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A novel antifungal from an Actinomadurae with preferential activity against the mycelial phase of *Candida albicans*

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SUMMARY

Sch 40873, a novel antifungal compound isolated from the fermentation broth of an *Actinomadura* spp. was discovered in an assay designed to detect compounds with preferential activity against the invasive mycelial form of *Candida albicans*. The geometric mean MIC of Sch 40873 against seven *Candida* spp. in Sabouraud dextrose broth (yeast phase) was ≥ 58 μg/ml and in Eagles minimum essential medium (mycelial phase) was < 0.03 μg/ml. Sch 40873 demonstrated slight in vivo topical activity in a hamster vaginal model.

INTRODUCTION

Candida albicans is an important fungal pathogen that affects man. The ability of *C. albicans* to grow in either a yeast or mycelial form (dimorphism) has been linked to pathogenicity, the mycelial form being associated with systemic candidiasis and tissue invasion [12]. A screen has been developed to detect natural products that specifically inhibit the mycelial form of *C. albicans*. Employing this screen, a culture identified as Schering Culture Collection 1838, was found to produce in fermentation a compound with preferential activity against the mycelial form of *C. albicans*. Purification of the active component yielded a novel antifungal agent [2,3]. This paper describes the assay system, taxonomy and fermentation of the producing organism, and the isolation and biological properties of the active component, Sch 40873.

MATERIALS AND METHODS

Culture

The culture was isolated from a Florida soil which was suspended in distilled water (1 g/10 ml), serially diluted and plated on YS medium (yeast extract, 1.0 g; soluble starch, 1.0 g; tap water, 1000 ml) containing 5 μg/ml neomycin. Colonies of SCC 1838 appeared after 2 to 3 weeks incubation at 30 °C.

Inoculum for morphological analysis was prepared according to the method of Horan and Brodsky [4]. Inoculated plates of AV agar [8], ATCC medium 172 [1], inorganic salts-starch agar [11] and water agar (agar, 1.0 g; tap water, 1000 ml) were incubated for 4 to 6 weeks prior to examination. Whole cells were analyzed by the method of Lechevalier [5].

Assay

C. albicans (strain Wisconsin, C-43) was grown in 20 ml of Sabouraud dextrose broth (SDB) incubated overnight at 37 °C, and diluted 1 : 50 in sterile saline. This inoculum (2.25 ml) was used to inoculate 150 ml of the following seed medium: 65.25 ml Eagles minimum essential medium (EMEM) [10], 1.5 ml non-essential amino acid mixture, 3.0 ml L-glutamine, 15 ml foetal bovine serum (all from M.A. Bioproducts) and 65.25 ml pre-autoclaved 3% Noble agar (Difco) in distilled water warmed at 48 °C. Plates were prepared using a base layer of 1.5% Noble agar overlaid with the inoculated seed medium in a ratio of 2 : 1 (v/v). On this medium *C. albicans* grows in the mycelial phase. Paper discs (6 mm) saturated with test solutions were placed on the plates, incubated overnight at 37 °C in 5% CO_2/air, and examined for zones of inhibition. Control assay plates representing the yeast phase of *C. albicans* were prepared from the same inoculum using SD agar as the medium and incubated at 37 °C.

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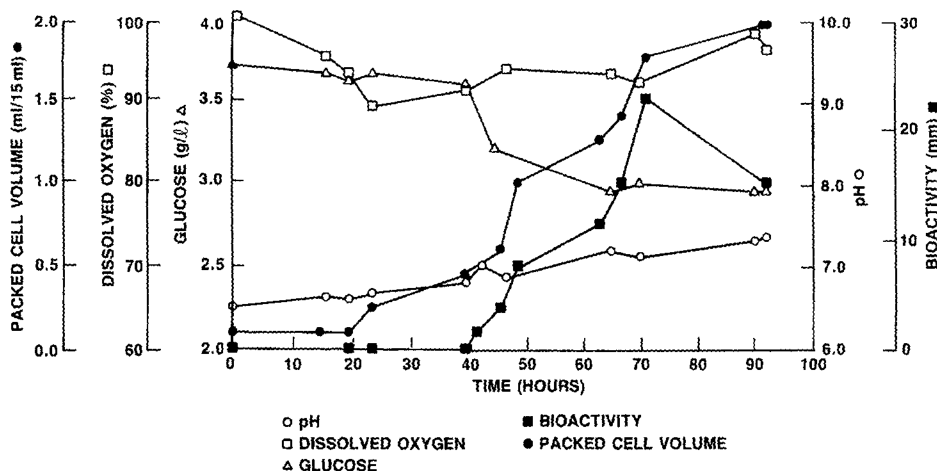

Fig. 1. Fermentation profile of culture SCC 1838.

Production of Sch 40873

The germination medium consisted of (w/v): beef extract 0.3%, tryptone 0.5%, yeast extract 0.5%, cerelose 0.1%, potato starch 2.4%, calcium carbonate 0.2% and antifoam 0.01% in tap water. Stock cultures were maintained as frozen broths at − 135 °C in 10% glycerol. A 250-ml Erlenmeyer flask containing 50 ml of germination medium was inoculated with 2.5 ml of a stock culture and incubated at 30 °C on a rotary shaker at 300 rpm for 48 h. 25 ml of this germination culture was used to inoculate a 2-l Erlenmeyer flask containing 350 ml of the same germination medium and incubated as above. The entire contents of this second stage seed inoculum were used to inoculate a 14-l New Brunswick Scientific fermenter containing 10 l of fermentation medium consisting of (w/v): beef extract 0.25%, yeast extract 0.2%, starch 2.0%, cerelose 0.25%, maltose 0.25%, casamino acid 0.5% and 0.1% (v/v) of 0.014 g/l $FeSO_4$ solution in

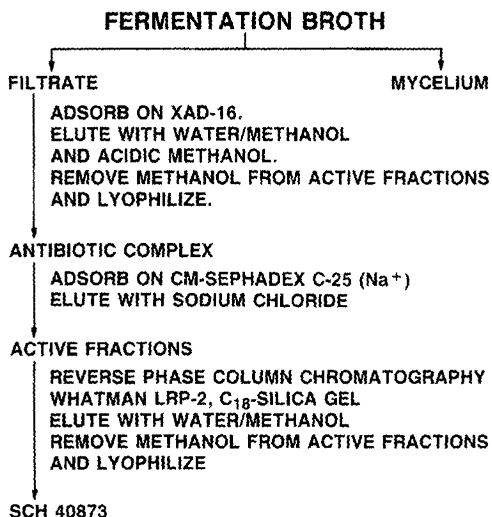

Fig. 2. Isolation of Sch 40873.

TABLE 1

Zones of inhibition (mm) against *C. albicans* (C-43)

Compound tested	μg/disk	Yeast form	Mycelial form
Ketoconazole	40	13H	25H (15)
	20	11H	24H (14)
	10	–	22H (12)
Amphotericin B	40	14	16
	20	12	15
	10	10	14
Candicidin	40	15	17
	20	13	15
	10	12	13
Cerulenin	20	30	30
	10	24	28
	5	19	24
Filipin	40	14	10
	20	10	–
	10	–	–
Nystatin	20	16	15
	10	12	13
	5	10	10
Papulacandin	20	16	20
	10	14	16
	5	12	12
Tunicamycin	20	10H	10
	10	–	–
	5	–	–
Sch 40873	20	8	22
	10	±	20
	5	±	18

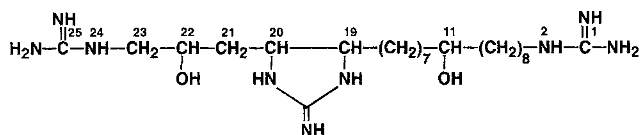

Fig. 3. Structure of Sch 40873.

tap water. The pH was adjusted with sulfuric acid to pH 7.5 prior to the addition of 3-(*N*-morpholino)-propane sulfonic acid (MOPS) 1.0%. The fermentation was carried out at 30 °C at 400 rpm with aeration at 4 l per min for 72 h (Fig. 1).

Purification of Sch 40873

Sch 40873 was isolated from the fermentation broth of 100 l of whole broth as shown in Fig. 2. The culture broth was filtered and the antifungal compound was adsorbed from the filtrate onto XAD-16 (Rohm & Haas). The antifungal was eluted with methanol and acidic methanol, the methanol was evaporated and the remaining aqueous solution was lyophilized. Further purification was achieved by adsorption on CM-Sephadex C-25 Na^+ (Pharmacia) followed by elution with 1 M NaCl. Chromatography on C-18 silica gel (Whatman) yielded 101.6 mg of pure Sch 40873. The detailed structural determination of Sch 40873 (Fig. 3) and the physico-chemical properties have been reported elsewhere [3].

Biological evaluation

Geometric mean MICs were determined against seven *Candida* spp. and six dermatophytes spp. using: SDB, pH 5.7, 28 °C [7], MA medium [8], pH 5.7 with CO_2 incubation at 37 °C for yeasts, and 28 °C for dermatophytes, and EMEM, pH 7.0, 37 °C, CO_2 [10]. In vivo antifungal activity was determined topically in a vaginal *C. albicans* infection model using hamsters [6]. Acute toxicity tests were carried out with groups of mice and LD_{50} values were calculated by probit analysis.

RESULTS AND DISCUSSION

SCC 1838 is a filamentous actinomycete which forms a compact mycelium that penetrates agar, and an aerial mycelium that develops from the substrate mycelium.

TABLE 2

The in vitro antifungal activity of Sch 40873 against various strains of *C. albicans* and dermatophytes

Strains	No.	MICs (μg/ml)				
		SDB[a]		MA[b]		EMEM[c]
		48 h	72 h	48 h	72 h	48 h
Candida albicans	C40	>64		4.0		<0.03
Candida albicans	C41	64		8.0		<0.03
Candida albicans	C42	64		2.0		<0.03
Candida albicans	C43	64		4.0		<0.03
Candida albicans	C60	64		2.0		<0.03
Candida tropicalis	C44	2.0		1.0		<0.03
Candida stellatiodea	C45	32		<0.03		<0.03
Geometric mean		≥58		≤1.4		<0.03
Trichophyton mentagrophytes	D23		0.25		8.0	
Trichophyton mentagrophytes	D24		<0.03		0.06	
Trichophyton mentagrophytes	D30		1.0		8.0	
Microsporum canis	D18		<0.03		4.0	
Microsporum gypseum	D16		4.0		16.0	
Epidermophyton floccosum	D58		<0.03		1.0	
Geometric mean			≤0.13		2.5	

[a] SDB = Sabouraud Dextrose Broth, pH 5.7, 28 °C.
[b] MA = MA medium pH 5.7, CO_2, 37 °C, yeasts, 28 °C, dermatophytes.
[c] EMEM = Eagles Minimum Essential Medium, pH 7.0, 37 °C, CO_2.

Spores occur along the length of the aerial mycelium and are arranged in tightly appressed spirals forming pseudosporangia. The color of the substrate mycelium is gray to yellow-brown. On ATCC medium 172 the colony reverse is violet-red and a violet-red slightly soluble pigment is formed. The aerial mycelium, in mass, is pink to yellow-pink. Whole cells characteristically contain meso-diaminopimelic acid and madurose. The producing culture was tentatively identified as a species of *Actinomadura*.

Fermentation of SCC 1838 was performed at 30 °C with an aeration of 4.0 l/min and an agitation rate of 400 rpm. The pH and dissolved oxygen levels were continuously monitored by submerged probes. Microbial growth was determined by packed cell volume. The production of Sch 40 873 was determined by a paper disk agar diffusion assay using. *C. albicans* as the test organism. Production of Sch 40 873 peaked at 70 h. A typical fermentation profile is shown in Fig. 1. The measured parameters do not exhibit any unusual trends or characteristics. A fermentation sample of the culture, SCC 1838, showed larger zones of inhibition against the mycelial than yeast form of *C. albicans*.

The ability of *C. albicans* to grow in the mycelial phase has been associated with pathogenicity. Studies have shown that in vivo activity of azole compounds correlate well with in vitro activity against the mycelial phase of *C. albicans* [9]. Table 1 shows the antifungal activity of a variety of microbial products and the synthetic azole, ketoconazole, against the yeast and mycelial form of *C. albicans* (C-43). None of the microbial products tested demonstrated the significant differential activity observed for ketoconazole. This assay was therefore used to search for microbial products with selective activity against the mycelial form of *C. albicans*. The in vitro activity of Sch 40 873 is shown in Table 2. Sch 40 873 was active against the seven strains of *Candida* spp., tested. In SDB the geometric mean MIC was ≥ 58 μg/ml against the yeast phase of *Candida* spp. However, in EMEM the geometric mean MIC was significantly lower, < 0.03 μg/ml. Sch 40 873 was also very active against dermatophytes in both SDB and MA medium.

The IV LD_{50} of Sch 40 873 in mice was ≤ 1.5 mg/kg. In vivo activity against *C. albicans* was determined topically using a hamster vaginal model. Slight activity was observed with an intravaginal application 2.5% given for 8 days.

In conclusion, utilizing an assay which identifies compounds with preferential activity against the mycelial form of *C. albicans*, a novel antifungal, Sch 40 873, was detected. The compound was extremely active against the mycelial phase of *C. albicans*, in vitro, and demonstrated slight in vivo topical activity in a hamster vaginal model.

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