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Compound IKD-8344, a Selective Growth Inhibitor Against the Mycelial Form of *Candida albicans*, Isolated from *Streptomyces* sp. A6792

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Abstract In the course of screening for selective growth inhibitors against the mycelial form of *Candida albicans*, we isolated a *Streptomyces* sp. A6792 from soils. The inhibitor was isolated from the above bacterium and identified through several spectral analyses with UV and mass spectrophotometries, and various NMR. The compound was determined to be a macrocyclic dilactone antibiotic, IKD-8344 (molecular weight: 844, molecular formula: $C_{48}H_{76}O_{12}$). The compound selectively inhibited the growth of mycelial form of *C. albicans* with an MIC of 6.25 μg/ml. It also exhibited strong inhibitory effect preferentially on the mycelial form of various *Candida* spp. including *C. krusei*, *C. tropicalis*, and *C. lusitaniae*, with MICs ranging from 1.56 to 25 μg/ml. Furthermore, the compound showed no significant toxicity against SPF ICR mice up to 60 mg/kg. These results suggest that IKD-8344 is a useful lead compound for the development of novel antifungal agents, based on the preferential growth inhibition against *Candida* spp.

Key words: Dimorphism, *Candida albicans*, morphological change, virulence, selectivity, IKD-8344

Candida albicans is an opportunistic pathogen and is a member of the normal microflora in most healthy people, where it remains at the mucosal surfaces of the oral cavity, gastrointestinal tract, and genitalia. However, especially in immunocompromised patients, *C. albicans* can cause superficial as well as life-threatening systemic infections [1–3]. In addition, *C. albicans* is one of the four most common causes of bloodstream infections in the United States and elsewhere in the world, and the attributable mortality rate is 40% [7] in those patients with candidemia [4–6]. At present, the most commonly used antifungal agents are fluconazole, flucytosine, and amphotericin B [8]. However, there are some problems of acquired resistance to azoles and considerable toxicity with flucytosine and amphotericin B. As a result, there is an increasing demand for the development of novel antifungal agents that have few side effects and potent activity against resistant strains.

A striking feature of *C. albicans* is its ability to grow in a variety of morphological forms. Morphological switching from yeast to filamentous forms can be induced by various environmental factors, such as serum, high temperature, nutrient-poor medium, neutral pH, and N-acetylglucosamine [1]. The ability to switch between yeast and hyphal growth is often considered to be necessary for virulence [9–11]. Recently, it was reported that *C. albicans* mutants lacking the filamentous form have markedly attenuated virulence [12, 13], suggesting that the ability to form a hyphal state may play an important role in the pathogenesis of *C. albicans*. Therefore, specific inhibitors for the mycelial form of *C. albicans* can be used as effective antifungal agents to prevent the candidiasis.

In the course of our screening program to search preferential growth inhibitors for the mycelial form of *C. albicans* from natural resources, we isolated a bioactive compound, IKD-8344, from the culture broth of *Streptomyces* sp. A6792. Here, we report the isolation, structure determination, and inhibitory activities of the compound (Fig. 1).

To search and develop a selective growth inhibitor for the mycelial form of *C. albicans* from natural resources, we performed assays by using the mycelial or yeast form plates of various *Candida* strains, including *C. albicans* ATCC 10231, *C. krusei* ATCC 6258, *C. tropicalis* ATCC 13803, and *C. lusitaniae* ATCC 42720. The assay plates of

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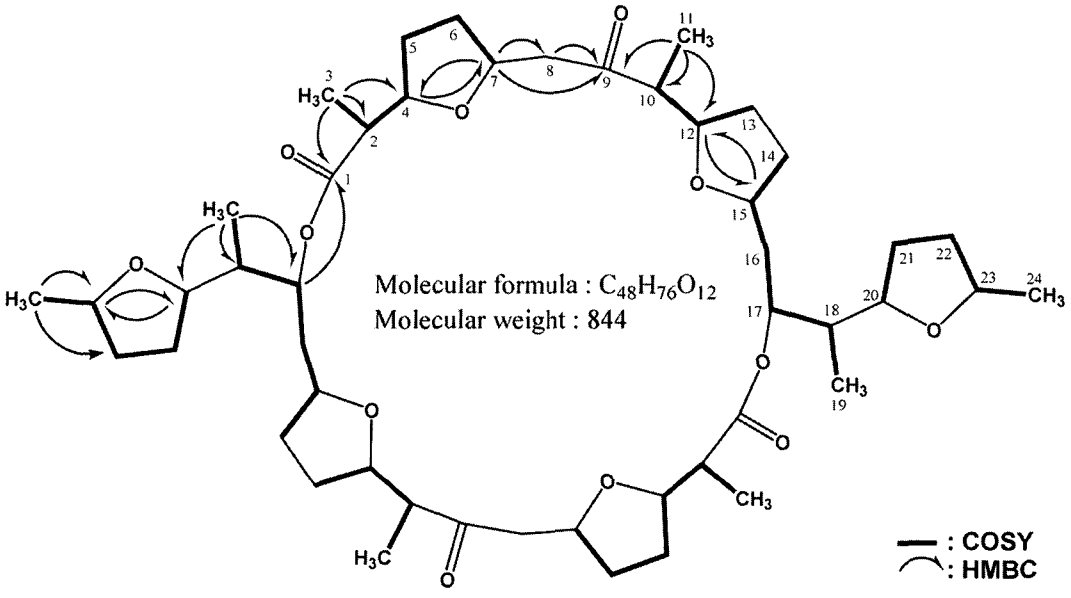

Fig. 1. Structure of isolated compound IKD-8344 from *Streptomyces* sp. A6792.

yeast and filamentous forms were prepared as described previously [14]. Paper discs saturated with test solution were simultaneously placed on both yeast and mycelial plates. The mycelial form plates were incubated overnight at 37°C in a 5.0% CO_2 incubator, whereas the yeast form plates were incubated overnight at 30°C. The strains exhibiting preferential growth inhibition against the mycelial form plates, but not against the yeast form plates, were selected.

The minimum inhibitory concentrations (MICs) were determined by the two-fold serial dilution method [15]. The yeast and mycelial form of *Candida* spp. were grown on Sabouraud dextrose medium (Difco, Detroit, MI, U.S.A.) and Eagle's Minimum Essential Medium (GibcoBRL, Grand Island, NY, U.S.A.), respectively. Antifungal activities against the yeast and mycelial form of *Candida* spp. were observed after 24 h incubation at 30–37°C [14].

The producing microorganism, the strain of *Streptomyces* sp. A6792, was isolated from soil collected from Seolak mountain, Gangwon Province, Korea. The strain was grown on YM medium (0.4% yeast extract, 1.0% malt extract, 0.4% glucose, 2.0% agar, pH 7.2) and stored in 20% glycerol at −70°C.

To confirm the genus of actinomycetes strain A6792, the determinations of the morphological characteristics and chemotaxonomy, in addition to physiological and biochemical characteristics and electron microphotography described previously [14], were performed by the methods of the International *Streptomyces* Project (ISP) and *Bergey's Manual of Systematic Bacteriology* [16]. Depending on the medium used, the color of the aerial mycelium was yellow-white to pinkish-gray, while the color of the reverse side was moderate yellow to grayish yellow. No soluble pigment was produced in the media. The diaminopimelic acid (DAP) in the cell wall of the strain was found to be an LL-type, and the strain contained ribose (73.9%) and glucose (26.1%) in the cell wall. The cellular fatty acids, including anteiso-$C_{15:0}$ (20.97%), anteiso-$C_{17:0}$ (19.38%), iso-$C_{16:0}$ (12.06%), and $C_{16:0}$ (10.22%), were detected by gas chromatography. From these results (Table 1), the strain was confirmed as the genus *Streptomyces* and designated as *Streptomyces* sp. A6792. Interestingly, another bioactive compound, which is different from 7-oxostaurosporin described previously [14], was found from this strain, and the compound was subsequently isolated.

The fermentation broth of *Streptomyces* sp. A6792 was extracted twice with 10 l of ethyl acetate and partitioned between ethyl acetate and water. The ethyl acetate layer was evaporated, and the preparation was applied to

Table 1. Morphological characteristics and chemotaxonomy of the isolate A6792.

Characteristics	A6792
Morphology	
Spore chain	Retinaculum-flexibilis
Spore size	0.5–0.6×0.8–1.0 μm
Spore surface	Smooth
Chemotaxonomy	
Cell-wall composition	LL-DAP[a]
Phospholipid fatty acids	Anteiso-$C_{15:0}$ (20.97%), anteiso-$C_{17:0}$ (19.38%) iso-$C_{16:0}$ (12.06%), $C_{16:0}$ (10.22%),
Whole-cell sugars	Ribose (73.9%), glucose (26.1%)

[a]DAP: Diaminopimelic acid.

silica gel column chromatography (Kieselgel 60, 230–400 mesh, Merck, Darmstadt, Germany,) with a gradient of chloroform:methanol (98:2 to 8:2, v/v) to give the active fractions. The active fractions were combined and concentrated *in vacuo*, yielding a yellow-brown residue. The residue was isolated on an ODS column (Lichroprep RP-18, 40–63 μm, Merck, Darmstadt, Germany) by eluting with a gradient of methanol:water (7:3 to 0:100, v/v) to yield the active fraction, and then subjected to silica gel column, eluting with a mixture of ethyl acetate:methanol (98:2, v/v) to give a yellow residue. Finally, the crude compound was further purified by a Sephadex LH-20 (Lipophilic LH-20, 25–100 μm, Sigma, St. Louis, MO, U.S.A.) column, eluted with methanol. The active fraction was collected and concentrated *in vacuo* to produce a pure active compound of white powder (4 mg). Spectral and physicochemical data on the isolated compound were obtained using the following instruments: UV, Shimadzu UV265 UV-Visible spectrophotometer, Kyoto, Japan; HIFAB-MS, Hewlett Packard 5989A, Palo Alto, U.S.A.; NMR, Varian UNITY 500 spectrometer, Palo Alto, U.S.A. The UV-visible spectrum showed the maxima at 278 nm in methanol. The IR spectrum showed ketone (1,708 cm^{-1}) and ester (1,731 cm^{-1}). The molecular formula of the isolated compound was determined to be $C_{48}H_{76}O_{12}$ on the basis of *high-resolution fast atom bombardment mass spectra* (HIFAB-MS) (*m/z* 867.4828 $[M+Na]^+$). In addition, ^1H- and ^{13}C-NMR spectra of isolated compound displayed only 38 protons and 24 carbons, respectively. ^1H-NMR (500 MHz, chloroform-*d*) δ: 2.35 (1H, m, H-2), 1.09 (3H, d, H-3), 4.12 (1H, dt, H-4), 2.26 (1H, m, H-5), 1.45 (1H, m, H-5'), 2.29 (1H, m, H-6), 1.75 (1H, m, H-6'), 4.35 (1H, m, H-7), 2.90 (2H, m, H-8), 2.42 (1H, m, H-10), 1.01 (3H, d, H-11), 3.79 (1H, m, H-12), 2.00 (1H, m, H-13), 1.45 (1H, m, H-13'), 2.00 (1H, m, H-14), 1.45 (1H, m, H-14'), 3.94 (1H, m, H-15), 1.78 (1H, m, H-16), 1.65 (1H, m, H-16'), 5.30 (1H, m, H-17), 1.95 (1H, m, H-18), 0.83 (3H, d, H-19), 3.90 (1H, m, H-20), 1.95 (1H, m, H-21), 1.65 (1H, m, H-21'), 2.00 (1H, m, H-22), 4.06 (1H, m, H-22'), 4.06 (1H, m, H-23), 1.19 (3H, d, H-24). ^{13}C-NMR (125 MHz, chloroform-*d*) δ: 175.0 (C-1), 45.5 (C-2), 14.3 (C-3), 80.5 (C-4), 30.8 (C-5), 32.1 (C-6), 74.9 (C-7), 45.6 (C-8), 211.9 (C-9), 53.3 (C-10), 13.7 (C-11), 80.0 (C-12), 29.6 (C-13), 30.7 (C-14), 75.2 (C-15), 36.2 (C-16), 72.2 (C-17), 41.1 (C-18), 10.8 (C-19), 79.5 (C-20), 29.4 (C-21), 33.9 (C-22), 74.7 (C-23), 21.2 (C-24). These results indicated that the isolated compound consisted of a symmetrical dimer. ^1H- and ^{13}C chemical shift assignments were made by standard NMR techniques, such as DEPT, ^1H-^1H COSY, HMQC, and HMBC. The DEPT experiment revealed the presence of 10 methine carbons, 8 methylene carbons, 4 methyl carbons, and 4 quaternary carbons, and an HMQC experiment established all direct ^1H-^{13}C connectivities. The partial structures of this compound were confirmed with ^1H-^1H COSY, and 3 tetrahydrofuran rings were determined, based on the HMBC spectrum (Fig. 1). Through the HIFAB-MS and NMR spectral data, the structure of the isolated compound was determined to be macrocyclic dilactone (macrolide), possessing a 28-membered ring. On the basis of the above spectral data, the isolated compound was designated as compound IKD-8344 by comparison of their spectral data with those published previously [17].

Table 2. The antifungal activities of compound IKD-8344 against various *Candida* strains.

Fungi	MICs (μg/ml)	
	EMEM[a]	SD[b]
Candida albicans ATCC 10231	6.25	>200
Candida krusei ATCC 6258	1.56	>200
Candida lusitaniae ATCC 42720	25	>200
Candida tropicalis ATCC 13803	12.5	>200

The experiments were repeated twice, with essentially the same results.
[a]EMEM: Eagle's Minimum Essential Medium.
[b]SD: Sabouraud Dextrose Medium.

The compound at 1.56 to 25 μg/ml concentration exhibited potent antifungal activities against the mycelial form of *Candida* spp., whereas no inhibition was observed against the yeast form of microorganisms by up to 200 μg/ml of the compound (Table 2). In particular, the compound exhibited a strong antifungal activity against the mycelial form of *C. krusei*, with an MIC of 1.56 μg/ml. The results indicated that IKD-8344 was a selective growth inhibitor against the mycelial form of *Candida* spp.

Previously, the compound IKD-8344 was reported to have a weak antifungal activity against *C. albicans* (13 mm inhibition zone on loading of 1 mg/ml), anthelmintic activities against *Trichinella spiralis in vitro* and *in vivo*, and antitumor activity [17]. In the present study, we found for the first time that IKD-8344 selectively inhibits the mycelial form of *Candida* spp. In addition, the compound showed no significant toxicity against SPF ICR mice up to 60 mg/kg (data not shown), although IKD-8344 exhibited a strong cytotoxicity against the mouse leukemia L5178Y cell with an IC_{50} of 0.54 ng/ml [17].

Antifungal activities of commercial antifungal compounds, including amphotericin B, cycloheximide, clotrimazol, and ketoconazol, were compared with those of IKD-8344 against the yeast and mycelial form of *Candida* spp. Unlike IKD-8344, all of the commercial compounds showed strong growth inhibition against both the yeast and the mycelial form of *Candida* spp. These results indicate that the compound IKD-8344 may have a different mechanism against the growth inhibition of *Candida* spp., when compared with the test compounds described above. Besides, IKD-8344 did not inhibit the chitin synthase 1 of *Candida albicans*. Therefore, IKD-8344 may be a new class of antifungal agent, having specific growth inhibitory activity

against the mycelial form of *Candida* spp. In conclusion, although the mode of action of this compound is not clear, IKD-8344 may be a useful lead compound for development of potential antifungal agents, due to its preferential activity against the mycelial form of *Candida* spp.

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