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Chaetoatrosin A, a Novel Chitin Synthase II Inhibitor Produced by *Chaetomium atrobrunneum* F449

EUI-IL HWANG, BONG-SIK YUN, YOUNG-KOOK KIM[a], BYOUNG-MOG KWON, HONG-GI KIM[b], HYANG-BOK LEE, KYUNG-SOOK BAE[c] and SUNG-UK KIM*

Antibiotics Research Lab., [a] Cardiovascular Research Lab., and [c] Korean Collection for Type Cultures, Korea Research Institute of Bioscience & Biotechnology, P. O. Box 115, Yusung, Taejon 305-333, Korea
[b] Division of Applied Biology, Chemistry and Food Science, Chungnam National University, Yusung, Taejon 305-764, Korea

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Chaetoatrosin A, a novel chitin synthase II inhibitor, was isolated from the culture broth of fungus F449, which was identified as *Chaetomium atrobrunneum* F449. Chaetoatrosin A was purified by solvent partition, silica gel, ODS, preparative TLC, and Sephadex LH-20 column chromatographies, consecutively. The structure of chaetoatrosin A was assigned as 1,8-dihydroxy-3(2-hydroxypropionyl)-6-methoxynaphthalene on the basis of various spectroscopic analyses including UV, IR, mass spectral, and NMR. Its molecular weight and formula were found to be 262 and $C_{14}H_{14}O_5$, respectively. Chaetoatrosin A inhibited chitin synthase II by 50% at the concentration of 104 μg/ml in an enzyme assay system. This compound showed antifungal activities against *Rhizoctonia solani*, *Pyricularia oryzae*, *Botrytis cinerea*, *Cryptococcus neoformans* and *Trichophyton mentagrophytes*.

Chitin, the β-(1,4)-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc), is an important structural component of the cell walls of nearly all pathogenic and phytopathogenic fungi and plays a major role in the determination of cell morphology[1~3]. Its synthesis constitutes a model for studying fungal morphogenesis and is a potential target in manipulating fungal growth[4]. Chitin is synthesized by chitin synthase I, II, and III in *Saccharomyces cerevisiae*[1~5]. Chitin synthase I is a nonessential repair enzyme of damaged chitin[5~7]. Chitin synthase II is an essential enzyme for primary septum formation and cell division[7~10], whereas chitin synthase III is responsible for chitin in the ring at bud emergence and in the lateral cell wall[11~13]. Therefore, specific inhibitors of chitin synthase II and III might block the formation of fungal cell wall, and could be used as effective antifungal agents.

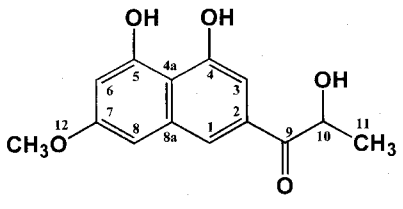
During our continuing search for potent inhibitors of chitin synthase II from natural sources[14,15], we isolated a new compound, chaetoatrosin A (Fig. 1) from the culture broth of *Chaetomium atrobrunneum* F449. In this paper, we report the taxonomy and fermentation of the chaetoatrosin A-producing strain, isolation, structure determination, and biological activity of chaetoatrosin A.

Materials and Methods

Producing Organism and Taxonomy

The producing microorganism, strain F449, was isolated from soil collected at mountain Odae, Gangwon province, Korea. The strain was examined mainly according to

Fig. 1. Structure of chaetoatrosin A.



DE HONG and GUARRO[16]. For the evaluation of cultural characteristics, the strain was incubated in CYA (yeast extract 0.5%, $NaNO_3$ 0.3%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $ZnCl_2 \cdot 7H_2O$ 0.001%, sucrose 3.0%, agar 1.5%), CYA20S (CYA medium with sucrose increased to 20%), Czapek ($NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, sucrose 3.0%, agar 1.5%), MEA (malt extract 2.5%, agar 1.5%), MEA1C (MEA medium with cellulose added to 1.0%), PDA (Difco Co.), YMA (yeast extract 0.3%, malt extract 0.3%, tryptone 0.5%, agar 1.5%) media, for 14 days at 25°C to 42°C. Morphological observation was carried out using a light microscope (Nikon, EFD3). The color names used were based on the ISCC-NBC Color-Name Chart Illustrated with Centroid Colors.

Spectral Analysis

The UV spectrum was recorded on a Shimadzu UV265 UV-Visible spectrophotometer. pH was measured with an Orion research digital pH/millivolt meter 611 and Ross electrode. EI and HREI mass spectra were recorded on a Hewlett Packard 5989A and JMS SX-102X, respectively. ^1H- and ^{13}C-NMR spectra were obtained on a Varian UNITY 500 spectrometer using CD_3OD. Chemical shifts are given in ppm using TMS as internal standard.

Chitin Synthase II and III Assay

The strain used in this study is a *Saccharomyces cerevisiae* ECY38-38A (*MATa chs1-23 chs2::LEU2 cal1/csd2 ura3-52 trp1-1 leu2-2* pAS6), which can only overexpress the chitin synthase II, that was grown in YPG (yeast extract 1.0%, peptone 2.0%, galactose 2.0%).

The cells suspended in 50 mM Tris-HCl (pH 7.5) containing 5 mM magnesium acetate were broken by vortex mixing with glass beads[17]. And the cell walls were sedimented at 3,000×*g* for 5 minutes and supernatant fluid was centrifuged at 130,000×*g* for 45 minutes. The membrane pellet was resuspended in the 50 mM Tris-HCl (pH 7.5) containing 33% glycerol used in the breakage, to a final volume of 1.6 ml/g (wet weight) of cells used. The activity of chitin synthase II was measured by the described procedure[17]. For the proteolytic activation step, reaction mixtures contained 32 mM Tris-HCl (pH 8.0), 1.6 mM cobalt acetate, 1.1 mM UDP-[^{14}C]-GlcNAc (400,000 cpm/mmol, NEN), 2 ml of trypsin at the optimal concentration for activation (2.0 mg/ml), 20 μl of membrane suspension, and 14 μl of samples in a total volume of 46 μl. The mixtures were preincubated for 15 minutes at 30°C. Proteolysis was stopped by adding 2 μl of a soybean trypsin inhibitor (4.0 mg/ml) at a concentration 2 times that of trypsin used, and mixtures were placed on ice for 10 minutes. GlcNAc was added to a final concentration of 32 mM and incubation at 30°C was carried out for 90 minutes. The insoluble chitin formed was assayed by measurement of radioactivity after addition of 10% trichloroacetic acid and filtration through glass fiber filter (GF/C, Whatman). The concentration of protein was measured by the method of LOWRY[18]. For chitin synthase III activity, the membrane was prepared from *Saccharomyces cerevisiae* ECY38-38A (*MATa chs1-23 chs2::LEU2 cal1/csd2 ura3-52 trp1-1 leu2-2* pWJC6), which can only overexpress the chitin synthase III under the control of the *GAL1* promoter[19]. The membrane from 1 g of cells was homogenized with 2 ml of 50 mM Tris-HCl (pH 7.5) containing 5 mM magnesium acetate and 2% Tergitol Nonidet P-40 (TNP40). Homogenized membranes were pelleted by ultracentrifugation at 130,000×*g* for 45 minutes. The pellet was suspended in 1.6 ml of 50 mM Tris-HCl (pH 7.5) containing 5 mM magnesium acetate and 33% glycerol[12], and the assay was performed the same as chitin synthase II except that 32 mM Tris-HCl (pH 7.5) and 4.3 mM magnesium acetate[17]. Blank values were measured with addition of 25% aqueous MeOH instead of both enzyme and sample. Percent inhibition of chitin synthase II activity was calculated by substracting the blank values from both control and test sample values.

$$\% \text{ Inhibition} = \left[1 - \frac{\text{Sample (cpm)} - \text{Blank (cpm)}}{\text{Control (cpm)} - \text{Blank (cpm)}}\right] \times 100$$

The chitin synthase II and III activities of the enzyme were confirmed by positive control with polyoxin D and nikkomycin Z (Calbiochem Co.), respectively.

Antifungal Activity

Human pathogenic fungi were grown on Sabouraud's agar medium, and plant pathogenic fungi were grown on Potato dextrose agar medium. Antifungal activity was tested using agar dilution method[20] and was observed after a 24-hour incubation at 30°C for yeasts and a 48-hour incubation for fungi, respectively.

Results

Taxonomy of the Producing Organism

The cultural characteristics of the strain F449 after incubation on six agar media at 25°C to 42°C for 14 days are summarized in Table 1. The optimal temperature for

Table 1. Cultural characteristics of fungus F449 on several media.

Media	Colony size(mm)[a]/℃ /25	/30	/37	/42	Color of mycelium[b]	Color of reverse side[b]
CYA	35	26	9	-	Pale orange yellow	Deep yellowish brown
CYA20S	32	29	9	-	Pale orange yellow	Deep yellowish brown
Czapek	24	32	13	-	Yellowish gray	Yellowish gray
MEA	19	24	10	-	Pale orange yellow	Strong orange yellowish brown
MEA1C	23	23	11	-	Pale orange Yellowish gray	Strong brown
PDA	18	27	11	-	Pale orange Yellowish gray	Strong brown
YMA	28	27	11	-	White	Deep reddish brown

[a]Colony size measured after 14 days.

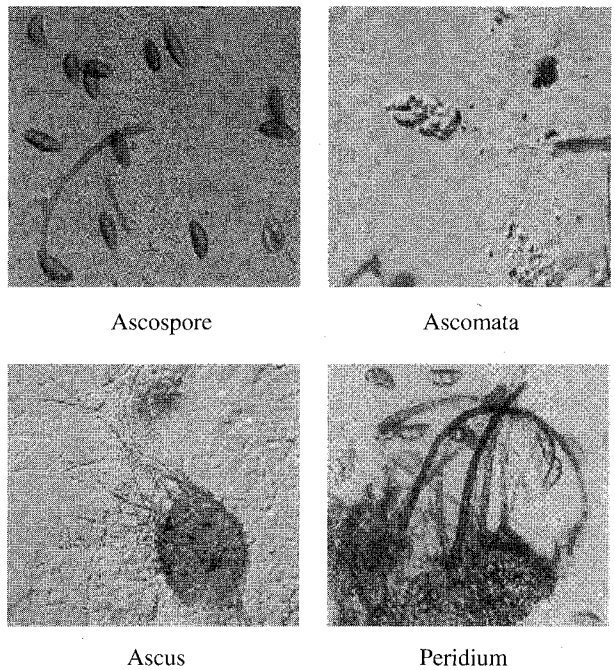
[b]ISCC-NBC Color-Name Chart illustrated with centroid colors, U. S. Dept. of Comm. Suppl. to cir. 553. Washington, D. C., 1976

growth of the strain F449 was between 25°C to 30°C. The mycelium was pale orange yellow to pale orange yellowish gray and the reverse side was deep yellowish brown to deep brown in color.

As shown in Fig. 2, morphological observation was carried out using a light microscope. The strain F449 formed a ostiolate with long hairs (setae) which color of olivaceous or black and peridium as *Textura angularis*. This ascus was cleaved and consist of 8 ascospores. The ascospore exhibited elongate pyriform or greyish fusiform. Ascomata was globose to subglobose, 40~70 μm, with straight and septate.

Based on the taxonomic data of known genus *Chaetomium* in Atlas of clinical fungi by De HONG and GUARRO[16], this strain F449 was closely related to *Chaetomium atrobrunneum*. Therefore, this strain was identified as *Chaetomium atrobrunneum* and designated as *Chaetomium atrobrunneum* F449. It was deposited in the Korean Collection for Type Culture (KCTC), as KCTC 0612BP.

Fig. 2. Light micrographs of *Chaetomium atrobrunneum* F449 (magnification : 800×).



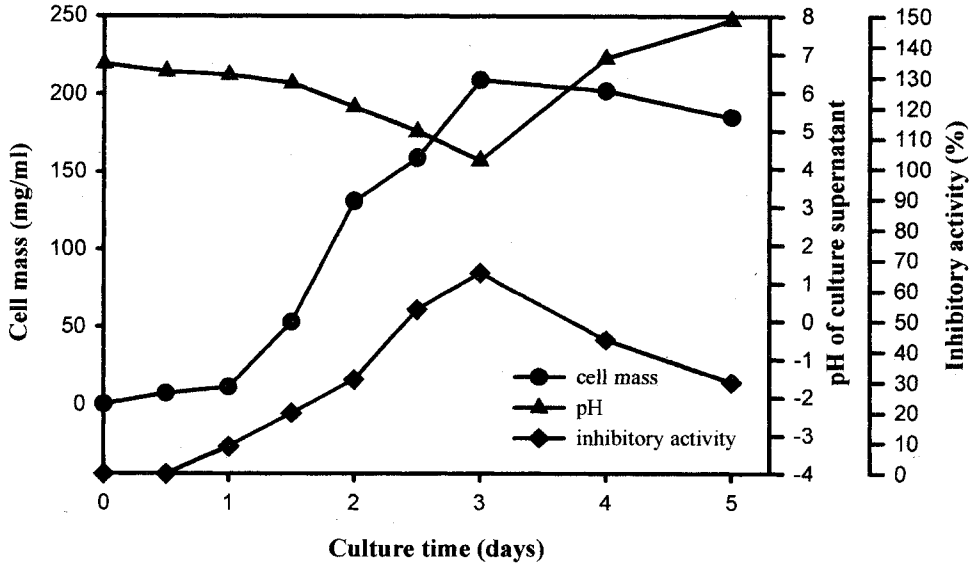
Cultivation was carried out at 30°C for 14 days in MEA medium.

Fermentation

A vial of the fungal spore suspension stocked at −80°C was thawed and inoculated into a 500 ml baffled flask containing of seed medium (glucose 0.5%, soluble starch 1.5%, yeast extract 0.2%, polypeptone 0.5%, KH_2PO_4 0.1%

Fig. 3. Time course of chaetoatrosin A production by *Chaetomium atrobrunneum* F449.

C. atrobrunneum F449 was cultivated at 26°C in 50 ml YM medium.



and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 5.8 prior to autoclaving). Distilled water was used in preparation of seed and production media. The seed culture was incubated for 4 days at 26°C on a rotary shaker at 150 rpm (radius 7 cm). Twenty ml of the seed culture was transferred into a 5-liter baffled flask containing 1 liter of production medium (YM medium; yeast extract 0.3%, malt extract 0.3%, tryptone 0.5%). The fermentation was carried out for 5 days at 26°C on a rotary shaker at 150 rpm. To determine the cell mass, mycelia were collected by filtration using Whatman No. 1 filter paper, then washed twice with distilled water and oven dried to a constant weight. The fermentation was carried out by monitoring chitin synthase II inhibitory activity. A typical time course of chaetoatrosin A production is shown in Fig. 3. The inhibitory activity for chitin synthase II started after 1 day and reached a maximum after 3 days of cultivation. Also, the cell mass reached a maximum after 3 days of fermentation.

Isolation

The fermentation broth of *C. atrobrunneum* F449 (10 liters) was filtered with Whatman No.2 filter paper. The filtrate was extracted twice with 10 liters of ethyl acetate and partitioned between ethyl acetate and water. The ethyl acetate layer was evaporated and applied to a column of silica gel (Merck, Kieselgel 60, 230~400 mesh) and eluted

Fig. 4. Purification steps of chaetoatrosin A from *Chaetomium atrobrunneum* F449.

Fermentation broth of
Chaetomium atrobrunneum F449(10L)
↓ Extracted with EtOAc
EtOAc extract
↓
Silica gel column chromatography
↓ $CHCl_3$:MeOH, 97:3(v/v)
C_{18} column chromatography
↓ MeOH:H_2O, 50:50(v/v)
Silica gel column chromatography
↓ $CHCl_3$:MeOH, 49:1(v/v)
Preparative silica gel TLC
↓ $CHCl_3$:MeOH:*i*-PrOH, 8:4:1(v/v)
Sephadex LH-20 column chromatography
↓ Eluted with MeOH
Chaetoatrosin A (5 mg)

Table 2. Physico-chemical properties of chaetoatrosin A from *Chaetomium atrobrunneum* F449.

Properties	Chaetoatrosin A
Appearance	Yellow powder
EI-MS (*m/z*)	262 [M^+, 51%], 217[100%], 189[80%]
HREI-MS (m/z) found.	262.0841
Calcd.	262.0841
Molecular formula	$C_{14}H_{14}O_5$
$[\alpha]^{25}_D$	0 (*c* 0.02, MeOH)
UV λ_{max}^{MeOH} nm(log ε)	408(2.40), 272(3.21), 225(3.38)
IR νcm^{-1} (KBr, disk)	3400, 2921, 2851, 1500-1750, 1386, 1200, 1157
Rf value[a]	0.33
Soluble	EtOAc, MeOH, DMSO, H_2O
Insoluble	$CHCl_3$, *n*-hexane, CH_2Cl_2, EtOH, CH_3CN, *i*-PrOH, Acetone

[a]On the TLC plate (Merck, Kieselgel 60, 230-400mesh), *n*-hexane:EtOAc:*i*-PrOH(8:4:1, v/v).

with chloroform-methanol, 97:3 (v/v) to give active fractions. The active fractions were combined and concentrated *in vacuo* yielding an yellow brown residue. The residue was isolated on an ODS column (Merck, Lichroprep RP-18, 40~63 μm) eluting with 50% aqueous methanol. And then the active fractions were further isolated on silica gel column with chloroform-methanol, 99:1 (v/v). The crude chaetoatrosin was further purified by preparative silica TLC developed with chloroform-methanol-isopropyl alcohol, 8:4:1 (in volume) and by Sephadex LH-20 (Sigma, Lipophilic LH-20, 25~100 μm) column with methanol. The active fraction was collected and concentrated *in vacuo* to give a yellow powder of pure chaetoatrosin A (5 mg) (Fig. 4).

Physico-chemical Properties and Structure

The physico-chemical properties of chaetoatrosin A are summarized in Table 2. Chaetoatrosin A was obtained as yellow powder and showed a Rf value of 0.35 in *n*-hexane: EtOAc: *i*-PrOH, 8:4:1 (in volume). Chaetoatrosin A was readily soluble in ethyl acetate, methanol, dimethyl sulfoxide, and water whereas insoluble in chloroform or *n*-hexane. The UV spectrum (in methanol) exhibited three absorption maxima at 225, 272, and 408 nm, suggesting that chaetoatrosin A had a naphthalene moiety. The IR spectrum suggested the existence of a hydroxyl (3400 cm^{-1}) and carbonyl (1750 cm^{-1}) group. The molecular formula was established as $C_{14}H_{14}O_5$ by HREI-Mass spectrum (found *m/z* 262.0841, calcd *m/z* 262.0841). The structure determination was carried out by analyses of ^1H-, ^{13}C-NMR, COSY, HMQC and HMBC. This compound was unstable in $CHCl_3$ and MeOH at room temperature, therefore, we measured all NMR spectral at low temperature of 8°C to delay decomposition.

In the ^1H-NMR spectrum (CD_3OD, 500 MHz), one methyl (δ_H 1.34), one oxygenated methine (δ_H 5.19), one methoxy proton (δ_H 3.77) and four aromatic methine protons (δ_H 6.39, δ_H 6.79, δ_H 6.93, δ_H 7.79) were observed. The ^{13}C-NMR (CD_3OD, 125 MHz) spectrum showed fourteen carbon signals, which were assigned as two methyls, five methines, and seven quarternary carbons by DEPT spectra. The ^1H-^1H COSY spectrum established three partial structures containing two correlations with *meta*-coupling between δ_H 6.39 and δ_H 6.79, and δ_H 6.93 and δ_H 7.79 together with a correlation between methyl proton at δ_H 1.34 ppm and oxygenated methine proton at δ_H 5.19. The ^{13}C-^1H connectivities were established by HMQC

Table 3. ^{1}H- and ^{13}C-NMR spectral data of chaetoatrosin A from *Chaetomium atrobrunneum* F449.

No	^{1}H-NMR (δ_H, J=Hz)	^{13}C-NMR (δ_C, ppm)
1		156.7
2	6.93 (1H, d, 2.0)	104.5
3		134.4
4	7.79 (1H, d, 1.5)	121.8
4a		138.5
5	6.79 (1H, d, 2.5)	100.4
6		160.9
7	6.39 (1H, d, 2.0)	103.8
8		157.1
8a		114.1
9		203.7
10	5.19 (1H, q, 7.0)	69.6
11	1.34 (3H, d, 7.0)	21.0
12	3.77 (3H, s)	55.3

· Measured in CD_3OD. s; singlet, d; doublet, q; quartet.

(Heteronuclear multiple quantum coherence) experiment as summarized in Table 3. Gross structure of this compound was determined by the HMBC (Heteronuclear multiple bond connectivity) experiment optimized for 8 Hz. The methyl protons (δ_H 1.34) showed long-range couplings to the carbonyl carbon (δ_C 203.7) and oxygenated methine carbon (δ_C 69.6). In addition, two aromatic methine protons (δ_H 6.93, δ_H 7.79) *meta*-coupled each other correlated to carbonyl carbon (δ_C 203.7) establishing the attacted position of hydroxy propionyl group to C-2 (Fig. 5). The HMBC peaks observed from four aromatic methine protons unambiguously revealed the presence of naphthalene moiety in chaetoatrosin A and assigned the structure of chaetoatrosin A as 1,8-dihydroxy-3-(2-hydroxypropionyl)-6-methoxynaphthalene, as shown in Fig. 1.

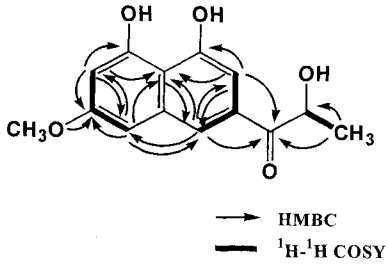
Biological Activity

Inhibition of Chitin Synthase II by Chaetoatrosin A

The chaetoatrosin A inhibited chitin synthase II in a dose-dependent manner on the concentration up to 280 μg/ml. The IC_{50} value of chaetoatrosin A for chitin synthase II was 104 μg/ml, representing 1.5 times weaker

Fig. 5. ^{1}H-^{1}H COSY and HMBC experiments of chaetoatrosin A (CD_3OD).

Arrows are directing H to C.



inhibitory activity than that of polyoxin D, the well-known chitin synthase II inhibitor (IC_{50}=70 μg/ml) (Fig. 6). But chaetoatrosin A did not exhibit inhibitory activity against chitin synthase III from *S. cerevisiae* ECY38-38A (pWJC6) (Data not shown).

Antifungal Activity

Antifungal activities of the chaetoatrosin A against plant pathogen *Rhizoctonia solani* was strong (MIC=50 μg/ml), which was the same as that of polyoxin D, whereas it exhibited very weak antifungal activity against human pathogens, such as *Trichophyton mentagrophytes*, and *Cryptococcus neoformans* (MIC=100 μg/ml). This compound exhibited no antifungal activity at a concentration of 100 μg/ml against *Candida albicans*, *C. krusei*, *Coccidioides immitis*, *Colletotrichum lagenarium*, *Fusarium oxysporum*, and *Phytophthora capsici* (Table 4).

Discussion

Much attention has been paid to the chitin biosynthesis as an ideal target for the search of novel antifungal agents. While chitin synthesis is an essential function in fungi, the presence of multiple isozymes adds a level of complexity. In fact, recent studies of the yeast and fungi chitin synthases by immunofluorescence microscopy and genetic engineering demonstrated that the localization and expression of chitin synthase II and III are regulated in distinct manners[9~12]. Therefore, while it is possible that specific inhibitors of chitin synthase II and III can be used as potential antifungal agents, the potency of an inhibitor may depend on the relative effectiveness of isozymes in

Fig. 6. Inhibition of chitin synthase II by chaetoatrosin A.

●, Polyoxin D; ○, chaetoatrosin A.

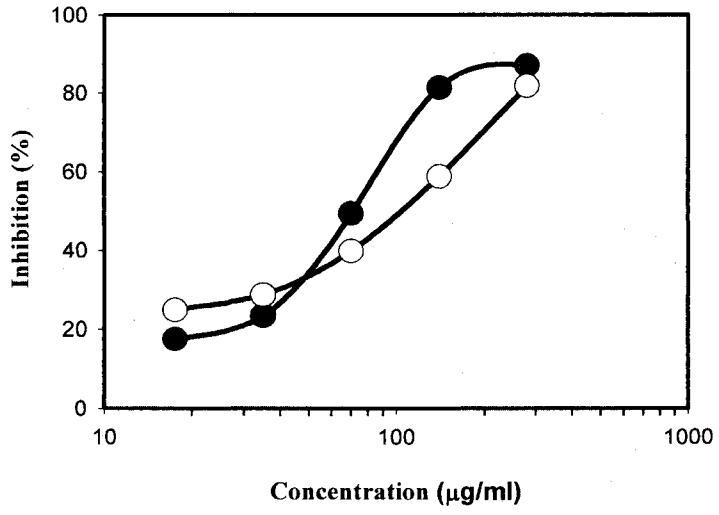


Table 4. The antifungal activities of chaetoatrosin A against various human and plant pathogens.

(Unit : μg/ml)

Pathogens		Chaetoatrosin A	Polyoxin D
Human	*Candida albicans* ATCC 10231	>100	>100
	C. krusei ATCC 6258	>100	>100
	Coccidioides immitis ATCC 34020	>100	>100
	Cryptococcus neoformans ATCC 36556	100	>100
	Trichophyton mentagrophytes ATCC 9533	100	>100
Plant	*Botrytis cinerea*	100	>100
	Colletotrichum lagenarium	>100	>100
	Fusarium oxysporum	>100	>100
	Phytophthora capsici	>100	>100
	Pyricularia oryzae	100	100
	Rhizoctonia solani	50	50

building a cell wall as well as its affinity to a given isozymes[21].

Recently several types of compounds affecting the chitin synthesis other than polyoxin and nikkomycin, the well known chitin synthase inhibitors[22], have been discovered; for example, protoberberine[23], which inhibits chitin and sterol biosynthesis of *Candida albicans*, and catechin[14] and ursolic acid[15], which inhibit chitin synthase II of *S. cerevisiae*.

In the course of our continuous screening program to find a potent chitin synthase II inhibitors from microorganism, we have isolated a novel compound, chaetoatrosin A, possessing the naphthalene skeleton from *Chaetomium atrobrunneum* F449, as an inhibitor of chitin synthase II responsible for the synthesis of fungal cell wall. The structure of this compound is not similar to those of

chitin synthase inhibitors previously reported such as polyoxins and nikkomycins[22]. Chaetoatrosin A is slightly less active than the polyoxin D for chitin synthase II inhibitory activity as shown in Fig. 6, but antifungal activity of chaetoatrosin A is similar to that of polyoxin D against plant pathogen *Rhizoctonia solani*. This compound did not exhibit inhibitory activity against chitin synthase III at the concentration of 140 μg/ml. Consequently, chaetoatrosin A is a specific inhibitor for chitin synthase II. Therefore, we consider that chaetoatrosin A may be a useful lead compound for development of new antifungal agents through the control of chitin biosynthesis. The structural modification and synthesis of chaetoatrosin A and its analogues are now in progress.

Acknowledgments

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