Characterization and Optimization of *In Vitro* Assay Conditions for (1,3)β-Glucan Synthase Activity from *Aspergillus fumigatus* and *Candida albicans* for Enzyme Inhibition Screening

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(1,3)β-D-Glucan synthase (E.C.2.4.1.34. UDP-glucose: 1,3-β-D-glucan 3-β-glucosyl transferase) catalyzes the polymerization of glucose ([1-3]-β-linkages) using UDP-glucose as substrate. We have determined optimal *in vitro* conditions for the assay of (1,3)β-glucan synthase activity from *Aspergillus fumigatus* and *Candida albicans*. These included lysis of cells in the following for *C. albicans*, 100 mM HEPES, pH 8.0, 10 μM guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS), 2 mM ethylenediaminetetraacetic acid (EDTA), disodium salt, 5 mM NaF, 250 mM sucrose, and 10 mM NaH_2PO_4; and for *A. fumigatus*, 50 mM HEPES, 10 mM EDTA, 750 mM sucrose, 10 mM NaH_2PO_4, 100 mM cellobiose and 50 μM GTPγS. Resulting low-speed supernatants were used as enzyme sources to determine the optimal *in vitro* assay conditions. We have characterized the resulting enzyme activities and tested the optimized assays with known (1,3)β-glucan synthase inhibitors including cilofungin, papulacandin, aculeacin A, and echinocandin B. We have used both optimized assays to screen >1000 extracts of marine macroorganisms and, using bioassay-guided purification, have identified (1,3)β-glucan synthase inhibitors.

During the last three decades there has been a dramatic increase in the frequency of fungal infections, especially disseminated systemic mycoses in immunodeficient hosts[1,2]. Mycoses in compromised hosts are mainly the result of opportunistic infections by organisms that are normally harmless, asymptomatic commensals which can be, under certain conditions, pathogenic[3,4]. Species of *Aspergillus*, *Candida*, *Coccidioides*, *Cryptococcus*, *Histoplasma*, and *Sporothrix* are important causative agents; of these, *Candida* species, especially *C. albicans*, and *A. fumigatus* are the most common. Presently, treatments for fungal infections are limited by few therapeutic options[5]. Current drugs include amphotericin B and a variety of azoles. Unfortunately, amphotericin B is toxic to humans and clinical resistance to azoles is increasing[6]. These observations underscore the clear need for new antifungal therapeutics[7].

The most obvious difference between fungal cells and human cells is that fungal cells are encased in a wall that protects them from an osmotically and, in the case of pathogens, an immunologically hostile external environment. One of the essential steps in fungal wall assembly is the synthesis of (1,3)β-linked glucan and its subsequent incorporation into the cell wall[8,9]. (1,3)β-D-Glucan synthase (E.C.2.4.1.34. UDP-glucose: 1,3-β-D-glucan 3-β-glucosyl transferase) catalyzes the polymerization of glucose ([1-3]-β-linkages) using UDP-glucose as substrate. Enzyme activity is localized to the plasma membrane, does not require either a divalent metal ion or a lipid-linked intermediate, and activity is not zymogenic[7,10,11].

Recently, we published a prototype high throughput screen for (1,3)β-glucan synthase inhibitors using *Neurospora crassa* crude lysates as enzyme sources and optimized *in vitro* enzyme assay conditions[12]. However, the morphologies, virulence, and (1,3)β-glucan synthase

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activities of *N. crassa* (non-virulent), *A. fumigatus* and *C. albicans* differ (this manuscript). We believe that a screen to discover novel (1,3)β-glucan synthase inhibitors to be used in the treatment of *C. albicans* and/or *A. fumigatus* infections should use enzyme activity from each pathogen.

A number of manuscripts have reported various *in vitro* assays for *C. albicans* (1,3)β-glucan synthase activity using microsomal membrane preparations (100,000 *g* particulate fractions) as enzyme sources[8,10,13,14]. None of the current *C. albicans* enzyme assays uses crude lysates or low-speed supernatants and optimized *in vitro* conditions. *A. fumigatus* is a filamentous Ascomycete with a cell wall similar in structure and composition to that of *N. crassa*[15]. Unfortunately, very little is known concerning (1,3)β-glucan synthase activity from *A. fumigatus*, and to our knowledge, there are no screens in place for inhibitors of enzyme activity. Although there are a number of reports concerning (1,3)β-glucan synthase activity of *A. fumigatus*[16~19], there are no reports in which the optimum *in vitro* enzyme assay conditions were systematically determined.

In this paper, we report the optimization of *in vitro* assay conditions for (1,3)β-glucan synthase activity from both *C. albicans* and *A. fumigatus* and characterization of each enzyme activity. In addition, we show that low-speed supernatants are suitable as enzyme sources in high throughput screens for (1,3)β-glucan synthase inhibitors. Finally, we present the results of screening >1000 natural product extracts for enzyme inhibitors.

Materials and Methods

Chemicals

Novozym 234 was obtained from Novo Biolabs (Bagsvaerd, Denmark). Pronase was purchased from Calbiochem (La Jolla, CA). UDP-[^{14}C]glucose was purchased from ICN (Costa Mesa, CA). Peptone, malt extract, yeast extract, Bacto-Peptone and agar were purchased from Difco (Detroit, MI). RPMI tissue culture medium with glutamate and without bicarbonate was purchased from Gibco (Baltimore, MD). Cilofungin was a gift from Eli Lilly and Company (Indianapolis, IN); L-733,560 (a lipopeptide) was a gift from Merck & Co., Inc (Rahway, NJ); papulacandin B was a gift from Professor J. NEUSCH, Ciba-Geigy (Basal, Switzerland); aculeacin A was a gift from Dr. T. SAITOH, Toyo Jozo Co., Ltd. (Tokyo, Japan); and echinocandin B was a gift from Dr. A. VON WARTBURG, Sandoz AG (Basel, Switzerland). Laminarinase (from Mollusk), α-amylase (Type II-A), and all other chemicals were of reagent grade from Sigma Chemical Co. (St. Louis, MO).

Culture Conditions

Candida albicans ATCC strain 44807, a clinical isolate, was obtained from the American Type Culture Collection (Rockville, MD). Freeze-dried cells were rehydrated in PYG medium [1% (w/v) peptone, 0.3% (w/v) yeast extract, 2% (w/v) glucose], transferred to malt extract agar slants [2% (w/v) malt extract, 2% (w/v) glucose, 0.1% (w/v) peptone, 1.5% (w/v) agar] and incubated at 25°C for seven days. Stock agar slants were stored at −20°C. Yeast cells were inoculated from slants into liquid PYG medium (50 ml in 250 ml Erlenmeyer flasks) and incubated at 25°C for 37 hours with rotary shaking (180 rpm). Fresh PYG medium was inoculated with 8.5×10^6 cells/ml, final concentration, and incubated at 25°C for 16 to 18 hours with rotary shaking (180 rpm). Stock inocula were prepared by adding sterile glycerol, 11% (v/v) final concentration, to the cells and placing 5×10^7 cells per tube, freezing immediately in dry ice, and storing at −70°C.

Cell stocks were thawed, inoculated into PYG medium (1×10^6 cells/ml, final concentration) and incubated at 25°C for 16 to 22 hours with rotary shaking (180 rpm). Five hundred milliliters (in 2 liter Erlenmeyer flasks) of Manning and Mitchell's basic salts medium[20] [0.5% (w/v) $(NH_4)_2SO_4$, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 1.4% (w/v) K_2HPO_4, 0.6% (w/v) KH_2PO_4, 0.5% (w/v) NaCl, 1.25% (w/v) glucose, 1×10^{-4}% (w/v) biotin] were inoculated with 1.0×10^7 cells/ml (final concentration) and cells incubated at 25°C with shaking (180 rpm) for 8 hours (mid-log phase). Cells were harvested by centrifugation (10,000 *g*, 5 minutes, 4°C) and protoplasts were released using the procedure of TAFT *et al.*[21]. Protoplasts were harvested by centrifugation (1,500 *g*, 5 minutes, 4°C) and washed twice with ice-cold 1.2 M sorbitol. Protoplast pellets were frozen in dry ice and stored at −70°C.

Aspergillus fumigatus ATCC 16424, a clinical isolate from a human aspergillosis patient, was obtained from the American Type Culture Collection (Rockville, MD). Freeze dried cells were resuspended in 0.3~0.4 ml sterile H_2O, transferred to several 5~6 ml MPG slants [2% (w/v) malt extract, 2% (w/v) glucose, 0.1% (w/v) Bacto peptone, 1.5% (w/v) Bacto agar] and incubated at 32°C for four days. Stock agar slants were stored at −20°C. Conidia were resuspended in 2~3 ml sterile H_2O, transferred to 50 ml MPG solid medium in 250 ml flasks and incubated at 32°C for four days. Agar stock flasks

were stored at −20°C.

Agar stock flasks were thawed and conidia resuspended in 25 ml YG [0.5% (w/v) yeast extract, 1% (w/v) glucose]. Conidial suspensions were used to inoculate liquid YG cultures (200 ml in 1 liter flasks) at 1×10^7 conidia per ml, final concentration, and incubated at 32°C with shaking (250 rpm) until mid log phase (~20 hours). Resulting hyphae were harvested by filtration, washed twice with ice cold water, ~10 mg (wet weight) was aliquoted into 2 ml screw cap tubes, frozen rapidly in dry ice and stored at −70°C.

(1,3)β-Glucan Synthase Assays

C. albicans: Frozen protoplasts were thawed on ice and were lysed in various hypotonic buffers by vortexing two times for 10 s with a 30 s cooling interval on ice. Crude lysates or low-speed supernatants (1,000 *g*, 10 minutes, 4°C) were used as enzyme sources. Reactions were performed in 96-well V-bottom microtiter plates (Dynatech) containing 1 mM UDP-[^{14}C]glucose (0.05 μCi/assay), 50 μg α-amylase, various additions and cell lysate (30~70 μg protein) in a final volume of 26 μl. Reactions were started by the addition of cell lysate to ice-cold reaction mixtures, incubated at 25°C for various times, and stopped by the addition of 50 μl 5% (w/v) trichloroacetic acid (TCA). Reactions were filtered through glass fibre filters (Printed Filtermat A, Wallac) that had been previously washed with 1% (w/v) tetrasodium pyrophosphate and 5% (w/v) TCA using a Milliblot™-D (Millipore) apparatus and washed twice with water[12]. Filters were air dried, wrapped in one layer of Saranwrap and exposed to a Molecular Dynamics phosphor screen. The amount of radioactive glucan bound to filters was quantitated with a Molecular Dynamics PhosphorImager SI. Pixels were converted to nmol glucose incorporated into TCA-insoluble product; the number of cpm per pixel was determined by exposing a reaction mixture to a phosphorscreen and determining the number of cpm in an identical mixture using a liquid scintillation counter. Units of (1,3)β-glucan synthase activity are defined as nmol glucose incorporated into TCA-insoluble glucan per minute incubation at 25°C.

A. fumigatus: Frozen hyphae were thawed on ice and resuspended in various buffers for lysis. Hyphae were disrupted by beating with zirconium beads in a Bead Beater (Bartlesville, OK) for six 30 s cycles with two-minutes cooling intervals on ice. Crude lysates were centrifuged (8,000 × *g*, 5 minutes, 4°C) and resulting supernatants used as enzyme sources. (1,3)β-glucan synthase activity was assayed as described above for *C. albicans* except that reaction mixtures contained 2.5 mM UDP-[U-^{14}C]-glucose (0.02 μCi/mM).

Enzyme kinetics

Enzyme kinetic data were processed using EZ-Fit 4.0 Enzyme Inhibition Analysis[22] for MS Windows 3.1 by Perrella Scientific, Inc. (Amherst, NH). Enzyme inhibitor data were tested against twelve models and the best fit reported.

Product Characterization

The characterization of the radioactive products was conducted as previously described with several modifications[11]. (1,3)β-glucan synthase reaction mixture volumes were increased 10-fold and contained 1 mM UDP-[^{14}C]-glucose (1.0 μ(Ci), 500 μg α-amylase and 300~600 μg cell protein. Reactions were performed in siliconized microfuge tubes, started by the addition of lysate to reaction mixtures, incubated at 25°C for 15 minutes, stopped by boiling 10 minutes and cooled on ice. Reactions were centrifuged (16,000 *g*, 30 minutes, 4°C), pellets washed once with ice-cold 95% (v/v) ethanol, twice with ice-cold water by centrifugation, and incubated at 37°C overnight in 200 μl 50 mM acetate buffer, pH 5.4, containing: a). 2 mg α-amylase; b). 2 mg BSA; c). 2 mg laminarinase; or d). buffer only. Reactions were stopped by the addition of 50 μl 50% (v/v) acetic acid and the incorporation of radioactive glucose into (1,3)β-glucan determined using the Millipore filter method described by GOODAY and DE ROUSSETT-HALL[23].

Protein assays

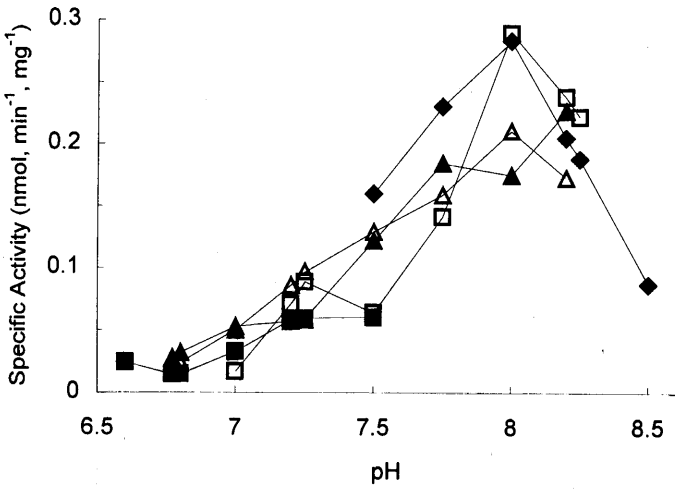
Protein concentrations were determined using BSA fraction V as standard by the method of BRADFORD[24].

Results and Discussion

There are numerous reports in the literature concerning *in vitro* conditions for (1,3)β-glucan synthase activity from a variety of fungi (*e.g.*[8,10~14,25]). Various compounds have been reported to stimulate or stabilize enzyme activity *in vitro*: for example, GTPγS stimulated enzyme activity, likely by activating the GTP-binding subunit, *rho1*p[26,27]; EDTA (or EGTA) stabilized enzyme activity by chelating Ca^{2+} and Mg^{2+} to prevent protease activity; NaF stabilized activity by inhibiting phosphatases; glycerol, sucrose, dithiothreitol or 2-mercaptoethanol, BSA and inorganic phosphate stimulated enzyme activity by unknown mechanisms[8,10,14]. Unfortunately, there are no reports that determined the

Fig. 1. Effect of pH of lysis buffer on *C. albicans* (1,3)β-glucan synthase activity.

Protoplasts were prepared as described in Materials and Methods and were lysed by vortexing in 100 mM Tris (◆), HEPES (□), MOPS (▲), phosphate (△) or PIPES (■) buffer containing 25 μM GTPγS, 3 mM EDTA, and 10 mM NaF adjusted to the indicated pH's.



Reactions contained 1 mM UDP-[^{14}C]glucose (0.05 μCi), 50 μg α-amylase, and crude cell lysate (50 ~ 70 μg protein) in a final volume of 26 μl. Reactions were incubated for 15 minutes at 25°C and the amount of glucan formed determined as described in Materials and Methods. Each data point represents the average of 4 determinations.

optimal *in vitro* conditions of (1,3)β-glucan synthase from either *C. albicans* or *A. fumigatus* crude lysates or low-speed supernatants. Thus, we began to systematically determine the optimal conditions for assay of each enzyme activity.

(1,3)β-Glucan Synthase Activity from *Candida albicans*

Optimal Buffer and pH

To determine the effect of various buffers on (1,3)β-glucan synthase activity, we lysed *C. albicans* protoplasts as described in Materials and Methods in 70 mM Tris, MOPS, PIPES, HEPES, or phosphate buffer over their pK_a ranges. Low-speed supernatants were obtained and the (1,3)β-glucan synthase activity of each supernatant determined as described in Materials and Methods. The results presented in Figure 1 show that the optimal pH for enzyme activity was pH 8.0. Tris and HEPES buffers equivalently produced the highest specific activities for (1,3)β-glucan synthase at pH 8.0. Because the pH of HEPES is more stable at various temperatures than Tris, HEPES was used in subsequent assays. Varying HEPES buffer (pH 8.0) concentration from 25 to 150 mM had no effect on enzyme activity (data not shown).

Buffer Additions

A range of concentrations of GTPγS, EDTA, and NaF were tested for their effects on enzyme activity by varying the concentration of one of the compounds in 100 mM HEPES buffer, pH 8.0, while the concentrations of the other two compounds remained constant (Table 1). Enzyme activity doubled with the addition of 10 μM GTPγS compared to controls without GTPγS, but concentrations of GTPγS from 10 to 80 μM were without further effect (results not shown). Enzyme activity was maximal at 2 mM EDTA and 5 mM NaF; ranges tested were from 0 to 20 mM (results not shown).

Other additions were tested in buffer containing the following: 100 mM HEPES, pH 8.0, 10 μM GTPγS, 2 mM EDTA, and 5 mM NaF (buffer A). If a compound was found to stimulate (1,3)β-glucan synthase activity, it was added at its optimal concentration in buffer used to test subsequent additions. The compounds listed in Table 1 were added individually to (1,3)β-glucan synthase reactions, over a range of concentrations, to determine the optimal concentration of each compound in the lysis/assay buffer resulting in maximum enzyme activity. The high osmolarity of buffers containing ≥ 500 mM sucrose or glycerol prevented lysis of protoplasts by vortexing (not shown), so protoplasts were lysed by sonication. With addition of either sucrose or glycerol at concen-

Table 1. Optimal concentrations of various compounds for maximal *C. albicans* (1,3)β-glucan synthase activity[a].

Addition	Optimal concentration	Concentrations tested
GTPγS	10 μM	10~80 μM
EDTA	2 mM	1~10 mM
NaF	5 mM	5~20 mM
Glycerol	250 mM	50~1000 mM
Sucrose	250 mM	50~1000 mM
EGTA	2 mM	1~10 mM
NaH_2PO_4	10 mM	1~50 mM
KCl	No Effect	1~5 mM
NaCl	No Effect	1~5 mM
$MgCl_2$	No Effect	1~5 mM
BSA	0.4% (w/v)	0.2~1.0% (w/v)

[a] Protoplasts were lysed by vortexing in buffer containing 100 mM HEPES, pH 8.0, and various concentrations of GTPγS, EDTA, NaF, glycerol, sucrose, EGTA, NaH_2PO_4, KCl, NaCl, $MgCl_2$ and BSA as described in Materials and Methods. Reactions contained 1 mM UDP-[^{14}C]glucose (0.05 μCi), 50 μg α-amylase, and cell lysate (30~70 μg protein) in a final volume of 26 μl. Reactions were incubated for 0 and 15 minutes at 25°C, and the amount of glucan formed determined as described in Materials and Methods. Specific activities (nmol glucose incorporated into TCA-insoluble product/minute/mg protein) of lysates were compared to determine the optimal concentration of each addition.

trations up to 250 mM, enzyme activity increased and then plateaued. Buffer containing 250 mM glycerol doubled enzyme activity while the same concentration of sucrose tripled enzyme activity (results not shown). EGTA from 0 to 10 mM was added to either buffer A with 250 mM sucrose or buffer A minus EDTA plus 250 mM sucrose. The addition of EGTA to buffer A containing EDTA had no effect on (1,3)β-glucan synthase activity (results not shown). The addition of EGTA alone in lysis buffer had a similar effect on enzyme activity as the addition of EDTA. Addition of dithiothreitol up to 10 mM in buffer A with 250 mM sucrose slightly inhibited (1,3)β-glucan synthase activity (results not shown). Inorganic phosphate added to buffer A containing 250 mM sucrose increased enzyme activity at concentrations from 0 to 10 mM and slightly decreased activity at concentrations from 10 to 50 mM. KCl, NaCl, and $MgCl_2$ at concentrations up to 5 mM in buffer A with 250 mM sucrose and 10 mM NaH_2PO_4 did not affect enzyme activity. BSA, when added from 0 to 1.0% (w/v) to buffer A containing 250 mM sucrose and 10 mM NaH_2PO_4, increased enzyme activity by ~20% (0.4% [w/v] BSA).

Table 2. Summary of *in vitro* properties of *C. albicans* (1,3)β-glucan synthase activity.

Property	
Linear with time	0~60 minutes
Linear with protein	9~80 μg
Specific activity	1.26±0.16 nmol, min^{-1}, mg^{-1}
Half life at 4°C	2.8±0.6 hours
Lysate activity after freeze/thaw	100%
100% lysate activity, −70°C	>25 weeks
Assay to assay variability	<±10%
(1,3)β-glucan in product	>95%
$K_{m\ app}$	0.4±0.04 mM
$V_{max\ app}$	0.02±0.0008 nmol, min^{-1}

When BSA was added to cell lysates, no effect on enzyme activity was observed (results not shown). Although BSA slightly stimulated enzyme activity, it also interfered with protein quantitation in lysates and subsequent filtration. For these reasons, BSA was not added to buffer in subsequent experiments.

Properties of (1,3)β-Glucan Synthase Activity from *C. albicans*

In order to use the optimized (1,3)β-glucan synthase assay as a screen for enzyme inhibitors, the *in vitro* properties of the enzyme activity were determined. The optimal buffer and additions (*e.g.*, 100 mM HEPES, pH 8.0, 10 μM GTPγS, 2 mM EDTA, 5 mM NaF, 250 mM sucrose, and 10 mM NaH_2PO_4) were used for these experiments. Acid-insoluble radioactive material synthesized using either crude lysate from vortexed protoplasts or 1,000*g* supernatants from bead-beaten protoplasts as enzyme sources was >95% hydrolyzed by (1,3)β-glucanase, showing that the product was (1,3)β-linked glucan (results not shown). Enzyme activity was linear for at least 60 minutes of incubation at 25°C and was linear with protein from 9 μg to 80 μg per assay (15 minutes of incubation-Table 2). These results are similar to previous reports of enzyme activity from microsomal membrane preparations (activity linear for ~75 minutes, and with up to 110 μg protein[10]). (1,3)β-Glucan synthase activity from low-speed supernatants of bead-beaten protoplasts had a specific activity of 1.26 units (mg protein)$^{-1}$ with a standard deviation of ±0.16 (n=6). The enzyme had a half life of 2.8±0.6 hours at 4°C

(results not shown).

Unexpectedly, enzyme activity survived freeze-thawing; cell lysates stored frozen at −70°C for up to 25 weeks retained 100% of the initial enzyme activity (results not shown). This is the first report of a *C. albicans* (1,3)β-glucan synthase enzyme preparation retaining activity after freeze-thawing. A highly purified (>1300-fold) preparation of enzyme from *N. crassa* retained activity after freeze-thawing (Awald, unpublished results), but a crude preparation of *N. crassa* (1,3)β-glucan synthase was inactivated by freeze-thawing (SELITRENNIKOFF and QUIGLEY, unpublished results). The ability to freeze and thaw lysate preparations is especially important in developing a screening assay, since one large batch of lysate may be made and then used for many screening assays making screening easier and more reproducible.

Enzyme Kinetics

The $K_{m\,app}$ of (1,3)β-glucan synthase activity with respect to UDP-glucose was 0.4±0.04 mM which is within the range reported previously (0.2 to 1.9 mM[10,13,14]) and the V_{max} was 0.02±0.0008 nmol, min^{-1}.

Assay reproducibility

C. albicans protoplasts were lysed by bead beating in the optimal buffer, centrifuged at 1,000 *g*, 10 minutes, 4°C and the supernatant used as the enzyme source in 24 replicate assays. The standard deviation of enzyme activity of these replicate assays was 6.8% (results not shown). Three batches of *C. albicans* protoplasts were lysed in the optimal buffer described above and the low-speed supernatant aliquoted, frozen immediately in dry ice and stored at −70°C. The frozen lysates from the three batches of *C. albicans* were used in 22 separate experiments in which the effect of 60 μM cilofungin on enzyme activity was assayed ~60 times. The assay-to-assay variability was ~10% (65%±7% inhibition). A summary of the *in vitro* properties of (1,3)β-glucan synthase activity from *C. albicans* low-speed supernatants is presented in Table 2.

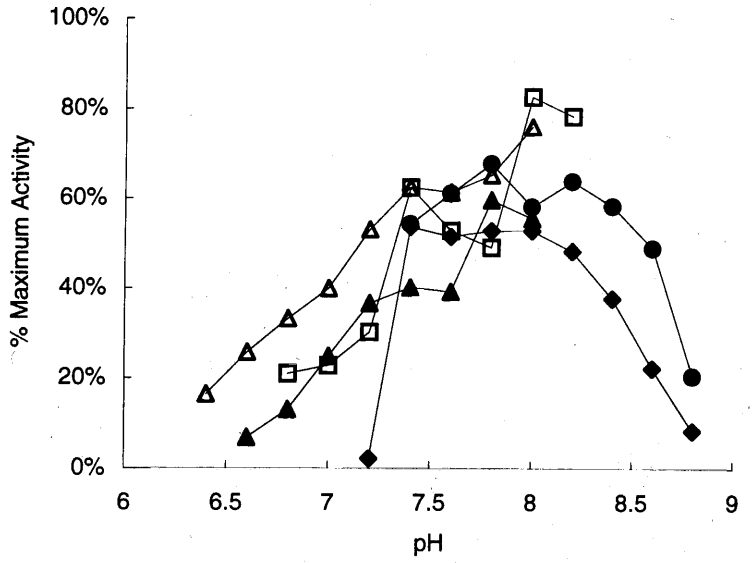
(1,3)β-Glucan Synthase Activity from *Aspergillus fumigatus*

Optimal Buffer and pH

To test the effect of various buffers on enzyme activity, hyphae were lysed and low-speed supernatants were prepared (as described in Materials and Methods) in 50 mM Tris, HEPES, MOPS, Tricine, and phosphate buffers at pHs ranging from pH 6.4~8.8 according to the pK_a ranges of the individual buffers. All buffers additionally contained 1 mM EDTA, 1 M sucrose, and 20 μM GTPγS (no enzyme activity was detected without these compounds-results not shown). Enzyme activity of

Fig. 2. Effect of pH of lysis buffer on *A. fumigatus* (1,3)β-glucan synthase activity.

Hyphae were obtained as described in Materials and Methods and lysed in 50 mM Tris (◆), MOPS (▲), TRICINE (■), HEPES (□) or phosphate (△) buffer containing 20 μM GTPγS, 1 M sucrose, and 1 mM EDTA adjusted to the indicated pH.



(1,3)β-glucan synthase specific activity of each reaction was determined as described in Materials and Methods and the percent maximum enzyme activity calculated. Each data point represents the average of 4 determinations.

each extract was determined as described in Materials and Methods and these results are shown in Figure 2. Note that hyphae lysed in HEPES, pH 8.0, resulted in extracts with the highest enzyme activity. To determine the optimal concentration of HEPES for enzyme activity, the concentration of HEPES, pH 8.0, in the lysis and assay buffer was varied from 25 to 500 mM. The highest specific activity was found using 50 mM HEPES (results not shown).

Buffer additions

The effect of various compounds on enzyme activity was determined using a strategy similar to that used for *C. albicans* glucan synthase activity. Hyphae were lysed in 50 mM HEPES, pH 8.0, containing various concentrations of a number of compounds (Table 3). Enzyme activity increased 185% with the addition of 5 μM GTPγS to lysis buffer. Enzyme activity continued to increase with the addition of GTPγS up to 50 μM (370%), at which point no further stimulation was observed (results not shown). We observed a 3-fold greater stimulation by GTPγS than previously reported for (1,3)β-glucan synthase activity from *A. fumigatus*[16,17]. This increased stimulation of activity is most likely due to lysing cells in the presence of GTPγS instead of adding GTPγS to the assay buffer after cell lysis.

Sucrose and glycerol were tested separately at concentrations ranging from 0~1 M. Enzyme activity increased up to 220% with increasing amounts of sucrose (results not shown). Although enzyme activity was greatest with 1 M sucrose, we chose 750 mM sucrose because the buffer was less viscous and cells lysed completely. Glycerol also increased enzyme activity, but less than observed for the equivalent concentration of sucrose (results not shown). EDTA and EGTA, were tested at concentrations from 0 to 100 mM. EDTA increased enzyme activity by ~350%; EGTA was less effective (results not shown). The optimal concentration of EDTA, 10 mM, was added to buffer for subsequent experiments.

Several other compounds were tested for their effect on (1,3)β-glucan synthase enzyme activity in buffer containing 50 mM HEPES, pH 8.0, 750 mM sucrose, 10 mM EDTA, and 50 μM GTPγS (buffer B). Compounds that significantly stimulated enzyme activity were used at their optimal concentration in subsequent buffers. Compounds listed in Table 3 were added individually over a range of concentrations to buffer B. NaH_2PO_4 increased enzyme specific activity by ~40% at 10 mM. $MgCl_2$, NaCl and KCl were added in concentrations from 0 to 5 mM in buffer lacking EDTA. Each compound increased enzyme activity by ~80% at 1 mM; higher concentrations produced no further stimulation (results not shown). The salts increased enzyme activity, but the addition of EDTA (which increased enzyme activity by ~350%) would chelate them, negating their activating effect. Therefore, salts were not added to lysis buffer in subsequent experiments. Cellobiose[28] added to lysis buffer was varied from 0 to 100 mM. The optimal concentration was 100 mM, which increased enzyme specific activity by 200% (results not shown). NaF was added to buffer B from 0~200 mM. Although it has been reported that NaF increased the specific activity of (1,3)β-glucan synthase from *A. fumigatus* by 72%[16], we found that NaF did not significantly increase enzyme activity (not shown). BSA was tested in 0.2% increments from 0~1% for its effect on enzyme activity. The addition of BSA to either lysis buffer or reactions did not significantly increase enzyme activity (not shown). Sodium tartrate and DTT slightly inhibited enzyme

Table 3. Optimal concentration of various compounds for maximal *A. fumigatus* (1,3)β-glucan synthase activity[a].

Addition	Concentrations tested	Optimal concentration
HEPES	25~500 mM	50 mM
EDTA	0~100 mM	10 mM
Sucrose	0~1 M	750 mM
GTPγS	0~200 μM	50 μM
NaH_2PO_4	0~200 mM	10 mM
Cellobiose	0~100 mM	100 mM
NaF	0~200 mM	Minimal Effect
EGTA	0~100 mM	1 mM
BSA	0~1%	Minimal Effect
Ficoll	0~3%	No Effect
DTT	0~100 mM	Inhibitory
Glycerol	0~1 M	1 M
Sodium tartrate	0~100 mM	Inhibitory
Cations	0~5 mM	1 mM

[a] Hyphae were lysed in buffer containing 100 mM HEPES, pH 8.0, and various concentrations of the indicated compounds as described in Materials and Methods. Reactions contained 1 mM UDP-[^{14}C]glucose (0.05 μCi), 50 μg α-amylase, and cell lysate (30~70 μg protein) in a final volume of 26 μl. Reactions were incubated for 0 and 15 minutes at 25°C, and the amount of glucan formed determined as described in Materials and Methods. Specific activities (nmol glucose incorporated into TCA-insoluble product/minute/mg protein) of lysates were determined.

activity while Ficoll did not stimulate or inhibit enzyme activity (not shown).

Properties of *A. fumigatus* (1,3)β-Glucan Synthase Activity

Based on the above results, the final, optimal buffer for enzyme activity included 50 mM HEPES, pH 8.0, 750 mM sucrose, 10 mM EDTA, 50 μM GTPγS, 100 mM cellobiose, and 10 mM NaH_2PO_4 and was used in subsequent experiments. Acid-insoluble radioactive material synthesized using either fresh or frozen cell-lysates was >97% hydrolyzed by (1,3)β-glucanase treatment, but resistant to α-amylase treatment, indicating that the product was (1,3)β-linked glucan (not shown). Enzyme activity was linear from 0 to 90 minutes and linear with 5~120 μg lysate protein (15 minute incubations). The half-life of enzyme activity at 4°C was ~5 days. Lysates could be stored at −70°C for at least 26 weeks (85% of original activity-results not shown).

Enzyme kinetics

The $K_{m\,app}$ for (1,3)β-glucan synthase activity of *A. fumigatus* was 0.43±0.013 mM and the $V_{max\,app}$ was 18±2.2 nmol, min^{-1}. The $K_{m\,app}$ is within the range of published values (0.4 to 1.9 mM[16,18,19]).

Assay Reproducibility

To determine the assay to assay variability, we prepared cell extracts as described in Materials and Methods and assayed 96 identical reactions. The standard deviation was 12% and was independent of individual laboratory personnel (not shown). The effect of cilofungin on enzyme activity was tested 12 times; the assay to assay variability was 12% (not shown). A summary of the properties of (1,3)β-glucan synthase activity from *A. fumigatus* is presented in Table 4.

Assay Application

In Vitro Enzyme Assays are Sensitive to Known Glucan Synthase Inhibitors

We used the optimal assay conditions determined above to test the effect of several well-characterized inhibitors[7] of (1,3)β-glucan synthase on enzyme activity. We determined the inhibition constants of cilofungin and L-733,560 (MK991) for each enzyme activity as well as IC_{50}s for papulacandin B, aculeacin A and echinocandin B. These results are shown in Table 5 (data for (1,3)β-glucan synthase activity from *N. crassa* are presented for comparison). MK991 was a non-competitive inhibitor[29].

Table 4. Summary of *in vitro* properties of *A. fumigatus* (1,3)β-glucan synthase activity.

Property	
Linear with time	0~90 minutes
Linear with protein	5~120 μg
Specific activity	4.8±1.3 nmol, min^{-1}, mg^{-1}
Half life at 4°C	~5 days
Lysate activity after freeze/thaw	100%
85% lysate activity, −70°C	>26 weeks
Assay to assay variability	±12%
(1,3)β-glucan in product	>97%
$K_{m\,app}$	0.43±0.013 mM
$V_{max\,app}$	18±2.2 nmol, min^{-1}

Table 5. Inhibition constants for (1,3)β-glucan synthase inhibitors[a].

Compound	*C. albicans*		*A. fumigatus*		*N. crassa*[b]	
	$K_{I\,app}$ (μM)	IC_{50}[c]	$K_{I\,app}$ (μM)	IC_{50}[c]	$K_{I\,app}$ (μM)	IC_{50}[c]
Cilofungin	56±7.2	~8	4.8±0.7	nt	13	~20
MK 991	2.3±0.4	nt	15±1.7	nt	nt	nt
Papalucandin B	nt	~8	nt	~0.09	nt	~500
Echinocandin B	nt	~31	nt	~0.2	nt	~4
Aculeacin A	nt	~16	nt	~0.01	nt	nt

[a] *C. albicans* and *A. fumigatus* extracts were prepared and (1,3)β-glucan synthase activity determined as described in Materials and Methods with various concentrations of the above (1,3)β-glucan synthase inhibitors dissolved in water (MK991 only) or DMSO.

[b] Data for *N. crassa* are from TAFT *et al.*[30]

[c] IC_{50} is the concentration that gave ~50% inhibition of enzyme activity compared to control-reported in μg/ml.

nt: not tested.

Table 6. Screening of marine macroorganism extracts for (1,3)β-glucan synthase inhibitors[a].

Glucan synthase inhibition[b]		Number of extracts	Antifungal activity[c]	
C. albicans	*A. fumigatus*		*C. albicans*	*A. fumigatus*
−	−	787	57	64
+	−	106	23	nt
−	+	36	nt	2
+	+	71	29	28

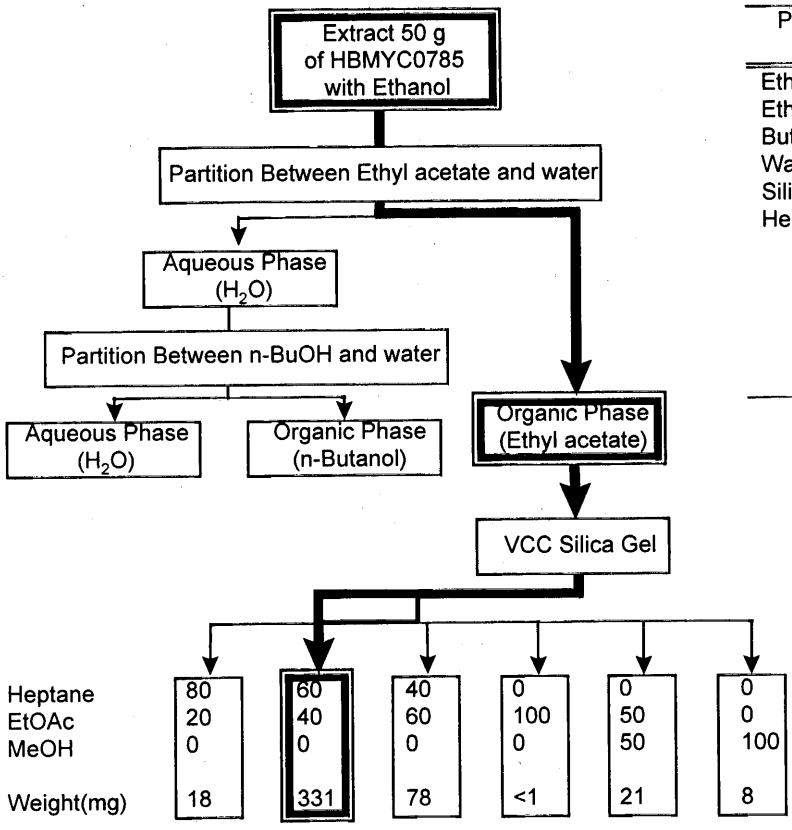
[a] Marine samples were extracted with ethanol, dried and resuspended in DMSO. Extracts were added to lysates of *C. albicans* and *A. fumigatus* (prepared as described in Materials and Methods) and tested for inhibition of (1,3)β-glucan synthase activity.

[b] (−) indicates no significant effect on enzyme activity; (+) indicates >35% inhibition.

[c] Antifungal activity-Samples inhibiting (1,3)β-glucan synthase activity were further tested for inhibition of fungal growth using either microtiter broth or agar diffusion assays[31]. The numbers indicate the number of extracts that inhibited growth of the indicated fungi.

nt: not tested.

Fig. 3. Bioassay guided purification of extract HBMYCO 785.



Purification Fraction	*C. albicans* G.S. IC_{50} (μg/mL)
Ethanol Extraction	~100
Ethyl Acetate Partition	~84
Butanol Partition	>480
Water Partition	>480
Silica Gel VCC Heptane:EtOAc:MeOH	
80 : 20 : 0	~225
60 : 40 : 0	~52
40 : 60 : 0	~60
0 : 100 : 0	~80
0 : 50 : 50	>480
0 : 0 : 100	>480

The ethanol extract of sample HBMYCO 785 was partitioned between ethyl acetate and water and the aqueous phase again partitioned between water and *n*-butanol (unpublished method of WRIGHT *et al.*). The concentration that inhibited *C. albicans* (1,3)β-glucan synthase activity by 50% was determined for the original ethanol extract and the three partitions by testing each fraction at 5 concentrations from 0.8 μg/ml to 480 μg/ml as described in Materials and Methods (see inset). The ethyl acetate partition was further separated by vacuum column chromatography (VCC), fractions collected and each fraction tested for enzyme inhibition. Note that the majority of the inhibitory activity was found in the fraction which eluted with heptane : ethylacetate (60 : 40 [v/v]-see inset).

of both enzyme activities and, consistent with previous results, had a ~6-fold lower $K_{i\,app}$ against glucan synthase from *C. albicans* than that from *A. fumigatus*. In contrast, cilofungin was >10-fold more effective against enzyme activity from *A. fumigatus*. Similar results were found for the remaining inhibitors, *i.e.*, enzyme activity from *A. fumigatus* was significantly more sensitive to each inhibitor. We conclude from these data that the optimal *in vitro* conditions determined above result in (1,3)β-glucan synthase preparations sensitive to known (1,3)β-glucan synthase inhibitors.

Screening of Natural Product Extracts for (1,3)β-Glucan Synthase Inhibitors

We screened over 1000 extracts of marine macroorganisms for inhibitors of (1,3)β-glucan synthase activity from *C. albicans* and *A. fumigatus*. Samples found to inhibit either enzyme activity were further tested for inhibition of growth of *C. albicans* and *A. fumigatus*. A summary of these results is presented in Table 6. Note that 787 samples did not inhibit enzyme activity from either fungus although 121 of these extracts inhibited fungal growth (presumably by acting *via* a mechanism[s] other than inhibition of (1,3)β-glucan synthase activity). In contrast, 71 extracts inhibited both enzyme activities and 29 of these, inhibited the growth of both organisms. Thirty-six extracts inhibited only glucan synthase from *A. fumigatus* and two of these also inhibited *A. fumigatus* cell-growth. One hundred six extracts inhibited (1,3)β-glucan synthase activity from *C. albicans* only and 23 of these inhibited *C. albicans* cell growth. As an important aside, the observation that certain extracts inhibited enzyme activity of one but not both fungi may underscore intrinsic differences in enzyme activity from each fungus.

Bioassay Guided Purification of an Active Extract

Based on the results presented in the previous section, we purified the active component from a number of extracts. Figure 3 presents the fractionation scheme of one extract and the inset shows the results of assay of each fraction. Note that the majority of inhibitory activity was found in the organic phase of the ethyl acetate/water partitioning and that this activity was eluted primarily in the heptane/ethyl acetate 60/40 fraction. Subsequent NMR analysis of this fraction revealed a complex mixture of compounds which were further separated by reversed phase HPLC (not presented). This revealed a family of seven compounds and will be the subject of a subsequent manuscript (WRIGHT *et al.*, in preparation).

Conclusions

We have for the first time developed optimized (1,3)β-glucan synthase assays using both *C. albicans* and *A. fumigatus* extracts which can be used easily for screening samples with potential antifungal activity. By using frozen low-speed supernatants as enzyme sources, the assays are simple and time efficient. These assays are reproducible from assay to assay and from batch to batch of lysate. In its present form, the assay is useful for screening thousands of samples for inhibitors, and with a few modifications, can be developed into a high-throughput assay.

We have used the optimized assay to screen thousands of samples for (1,3)β-glucan synthase activity inhibition and we have discovered a number of (1,3)β-glucan synthase inhibitors (WRIGHT *et al.*, in preparation). Several extracts have been fractionated and the active component(s) identified. In several cases, the enzyme inhibitor was novel and each will be described in subsequent publications.

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References

1) HOEPRICH, P.: Antifungal chemotherapy. Prog. Drug Res. 44: 87~127, 1995
2) SAMONIS, G. & D. BAFALOUKOS: Fungal infections in cancer patients: an escalating problem. *In Vivo* 6: 183~194, 1992
3) RHODES, J.; H. JENSEN, A. NILIUS, C. R. CHITAMBAR, S. G. FARMER, R. G. WASHBURN, P. E. STEELE & T. W. AMLUNG: *Aspergillus* and aspergillosis. J. Med. Vet. Myc. 30: 51~57, 1992
4) SARAL, R.: Candida and aspergillus infections in immunocompromised patients: an overview. Rev. Infect. Dis. 13: 487~492, 1991
5) LORTHOLARY, O. & B. DUPONT: Antifungal prophylaxis during neutropenia and immunodeficiency. Clinical Microbiol. Reviews 10: 477~504, 1997
6) ODDS, F.: Resistance of clinically important yeasts to antifungal agents. Internat. J. Antimicrob. Agents 6: 145~147, 1996
7) KURTZ, M.: New antifungal drug targets: A vision for the future. ASM News 64: 31~39, 1988
8) ORLEAN, P. A. B.: (1,3)-β-D-Glucan synthase from budding and filamentous cultures of the dimorphic fungus

Candida albicans. Eur. J. Biochem. 127: 397～403, 1982

9) RUIZ-HERRERA, J.: Biosynthesis of β-glucans in fungi. Antonie van Leeuwenhoek. 60: 73～81, 1991

10) FROST, D. J.; K. BRANDT, J. CAPOBIANCO & R. GOLDMAN: Characterization of (1,3)-β-glucan synthase in *Candida albicans*: microsomal assay from the yeast or mycelial morphological forms and a permeabilized whole-cell assay. Microbiol. 140: 2239～2246, 1994

11) HRMOVA, M.; C. S. TAFT & C. P. SELITRENNIKOFF: 1,3-β-Glucan synthase of *Neurospora crassa*: partial purification and characterization of solubilized enzyme activity. Exp. Mycol. 19: 153～161, 1989

12) TAFT, C. S.; C. S. ENDERLIN & C. P. SELITRENNIKOFF: A high throughput *in vitro* assay for fungal (1,3)β-glucan synthase inhibitors. J. Antibiotics 47: 1001～1009, 1994

13) ORLEAN, P. A. B. & S. M WARD: Sodium fluoride stimulates (1,3)-β-D-glucan synthase from *Candida albicans*. FEMS Microbiol. Lett. 18: 31～35, 1983

14) TANG, J. & T. R. PARR, Jr.: W-1 solubilization and kinetics of inhibition by cilofungin of *Candida albicans* (1,3)-β-glucan synthase. Antimicrob. Agents Chemother. 35: 99～103, 1991

15) BARTNIKI-GARCIA, S.: Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. 22: 87, 1968

16) BEAVAIS, A.; R. DRAKE, K. NG, M. DIAQUIN & J. LATGE: Characterization of the 1,3β-glucan synthase of *Aspergillus fumigatus*. J. Gen. Microbiol. 139: 3071～3078, 1993

17) BEAULIEU, D.; J. TANG, D. ZECKNER & T. PARR: Correlation of cilofungin efficacy with its activity against *Aspergillus fumigatus* (1,3,)-β-D-glucan synthase, FEMS Microbiol. Letters. 108: 133～138, 1993

18) BEAULIEU, D.; J. TANG, S. B. YAN, J. M. VESSELS, J. A. RADDING & T. R. PARR: Characterization and cilofungin inhibition of solubilized *Aspergillus fumigatus* (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother 38: 937～944, 1994

19) KURTZ, M. B.; I. B. HEATH, J. MARRINAN, S. DREIKORN, J. ONISHI & C. DOUGLAS: Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother 38: 1480～1489, 1994

20) MANNING, M. & T. G. MITCHELL: Strain variation and morphogenesis of yeast- and mycelial-phase *Candida albicans* in low-sulfate, synthetic medium. J. Bacteriology 142: 714～719, 1980

21) TAFT, C. S.; T. STARK & C. P. SELITRENNIKOFF: Cilofungin (LY121019) inhibits *Candida albicans* (1-3)-β-D-glucan synthase activity. Antimicrob. Agents Chemother 32: 1901～1903, 1988

22) PERRELLA, F. EZ-FIT: A practical curve-fitting microcomputer program for the analysis of enzyme kinetic data on IBM-PC compatible computers. Anal. Biochem. 174: 437～447, 1988

23) GOODAY, G. W. & A. DE ROUSSETT-HALL: Properties of chitin synthetase from *Coprinus cinereus*. J. Gen. Microbiol. 89: 137～145, 1975

24) BRADFORD, M. M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248～2541, 1976

25) TKACZ, J.: Glucan biosynthesis in fungi and its inhibition, *In* Emerging targets in antibacterial and antifungal chemotherapy, *Ed.*, J. SUTCLIFFE and N. H. GEORGOPAPADAKOU, pp. 495～523. Chapman and Hall, New York, 1992

26) DRGONOVA, J.; T. DRGON, K. TANAKA, R. KOLLAR, G. C. CHEN, R. A. FORD, C. S. CHAN, Y. TAKAI & E. CABIB: Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. Science 272: 277～279, 1996

27) MAZUR, P. & W. BAGINSKY: *In vitro* activity of 1,3-β-D-glucan synthase requires the GTP-binding protein Rho1. J. Biol. Chem. 271: 14604～14609, 1996

28) QUIGLEY, D. R. & C. P. SELITRENNIKOFF: β-Linked disaccharides stimulate, but do not act as a primer for (1,3)-glucan synthase of *Neurospora crassa*. Current Microbiol. 15: 181～184, 1987

29) CLELAND, W. W.: The kinetics of enzyme catalyzed reactions with two or more substrates or products. Rate equations and I Nomenclature. Biotic. Biophys. Acta. 67: 104～137, 1963

30) TAFT, C. S.; M. ZUGEL & C. P. SELITRENNIKOFF: *In vitro* inhibition of stable 1,3-β-D-glucan synthase activity from *Neurospora crassa*. J. Enzyme Inhibition 5: 41～49, 1991

31) CORMICAN, M. & M. PFALLER: Standardization of antifungal susceptibility testing. J. Antimicrob. Chemother. 38: 561～578, 1996