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Evidence for the Mechanism of Action of the Antifungal Phytolaccoside B Isolated from *Phytolacca tetramera* Hauman

Andrea Escalante,[†] Martha Gattuso,[‡] Pilar Pérez,[§] and Susana Zacchino*[,†]

Área Farmacognosia and Área Biología Vegetal, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina, Suipacha 531, (2000) Rosario, Argentina, and Instituto de Microbiología Bioquímica (CSIC), Departamento de Microbiología y Genética, Universidad de Salamanca, Avenida Campo Charro s/n, (37007) Salamanca, Spain

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Phytolaccoside B (**1**), an antifungal monodesmoside triterpenoid glycoside isolated from berries of *Phytolacca tetramera* Hauman (Phytolaccaceae), alters the morphology of yeasts and molds. The malformations were similar to those produced by enfumafungin, a known inhibitor of (1→3)-*β*-D-glucan synthase, an enzyme that catalyzes the synthesis of (1→3)-*β*-D-glucan, one of the major polymers of the fungal cell wall. However, enzymatic assays revealed that **1** did not inhibit (1→3)-*β*-D-glucan synthase, but it did produce a notable enhancement of the chitin synthase 1 activity and, concomitantly, a rise in chitin, another important polymer of the fungal cell walls. This finding was corroborated by fluorescence microscopy and also by quantification of the chitin. In addition, a 2-fold increase in the thickness of the fungal cell wall was observed with transmission electronic microscopy. On the other hand, **1** neither bound to ergosterol nor caused hemolysis of red blood cells, although some fungal membrane damage was observed at the MIC of **1**.

Over the past two decades, fungi have emerged as a major cause of serious infections in immunocompromised patients.[1,2] A matter of concern in the treatment of fungal infections is the limited number of efficacious antifungal drugs. Many of the currently available drugs are toxic, are inefficient, or lead to the development of resistance due in part to their intensive prophylactic use.[3] There is, therefore, a clear need for new antifungal compounds that could constitute alternatives for the management of mycoses especially in immunocompromised hosts.[4,5]

Phytolaccoside B (**1**), 3-*O*-*β*-D-xylopiranosylphytolaccagenin, a monodesmoside triterpenoid glycoside isolated from berries of *Phytolacca tetramera*,[6] showed antifungal activity in agar dilution assays, inhibiting human opportunistic and pathogenic fungi.[6] Also, **1** had previously shown antifungal properties against phytopathogenic fungal species.[7]

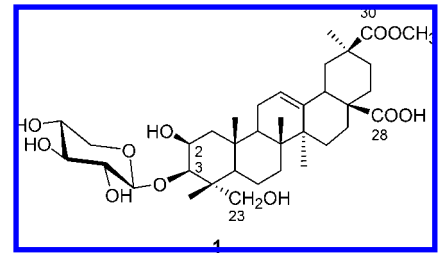
Phytolaccoside E [3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-xylopiranosylphytolaccagenin], also isolated from *P. tetramera* berries, showed a lower inhibition capacity and a narrower spectrum of activity, indicating that the number and kind of sugar residues of phytolaccagenin glycosides influence their antifungal properties.

In this study, we report the antifungal activities of **1** by using the standardized methods recommended by the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical and Laboratory Standards, NCCLS).[8] In addition, we performed studies to determine the mode of action of **1** by monitoring the induced morphological abnormalities in fungal cells. This type of study has previously provided insight into the mechanism of action of antifungal compounds.[9,10]

According to the observed malformations, several studies to check the effect of **1** on fungal cell walls were performed. Finally, considering that the antifungal activity of triterpenoid glycosides is sometimes associated with their ability to complex with sterols and cause loss of membrane integrity, **1** was tested for its capacity to bind to ergosterol and to disrupt fungal membranes.[11−13]

Results and Discussion

Antifungal evaluation of **1** was carried out according to CLSI (formerly NCCLS) guidelines against both filamentous fungi and yeasts.[8] Compound **1** was shown to be active against the filamentous fungi *Neurospora crassa*, *Aspergillus niger*, *A. flavus,* and *A. fumigatus* and the yeasts *Candida albicans, Saccharomyces cerevisiae*, and *Cryptococcus neoformans* with minimum inhibitory concentrations (MICs) between 74−188 *μ*M. Although these values are higher than those of the MICs of positive controls, **1** appeared to be an interesting antifungal compound considering the second-order studies reported herein.



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In addition to MIC (MIC_{100}), the concentration of **1** that inhibits either 80% or 50% growth (MIC_{80} or MIC_{50}, respectively) was determined for yeasts. The application of less stringent end points such as MIC_{80} or MIC_{50} for yeasts is recommended by CLSI[8] and often provides a better correlation with other measurements of antifungal activity. Among the results obtained (Table 1), it is noteworthy that the MIC_{50} values of **1** against *S. cerevisiae* and *C. neoformans* were very low (18 and 9 *μ*M, respectively). Interestingly, **1** was fungicidal against all yeasts tested with minimun fungicide concentration (MFC) values between 112 and 150 *μ*M. To be fungicidal rather than fungistatic is an important finding since antifungal agents that kill fungi (cidal) have demonstrated to be, in most cases, clinically more useful than those that merely inhibit (static) fungal growth.[14]

Comparison of MIC_{100} values of **1** with its MFCs against yeasts indicates that **1** is fungicidal rather than fungistatic (MIC_{100} = MFC) against *C. neoformans* and is fungistatic at 74 *μ*M and turned fungicidal at 150 *μ*M against *C. albicans* and *S. cerevisiae.*

Studies on Mechanisms of Action of 1. Changes in morphology of filamentous fungi and yeasts caused by antifungal compounds have often provided insight into the mechanism of antifungal action.[9,10] We examined the effect of **1** on a single representative species each of filamentous fungi and yeasts.

Among filamentous fungi, the sensitive species *N. crassa* was chosen because it has been shown to be an excellent model for

* Corresponding author. Tel and fax: +54-341-4375315. E-mail: szaabgil@citynet.net.ar.
[†] Área Farmacognosia, Universidad Nacional de Rosario, Argentina.
[‡] Área Biología Vegetal, Universidad Nacional de Rosario, Argentina.
[§] Instituto de Microbiología Bioquímica, Universidad de Salamanca.

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Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) in μM of Phytolaccoside B for the Yeasts *Candida albicans* (*C.a.*), *Saccharomyces cerevisiae* (*S.c.*), and *Cryptococcus neoformans* (*C.n.*) and for the Filamentous Fungi *Neurospora crassa* (*N.c.*), *Aspergillus niger* (*A.n.*), *A. fumigatus* (*A.fu.*), and *A. flavus* (*A.fl.*)[a]

		phytolaccoside B				Amph	Ket
fungal spp.	voucher specimen	MIC_{100}	MIC_{80}	MIC_{50}	MFC	MIC_{100}	MIC_{100}
C.a.	ATCC 10231	74	74	74	150	0.84	1
S.c.	ATCC 9763	74	37	18	150	1.62	1
C.n.	ATCC 32264	112	74	9	112	0.27	0.50
N.c.	ATCC 9279	74	n.t.	n.t.	n.t.	1	2
A.n.	ATCC 9029	188	n.t.	n.t	n.t.	0.50	0.25
A.fu.	ATCC 26934	188	n.t.	n.t.	n.t..	0.50	0.13
A.fl.	ATCC 9170	74	n.t.	n.t.	n.t.	0.50	0.50

[a] MIC_{100}, MIC_{80}, and MIC_{50}: concentration of a compound that caused 100%, 80%, or 50% reduction of the growth control, respectively. ATCC = American Type Culture Collection (Peoria, IL); Amph = amphotericin B; Ket = ketoconazole; n.t. = not tested.

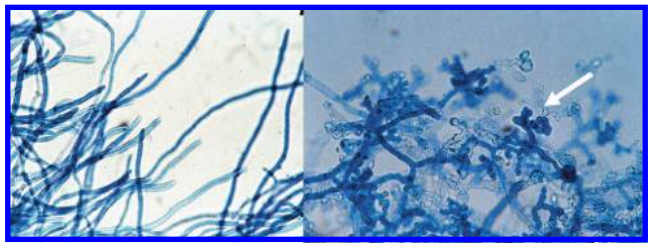

Figure 1. *Neurospora crassa* hyphae, untreated (left) and treated (right) with sub-MIC concentration of phytolaccoside B (37 μM), observed with optical microscopy (400×). The arrow shows the malformations.

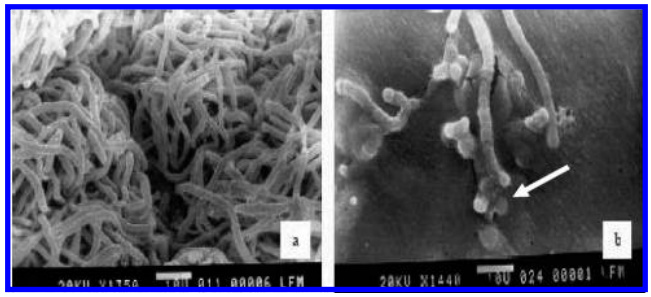

Figure 2. Scanning electron microscopy (SEM) of *Neurospora crassa* hyphae: (a) untreated; (b) treated with phytolaccoside B (37 μM/24 h incubation time in the culture medium). Bar = 10 μm.

cellular and biochemical research.[15] In addition, a recent paper has correlated the defects in *N. crassa* hypha elongation with the mechanism underlying the malformations observed.[16]

To study whether **1** produces morphological changes in *N. crassa* hyphae, microscopic observation of fungal colonies subjected to a subinhibitory concentration of **1** was performed because malformations were better detected at this concentration in previous reports.[10] In accordance to CLSI, a sub-MIC concentration can be defined as the concentration of an antimicrobial agent that is not active in microorganism growth, but that is still active in altering microorganism biochemistry and shape, *in vitro* or *in vivo*.[17,18] Results showed that **1** at 37 μM produced shortening of *N. crassa* hyphae and highly branched bulbous hyphal tips (Figure 1).

These results were corroborated by scanning electron microscopy (SEM), where shortening of the hyphae and bulbous tips were again clearly observed in *N. crassa* cultures (Figure 2).

In addition to testing sub-MIC levels of phytolaccoside B in filamentous fungi, we also analyzed its effect in *S. cerevisiae*. This yeast has been widely used as a model eukaryotic microorganism and has proven to be very useful for studies of mechanisms of action of antifungal compounds.[19−21] Results showed that **1** also modified the normal morphology of *S. cerevisiae*, producing aggregates and swollen cells in the culture medium (Figure 3).

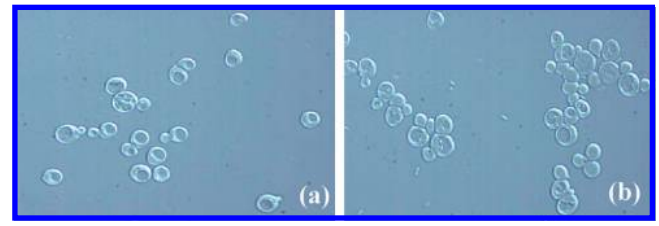

Figure 3. Differential interference contrast micrographs (400×) of *Saccharomyces cerevisiae* cells incubated during 24 h in (a) control medium; (b) medium with phytolaccoside B (37 μM).

Interestingly, the malformations produced by **1** in both types of fungi were similar to those caused by enfumafungin,[22] a natural triterpenoid monoglycoside that was reported by Merck as an inhibitor of (1→3)-β-D-glucan synthase.[23] This enzyme catalyzes the synthesis of (1→3)-β-D-glucan, one of the major polymers of the fungal cell wall. Mammalian cells are devoid of walls, thus making it an attractive target for antifungal drugs.[22]

Effect of Phytolaccoside B in the Fungal Cell Wall. A distinctive feature of the specific inhibitors of the fungal cell wall is that their antifungal effect is reversed in a medium containing an osmotic stabilizer such as sorbitol.[10] When the yeast *S. cerevisiae* was treated with **1** in a medium supplemented with sorbitol, MIC values did not shift to a higher value, thus suggesting that **1** would not act through the inhibition of cell wall synthesis or assembly. To further analyze whether **1** has an effect in the cell wall, it was tested *in vitro* for its capacity to inhibit *S. cerevisiae* (1→3)-β-D-glucan synthase and chitin synthase 1, enzymes that catalyze the synthesis of the major polymers of the fungal cell wall, (1→3)-β-D-glucan and chitin.[22−24] Results showed (Table 2) that **1** slightly inhibits the incorporation of UDP [^{14}C]-glucose into insoluble (1→3)-β-D-glucan (93.36% of residual activity of (1→3)-β-D-glucan synthase; 6.64% of inhibition at 0.50 μg/μL). Surprisingly, the incorporation of [^{14}C]-*N*-acetylglucosamine into insoluble chitin was increased. Moreover, phytolaccoside B activates the chitin synthesis through the increase of both basal and maximum chitin synthase 1 activities (measured in the absence and in the presence of exogenous proteolytic trypsin activation), showing 189.70% and 165.77% of residual activity, respectively.

Considering that an enhancement of chitin synthase 1 activity would lead to a higher content of chitin in the cell wall of treated fungi, we performed three different assays to corroborate this result: (i) staining of chitin fibrils with Calcofluor white (Cw); (ii) quantification of the chitin content; (iii) observation of the cell wall of treated cells by TEM.

(i) Cw is a negatively charged fluorochrome that exhibits a particularly high affinity for chitin, emitting a bright bluish fluorescence under UV light.[24] At low concentrations (20−50 μM) it is used as a vital dye for the fungal cell wall.[25] *S. cerevisiae* cells, untreated and treated with **1** at sub-MIC concentration (37 μM), were stained with Cw and examined by fluorescence microscopy. This experiment showed that **1** induced a significant

Table 2. Effect of Phytolaccoside B (20 μg/assay) on the *in Vitro* Incorporation of [^{14}C]-Glucose and [^{14}C]-*N*-Acetylglucosamine into Insoluble (1→3)-β-D-Glucan and Chitin Expressed as Percent of Residual Activity (% RA) of the Enzymes (1→3)-β-D-Glucan Synthase and Chitin Synthase 1 and IC_{50} (μg/μL) Values of the Test Compound, Respectively

	(1→3)-β-D-glucan synthase[a]		chitin synthase 1[b]			
			basal (−trypsin)		maximum (+trypsin)	
compd	% RA[c]	IC_{50}[d]	% RA[c]	IC_{50}[d]	% RA[c]	IC_{50}[d]
1	93.36 ± 5.58	>0.50	189.70 ± 11.78	>0.40	165.77 ± 2.89	>0.40
Pap	5.00 ± 0.54	0.01	n.t.	n.t.	n.t.	n.t.
Nik	n.t.	n.t.	n.d.	0.0002	n.d.	0.0002

[a] 40 μL total volume. [b] 50 μL total volume. [c] Expressed as mean ± SEM. [d] Concentration (μg/μL) that produces 50% inhibition of enzyme activity; Pap: papulacandin; Nik: nikkomycin Z; n.t.: not tested; n.d.: no detectable residual activity.

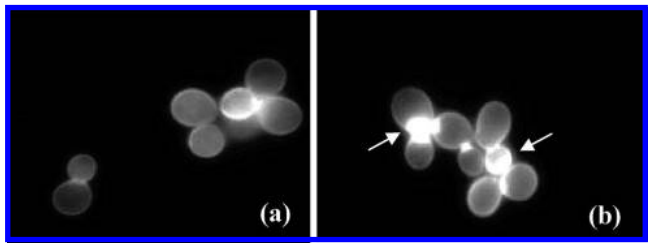

Figure 4. Fluorescence micrographs of Calcofluor-stained *Saccharomyces cerevisiae* cells in the absence (a) and in the presence (b) of sub-MIC levels of phytolaccoside B (37 μM). Both micrographs were taken with the same lenses (400×) and exposure times. Arrows show the accumulation of fluorescence around the *S. cerevisiae* cell surfaces.

accumulation of fluorescence around the *S. cerevisiae* cell surfaces of mother or daughter cells (Figure 4), suggesting an increase in the amount of cell wall chitin.

(ii) The chitin content of *S. cerevisiae* cells grown in the absence or in the presence of sub-MIC levels of phytolaccoside B was determined with the Reissig method.[26] This is a colorimetric test that quantifies spectrophotometrically (585 nm) the *N*-acetylglucosamine (NAG) residues of chitin, through the reaction with *p*-dimethylaminobenzaldehyde (DMAB). Results showed that 100 mg each of control and **1**-treated cells produced 124 and 153 nmol of NAG, respectively, which represented a significant enhancement of chitin content (23%) in the treated cells with respect to the control cells, in accordance with the microscopic observations (Figure 4).

Observation of Cw-stained *N. crassa* hyphae also showed a high accumulation of chitin in **1**-treated cells. The fluorescence was particularly increased in septa and hyphal tips (Figure 5).

(iii) *N. crassa* hyphae, untreated and treated with sub-MIC levels of **1**, were observed by TEM (Figure 6). The walls of treated hyphae showed a thickness of 400 nm (Figure 6b), which represents a 2-fold increase with respect to control hyphae (200 nm, Figure 6a). These results suggest that phytolaccoside B is altering the structure of the fungal cell wall.

Assays Related to the Interaction with the Fungal Membrane. One of the proposed mechanisms for triterpenoid glycosides acting as antifungal agents is their binding to the membrane ergosterol, which leads to fungal membrane disruption and loss of the intracellular content.[7]

To determine if **1** binds to the fungal membrane sterol, the MIC of this compound for *S. cerevisiae* was determined with and without the addition of ergosterol. If the activity of compound **1** was caused by binding to ergosterol, the exogenous ergosterol would prevent the binding to the fungal membrane's ergosterol. As a consequence, MIC enhancement of **1** in the presence of exogenous ergosterol with respect to the control assay should have been observed.[18,27]

Results showed (Figure 7) that the MIC of **1** for *S. cerevisiae* cells remained unchanged in the presence of different concentrations of exogenous ergosterol, suggesting that this compound would not act by binding to membrane ergosterol. In contrast, a 4-fold increase of MIC was observed for the positive control drug amphotericin B, whose interaction with ergosterol has been repeatedly demonstrated.[28,29]

Due to its amphipatic character, **1** could damage the fungal membrane by interacting with elements other than ergosterol. This harm could be detected by measuring the release of intracellular components to the medium from buffer-washed treated cells. Cellular components that absorb at 260 nm represent one class of leakage components, primarily nucleotides, of which uracil-containing compounds exhibit the strongest absorbance.[27]

Phytolaccoside B was added to cell suspensions of *S. cerevisiae*, and the samples were examined at several time intervals (6, 12, 24, and 48 h). Sodium dodecyl sulfate (SDS) (2%) was used to calculate 100% cellular leakage. Results showed (Figure 8) that **1** produced an increase of about 17% in OD_{260} at 6−48 h ($p < 0.001$).

The above study on the effect of **1** on fungal membranes was also performed with human erythrocytes. The ability of saponins to lyse erythrocyte membranes causing a release of hemoglobin has been extensively studied, with results varying considerably depending on the structure of the glycoside.[12]

Compound **1** did not show hemolytic capacity against mammalian erythrocytes up to 120 min (LC_{100} > 188 μM), which corroborates the previous findings that not all saponins disrupt the red cell membranes.[30] In contrast, amphotericin B showed a strong hemolytic effect at 120 min with LC_{100} = 13.5 μM.

The present work showed that subinhibitory concentrations of **1** induced morphological changes in yeasts and filamentous fungi similar to those produced by enfumafungin, a known inhibitor of (1→3)-β-D-glucan synthase. Nevertheless, the antifungal activity of **1** was not prevented by an osmotic stabilizer, and in addition **1** did not inhibit either (1→3)-β-D-glucan or chitin synthase 1 *in vitro*. In contrast, **1** led to a 1.6−2-fold increase in the level of chitin synthase 1 activities, measured in *S. cerevisiae*-permeabilized cell extracts. Concomitantly, the chitin content of fungal cell walls was enhanced by about 23% *in vivo* by **1** when it was quantified with the Reissig colorimetric method.

The fluorescence micrographs of Cw-stained cells allowed us to observe a high deposit of chitin in *S. cerevisiae* cells and *N. crassa* hyphae treated with **1**. Additionally, TEM observations of *N. crassa* showed abnormally thick septa and a 2-fold thickening of the cell walls compared to control cells.

Considering that the process of chitin synthesis occurs in the inner face of the plasma membrane,[31] it is possible that the low membrane damage produced by **1** could act as a primary mechanism of defense to the fungal cell,[32−34] triggering the activation of the zymogen form of chitin synthase 1 with concomitant enhancement of chitin synthesis. According to Roncero,[21] the high deposit of chitin would lead ultimately to the arrest of cell growth, as was previously reported for Calcofluor and Congo Red.

Experimental Section

Extraction, Isolation, and Purification of Phytolaccoside B (1). Phytolaccoside B was isolated from the *n*-butanol extract of *P. tetramera* collected in Magdalena (Buenos Aires province), identified by one of

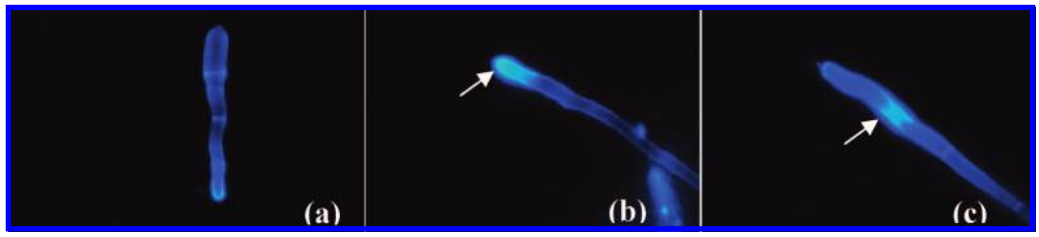

Figure 5. Fluorescence micrographs of Calcofluor-stained *Neurospora crassa* hyphae in the absence (a) and in the presence (b and c, see arrows) of phytolaccoside B (37 μM). The three micrographs were taken with the same lenses (400×) and exposure times.

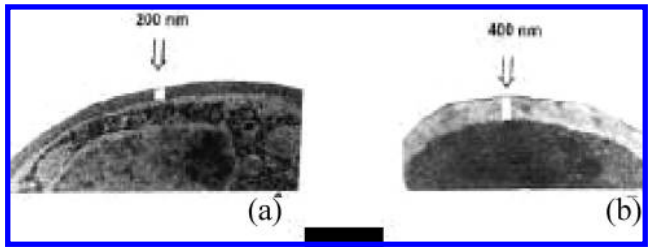

Figure 6. Transmission electron micrographs of medial sections through a *Neurospora crassa* hyphae untreated (a) and treated (b) with phytolaccoside B at 37 μM. Bar: 1 mm.

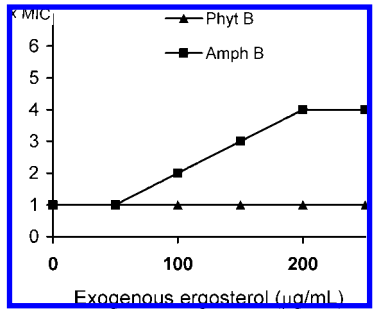

Figure 7. Effect of different concentrations of exogenous ergosterol (50−250 μg/mL) on the MIC of both phytolaccoside B (Phyt B) and amphotericin B (Amph B) for *Saccharomyces cerevisiae*.

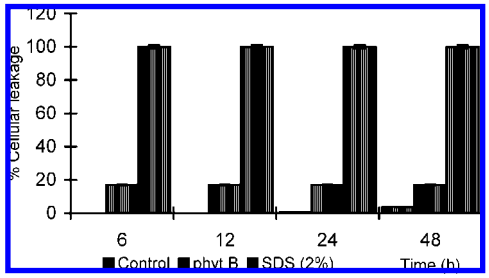

Figure 8. Percent of leakage of *Saccharomyces cerevisiae* cells untreated (control) or treated with phytolaccoside B at MIC (74 μM) and sodium dodecyl sulfate (SDS, 2%).

the authors (M.G.), and deposited in the Herbarium of the National University of Rosario (voucher specimen MG 134) as previously described.[6]

Phytolaccoside B (1). The ^{1}H NMR and ^{13}C NMR spectra [in pyridine-d_5 with TMS as internal standard on a Bruker AMX 500 operating at 500 MHz (^{1}H) and 125 MHz (^{13}C)] were similar to reported data:[6] $[\alpha]^{20}_D$ +59.2 (MeOH; *c* 0.250); FAB-MS *m/z* 687 $[M + Na]^+$; *anal*. calcd for $C_{36}H_{56}O_{11}$, C 65.04%, H 8.49%, found C 64.98%, H 8.45%.

Antifungal Evaluation. Microorganisms and Media. For the antifungal evaluation, standardized strains from the American Type Culture Collection (ATCC, Rockville, MD) were used: *C. albicans* ATCC 10231, *S. cerevisiae* ATCC 9763, *C. neoformans* ATCC 32264, *N. crassa* ATCC 9279, *A. niger* ATCC 9029, *A. fumigatus* ATCC 26934, *A. flavus* ATCC 9170. They were grown on Sabouraud chloramphenicol agar slants for 48 h at 30 °C and maintained on slopes of Sabouraud dextrose agar (SDA, Britania, Buenos Aires, Argentina). Inocula of cells or spore suspensions were obtained according to reported procedures and adjusted to 2.5 × 10^3 cells or spores with colony-forming units (CFU)/mL.[8]

Antifungal Susceptibility Testing. The MIC of compound **1** was determined by using broth microdilution techniques according to the guidelines of the CLSI for yeasts (M27-A2) and for filamentous fungi (M38-A).[8] The culture medium was RPMI-1640 (Sigma Chemical Co., St. Louis, MO), buffered to pH 7.0 with MOPS (Remel, Lenexa, KS). Microtiter trays were incubated at 35 °C in a moist, dark chamber, and MICs were visually recorded at 48 h. For the assay, a stock solution of **1** was 2-fold diluted with RPMI from 250 to 0.98 μg/mL (volume = 100 μL) and a final DMSO concentration of ≤1%. A volume of 100 μL of inoculum suspension was added to each well, with the exception of the sterility control, where sterile water was added to the well instead. Ketoconazole (Janssen Pharmaceutica, Beerse, Belgium) and amphotericin B (Sigma Chemical Co.) were used as positive controls. Two wells containing 100 μL of RPMI medium with a final DMSO concentration of ≤1% and 100 μL of inoculum suspension were used as negative controls.

End points were defined as the lowest concentration of drug resulting in total inhibition (MIC_{100}) or in 80% or 50% reduction (MIC_{80} and MIC_{50}) compared to the growth in the control wells containing no antifungal and were determined spectrophotometrically with the aid of a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA). The MFC against each isolate was determined as follows: after determining the MIC_{100}, an aliquot of a 5 μL sample was withdrawn from each clear well of the microtiter tray and plated onto a 150 mm RPMI-1640 agar plate buffered with MOPS. Inoculated plates were incubated at 30 °C and recorded after 48 h. The MFC was defined as the lowest concentration of each compound that resulted in total inhibition of visible growth.

Microscopic Studies. (a) Optical Microscopy (OM): An inoculum of 1 × 10^5 spores or cells/mL of each of *N. crassa* (ATCC 9279) and *S. cerevisiae* (ATCC 9763), prepared according to reported procedures,[35] was grown for 24 h at 28 °C with shaking (145 rpm) in YEPD medium (0.5% yeast extract, 0.5% peptone protease, and 4% dextrose), in the presence and absence of subinhibitory concentrations of **1** (37 μM). *N. crassa* hyphae were washed with cold 0.1 M phosphate buffer (pH 7), collected by filtration through Millipore type HA filters, and stained for 15 min with lactophenol blue.[36] Stained preparations were examined with a Zeiss Axiolab research optical microscope equipped with a MC 80 camera. Photographs were taken at a magnification of 400. (b) SEM: Both **1**-treated (37 μM) and untreated hyphae of *N. crassa* ATCC 9279, prepared as described above, were fixed with a solution of 4.0% glutaraldehyde in 0.1 M sodium cacodilate, at pH 7.2.[37] The fixed hyphae were dehydrated in a grade series of EtOH, then in *n*-butanol, and finally air-dried. The fixed and dehydrated hyphae were mounted onto SEM stubs, coated with gold−palladium (60:40) in a Vecco vacuum evaporator (1500 V, 70 mmHg in argon atmosphere, 10 min),[38] and viewed with a Jeol scanning microscope (JSM) 35C, equipped with a dispersive X-ray detector. Samples were exposed to a 20 kV acceleration voltage. (c) Differential Interference Contrast (DIC) Microscopy: *S. cerevisiae* ATCC 9763 cells, prepared as described above, were harvested, washed once, and resuspended in water to be observed under a Leica DM RXA DIC microscope (Wetzlar, Germany) with a magnification of 400×. (d) TEM: *N. crassa* ATCC 9279 mycelium was prepared as described above, using **1** at 37 μM. It was fixed for 2 h on ice and put into a fixative solution (4% glutaraldehyde

in 0.1 M sodium cacodylate buffer, pH 7.4, rt) for 1 h. For ultrastructural studies, the mycelium was removed, washed with H_2O, and replaced in 0.1 M phosphate-buffered saline (PBS), amending it with 10% sacarose in 0.1 M PBS. After 20 min at rt, the mycelium was washed with H_2O and cut into small blocks. For better visualization of septa, the mycelium was treated for 1 h with 1.5% OsO_4 in 0.1 M PBS (pH 7.2, rt) and then washed with H_2O, treated with 1.5% $KMnO_4$, dehydrated with acetone, and embedded in Spurr resins (Union Carbide International Co., Houston, TX). Ultrathin sections were cut in an LKB microtome and placed on carbon-coated nickel, single-hole grids. Each grid was incubated for 20 min in 2.5% uranyl acetate and for 4−5 min in a Reinolds solution[39] for the double-staining of mycelia. Finally, all samples were washed with H_2O and visualized with a Philips 300 electron microscope.

Sorbitol Protection Assay. MIC values were determined using *S. cerevisiae* ATCC 9763 by the standard broth microdilution procedure described above. Duplicate plates were prepared: one of them containing 2-fold dilutions of **1** and the other one containing **1** plus 0.8 M sorbitol as an osmotic support, in each of the wells. MICs were read at 2 and 7 days.[10]

Enzymatic Assays. The *S. cerevisiae* strain used was *MATa trpI ura3 leu2 his3 pep4::HIS3 nuc1::LEU2*. Routine yeast growth medium was as described.[40] Enzyme preparation: Cell extracts were obtained essentially as described previously.[41] Early logarithmic phase cells grown in 100 mL of YEPD medium were collected, washed once with 50 mM Tris-HCl pH 7.5, suspended in 100 mL of the same buffer, and broken with glass beads in a FastPrep FP120 apparatus (Savant, BIO 101, Inc., Cedex, France) (one pulse every 15 s at a speed of 6.0). Broken material was collected, and cell debris was removed by low-speed centrifugation (5000*g*, 5 min at 4 °C). The supernatant was centrifuged at 18000*g* for 30 min at 4 °C, and the pellet was resuspended in 50 mM Tris-HCl pH 7.5 containing 33% glycerol (at a concentration of approximately 3 mg protein/mL) and stored at −80 °C. Protein was quantified by the Bradford dye binding procedure[42] using the Bio-Rad protein assay dye reagent and bovine serum albumin as standard.

(1→3)-β-D-Glucan Synthase Assay. This assay was essentially as described previously.[41] The standard assay mixture contained 5 μL of enzyme (15 μg protein), in a total volume of 40 μL. Two microliters of MeOH or the corresponding compounds (kept in stock solution, 10 mg/mL) in MeOH at −20 °C was added to each reaction. The reaction was incubated for 30 min at 30 °C and stopped by addition of 1 mL of 10% trichloroacetic acid. All reactions were carried out in duplicate. The drug papulacandin B was used as standard drug, and it was a generous gift from K. Scheibli and P. Traxler (Novartis, Basel, Switzerland). The antibiotic was kept in stock solution (10 mg/mL in MeOH) at −20 °C.

Chitin Synthase 1 Assay. The assay was performed as described previously.[43] The standard assay mixture contained 10 μL of enzyme (30 μg of protein) in a total volume of 50 μL. Two microliters of MeOH or the corresponding compounds (kept in stock solution, 10 mg/mL in MeOH at −20 °C) was added to each reaction. Enzyme activation was performed by partial proteolysis of the reaction mixture with 2 μL of trypsin (0.25 μg/μL) during 15 min at 30 °C and stopped by the addition of 2 μL of trypsin inhibitor (0.375 μg/μL). The reaction was incubated for 90 min at 30 °C and stopped by addition of 1 mL of 10% trichloroacetic acid. All reactions were carried out in duplicate.

Calcofluor Assay. For Cw staining (M 2R New American Cyanamid Co., Bound Brook, NJ), untreated and **1**-treated (37 μM) exponentially growing *S. cerevisiae* (ATCC 9763) cells and *N. crassa* (ATCC 9279) hyphae (grown in YEPD liquid medium) were harvested, washed once, and resuspended in H_2O with Cw at a final concentration of 20 μM for 5 min at rt. After washing with H_2O, cells were observed under a Leica DM RXA fluorescence microscope (Wetzlar, Germany), using the appropriate UV filter. The localization of chitin was visualized as a bright bluish fluorescence.[24]

Chitin Quantification Assay (Reissig Reaction). Untreated and **1**-treated cells (37 μM) of *S. cerevisiae* (ATCC 9263) (100 mg each) were extracted with 6% KOH during 90 min. Digestion of cells with chitinase, cleavage of chitobiose with glusulase, and measurement of NAG were performed as described previously:[44] Each sample (0.5 mL) in a 13 × 100 mm test tube was treated with 0.1 mL of 0.8 M potassium tetraborate (Fisher Scientific Company, Pittsburgh, PA). The tubes were heated in vigorously boiling H_2O for exactly 3 min and cooled in tap H_2O. DMAB reagent (3.0 mL) (Eastman Kodak Company, Rochester, NY) was then added, and immediately after mixing, the tubes were placed in a bath at 36−38 °C. After precisely 20 min, the tubes were cooled in tap H_2O and read without delay at 585 nm. Optical density of the solution was measured with a Beckman quartz spectrophotometer (model DU), with Corex cells and a 1 cm light path. NAG (Nutritional Biochemicals Corporation, Cleveland, OH) was used as a control.

Ergosterol Effect. The MIC of **1** against *S. cerevisiae* ATCC 9763 was determined following the guidelines of CLSI as explained above, in the absence and in the presence of different concentrations (50−250 μg/mL) of ergosterol (Sigma Chemical Co.) added to the assay medium, in different lines of the same microplate.[27] Amphotericin B was used as a control drug. The MIC was determined after 24 h of incubation.

Leakage Effect. Cells of *S. cerevisiae* ATCC 9763 cultured by shaking at 30 °C to early stationary phase were washed twice and diluted to approximately (3−5) × 10^7 cells/mL with cold MOPS buffer, pH 6.0. Cells were aliquoted to tubes, and **1** (final concentration = 74 μM) was added. SDS (2%) was used as reference compound, which produces 100% cellular leakage. Cells were incubated at 30 °C, and samples were taken at time intervals (6, 12, 24, and 48 h) and spun at 3000 rpm for 5 min in microcentrifuge tubes. The supernatants were collected for absorbance analysis at 260 nm in a Beckman DU-600 spectrophotometer. Results are the means of values from at least two independent assays.[27,45]

Statistical Analysis. Data were statistical analyzed by the Student's test. A $p < 0.05$ was considered significant.

Hemolysis Assay. Freshly obtained heparinized human red blood cells (RBC) were washed three times by centrifugation (2500 rpm for 10 min) in isotonic PBS, pH 7.0 at rt. RBC (2.0 mL) was added to 50 mL of sterile 5% glucose to obtain a 4% suspension. Stock solutions of **1** solubilized in DMSO (Sigma Chemical Co.) were diluted with sterile 5% glucose to yield final test concentrations of 188 to 9 μM. One milliliter of RBC suspension was added, the solution was mixed, and tubes were incubated at 37 °C. The absorbance of the liberated hemoglobin was measured spectrophotometrically at 540 nm in a double-beam Beckman spectrophotometer. Hemolysis of erythrocytes was indicated by complete clearing (lysis).[45] DMSO alone causes no lysis. Lytic concentration (LC_{100}) is defined as the lowest concentration of **1** that produces complete lysis of erythrocytes. The experiment was done in duplicate.

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