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Identification of Phoslactomycin E as a Metabolite Inducing Hyphal Morphological Abnormalities in *Aspergillus fumigatus* IFO 5840

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Abstract In our survey for antifungal compounds, a fermentation broth of *Streptomyces* sp. HA81-2 was found to inhibit the *in vitro* growth of *Aspergillus fumigatus* IFO 5840 accompanied by hyphal morphological abnormalities. One of the isolated antibiotics was identified as phoslactomycin E based on LC-MS and NMR spectral data. In a preliminary assay using the membrane fractions of *A. fumigatus*, phoslactomycin E was found to inhibit the activity of 1,3-β glucan synthase.

Keywords antifungal activity, phoslactomycin E, *Aspergillus fumigatus*, hyphal morphological abnormalities, 1,3-β glucan synthase

Opportunistic fungal infections are a major cause of serious morbidity and mortality in immunocompromised patients. However, there are few drugs for antifungal chemotherapy of deep-seated mycosis. As both human and fungal cells are eukaryotes, it is difficult to develop antifungal drugs without side effects in human hosts. Current targets are limited to ergosterol, cell wall components, biosynthetic pathways of cell wall and cytosine deaminase [1, 2]. Therefore, there is a great need for novel antifungal drugs with new mechanisms of action. Morphological deformation of fungal hyphae is often induced by antifungal drugs [3]. As this phenomenon is specific to fungi, it could be used to screen for selective antifungal antibiotics [4]. In addition, this hyphal deformation is expected to reflect the disruption of cell wall biosynthesis, which is a specific target of antifungal agents. Moreover, investigating the mechanism of antifungal-induced hyphal morphological abnormalities is potentially useful for understanding the morphogenesis of fungal hyphae.

In the course of our search for antifungal antibiotics, we found that a fermentation broth of *Streptomyces* sp. HA81-2 exhibited antifungal activity against *Aspergillus fumigatus* IFO 5840 accompanied by hyphal abnormalities. The isolated active fraction was identified as phoslactomycin E (PLME) [5] based on LC-MS and NMR spectral data. We herein report the isolation procedures for PLME, its molecular characteristics, and its induction of morphological changes in fungal cells.

Active principles were isolated as follows; fermentation broth was filtered, the filtrate was applied on Diaion HP-20 column, and then eluted using an increasing gradient of aq MeOH (10～100%). The 80～90% MeOH fractions were concentrated *in vacuo* to afford crude extract, which was partitioned between water and $CHCl_3$. Purification of

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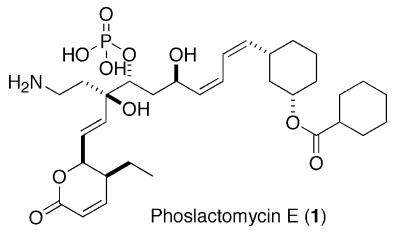

Fig. 1 Structure of phoslactomycin E (PLME, **1**).

$CHCl_3$ extract was performed on reversed-phase HPLC column (KANTO Mightysil RP-18 250-20, 5.0 μm) using as eluent 50% CH_3CN in water - 0.1% formic acid, a 5.0 ml/minute flow rate, and UV detection at 230 nm to afford the purified antibiotic (**1**). LC-MS analysis interfaced with ESI indicated the presence of a protonated molecular ion $(M+H)^+$ at *m/z* 640. Elemental composition analysis revealed the molecular formula of **1** to be $C_{32}H_{50}NO_{10}P$ based on HR-FAB-MS (calcd 640.3251 for $C_{32}H_{51}NO_{10}P$; found 640.3261). **1** was positive on ninhydrin test, indicating that **1** possessed a primary amino group. The ^{31}P-decoupled ^{1}H-NMR spectrum revealed an H-P correlation of protons at δ_H=4.32 with a phosphorus signal at δ_P=2.45, suggesting a monophosphate ester, with the phosphorus atom bonded to a secondary OH group. Complete assignments for the ^{1}H- and ^{13}C-NMR signals in MeOH-d_4 were accomplished using a combination of 2D NMR experiments, such as ^{1}H-^{1}H COSY, HOHAHA, HMQC and HMBC. Comparison of the ^{1}H- and ^{13}C-NMR data of **1** with those of PLME [6] led to identification of **1** as PLME (Fig. 1). The configuration of **1** was assigned to be identical to that of PLME due to their similar optical rotations; The optical rotation of **1** was determined on a Perkin-Elmer 241 polarimeter; $[\alpha]_D^{27}$ +57° (*c* 0.09, MeOH). Lit. [5] $[\alpha]_D^{25}$ +61° (*c* 0.2, MeOH) [7, 8].

PLME was then evaluated for *in vitro* antifungal activity. MIC values were estimated by the serial 2-fold broth dilution method [9] after 24 hours of cultivation in 2.5% malt extract broth. The results are summarized in Table 1. PLME inhibited the growth of all fungal strains tested. In particular, the MIC of PLME against 3 strains of *Aspergillus* spp. was 10^{-1} μM, but the MIC of PLME against *Candida albicans* was 19.6 μM. As shown in Fig. 2, PLME inhibited the hyphal growth of *A. fumigatus* IFO 5840, accompanied by significantly swollen or expanded hyphae when compared with control hyphae. In the other strains of *Aspergillus* spp. tested, similar morphological changes were observed (data not shown). PLME also induced spore swelling during germination of *A. fumigatus* (data not shown). On the other hand, these changes were not observed in the budding yeast *Saccharomyces cerevisiae* (data not shown).

Table 1 Antifungal spectrum of phoslactomycin E (PLME, **1**)

Strain	MIC (μM)
Aspergillus fumigatus IFO* 5840	0.15
A. niger ATCC** 6275	0.31
A. nidulans Glasgow 00***	0.61
Candida albicans IFO 1061	19.56
Mucor mucedo IFO 7684	2.45
Fusarium oxysporum IFO 7152	1.23
Saccharomyces cerevisiae BY 4741****	14.4

Inoculum of each strain tested had turbidity of 0.1 at 660 nm. Strains were incubated in 2.5% malt extract broth at 25°C for 24 hours. MIC was determined as the lowest concentration of PLME that showed no visible growth. *, Institute for Fermentation, Osaka; **, American Type Culture Collection;
***, gift from J. Clutterbuck at University of Glasgow, United Kingdom;
****, obtained from Open Biosystems.

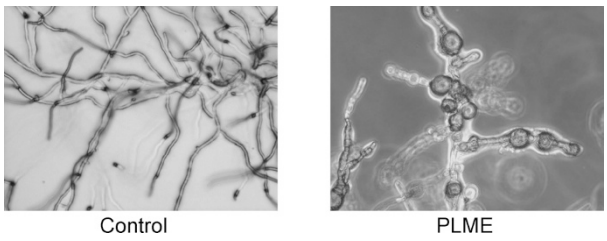

Fig. 2 Effects of PLME on hyphal morphology in *Aspergillus fumigatus*.

Germinated hyphae of *A. fumigatus* IFO 5840 were incubated in 2.5% malt extract medium with or without MIC of PLME at 25°C for 24 hours.

The cell wall of *Aspergillus* spp. including *A. fumigatus* is mainly composed of 1,3-β-glucan [10]. The metabolic abnormality of cell wall biosynthesis is one of the possible causes of morphological changes in *Aspergillus* spp. Therefore we examined the effect of PLME on three 1,3-β-glucan synthase-lacking mutants of *S. cerevisiae*, which has 1,3-β-glucan as one of the main components of cell wall. All *Δfks* mutants tested were more sensitive than a wild-type strain (Table 2). The *Δfks1* mutant was most sensitive to PLME among three *Δfks* mutants. In *S. cerevisiae*, FKS1p functions as the catalytic subunit of the 1,3-β-glucan synthase complex [11]. FKS2p also functions as FKS1p, but FKS2p is thought to be necessary for the limited functions such as the formation of the inner layer of spore wall. On the other hand, the sensitivity to micafungin, an inhibitor of 1,3-β-glucan synthase, is the same in the

Table 2 Effects of PLME, cantharidin and micafungin on deletion mutants of *S. cerevisiae*

S. cerevisiae strain	MIC (μM)		
	PLME	Cantharidin	Micafungin
Wild-type	14.4	200	0.13
*Δfks1** (YLR342w)	3.6	200	0.13
*Δfks2*** (YGR032w)	7.2	200	0.13
*Δfks3**** (YMR306w)	7.2	200	0.13

Inoculum of each strain tested had turbidity of 0.1 at 595 nm. BY4741 strains were incubated in 2.5% malt extract broth at 25°C for 24 hours. MIC was determined as the lowest concentration of each drug that showed no visible growth. *, Fks1p is a major catalytic subunit of 1,3-β-glucan synthase; **, Fks2p is a catalytic subunit of 1,3-β-glucan synthase, involved in formation of the inner layer of spore wall; ***, Fks3p is an unknown functioned protein, has similarity to 1,3-β-glucan synthase catalytic subunits Fks1p and Fks2p.

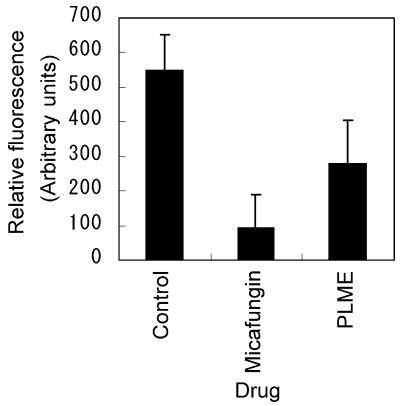

Fig. 3 Effects of PLME on increase in fluorescence derived from aniline blue-glucan complex in membrane fractions of *A. fumigatus* IFO 5840.

Micafungin and PLME were added to a reaction mixture at 10 μM. Values are means±standard deviations (n=3).

wild-type, *Δfks1*, *Δfks2*, and *Δfks3* strains (Table 2). The above results suggest that the antifungal mechanism of action of PLME on three *Δfks* strains is different from that of micafungin.

1,3-β-Glucans are synthesized from UDP-glucose *via* the addition of monomeric glucose by a membrane protein complex, 1,3-β-glucan synthase [12]. In order to gain insight into the mode of action of PLME, we preliminarily examined the effect of the drug on the glucan production in membrane fractions of *A. fumigatus*. Glucan production was estimated using a fluorometric assay using aniline blue dye, which specifically binds to glucan to yield fluorescence [13]. The membrane fractions of *A. fumigatus* were prepared by the method of Shedletzaky *et al.* [13]. The reaction mixture contained 50 mM Tris/HCl (pH 7.5), 20 μM GTP, 4 mM EDTA, 0.5% Brij 35, 6.6% glycerol, 0.2 mM UDP-glucose, and the membrane fraction. The mixture was incubated at 30°C for 30 minutes. The reaction was terminated by the addition of 0.2 volume of 6.0 M NaOH. Glucan produced was solubilized at 80°C for 30 minutes followed by the addition of aniline blue mixture [13]. Fluorescence from glucan-aniline blue complex was quantified at an excitation wavelength of 400 nm and an emission wavelength of 460 nm. PLME at 10 μM inhibited 50% of glucan production as shown in Fig. 3. The 1,3-β-glucan synthase inhibitor, micafungin, also inhibited 87% of the production at the same concentration. Inhibition of glucan production would probably depend on the enzyme inhibition of 1,3-β-glucan synthase by PLME thereby inducing morphological changes of hyphae. The high activity of PLME against *Δfks1* mutant of *S. cerevisiae* (Table 2) and the antifungal activity of PLME can be explained by the inhibition of glucan production.

Phoslactomycins A~F exhibit a potent inhibitory activity against protein serine/threonine phosphatase 2A (PP2A) [14]. However, there have been few reports on the physiological role of PP2A in filamentous fungi [15, 16]. Cantharidin, an inhibitor of PP1A and PP2A, inhibits the hyphal growth of *Neurospora crassa* accompanied by swollen hyphal tips and an increase in hyphal branching [15]. The antifungal spectrum and potency of cantharidin against *Δfks* mutants of *S. cerevisiae* differed from those of PLME. The appearance of balloon-like hyphae induced by PP1A and PP2A inhibitors was reported to be limited at the hyphal tips in *N. crassa* [15]. With PLME, expansion in diameter was observed throughout the hyphae of *A. fumigatus* (Fig. 2). Moreover, there was frequent appearance of balloon-like hyphae, particularly at branching points. PLME may also affect the maintenance of preexisting cell wall, in addition to morphogenesis at the branching points of hyphae.

Further investigations are in progress to elucidate the detailed mechanism for induction of changes in hyphal morphology and their role in the inhibition of 1,3-β-glucan synthase and PP2A.

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