A HIGH THROUGHPUT *IN VITRO* ASSAY FOR FUNGAL (1,3)β-GLUCAN SYNTHASE INHIBITORS

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(Received for publication April 8, 1994)

An *in vitro* assay for (1,3)β-glucan synthase activity from the filamentous ascomycete *Neurospora crassa*, suitable for use as a high throughput screen for enzyme inhibitors is described. Samples were added to 25 μl reaction mixtures in 96-well V-bottom microtiter plates and plates incubated at 22°C. Reactions were terminated by the addition of TCA and the contents transferred to a Milliblot D apparatus containing Inotech 201-A glass fiber filters. Filters were washed to remove unincorporated substrate and the amount of ^{14}C-labeled (1,3)β-glucan formed by each reaction was quantitated using a Phosphorlmager. As little as 50 ng of an inhibitor with a *Ki* of 4 μM was detected by this assay. This assay is rapid and cost effective, permitting its use as a screen to detect compounds that inhibit (1,3)β-glucan synthase activity.

Human fungal infections are serious and often life-threatening, particularly for immunocompromised patients. For example, the mean survival time of AIDS patients with cryptococcal infections is 8.4 months (NIH meeting; Medical Mycology, June, 1993). Infections are often the result of opportunistic fungi that are usually asymptomatic commensals[1,2]. Fungi of particular importance include: *Candida albicans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Aspergillus fumigatus*, *Sporothrix schenckii*, *Blastomyces dermatitidis*, *Penicillium marneffei*, *Alternaria* sp. and even *Fusarium* sp. Of these, *Candida* infections are the most common.

Treatment of mycotic infections is difficult due to the lack of effective and safe antifungal antibiotics[3~7]. Even after 29 years of use, amphotericin B (a polyene) is the drug of choice to treat systemic fungal infections[6,7]. However, amphotericin B is toxic to humans and therapy is fraught with side effects including renal failure, fever, chills, and hypotension. Imidazoles inhibit fungal growth by inhibiting sterol biosynthesis, but are fungistatic rather than fungicidal; resistance to azoles is becoming an increasing problem as their use increases. Other antifungal agents are available (for example, flucytosine, cilofungin, papulacandin B, aculeacin A), but they are limited by either a narrow spectrum of activity, toxicity, or both.

One striking difference between fungal and human cells is that fungal cells are encased in a wall that protects them from an osmotically and immunologically hostile external environment. The cell wall of fungi has a complex composition and structure and is the subject of a number of recent reviews[8~10]. In general, human pathogenic fungi (both yeast and filamentous forms) contain chitin (GlcNAc polymer), (1,3)β-glucan, other glucans (some even contain cellulose), peptides, lipids, and a small amount of unknown material. For filamentous fungi, growth and cell wall assembly occur only at each hyphal apex[8,10]. That normal cell wall assembly is essential for growth and viability of a large number of fungi has been convincingly demonstrated using cell wall acting drugs and by the analysis of mutants defective in key wall-assembly steps[11,12].

To date, only three enzyme activities have been shown to be essential for cell wall assembly, namely,

chitin synthase, (1,6)β-glucan synthase and (1,3)β-glucan synthase[13~15]. Since mammalian cells lack these carbohydrates, the fungal pathways for their syntheses are attractive targets for antifungal drugs. The recent report that *Pneumocystis carinii* pneumonia in a rat model was treated successfully with a (1,3)β-glucan synthase inhibitor[16] has renewed interest in the discovery and development of new (1,3)β-glucan synthase inhibitors.

(1,3)β-Glucan synthase activity is required for normal cell wall assembly, growth, and development of a number of fungi, including species of *Candida*, *Aspergillus*, and *Neurospora*[10,17~24]. In each case, when the level of (1,3)β-glucan synthesis was reduced either by drug treatment or by mutations that alter the level of substrate or enzyme activity, resulting cell wall assembly and morphogenesis were abnormal, that is, cells either grew poorly or lysed (*e.g.*,[24]).

(1,3)β-D-Glucan synthase (EC.2.4.1.34 UDP-glucose: 1,3-β-D-glucan, 3-β-glucosyl transferase) catalyzes the formation of (1,3)β-linked glucose and UDP from UDP-glucose (K_m value for the enzyme from *Neurospora crassa* was 0.7 mM UDP-glucose[25,26]). Enzyme activity is localized at the plasma membrane[27]. A GTP-binding protein seems to play an important regulatory role and can be dissociated from "core" enzyme activity[28~31]. β-Linked disaccharides are activators while inhibitors include uridine nucleotides, neopeptins, aculeacin A, echinocandin B, gluconolactone, papulacandin B, sorbose and cilofungin[20~23,26,27,32~36]. Unfortunately, none of these inhibitors has proven useful as therapeutic agents due to host toxicity.

In this manuscript, we describe a high throughput, sensitive, and inexpensive *in vitro* (1,3)β-glucan synthase assay that is suitable for large-scale screening of compounds for enzyme inhibitors. The availability of this assay will stimulate further interest in the search for new (1,3)β-glucan synthase inhibitors that will be useful clinically.

Materials and Methods

Chemicals

UDP-^{14}C-Glucose was obtained from ICN (Irvine, CA). Zeta-Probe GT and Bradford Protein Assay[37] reagent were purchased from Bio-Rad Laboratories (Richmond, CA). BioTrace nitrocellulose was from Gelman Sciences, Ann Arbor, MI. Nitrocellulose filter type HAWP, 0.45 μm, 25 mm were from Millipore Corp., Bedford, MA. Glass fiber filters IH-201A, IH-201B, IH-201C, IH-201-A21 and IH-201-AH were from Inotech Biosystems International, Lansing, MI. Cilofungin was obtained from Dr. R. GORDEE of Eli Lilly, Indianapolis, IN. Papulacandin B was a gift from Professor J. NEUSH, Ciba-Geigy, Basel, Switzerland, and echinocandin B was a gift from Dr. A. VON WARTBURG, Sandoz AG, Basel, Switzerland. Tunicamycin, monensin, nikkomycin, penicillin G, gentamicin sulfate, and polymyxin B were from CalBiochem Co., San Diego, CA. Validamycin was a gift of Takeda Chemical Industries, Osaka, Japan. Scintillation fluid (Biodegradable Counting Cocktail, Econo-safe) was from RPI (Mt. Prospect, IL). Other chemicals and reagents were obtained from Sigma Chemical Co (St. Louis, MO). Distilled water was used throughout.

Growth and Harvesting of *Neurospora crassa*

Wild-type *N. crassa* (OR-74-8a) was obtained from the Fungal Genetics Stock Center, Kansas City, KS. Cultures were grown in 250-ml flasks containing 50 ml agar-solidified Vogel's medium N[38] containing 1.5% (w/v) sucrose for 7~10 days at 25°C. Flasks containing macroconidia were stored at −20°C until used.

Flasks were thawed at room temperature and macroconidia harvested with sterile water. Macroconidia were added to 6 liters of Vogel's medium N containing 1.5% (w/v) sucrose ($1 \sim 5 \times 10^6$ conidia/ml, final concentration). Cultures were incubated for 16~18 hours at 22°C with constant stirring and aeration (1~2 liters air/minute). Resulting hyphae were harvested and washed with ice-cold water by vacuum filtration.

Hyphae were frozen in dry ice in 4~5 g aliquots and stored at −70°C until used.

Assay of (1,3)β-Glucan Synthase Activity

(1,3)β-Glucan synthase activity was assayed using modifications of a procedure previously described[25]. Briefly, 4~5 g of hyphae were thawed on ice and 20 ml of 25 mM HEPES (pH 7.4), 10 mM NaH_2PO_4, 600 mM glycerol, 5 mM EDTA, 10 mM NaF and 10 μM GTPγ-S added. Hyphae were disrupted at 4°C using a Bead Beater (Biospec Products, Bartlesville, OK) with 0.5 mm zirconium beads (5 × 30 seconds pulses with 2 minutes cooling between each pulse). Crude lysates were centrifuged at 1,000 × *g* for 10 minutes at 4°C and the resulting supernatants were used as enzyme sources. The BioRad protein assay was used to determine the amount of protein in low-speed supernatants[37].

In vitro reactions contained 50 μg α-amylase (type IIA; Sigma), 1.2 mM UDP-^{14}C-glucose (50,000 cpm/assay), and 15 μl cell extract in a final volume of 25 μl. Reactions were carried out in either glass tubes (6 × 50 mm) or V-bottom 96-well microtiter plates (Dynatec Laboratories, Chantilly, VA) and incubated at 22°C for 20 minutes. Reactions in glass tubes were terminated by the addition of 50 μl 5% (w/v) TCA, followed by the addition of 200 μl water. The amount of radioactive (1,3)β-glucan formed in individual glass tubes was determined by a Millipore filter method[39]. Similarly, individual reactions in microtiter plates were terminated by the sequential addition of 50 μl 5% (w/v) TCA and 200 μl H_2O. The contents of each well were transferred to a Milliblot D apparatus (Millipore Corp.) containing an Inotech 201A glass fiber filter previously washed with 5% TCA containing 1% (w/v) sodium pyrophosphate to reduce nonspecific adsorption of radioalabeled UDP-glucose. Each well was washed twice with 500 μl water, briefly air-dried, and wrapped in one layer of plastic wrap.

The amount of radioactive (1,3)β-linked glucan retained by Millipore filters was determined by liquid scintillation counting. Wrapped Inotech glass fiber filters were placed for 4~18 hours on a Molecular Dynamics Phosphor Screen. The amount of radioactivity in areas corresponding to individual microtiter plate wells was determined using a Molecular Dynamics Phosphorlmager Model 400E. To convert the number of photons recorded by the Phosphorlmager to the amount of nmoles of radioactive (1,3)β-glucan present, we cut selected areas out of filters and determined the amount of radioactivity present by liquid scintillation counting. We calculated the pixel density per cpm (Phosphorlmager signal/cpm present) and converted this number to the number of pixels per nmol (1,3)β-glucan formed. This calculation was performed for each experiment to correct for differences in exposure times to the Phosphor Screen.

Results and Discussion

Current *in vitro* (1,3)β-glucan synthase assays use individual Millipore HAWP or glass fiber filters in the quantitation of radiolabeled (1,3)β-glucan formed from radioactive UDP-^{14}C-glucose, *e.g.*[25]. These assays are sensitive (as little as ~0.2 nmol product is detectable) and reproducible, yet they are expensive (about $1.40 per assay excluding labor costs) and labor intensive, making them unsuitable for mass screening for (1,3)β-glucan synthase enzyme inhibitors. However, in an abstract, FROST *et al.*,[40] reported an assay for (1,3)β-glucan synthase activity of *C. albicans* using a Milliblot D apparatus, enabling the simultaneous determination of the amount of (1,3)β-glucan formed in 96 reaction mixtures. Building on this idea, we have developed a high throughput *in vitro* (1,3)β-glucan synthase assay that is suitable for the mass screening of samples for inhibitors of enzyme activity.

We first compared the amount of (1,3)β-glucan retained by a number of different filters to that retained by Millipore HAWP filters. Reaction mixtures were prepared in 6 × 50 mm glass tubes and *N. crassa* low-speed supernatant added as the source of (1,3)β-glucan synthase activity. Mixtures were incubated for 20 minutes, reactions terminated by the addition of TCA and the contents transferred to individual filters of the type shown in Table 1. The amount of radioactivity retained by each filter was determined by liquid scintillation counting. In addition, we measured the flow rates for each type of filter, including the MultiScreen Assay Plate System (Table 1). The MultiScreen Assay plates and individual filters of

Table 1. Comparison of various filter matrixes for retention of radioactive (1,3)β-glucan[a].

Filter	Filter type	(1,3)β-glucan retained (% Millipore HAWP)	Filtration rate
Millipore HAWP	Nitrocellulose	100%	Slow
Multi screen membrane-bottom plate	Nitrocellulose	105%	Very slow
IH-201A	Glass fiber	105%	Fast
IH-201B	Glass fiber	103%	Fast
IH-201C	Glass fiber	103%	Intermediate
IH-201 A21	Glass fiber	97%	Slow
IH-201 AH	Glass fiber	101%	Slow
Zetaprobe GT (BioRad)	Positively changed nylon	Not determined	Very slow
Biotrace (gelman Sciences)	Nitrocellulose	Not determined	Very slow

[a] Low-speed supernatant fractions were obtained as described in Materials and Methods. *In vitro* reactions were prepared in 6×50 mm glass tubes and incubated as described in Materials and Methods. The amount of radioactive (1,3)β-glucan formed in each reaction mixture was determined by liquid scintillation counting using the above filters (circles were cut from sheets of Inotech filters, Zetaprobe and Biotrace) or MultiScreen membrane bottom plates (Millipore Corporation). The amounts of radioactive (1,3)β-glucan formed in reaction mixtures using Zetaprobe or Biotrace were not determined due to the very slow flow rates. The amount of radioactive (1,3)-β-glucan retained by each filter type was compared to that retained by Millipore HAWP nitrocellulose filters. Values represent the means of two determinations.

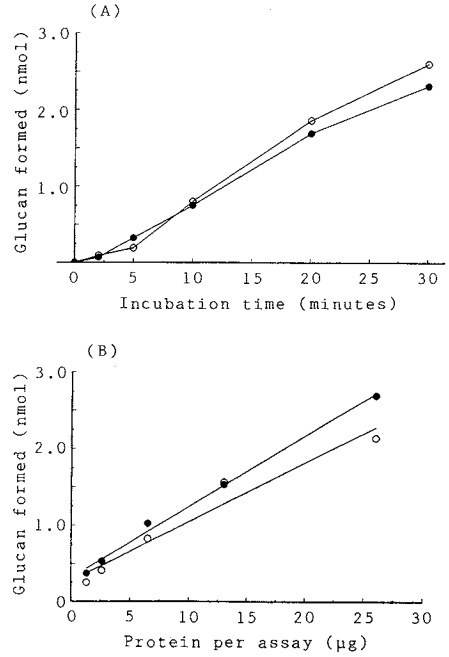
Zeta-Probe and Biotrace had very slow flow rates while Inotech Filters types IH-201A and IH-201B had very rapid filtration rates and retained radiolabeled glucan as efficiently as Millipore HAWP filters (Table 1).

We then compared (1,3)β-glucan synthase reactions in V-bottom 96-well microtiter plates to those incubated in individual glass tubes. Low-speed supernatant fractions of *N. crassa* hyphae were used as enzyme sources and added to reaction mixtures in glass tubes or in V-bottom 96-well microtiter plates as described in Materials and Methods. Mixtures were incubated for various times and terminated by the addition of TCA and water. Reactions performed in glass tubes were filtered and washed through individual HAWP filters while those performed in V-bottom 96-well microtiter plates were processed through Inotech 201A filters in a Milliblot D apparatus. The radioactivity retained by individual HAWP filters was determined by liquid scintillation counting while that retained by Inotech 201A filters was determined with a PhosphorImager. These results are shown in Fig. 1A. Note that the amounts of radioactive (1,3)β-glucan formed by reaction mixtures incubated for various times and determined by the Millipore filter method (●) or by the 96-well microtiter plate-Milliblot D-PhosphorImager method (○) were very similar. When the amount of cell extract protein per assay was varied and the amounts of (1,3)β-glucan formed determined by both methods, the results shown in Fig. 1B were obtained. Note that the two curves are very similar.

The results presented above in Fig. 1 showed that the incorporation of ^{14}C-glucose from UDP-^{14}C-glucose into(1,3)β-linked glucan was linear from 5 to 20 minutes of incubation at 22°C using 25 μg protein per assay. In addition, incorporation was linear for up to 25 μg protein per assay for 20 minutes at 22°C. Based on these results, subsequent assays routinely used 25 μg protein per assay and mixtures were incubated for 20 minutes at 22°C. This provided for a relatively short incubation time, used a very small fraction of the cell extract (about 1,000 assays could be performed using an initial 4 g of hyphae) and resulted in an easily detectable PhosphorImager signal.

Previous work by our laboratory has shown that radioactive ^{14}C-glucose incorporated into TCA-precipitable material by (1,3)β-glucan synthase reaction mixtures is greater than 90% digestible by (1,3)β-glucanase, demonstrating that the product is (1,3)β-linked glucan. To confirm that the radioactivity retained by Inotech 201A filters was (1,3)β-linked glucan, we incubated (1,3)β-glucan synthase reaction mixtures for 20 minutes and terminated reactions by boiling. BSA, α-amylase, or (1,3)β-glucanase was added to each mixture, mixtures were incubated overnight, and processed using a Milliblot D apparatus with an Inotech 201A filter. The amount of radioactivity in each reaction mixture was determined using a PhosphorImager. Less than 5% of the radioactive product was digestible by treatment with BSA or with α-amylase; in contrast, 90% of the radioactive product was digestible with (1,3)β-glucanase (results not shown). These results are identical to previous results using individual glass tubes as reaction vessels and the Millipore filter assay[39] and indicate that radioactivity retained on Inotech 201A filters is (1,3)β-linked glucan.

Fig. 1. Comparison of the amount of radioactive (1,3)β-glucan formed as a function of time and amount of cell extract per assay.

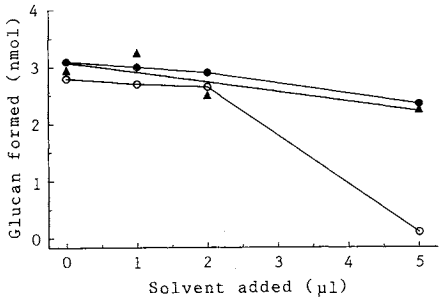


Low-speed supernatant fractions were prepared and used as enzyme sources as described in Materials and Methods. (A): Reaction mixtures containing 15 μl (27 μg) low-speed supernatant fraction were prepared in glass tubes or in 96-well microtiter plates and incubated at 22°C for the indicated times. Reactions were processed either using Millipore filters and the amount of radioactivity on filters determined by liquid scintillation counting (○) or using a Milliblot D with an Inotech 201A filter and the amount of radioactivity on areas of filters determined with a PhosphorImager (●). The number of nmoles of product formed in each reaction was determined as described in Materials and Methods. (B): Reactions containing the indicated amounts of cell extract protein were incubated for 20 minutes at 22°C and processed as in (A). Open symbols are individual determinations and closed symbols are the averages of two determinations.

The experiments described above used ~50,000 cpm of UDP-^{14}C-glucose per assay or a radiospecific activity of about 1,200 cpm/nmol glucose. Because the radiolabel is the most expensive item per assay ($0.40), we determined whether fewer cpm per assay could be used without compromising sensitivity. Reaction mixtures containing 50,000 cpm, 25,000 cpm or 12,500 cpm UDP-^{14}C-glucose (each at a final concentration of 1.2 mM) in 96-well microtiter plates were incubated for 20 minutes and the amount of radioactive (1,3)β-glucan formed by each reaction mixture determined using a Milliblot D and a PhosphorImager. As expected, there was a linear decrease in the number of cpm incorporated in reaction mixtures containing 25,000 cpm and 12,500 cpm as compared to those containing 50,000 cpm (results not shown). Importantly, the PhosphorImager image from reactions containing less than 50,000 cpm UDP-^{14}C-glucose per assay did not have sufficient intensity to be useful for the reliable detection of inhibitors.

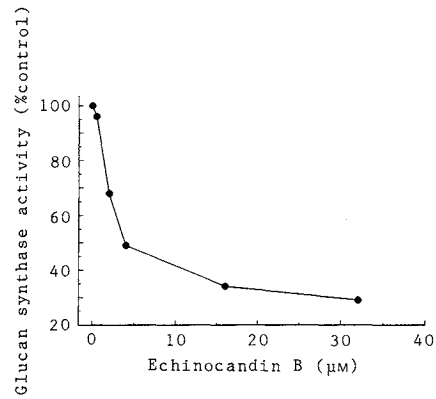
Since samples for mass screening are routinely dissolved in organic solvents, we tested the effect of DMSO, ethanol, or methanol on (1,3)β-glucan synthase activity. Previously, we found that 1 μl of 50%

Fig. 2. Effect of the addition of solvents on (1,3)β-glucan synthase activity.



(1,3)β-glucan synthase reactions (25 μl) containing 25 μg (15 μl) low-speed supernatant protein and the indicated amounts of 50% DMSO (●), 50% ethanol (▲) or 50% methanol (○) were incubated in V-bottom plates for 20 minutes at 22°C. Reactions were terminated by the sequential addition of TCA and water and filtered using a Milliblot D with an Inotech 201A filter. The amount of radioactive (1,3)β-glucan formed was determined using a PhosphorImager as described in Materials and Methods. Symbols represent the average of two determinations.

Fig. 3. Effect of echinocandin B on (1,3)β-glucan synthase activity.



(1,3)β-glucan synthase reactions (25 μl) containing 25 μg (15 μl) low-speed supernatant fractions and the indicated concentrations of echinocandin B dissolved in 50% DMSO were incubated in V-bottom plates for 20 minutes at 22°C. Reactions were terminated by the sequential addition of TCA and water and processed using a Milliblot D with an Inotech 201A filter. The amount of radioactive (1,3)β-linked glucan was determined with a PhosphorImager as described in Materials and Methods. Controls contained solvent only (0 μM inhibitor). Symbols are averages of two determinations.

acetone added to 25 μl reaction mixtures inactivated enzyme activity (TAFT, unpublished results). In contrast, we found that 5 μl of 50% DMSO (● in Fig. 2) or 5 μl 50% ethanol (▲) had only a slight inhibitory effect. Five μl of 50% methanol (○) inactivated enzyme activity; however, addition of 2 μl 50% methanol had no effect. These results indicate that samples dissolved in 2 μl 50% methanol, in 5 μl of 50% DMSO or 50% ethanol per 25 μl assay can be added to reaction mixture without solvent significantly affecting enzyme activity.

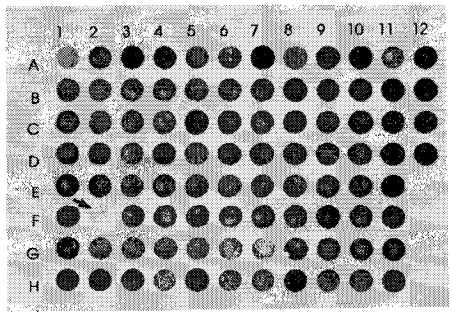
In order for an *in vitro* enzyme inhibitor screen to be useful it must be sensitive, that is, small quantities of inhibitors present in test samples must be detected, so as to minimize the percentage of false negatives. In order to determine if the (1,3)β-glucan synthase assay we had developed had sufficient sensitivity, we incubated reaction mixtures with various concentrations of the (1,3)β-glucan synthase inhibitors, cilofungin, echinocandin B and papulacandin B. Previous work from our laboratory showed that cilofungin, papulacandin B, and echinocandin B were noncompetitive inhibitors of *N. crassa* (1,3)β-glucan synthase activity with *Ki*'s of 13 μM, 360 μM, and 4 μM, respectively[13,26]. Fig. 3 shows the effect of various concentrations of echinocandin B on (1,3)β-glucan synthase activity. Plots for the other two inhibitors were similar (not shown). Note that 2 μM echinocandin B resulted in a ~35% decrease in enzyme activity. One hundred μM papulacandin B and 4 μM cilofungin resulted in similar decreases (results not shown). These concentrations corresponded to ~100 ng cilofungin, ~50 ng echinocandin B and ~2.5 μg papulacandin B per assay. Therefore, as little as 50 ng of an inhibitor with a *Ki* of 4 μM was detected by this assay. In contrast, a number of compounds that are not (1,3)β-glucan synthase inhibitors were tested and none had any significant inhibitory effect on enzyme activity, even at amounts 1,000-fold higher than the (1,3)β-glucan synthase inhibitors tested. These results are shown in Table 2. Values for echinocandin

Table 2. Effect of various compounds on (1,3)β-glucan synthase activity[a].

Compound	Amount (μg/assay)	(1,3)β-glucan synthase activity (% control)
Echinocandin B	0.1	52
Cilofungin	1	33
Papulacandin B	10	63
Tunicamycin	6.5	118
Kanamycin	18	84
Griseofulvin	12	110
Monensin	6	115
Cephalosporin C	16	115
Amphotericin B	14	96
Nystatin	4	110
Bacitracin	11	127
Validamycin	8	107
Nikkomycin Z	10	118
Penicillin G/gentamicin	12/10	90
Unknown A-1	10	62
Unknown A-2	10	55

[a] (1,3)β-glucan synthase reaction mixtures were prepared as described in Materials and Methods in V-bottom plates and contained the above compounds at the indicated concentrations. Samples were dissolved in 50% (v/v) DMSO except for nikkomycin, and penicillin/gentamicin which were prepared in water. Reactions were incubated for 20 minutes at 22°C and the amount of (1,3)β-glucan formed determined using a Milliblot D with an Inotech 201A filter and a PhosphorImager 400E. The amount of radioactive (1,3)β-glucan formed was compared to controls (solvent only). Unknowns A-1 and A-2 are natural products tested for inhibition of (1,3)β-glucan synthase as described in the text. Unknown A-1 is from well 2F of the plate shown in Fig. 4 while A-2 is from the second set of samples.

Fig. 4. Screening of natural products.



(1,3)β-glucan synthase reaction mixtures (25 μl) contained low-speed crude supernatant from *N. crassa* (25 μg protein; 15 μl), UDP-^{14}C-glucose (1.2 mM; 50,000 cpm) and 1 μl 50% DMSO containing 10 μg of each test sample. Mixtures were incubated for 20 minutes at 22°C in V-bottom plates and terminated by the addition of TCA and water. Mixtures were filtered using a Milliblot D with an Inotech 201A filter. The amount of radioactive glucan formed by each mixture was determined using a PhosphorImager as described in Materials and Methods. Column 12, wells A and B, contained no additions; wells C and D, 1 μl 100% DMSO; wells E and F, 75 μM (final concentration) cilofungin in 1 μl 100% DMSO.

B, cilofungin and papulacandin B are included for comparison as are values for two natural products (see below), designated Unknown A-1 and Unknown A-2.

A major difficulty with *in vitro* enzyme screens is that the number of samples that test positive is often in excess of 5%, indicating that the screen is not selective enough to be useful. To determine if this were the case for the *in vitro* (1,3)β-glucan synthase enzyme assay that we developed, we obtained one hundred and seventy six samples of natural products from a variety of organisms that were part of another company's screening program for antifungals. These samples were screened for (1,3)β-glucan synthase inhibitors using the equipment and optimal *in vitro* conditions described above. Fig. 4 shows a PhosphorImager image of the amount of radioactive (1,3)β-glucan formed by reaction mixtures for one 96-well microtiter plate. Note that well 2F (arrow) as well as the two controls containing cilofungin (12E and 12F) had significantly less radioactive (1,3)β-glucan than other wells. Quantitation of the amount of radioactivity in each well revealed that well 2F had 62% of control values (wells 12C, 12D) while wells 12E and 12F had ~10% of control values—see Table 2, unknown A-1. It is important to note that the number of positive samples (excluding our controls) was 1 out of 88 samples. Similar results, *i.e.*, one positive per plate, were noted for the other 88 samples (Table 2, Unknown A-2 and results not shown).

The search for (1,3)β-glucan synthase inhibitors has been in part hampered by the lack of a sensitive, specific, and inexpensive *in vitro* screen. The assay that we have developed permits the rapid screening of

many hundreds of individual compounds for the detection of inhibitors. We are currently determining the optimal *in vitro* conditions to assay (1,3)β-glucan synthase activity from *A. fumigatus*, *C. albicans*, and other human opportunistic fungal pathogens. This high throughput assay of fungal (1,3)β-glucan synthase will be a viable tool in the search for new enzyme inhibitors.

Acknowledgments

This work was supported by a Phase I SBIR award (NIAID 1R43 AI33749-1) to Mycotox, Inc. We would like to thank JOHN CAMPBELL and PAUL AWALD for their comments and helpful suggestions.

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