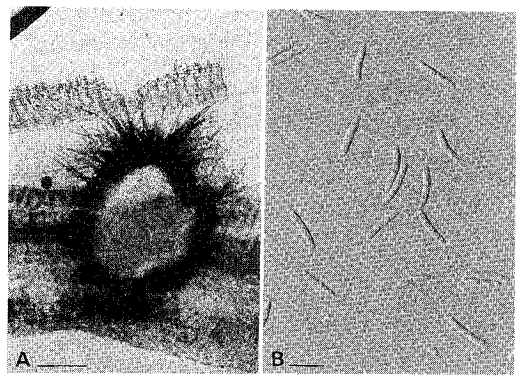
Colonies of NR 7127 grown on malt extract agar were dark brown to black and floccose in appearance. Exudates or soluble pigments were not produced. No conidiogenesis was observed under normal fluorescent or natural light. Under near UV light, however, we succeeded in inducing fruiting bodies. In addition, conidiomata were produced most effectively by cultivation on sterilized banana leaf agar. Distinct fungal colonies with a black margin appeared on the surface of banana leaves after several weeks at 20°C under black light illumination. When the inoculated leaf was sectioned vertically and examined microscopically, young conidiomata and hyphae were observed in the dead plant tissue. When matured, numerous conidiomata appeared half submerged in agar or protruding from the surface of the banana leaves.

Morphological Characteristics

The conidiomata were pycnidial, dark brown to black, globose to subglobose, and up to 450 μm in diameter, as shown in Fig. 1A. Ostioles were single, central, and slightly beaked. Numerous bristly setae were restricted to the region around the beak and were septate and straight. The conidiogenous cells with collarette were phialidic, cylindrical and hyaline, ranging in size from $6.0 \sim 10.5 \times 3.0 \sim 8.0$ μm, with thick periclinal walls. The conidia (Fig. 1B) were hyaline, one-septate, smooth, straight to slightly curved, and their apex and base were obtuse. Their size was $11.5 \sim 16.0 \times 1.5 \sim 3.5$ μm.

Fig. 1. Conidioma and conidia of NR 7127.

A, Conidioma. Bar 100 μm; B, Conidia. Bar 10 μm.



Identification

Characteristics such as pycnidia with numerous setae, phialides, and one-septate conidia, which were hyaline and straight to curved, clearly indicated that NR 7127 was included in the genes *Chaetosphaeronema* Moesz of Coelomycetes (SUTTON, 1980). Other properties including the sizes of conidiomata, conidiogenous cells, and conidia were in close agreement with those of *Chaetosphaeronema*

Table 1. Comparison of *Chaetosphaeronema hispidulum* strains.

	NR 7127	IMI 182342	CBS 826.88	Sutton (1980)
Conidiomata	~400 μm	~450 μm	~200 μm	~450 μm
Beaks	~100 μm	~250 μm	~450 μm	~200 μm
Setae	+	+	+	+
Conidia	Straight to slightly curved	Straight	Straight	Straight to slightly curved
	2-celled	2- to 4-celled	2- to 3-celled	2-celled
	11.5~16.0 × 1.5~3.5 μm	11.0~17.0 × 1.5~2.5 μm	12.5~23.3 × 5.0~6.5 μm	12~13 × 2 μm
Production of hispidospermidin	+	NT[a]	−	

[a] NT: Not tested (herbarium specimen).

hispidulum (Cda) Moesz described by SUTTON (1980). We then compared NR 7127 with a living culture of *C. hispidulum* CBS 826.88 and a herbarium specimen of *C. hispidulum* Herb-IMI 182342 (Table 1). The shape and size of CBS 826.88 conidia were slightly different from those in the literature, being rather cylindrical and larger ($12.5 \sim 23.0 \times 5.0 \sim 6.5\,\mu m$), while Herb-IMI 182342 conidia were almost the same shape and size as those of NR 7127. The beak size of these reference strains differed from that of NR 7127; they were much longer as seen in the description of *C. hispidulum* by SUTTON. In a personal communication with Dr. SUTTON of the International Mycological Institute, however, he suggested that the difference in the length of beaks is often observed and can be neglected. We therefore identified the strain NR 7127 as *Chaetosphaeronema hispidulum* (Cda) Moesz. This strain was deposited at the National Institute of Bioscience and Human-Technology, Tsukuba, with an accession number of FERM BP-4081.

Fermentation

A typical time course for the production of hispidospermidin in a 50-liter jar fermentor is illustrated in Fig. 2. After 48 hours of fermentation, the dissolved oxygen reached minimum, which was indicated by the luxuriant growth of the producing organism. Growth reached maximum after about 72 hours incubation and gradually decreased thereafter. The inhibitory activity against PLC also peaked at about 72 hours of fermentation. The overall time course of hispidospermidin production was, therefore, a typical profile of secondary metabolites.

PLC Inhibition Activity

The 50% inhibitory concentration (IC_{50}) of hispidospermidin for rat brain PLC was 16 μM in the standard assay. Hispidospermidin inhibited PLC in a dose dependent manner (Fig. 3). It showed no antibacterial activity against *Escherichia coli* and no antifungal activity against *Candida albicans* at

Fig. 2. Fermentation profile of hispidospermidin.

● Inhibition activity, □ packed cell volume, ■ PH, ○ dissolved oxygen (DO).

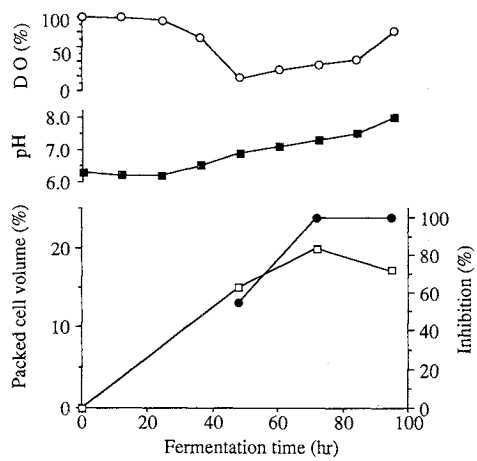


Fig. 3. Dose response of PLC inhibition by hispidospermidin.

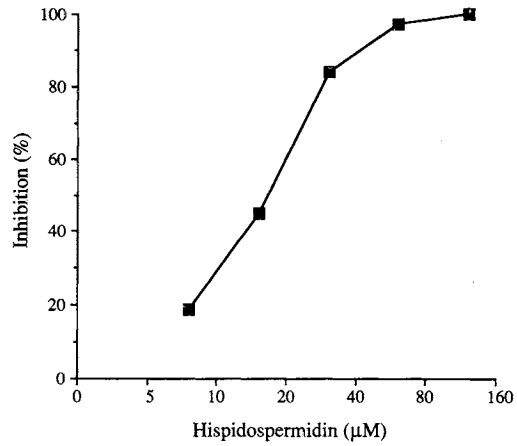


Table 2. Activities of hispidospermidin.

Activity	IC_{50} (μM)
Phospholipase C inhibition	16
Protein kinase C inhibition	>737
Phospholipase A_2 inhibition	737
DNA gyrase inhibition	>245
Antifungal activity against *Candida albicans*	>737
Antibacterial activity against *Escherichia coli*	>737
Cytotoxity against HeLa cell	36

737 μM. Although hispidospermidin showed a cytotoxic activity against HeLa cells (IC_{50} of 36 μM), it was found to be a specific inhibitor of phospholipase C (Table 2). Detailed biological activity of the compounds will be reported elsewhere.

Detection of Hispidospermidin by TLC

To investigate the prevalence of hispidospermidin production among the strains related to NR 7127, we examined 4 strains of the genus *Chaetosphaeronema*: *C. hispidulum* IMI 261026b, CBS 216.75, and CBS 826.88, and *C. narasimhani* CBS 335.65. None of these strains showed a Ninhydrin positive spot similar to that of the authentic sample of hispidospermidin from NR 7127. Since NR 7127 produced 400 μg/ml in the broth, and we did not detect this compound in the other strains, the production of this compound was thought to be specific to our original producer, NR 7127.

Conclusion

We discovered a novel PLC inhibitor, hispidospermidin, in the culture broth of the fungal strain, *Chaetosphaeronema hispidulum* NR 7127 from a screening of more than 5,000 fungal isolates. Hispidospermidin showed a potent and specific activity against phospholipase C. Notably, until now no secondary metabolite had been reported from this genus. This emphasizes the importance of searching for new bioactive metabolites from hitherto unexplored genera. Production of this compound was not species specific but strain specific. Isolation and structural elucidation of hispidospermidin will be reported in the ensuing paper.

Acknowledgment

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