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7-Oxostaurosporine Selectively Inhibits the Mycelial Form of *Candida albicans*

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Abstract In the course of screening for specific inhibitors against the mycelial form of *Candida albicans* from natural resources, we have isolated and identified A6792-1 from *Streptomyces* sp. A6792 by using several chromatographies. By spectral analyses, this compound was determined as 7-oxostaurosporine, having a structure of staurosporine aglycon moiety. 7-Oxostaurosporine exhibited a selective growth inhibitory activity against the mycelial form of *Candida* spp. up to 100 μg/disc in bioassay. It also exhibited a specific antifungal activity against the mycelial form of *Candida* spp. including *C. krusei*, *C. albicans*, *C. tropicalis*, and *C. lusitaniae* with MICs ranging from 3.1 to 25 μg/ml. 7-Oxostaurosporine demonstrated no *in vivo* toxicity in SPF ICR mice. Therefore, this compound may have a considerable potential as an antifungal agent based on the preferential inhibition against growth of the mycelial form of *Candida* spp., dimorphic fungi.

Key words: *Candida albicans*, morphological transition, 7-oxostaurosporine

In the last two decades, the need for more effective, novel antifungal agents has been increasing since most of the discovered antifungal antibiotics have a high toxicity level and various side effects. Recently, the deeply invasive mycoses have been frequently emerging through opportunistic infections with the advent of organ transplantation, cancer chemotherapy, or the human immunodeficiency virus infection. Therefore, the demand for the development of new antifungal agents having low side effects and broad spectrum activity against various fungi is greater than ever.

Candida albicans is an important pathogen, which causes a variety of superficial and deep-seated mycoses. The organism shows either yeast or mycelial form in response to different environmental conditions (Fig. 1), and the switch from a yeast to a filamentous form often correlates with pathogenicity [8]. The mycelial form is responsible for the pathogenicity of *C. albicans* that is associated with systemic candidiasis and tissue invasion [1]. A recent study indicated that mutants lacking mycelial are avirulent, in contrast to the virulent parental strain, suggesting that the ability to form mycelia may play an important role in the pathogenesis of *C. albicans* [5]. By viewing the potential role of this dimorphism, specific inhibitors for the mycelial form of *C. albicans* may control the candidiasis and tissue invasion, and can be used as effective antifungal agents.

To search and develop a preferential growth inhibitor for the mycelial form of *Candida* spp. from natural resources, we performed assays using the mycelial or yeast-form plates of *Candida* spp. *Candida krusei* ATCC 6258, *C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, and *C. lusitaniae* ATCC 42720 grown in Sabouraud's dextrose broth (Difco Co.) were left overnight at 30°C until A_{550}= 1.5. Each culture was then added into Eagle's Minimum Essential Medium (EMEM) (17.5 ml, fetal bovine serum 5 ml, 10% $NaHCO_3$ 10 ml, 4.0% preautocleaved Noble agar 17.5 ml, warmed at 48°C; all from M.A. Bioproducts). Plates were prepared using a base layer of 1.5% Noble agar overlaid with the inoculated EMEM in a ratio of 2:1 (v/v) [3]. Paper discs (8 mm) saturated with test solution were placed on the plates and incubated overnight at 37°C in 5.0% CO_2/air (Fig. 1). The yeast-form plates of *Candida* spp. were prepared as follows. Five ml of the same culture was added in 50 ml of overlay medium (0.75% agar in Sabouraud's dextrose broth). The base medium (Sabouraud's dextrose agar, Difco Co.) was then overlaid with the incubated overlay medium in a ratio of 2:1 (v/v) [3]. Plates were then incubated overnight at 30°C (Fig. 1). The preferential activities against the mycelial and yeast forms

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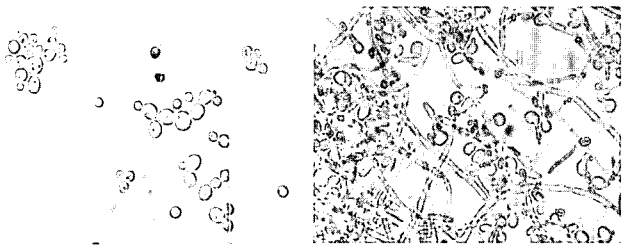

Fig. 1. Dimorphic growth pattern of *Candida albicans*.
Candida albicans ATCC10231 was incubated in the medium: Sabouraud's dextrose broth medium at 30°C for 24 h (left), Eagle's Minimum Essential Medium with 5.0% CO_2 at 37°C for 24 h (right).

were examined for zone of growth inhibition. The determination of MICs was conducted according to the serial dilution method [6]. Antifungal activity was determined against the mycelial form of *Candida* spp. using EMEM with 5.0% CO_2 incubation at 37°C for 24 h, and the yeast phase of all test organisms using Sabouraud's dextrose agar incubation at 30°C for 24 h [3].

The producing microorganism, a strain of *Streptomyces* sp. A6792, was isolated from soil collected at Mt. Seolak of Gangwon Province, Korea. The strain was designated as *Streptomyces* sp. A6792 by biochemical properties, physiological characteristics, and electron microphotography (Fig. 2). The extract of fermentation broth of strain A6792 selectively inhibited the mycelial form of *C. albicans* (inhibition zone: >40 mm at a concentration of 1 mg/disc) in our bioassay system. We have thus isolated an active compound from this strain with a selective activity against the mycelial form of *C. albicans*. The strain was incubated in fermentation medium (yeast extract 0.1%, starch 2.0%, soytone 0.4%, polypeptone 0.2%, phama media 0.3%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.1%, $CaCO_3$ 0.3%, $FeSO_4$ 0.002%, $MnCl_2$ 0.001%, $ZnSO_4$ 0.001%, $CoCl_2$ 0.0005%) for 4 days at 26°C on a rotary shaker at 150 rpm (radius 7 cm). The fermentation broth of *Streptomyces* sp. A6792 (10 l) was extracted twice with 10 l of ethyl acetate and partitioned between ethyl acetate and water. The ethyl acetate layer was evaporated (3.5 g), applied to a column of silica gel (Merck, Kieselgel 60, 230–400 mesh) and eluted with chloroform-methanol (98:2 to 90:10, v/v) to give active fractions. The active fractions were combined and concentrated *in vacuo*, yielding a yellow-brown residue (1.2 g). The residue was applied on an ODS column (Merck, Lichroprep RP-18, 40–63 μm), eluted with a gradient of methanol-water (70:30 to 10:90, v/v) to give the active fraction (600 mg), and then subjected to a silica gel column eluted with a mixture of ethyl acetate-methanol (98:2, v/v) to yield a yellow residue (70 mg). The residue was further purified by preparative silica gel TLC (Merck, HPTLC pre-coated plates Silica gel 60 F254) developed with ethyl acetate-methanol (98:2, v/v, R_f 0.3) (12 mg). The silica was washed several times and removed by centrifugation and filtration through a 0.2-μm-pore-size filter. Finally, the band with an R_f of 0.3 was further purified using a Sephadex LH-20 (Sigma, Lipophilic LH-20, 25–100 μm) column with methanol. The active fraction was collected and concentrated *in vacuo* to yield a yellow powder of pure active compound as A6792-1 (5 mg). Spectral and physicochemical data for A6792-1 were obtained using the following instruments: UV, Shimadzu UV265 UV-Visible spectrophotometer; ESI-MS, Hewlett Packard 5989A; NMR, Varian UNITY 500 spectrometer. The mass spectroscopic (ESI-MS; *m/z* 481.3$[M+H]^+$), UV spectral (λmax(MeOH); 238, 260, 287, 317, 410), and ^{1}H-, ^{13}C-NMR, ^{1}H-^{1}H COSY, and HMBC spectral data for the purified compound were in good agreement with the previously published spectral data for 7-oxostaurosporine (Fig. 3) [4]. ^{1}H-NMR (500 MHz, chloroform-*d*) δ: 7.32 (1H, brd, H-1), 7.57 (1H, m, H-2), 7.43 (1H, m, H-3), 9.24 (1H, brd, H-4), 7.40 (1H, s, H-6), 9.35 (1H, d, H-8), 7.37

Fig. 2. Scanning electron microphotograph of strain A6792 cultured in ISP-6 medium.

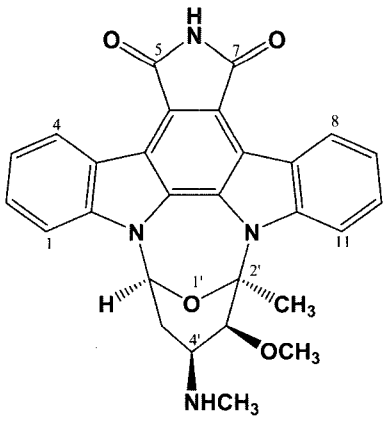

Fig. 3. Structure of 7-oxostaurosporine isolated from *Streptomyces* sp. A6792.

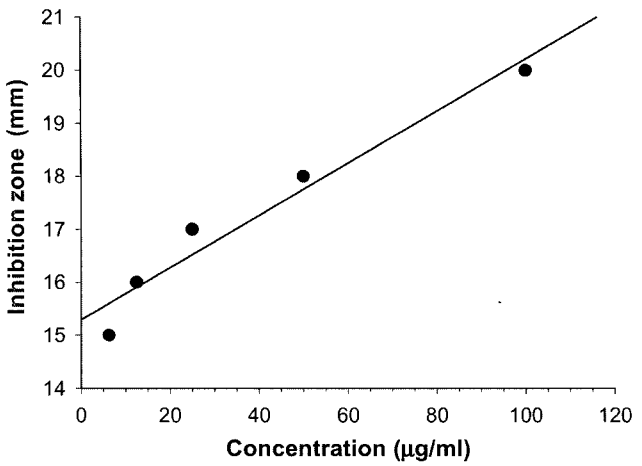

Fig. 4. The inhibitory activity of 7-oxostaurosporine on the mycelial form of *C. albicans*.
C. albicans ATCC10231 was incubated in the Eagle's Minimum Essential Medium with 5.0% CO_2 at 37°C for 24 h.

(1H, m, H-9), 7.48 (1H, m, H-10), 7.91 (1H, d, H-11), 2.37 (3H, s, 2'-C$\underline{H}_3$), 3.88 (1H, d, H-3'), 3.43 (3H, s, H-3'-OC$\underline{H}_3$), 3.36 (1H, m, H-4'), 1.56 (3H, s, 4'-NC$\underline{H}_3$), 2.39 (1H, ddd, H-5'a), 2.76 (1H, brdd, H-5'b), 6.52 (1H, brd, H-6'). ^{14}C-NMR (125 MHz, chloroform-*d*) δ: 107.44 (C-1), 26.67 (C-2), 120.84 (C-3), 126.09 (C-4), 122.31 (C-4a), 115.72 (C-4b), 119.20 (C-4c), 169.99 (C-5), 170.12 (C-7), 120.39 (C-7a), 117.11 (C-b), 123.65 (C-7c), 125.36 (C-8), 120.53 (C-9), 125.99 (C-10), 114.81 (C-11), 140.84 (C-11a), 131.69 (C-12a), 130.77 (C-12b), 137.95 (C-13a), 91.16 (C-2'), 30.19 (2'-$\underline{C}H_3$), 84.14 (C-3'), 57.26 (3'-O$\underline{C}H_3$), 50.34 (C-4'), 33.40 (4'-N$\underline{C}H_3$), 31.92 (C-5'), 80.07 (C-6').

Staurosporine and its analogs have been isolated from actinomycetes and reported with the biological activities as antimicrobial, antihypotensive, and as inhibitors of platelet aggregation and protein kinase C [9]. Among them, 7-oxostaurosporine was reported to have inhibitory activity for protein kinase C and weak antimicrobial activity against *Chlorella vulgaris* and *Magnaporthe grisea* [10]. Here, we report for the first time that 7-oxostaurosporine preferentially inhibits the mycelial form of *Candida* spp. As shown in Fig. 4, 7-oxostaurosporine exhibited a selective growth inhibitory activity against the mycelial form of *C. albicans* in a dose-dependent manner at a concentration up to 100 μg/disc, but not the yeast form of *C. albicans*. Also, 7-oxostaurosporine showed specific growth inhibitory activity and potent antifungal activity from 3.1 to 50 μg/ml against the mycelial form of *Candida* spp. (Table 1). In particular, this compound exhibited a strong antifungal activity against the mycelial form of *C. krusei* with MIC of 3.1 μg/ml. However, no inhibition was observed against the yeast form of the test organisms by up to 200 μg/ml of the compound. The results indicated that

Table 1. *In vitro* antifungal activity of 7-oxostaurosporine against *Candida* spp. using bioassay and agar dilution method.

Candida spp.	Bioassay[a] (Inhibition zone)		Agar dilution method (MICs, μg/ml)	
	EMEM[b]	SD[c]	EMEM	SD
C. krusei	+++	-	3.1	>200
C. albicans	+++	-	25	>200
C. tropicalis	+	-	50	>200
C. lusitaniae	++	-	12.5	>200

The experiments were repeated twice with essentially the same results.
[a]In bioassay loading 100 mg/disc, we established four classes, marked by the following signs: - no inhibition; + 10-15 mm; ++ 15-20 mm; +++ 20-2 mm.
[b]EMEM: Eagle's Minimum Essential Medium.
[c]SD: Sabourauds Dextrose Medium.

7-oxostaurosporine was a selective inhibitor for the mycelial form of *Candida* spp.

In conclusion, although the virulence of *C. albicans* is generated by diverse factors such as phospholipase, aspartyl protease, mycelial formation, and surface hydrophobicity, the key factor of virulence remains unknown [1]. We detected 7-oxostaurosporine utilizing a bioassay that identifies compounds with preferential activity against the mycelial-yeast form transition of *C. albicans*. Until now, several types of compounds having preferential inhibitory activity against the mycelial form of *C. albicans* have been discovered, including Sch 40873 [3] and tetaine, which is a well known glucosamine-6-phosphate synthetase inhibitor responsible for chitin and mannoprotein biosynthesis [7]. 7-Oxostaurosporine, being an indolocarbazole group antibiotic, however, has a different structure with no inhibitory activity against chitin synthases 1, 2, and 3 from *Saccharomyces cerevisiae* at a concentration of 140 μg/ml (data not shown). Although the mode of action for this compound is not clear, the compound showed a selective antifungal activity against the mycelial form of *Candida* spp. using bioassay and agar dilution method (Table 1). Furthermore, in our *in vivo* toxicity test, 7-oxostaurosporine exhibited no significant toxicity against SPF ICR mice up to 60 mg/kg (data not shown). Therefore, this compound may be a useful lead compound for development of potential antifungal agents based on its selective inhibition against the mycelial form of dimorphic fungi. The mechanism of the antifungal activity of 7-oxostaurosporine is under investigation in our laboratory.

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