COMMUNICATIONS TO THE EDITOR

A Novel Screen for the Detection of Chitin Acting Antifungal Compounds

Sir:

In the course of our antifungal screening programme, we have devised an efficient screening system for chitin synthesis inhibitors. Considering this as a therapeutic target, the fungal cell wall has a promising unique layer that fulfills the criteria for selective toxicity[1]. The major structural cell wall components of many medically important yeasts, filamentous fungi, and dimorphic fungi consist mainly of glucans and chitin[2]. It is also known that complete depletion of chitin synthesis is lethal to fungi. Thus we have taken a wild type *Saccharomyces cerevisiae* (strain SS 553) and its chitin synthetase I-defective mutant (strain EC 19) as key measures to screen for new antifungal antibiotics against chitin synthesis. We sought for new antifungal agents, which have no or little activity against the wild type strain SS 553 but has greater activity against mutant strain EC 19.

In addition to devising a new screening method, we sought for a unique source of microorganisms. Three hundred strains of ascomycetes isolated from dead stems of dicotyledonous plants were subjected to our screening system and two strains were picked up according to their activity. As shown in Table 1, two strains showed greater activity against the mutant than the wild type. Polyoxin D that is known as a chitin synthesis inhibitor showed a similar activity against our test strains. These producer strains were determined to be *Leptosphaeria* sp. MCI 2799 and *Ellisiodothis inquinans* L1558-A8, respectively. Taxonomic basis of their identification was summarized in Table 2. There were no report concerning the production of chitin inhibitors from these genus.

E. inquinans LI558-A8 was incubated for 3 days in 100 ml of the seed medium containing the following components (g/liter): fructose 30, soybean meal 5, KH_2PO_4 1, $MgSO_4$ 0.5, and $CaCl_2$ 0.1 (pH 6.0) in a Sakaguchi flask. After 3 days of incubation, 1 ml (1%v/v) of the seed culture was transferred into 100 ml of fermentation medium containing the following components (g/liter): glucose 3, glycerol 5, Polypepton 2, yeast extract 3, NaCl 2, and $CaCO_3$ 3 (pH 6.0) in a Sakaguchi flask and incubated at 27°C for 7 days. Culture supernatant was subjected to Diaion HP-20 resin (Mitsubishi Chemical Co., Ltd., Japan) column and silica gel (Wakogel C-300) chromatography and then to preparative thin layer chromatography to obtain partly purified samples. And then, samples were purified further by HPLC (Hitachi HPLC system comprising of a L-7100 pump, a L-3000 photo-diode array detector using Wakosil II 5C18). Purification procedures are summarized in Fig. 1.

The purified substance was subjected to physico-chemical analyses and the product was identified as ascosteroside[3] that was discovered as an antifungal antibiotic, but its action mechanism was first discovered as chitin synthesis as shown in Table 3.

Lepthosphaeria sp. MCI2799 was incubated for 3 days in 100 ml of the seed medium containing the following components (g/liter): fructose 30, soybean meal 20, KH_2PO_4 1, $MgSO_4$ 0.5, and $CaCl_2$ 0.1 (pH 6.0) in a Sakaguchi flask. After 3 days of incubation, 1 ml (1%v/v) of the seed culture was transferred into 100 ml of fermentation medium containing the following components (g/liter): malt extract 35, corn starch 30, corn steep liquor 15, sangrain 5, pharmamedia 15, and $CaCO_3$ 2 (pH 6.0) in a Sakaguchi flask and incubated at 27°C for 5 days. Culture supernatant was treated similarly as in the case of *E. inquinans* LI558-A8, except for some changes in conditions for elution as shown in Fig. 2.

The purified substances (AM-1, 2 and 3) were subjected to physico-chemical analyses as shown in Table 3. Although antibiotics (AM-1, 2 and 3) were not pure enough to determine the chemical structure as yet, all of them showed preferential activity against the mutant

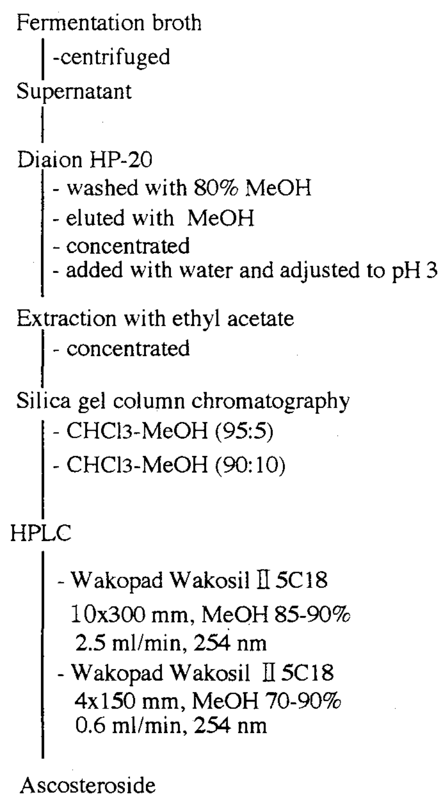
Table 1. The biological activity of culture broth produced from *Ascomyces* strains and polyoxin D.

	Zone diameter (mm)	
	Strain SS553	Strain EC19
Polyoxin D	10.5	29.2
MCI 2799	None	25.6
L1558-A8	None	12.5

Table 2. Morphological characteristics of the producing strains MCI 2799 and L1588-A8.

	MCI 2799	L1588-A8
Habitat:	Occurring on dead stem of dicotyledonous plant, collected at Okinawa Pref.	Occurring on dead stem of dicotyledonous plants collected at Okinawa Pref.
Fruit bodies:	Solitary, immersed in the substratum, with the papillate ostiolum, $230\sim380\,\mu$m in diameter.	$300\sim600\,\mu$m in diameter and $50\sim80\,\mu$m in height.
Peridia:	Thin and soft, $8\sim11\,\mu$m.	Thin and walled, $15\sim20\,\mu$m, $3\sim4$ stratum of polygonal cell, outside dark-brown and inside light-brown in color.
Asci:	Clavate, with along stipe, $120\sim132\,\mu m \times 20\sim24.5\,\mu$m, bitunicate, paraphysate.	Horizontally and/or cylindrical horizontally clavate, $58.5\sim66.0\,\mu m \times 26.4\sim33.0\,\mu$m, short strip, thick-bitunicate, paraphysate, colorless containing 8 ascospores.
Ascospores:	2 or 1 seriate, ellipsoid to slipper-shaped, deep yellowish brown, 3-septate, rarely 4-septate, somewhat constricted, $25.9\sim34.1\,\mu m \times 8.4\sim10\,\mu$m.	1 or 2 seriate, ellipsoid, $15.6\sim18.4\,\mu m \times 9.3\sim11.0\,\mu$m, colorless.

Fig. 1. Isolation and purification scheme of ascosteroside.



yeast as expected and also showed inhibitory activity for chitin synthesis as shown in Table 3. Macromolecular synthesis such as DNA, RNA, and protein were not affected very much with these compounds (data not shown).

In conclusion, our novel screen method using the chitin synthesis-defective mutant was proven very effective to pick up chitin synthesis inhibitor and candidate compounds with selective toxicity toward fungi were discovered.

Experimental

Organisms and Cultivation

Fungal strains were isolated in Okinawa prefecture and were grown at 27°C on potato - glucose agar (PGA) containing the following components (g/liter): potato extract 200, glucose 20, and agar 15 (pH 5.6) was used as a stock medium.

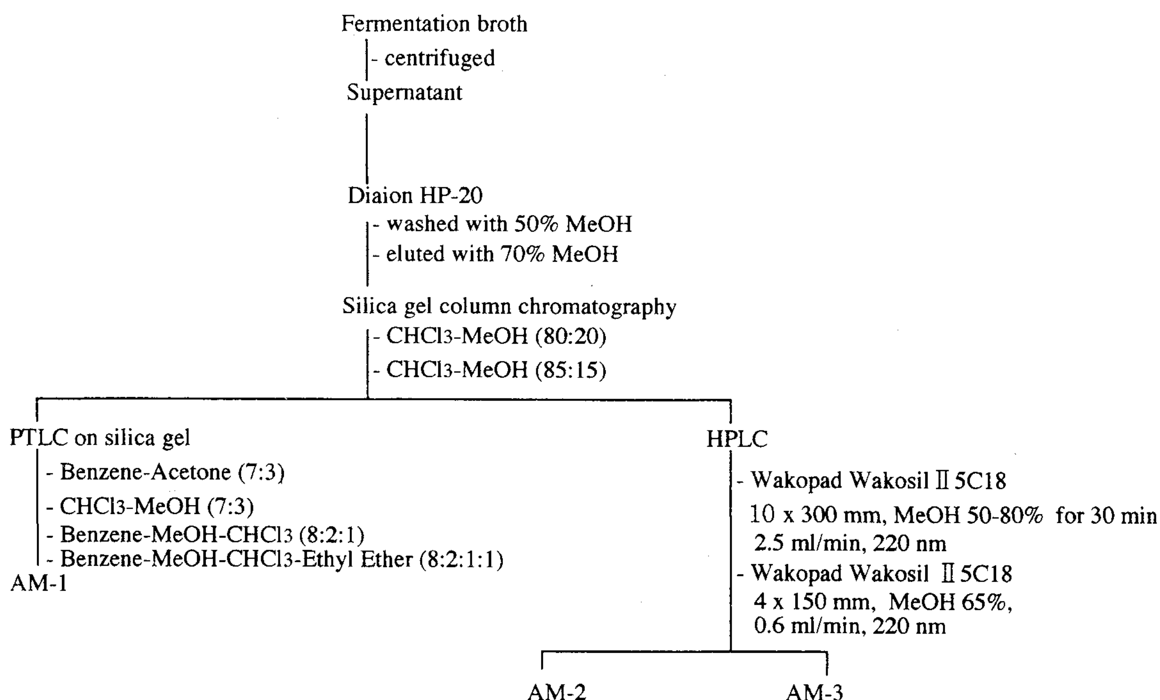
To determine the antibiotic activity of the samples, agar diffusion assay method was conducted using YPD medium containing the following components (g/liter): yeast extract 10, polypepton 20, glucose 20, and agar 15 (pH 7.0). Two strains of *Saccharomyces cerevisiae* strain SS 553 (*MATa*/*MATα*, *leu2*/*leu2*, *ura3*/*ura3*, *CAN*^{s}/*can*^{r}) and strain EC 19 (*MATa*/*MATα*, *leu2*/*leu2*, *ura3*/*ura3*,

Table 3. Properties of isolated compounds.

	Molecular weight	Molecular formula	MIC, against *Saccharomyces cerevisiae* RC19	Chitin synthetase inhibition IC_{50}
AM-1	337	$C_{22}H_{43}NO$	3.0 μg/ml	31 μg/ml
AM-2	303	$C_{21}H_{37}N$	4.5 μg/ml	38 μg/ml
AM-3	638	$C_{40}H_{79}O_4$	NT	NT
Ascosteroside	646	$C_{37}H_{58}O_9$	0.1 μg/ml	7 μg/ml

NT, not tested.

Fig. 2. Isolation and purification scheme of AM-1, AM-2, and AM-3.



trp1/*trp1*, *chs1*/*chs1*) were used as test organisms. These strains are generous gifts from Dr. CABIB[4,5]. Both strains were grown in 10 ml of YPD medium (without agar) in test tubes for overnight at 27°C with shaking. The growth of yeasts was measured by OD_{660}. One ml of each test organism (OD_{660} = 0.80 ~ 1.2 which is about $5.4 \times 10^7 \sim 3.1 \times 10^8$ cells/ml) was added to 200 ml of YPD medium, then poured into a plastic plate. The plate with paper discs was incubated overnight at 27°C for antibiotic assays.

Chitin Synthesis

Chitin synthesis were measured by the procedure of RUIZ-HERRERE and BARTNICKI-GARCIA[6] as incorporation of radioactive from UDP-*N*-acetyl [U-^{14}C]-glucosamine. Chitin synthetase was measured in an incubation mixture containing, unless otherwise stated: 0.5 mM-UDP-[^{14}C]-GlcNAc (0.2 μCi μmol^{-1} [13]), 20 mM-GlcNAc, 0.2 mM-ATP, 10 mM-$MgCl_2$, 50 mM-KH_2PO_4/NaOH buffer (pH 6.0), and 80 μl of prepared chitin synthetase enzyme (protein 9.90 μg/ml) as mentioned above, in a final volume of 0.25 ml. Appropriate amounts of samples were added into the mixture while the control

had the same volume of sterilized water instead. The mixtures were incubated for 30 minutes at 22°C and sampling was done at 15 minutes interval. The reaction was stopped by adding 20 μl glacial acetic acid.

The whole incubation mixture of total volume 0.25 ml was filtered through Whatman GF/C glass-fiber discs (2.4 cm) and washed with about 30 ml of 95% ethanol-1 M acetic acid (20:80, by vol.). The discs were dried in an oven at 70°C and placed in small (30 ml) vials containing 3 ml toluene scintillation fluid. The toluene scintillation fluid consisted of 3 g DPO (2,5-diphenyloxazole), 0.1 g POPOP (1,4-bis[2-(5-phenyloxazolyl)]-benzene) in 1 liter toluene. Radioactivity on each glass-fiber discs was counted in scintillation fluid as mentioned above by an Aloka scintillation counter. Protein was measured with Folin's phenol reagent by Lowry method[7].

Physico-chemical Data

The UV spectrum was recorded with a Milton Roy spectronic 3000 array and IR spectrum was obtained on a Perkin Elmer system 2000 FT-IR. FD-MS and FAB-MS were measured on a JEOL JMS-SX102A mass spectrometer. EI-MS and HREI-MS were measured on a JEOL JMS-AX500 mass spectrometer. ESI-MS was measured using a JEOL JMS-SX102A. The optical rotation was determined with a JASCO DIP-4 polarimeter.

Takako Sakurai
Naowarat Cheeptham
Takashi Mikawa†
Atsushi Yokota
Fusao Tomita*

Laboratory of Applied Microbiology,
Faculty of Agriculture, Hokkaido University,
Sapporo 060-8589, Japan
† Yokohama Research Center, Mitsubishi Chemical Corp.,
Yokohama 226-0000, Japan

(Received March 29, 1999)

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