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Isolation of *Candida albicans* Chitin Synthase 1 Inhibitor from *Streptomyces* sp. A6705 and Its Characterization

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Abstract In the course of searching for potent chitin synthase 1 inhibitors from natural resources, *Streptomyces* sp. A6705 was found to exhibit potent inhibitory activity against the chitin synthase 1 from *C. albicans* (CaCHS1p). As a result, the inhibitor was isolated and identified using a series of chromatographies. Through chemical analyses with UV spectrophotometry, MS spectrometry, and various NMR techniques, the inhibitor was identified as N,N-bis(2-phenylethyl)urea. The compound exhibited strong inhibitory activity against the chitin synthase 1 from *C. albicans* with an IC_{50} of 14 μg/ml, representing a similar inhibitory activity to that of the well-known chitin synthase inhibitor, polyoxin D (IC_{50}; 15 μg/ml). However, the compound showed no inhibitory activity against the chitin synthase 2 of *Saccharomyces cerevisiae* up to 280 μg/ml, which is structurally and functionally analogous to CaCHS1p. In addition, the compound exhibited weak antifungal activities against *Cryptococcus neoformans* and *Rhizoctonis solani.*

Key words: *Candida albicans*, chitin synthase 1 inhibitor, chemotaxonomy, N,N-bis(2-phenylethyl)urea

Candida albicans is an opportunistic pathogen that can cause serious infections in humans [1]. In healthy individuals, it remains in the oral cavity, gastrointestinal tract, and genitalia. However, it can also colonize and invade various tissues or organs, causing oral and vaginal thrush, and sometimes life-threatening systemic fungal infections, particularly in immunocompromised patients with AIDS, cancer, and after organ transplantation [2]. As the incidence of both mucosal and invasive fungal infections and the occurrence of resistant strains are increasing [3, 4], finding new compounds to treat these infections is important.

The fungal enzyme chitin synthase catalyzes the synthesis of the structural polysaccharide chitin, a β-1,4 homopolymer of N-acetylglucosamine, which is important for cell shape, strength, and viability [5]. Like *Saccharomyces cerevisiae*, *C. albicans* harbors three chitin synthases, encoded by the *CaCHS*1, *CaCHS*2, and *CaCHS*3 genes [6, 7, 8]. The chitin synthase 1 (CaCHS1p), 2 (CaCHS2p), and 3 (CaCHS3p) from *C. albicans* are considered to correspond to *S. cerevisiae* Chs2 (ScCHS2p), Chs1 (ScCHS1p), and Chs3 (ScCHS3p), respectively [2, 7]. CaCHS2p is needed for the repair of damaged chitin, but it is not essential for viability, whereas CaCHS1p and CaCHS3p are required for septum formation and for a large part of the lateral cell wall synthesis, respectively. Chitin synthases are found in all fungal pathogens, but not in humans. Therefore, these enzymes, especially CaCHS1p that is essential for the growth of *C. albicans*, are a potential specific target for antifungal drugs [9].

In the course of a continuous screening program to identify potent chitin synthase inhibitors from natural resources, strong inhibitory activity against CaCHS1p was exhibited by an extract from a fermentation broth of *Streptomyces* sp. A6705. Therefore, this paper describes the isolation, structure determination, and inhibitory activities of the bioactive compound.

The homozygous *chs2 chs3* mutant *(chs2 ::hisG/chs2 ::hisG chs3 ::hisG/chs3 :: hisG)* of *C. albicans* was used for the CaCHS1p activity [7], and the strain was grown in a Sabouraud dextrose medium (Difco Co.) at 30°C. The cell membrane was prepared from the *C. albicans chs2 chs3*

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mutant as described by Orlean [10], and the chitin synthase activity measured according to the method of Choi [11]. For the proteolytic step of chitin synthase 1 of *C. albicans*, the reaction mixtures contained 32 mM Tris-HCl (pH 7.5), 2 mM cobalt acetate, 1.1 mM UDP-[^{14}C]-N-acetylglucosamine (GlcNAc) (400,000 cpm/mol, Dupont, Boston, MA, U.S.A.), 2 µl of trypsin at the optimal concentration (2.0 mg/ml), 20 µl of the membranes, and 14 µl of the test sample in a total volume of 46 µl. The mixtures were incubated for 15 min at 30°C. The proteolysis was stopped by adding 2 µl of soybean trypsin inhibitor solution, and then the tubes were placed on ice. GlcNAc was added to a final concentration of 32 mM, followed by incubation at 30°C for 90 min. The percent inhibition of chitin synthase activity was calculated by subtracting blank values from both the control and the test sample values, as described previously [12].

The chitin synthase 1 activity of *C. albicans* was confirmed using a positive control with polyoxin D (Calbiochem Co., SanDiego, CA, U.S.A.). Each isolated and control compound was solubilized in 25% MeOH and distilled water to make stock solutions (1 mg/ml), and an aliquot (14 µl) of these stock solutions used for each reaction to give a final concentration of 280 µg/ml. The inhibitory activities were represented as average values based on two independent experiments. In addition, to measure the ScCHS1p, -2p, and -3p activities, the membrane preparation and assays were performed using a previously described method [12].

The producing microorganism, strain A6705, 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, and 2.0% agar) and stored in 20% glycerol at -70°C.

One ml aliquot of a spore suspension of strain A6705 in 20% glycerol was cultivated on the YM medium at 25°C. Spores that grew well on the YM medium were inoculated into a 500-ml baffled flask containing a fermentation medium (0.1% yeast extract, 2.0% starch, 0.4% soytone, 0.2% polypeptone, 0.3% pharmamedia, 0.2% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4, 0.3% $CaCO_3$, 0.002% $FeSO_4$, 0.001% $MnCl_2$, 0.001% $ZnSO_4$, 0.0005% $CoCl_2$) and incubated for 3 days at 26°C on a rotary shaker at 150 rpm (radius 7 cm). An aliquot (20-ml) of the culture was then transferred into a 5-l baffled flask containing 1 l of the same medium, and the fermentation carried out for 4 days at 26°C on a rotary shaker.

The structure analyses were performed using ESI-MS (Hewlett Packard 5989A, U.S.A.), NMR (Varian UNITY 300, U.S.A.), and UV spectra (Shimazu UV265, Japan).

The minimum inhibitory concentrations (MICs) were determined by the two-fold serial agar dilution method [13]. The human pathogens *C. albicans*, *C. lusitaniae*, *C. krusei*, and *C. neoformans* were grown on Sabouraud dextrose agar medium, while the plant pathogens *Alternaria kikuchiana*, *Botrytis cineria*, *Colletotrichum lagenarium*, *Fusarium oxysporum*, *Magnaporthe grisea*, and *Rhizoctonia solani* were grown on potato dextrose agar (Difco Co.) medium. All the human and plant pathogens were incubated for 1-7 days at 30°C and 25°C, respectively, and the antifungal activities observed after 1-5 days of incubation.

To identify the genus of actinomycetes strain A6705, the cultural, morphological, physiological characteristics, and chemotaxonomy were performed using the methods of the International *Streptomyces* Project (ISP) and *Bergey's Manual of Systematic Bacteriology* [14]. The color of the aerial mycelium was white to pink, whereas the color of the reverse side was brown to grey-brown depending on the medium used. The spore surface of the strain was found to be knobby, and the long-chain spore was connected as a retinaculum-apertum. 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 (Table 1). From these results, the strain was identified as the genus *Streptomyces* and designated as *Streptomyces* sp. A6705.

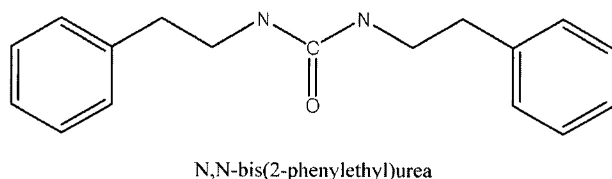
The cells of strain A6705 isolated from 10 l of the fermentation broth were extracted twice with 1 l of acetone at room temperature for 24 h. The extract was then concentrated under reduced pressure, and dissolved with hexane. Thereafter, the hexane layer was evaporated and silica gel column chromatography (Merck, kieselgel 60, 230-400 mesh) applied with a gradient of chloroform-methanol to yield the active fractions (97:3 fraction). Finally, the crude compound was further purified using Sephadex LH-20 (Sigma, Lipophilic LH-20, 25-100 µm).

The molecular formula was determined to be $C_{17}H_{20}N_2O$ on the basis of the EI-mass spectrum (m/z 268[M]$^+$) in combination with the ^{1}H-, ^{13}C-NMR, COSY, HMQC, and HMBC spectral data. Therefore, the compound was determined to be N,N-bis(2-phenylethyl)urea and alkaloid, and the

Table 1. Morphological and physiological characteristics, and chemotaxonomy of isolate A6705.

Characteristics	A6705
Morphology	
Spore size	0.75-1.0×1.0-1.2 µm
Spore chain	Retinaculum-apertum
Spore surface	Knobby
Chemotaxonomy	
Cell wall composition	LL-DAP[a]
Phospholipid fatty acids	Anteiso-$C_{15:0}$(30.05%), iso-$C_{16:0}$(17.15%) Anteiso-$C_{17:0}$(12.42%),iso-$C_{15:0}$(9.11%)
Physiology	
Starch hydrolysis	Positive
Melanine formation	Positive
Decomposition	
Adenine	Positive
Casein	Positive
Trypsin	Positive

[a]DAP: Diaminopimelic acid.



N,N-bis(2-phenylethyl)urea

Fig. 1. Structure of chitin synthase 1 inhibitor, N,N-bis(2-phenylethyl)urea, from *Streptomyces* sp. A6705.

spectral data were in good agreement with those previously published for N,N-bis(2-phenylethyl)urea (Fig. 1) [15, 16]. The compound strongly inhibited CaCHS1p activity in a dose-dependent manner with an IC_{50} of 14 µg/ml, and the IC_{50} of the compound represented similar inhibitory activity to that of a well-known chitin synthase inhibitor, polyoxin D. However, the compound exhibited little or no inhibitory activities against ScCHS1p, -2p, and -3p up to the concentration of 280 µg/ml (data not shown).

The antifungal activity of N,N-bis(2-phenylethyl)urea against various pathogens was examined using human and phytopathogenic fungi. The compound showed strong activity against the human pathogen *Cryptococcus neoformans*, whereas it exhibited no antifungal activity at a concentration of 100 µg/ml against *C. albicans*, *C. lusitaniae,* and *C. krusei* (Table 2). The failure of the compound to inhibit the cell growth of the *Candida* species was probably due to the low susceptibility of CaCHS1p to the compound. However, the exact mechanism of the antifungal activity of the compound remains to be investigated. In addition, N,N-bis(2-phenylethyl)urea also exhibited significant antifungal activities against the phytopathogenic fungus *Rhizoctonia solani*, which is the causal agent of sheath blast disease in rice plants, with an MIC of 50 µg/ml. However, the compound showed no activities against other phytopathogenic fungi, including *Alternaria kikuchiana*, *Botrytis cinerea*, and *Fusarium oxysporum*, at a concentration up to 100 µg/ml, indicating that the antifungal activities of the compound

Table 2. *In vitro* antifungal activities of isolated compounds against various pathogenic fungi.

(MIC, µg/ml)

Pathogens	N,N-bis(2-henyl-ethyl)urea	Polyoxin D
C. albicans ATCC 10231	>100	>100
C. lusitaniae ATCC 42720	>100	>100
C. krusei ATCC 6258	>100	>100
C. neoformans ATCC 36556	50	>100
A. kikuchiana	>100	>100
B. cinerea	>100	>100
C. lagenarium	100	>100
F. oxysporum	>100	>100
M. grisea	100	50
R. solani	50	50

The experiment was performed three times with essentially the same results.

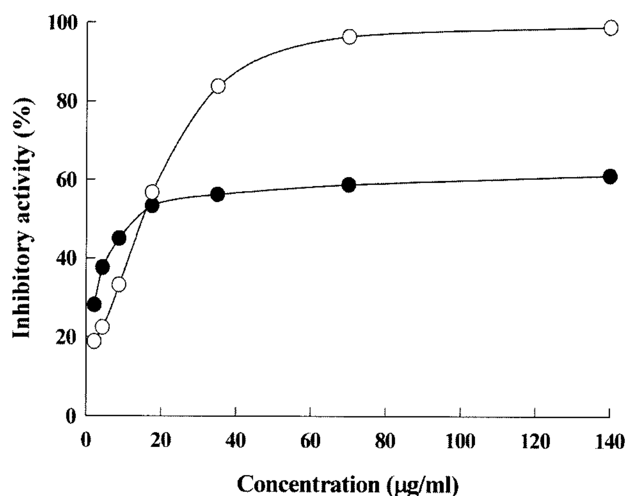


Fig. 2. Effect of N,N-bis(2-phenylethyl)urea on CaCHS1p activity *in vitro*.

The enzyme assay was carried out as described in Materials and Methods. Open circle: N,N-bis(2-phenylethyl)urea; closed circle: polyoxin D.

were caused by the inhibition of cellular targets other than chitin synthase. Considering the fact that N,N-bis(2-phenylethyl)urea may also have other biological activities, such as antidepressant activity [16], in addition to its inhibitory activity towards CaCHS1p, the compound may have multiple target sites.

Several types of compound, such as protoberberine [17], HWY-289 [18], R0-09-3143 [2], and obovatol [12], affecting the chitin synthesis of *C. albicans* have been reported recently. Among them, protoberberine, HWY-289, and R0-09-3143 are chemically synthesized compounds, whereas obovatol is isolated from the medicinal plant, *Magnolia obovata.* In tests, the HWY-289 and R0-09-3143 compounds have shown more potent inhibitory activity against CaCHS1p than against ScCHS2p, while obovatol was found to exhibit similar inhibitory activities against both ScCHS2p and CaCHS1p. However, in this study, N,N-bis(2-phenylethyl)urea only showed inhibitory activity against CaCHS1p, and its structure was found to be entirely different from that of other chitin synthase inhibitors reported to date. Of note, the compound showed no acute toxicity in mice after an intraperitoneal injection of 100 mg/kg [16].

Accordingly, N,N-bis(2-phenylethyl)urea with substantial inhibitory activity against chitin synthase may serve as a useful lead compound for the development of new antifungal agents through the control of chitin biosynthesis.

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