Hyalodendrosides A and B, Antifungal Triterpenoid Glycosides from a Lignicolous Hyphomycete, *Hyalodendron* Species

Gerald Bills,* Anne Dombrowski, Sandra A. Morris, Otto Hensens, Jerrold M. Liesch, Deborah L. Zink, Jan Onishi, Maria S. Meinz, Mark Rosenbach, John R. Thompson, and Robert E. Schwartz

Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065

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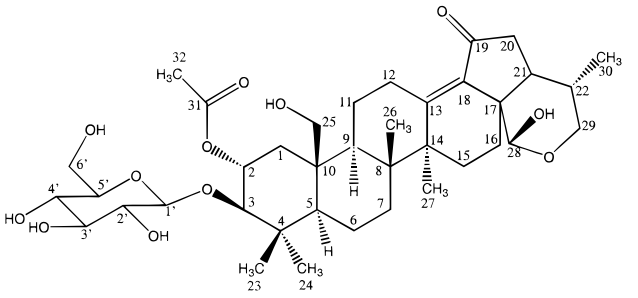
Two antifungal triterpenoid glycosides, hyalodendrosides A and B (**1** and **2**), were isolated from a solid matrix fermentation of a lignicolous hyphomycete, *Hyalodendron* sp. Their structures were determined based upon extensive examination of spectral parameters, particularly NMR and MS data. Both compounds have β-linked glucose moieties. Compounds **1** and **2** show weak to moderate antifungal activity against some clinically relevant fungi.

Screening extracts of filamentous ascomycetes for antifungal properties indicated that extracts of a strain of a wood-inhabiting *Hyalodendron* sp. exhibited in vitro antifungal activity toward clinically important yeasts and filamentous fungi. *Hyalodendron* is a genus of conidial fungi that has been applied to the asexual states of various unrelated ascomycetes.[1−3] To our knowledge no natural products have been reported from such fungi. Isolation of the active components was achieved by bioassay-guided fractionation to yield the novel metabolites, hyalodendrosides A (**1**) and B (**2**). This report describes the structure determination and antifungal activity of these new triterpene glycosides.

Results and Discussion

The UV spectrum of hyalodendroside A (**1**) exhibited a strong absorption at 263 nm, suggesting that the molecule contains an α,β-unsaturated carbonyl; the IR spectrum indicated the presence of carbonyl and hydroxyl functionalities. HRESIMS suggested a molecular formula of $C_{38}H_{58}O_{12}$ for **1** ([M+NH_4] was observed at *m*/*z* 724.4299, calcd 724.4274 for $C_{38}H_{58}O_{12} + NH_4$), indicating 10 degrees of unsaturation. MS fragmentation implied the presence of a hexose sugar ($C_6H_{11}O_6$) and an acetate group. The ^{13}C NMR/APT spectra revealed all 38 carbon atoms: six methyls, 11 methylenes, 12 methines, and nine quaternary carbons. The APT experiment gave an attached proton count of 52, suggesting six exchangeable protons.

^1H and ^{13}C NMR data for hyalodendroside A (**1**, Table 1) indicated the presence of (a) an acetate group [δ_H 2.06 (s); δ_C 21.8 (q), 173.3 (s)]; (b) a hexose sugar; (c) an α,β-unsaturated carbonyl [δ_C 211.3 (s), 136.6 (s), 157.4 (s)]; and (d) a doubly oxygenated methine carbon, likely belonging to an acetal/hemiacetal functionality [δ_H 4.98 (br s); δ_C 95.3 (d)]. These functionalities accounted for four of the 10 degrees of unsaturation (assuming a cyclized sugar); those remaining were attributed to the presence of six rings in the aglycon portion of **1**. Eleven of the 12 oxygen atoms in the molecule were accounted for by these features. The relationship of these functionalities was established using $^1H-^1H$ NMR connectivity experiments (COSY, LRCOSY, TOCSY) and $^1H-^{13}C$ NMR connectivity experiments (HMQC, HMBC), as depicted in the substructures in Figure 2. ^1H NMR coupling constant and ^{13}C NMR chemical shift data suggested that the sugar was glucose. The absolute stereochemistry of the glucose portion was not determined. The β-linkage was determined based on the ^1H NMR coupling constant of the anomeric proton (7.8 Hz) and the ^{13}C NMR chemical shift of the anomeric carbon (the anomeric carbon in a β-linked sugar has a much more downfield shift than that in the corresponding α-linked sugar). For example, the ^{13}C NMR chemical shift of C-1′ in **1** is δ 105.8, while the α-linkage in ascosteroside has been reported at δ 96.8 (d).[4,5]



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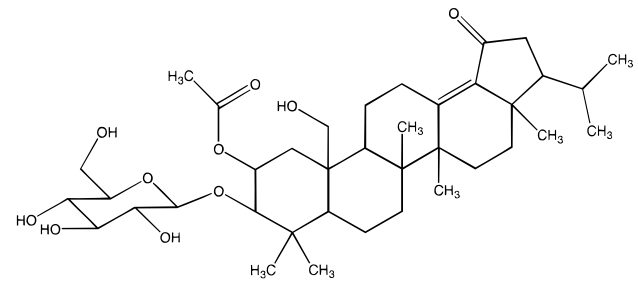

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Figure 1. Structures of hyaldendrosides A (**1**) and B (**2**).

The substructures in Figure 2 accounted for 11 of the 12 oxygen atoms in compound **1**; an isolated methylene group [δ_H 3.78 (d; J = 12.0 Hz), 3.74 (d; J = 12.0 Hz); δ_C 61.9 (t)] was associated with the remaining oxygen atom in the molecule. Further analysis of the HMBC data led to the proposed structure. Because this structure accounted for all 10 degrees of unsaturation in the molecule, the oxygen atoms on C-25 and C-28 were determined to be hydroxyl groups. These two hydroxyls, in addition to the four on the glucose moiety, accounted for the six exchange-

* To whom correspondence should be addressed. Tel.: (732) 594-3491. Fax: (732) 594-5468. E-mail: gerald_bills@merck.com.

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Table 1. NMR Data (CD_3OD, 500 MHz) for Hyalodendroside A (**1**) and Hyalodendroside B (**2**)[a]

	1		**2**	
position	δ ^{13}C	δ ^{1}H HMQC	δ ^{13}C	δ ^{1}H HMQC
1	41.6 (t)	a 2.43 (dd, 12.3, 5.1) b 1.04 (m)	41.6 (t)	a 2.43 (dd, 12.7, 5.0) b 1.04 (dd, 12.7, 10.9)
2	73.0 (d)	5.42 (ddd, 11.0, 10.3, 5.1)	73.0 (d)	5.43 (ddd, 10.9, 9.9, 5.0)
3	89.5 (d)	3.36 (d, 10.0)	89.5 (d)	3.37 (d, 9.9)
4	41.8 (s)		41.8 (s)	
5	56.5 (d)	1.02 (m)	56.5 (d)	1.03 (m)
6	18.8 (t)	a 1.65 (m) b 1.44 (m)	18.8 (t)	a 1.66 (m) b 1.44 (m)
7	35.7 (t)	a 1.71 (m) b 1.55 (m)	35.6 (t)	a 1.72 (m) b 1.55 (m)
8	44.2 (s)		44.0 (s)	
9	54.3 (d)	1.67 (m)	54.4 (d)	1.69 (m)
10	44.5 (s)		44.6 (s)	
11	24.4 (t)	a 1.50 (m) b 1.68 (m)	24.5 (t)	a 1.70 (m) b 1.51 (m)
12	25.4 (t)	a 4.05 (br d) b 1.93 (m)	25.6 (t)	a 4.05 (br d, 13.2) b 1.91 (ddd, 14.0, 13.2, 4.7)
13	157.4 (s)		158.6 (s)	
14	44.9 (s)		44.0 (s)	
15	30.2 (t)	a 2.02 (m) b 1.28 (m)	30.0 (t)	a 2.02 (m) b 1.33 (m)
16	35.0 (t)	a 2.18 (br d) b 1.36 (dd)	38.9 (t)	a 2.00 (m) b 1.44 (m)
17	45.1 (s)		44.5 (s)	
18	136.6 (s)		140.4 (s)	
19	211.3 (s)		210.4 (s)	
20	44.4 (t)	a 2.58 (dd, 18.5, 12.2) b 2.27 (dd, 18.5, 9.6)	43.7 (t)	a 2.37 (dd, 18.7, 7.9) b 2.08 (dd, 18.7, 7.7)
21	45.9 (d)	1.78 (m)	54.3 (d)	1.40 (m)
22	31.2 (d)	1.73 (m)	31.1 (d)	1.70 (m)
23	16.8 (q)	0.98 (s)	16.8 (q)	0.99 (s)
24	28.8 (q)	1.13 (s)	28.8 (q)	1.14 (s)
25	61.9 (t)	a 3.78 (d, 12.0) b 3.74 (d, 12.0)	61.9 (t)	a 3.78 (d, 12.3) b 3.74 (d, 12.3)
26	19.6 (q)	0.91 (s)	19.7 (q)	0.91 (s)
27	28.4 (q)	1.18 (s)	28.1 (q)	1.22 (s)
28	95.3 (d)	4.98 (br s)	18.8 (q)	0.96 (s)
29	59.3(t)	a 4.34 (dd, 11.4, 2.6) b 3.24 (m)	23.2 (q)	0.93 (d, 6.7)
30	21.1(q)	1.22 (d)	23.3 (q)	1.03 (d, 6.5)
31	173.3(s)	-	173.3 (s)	
32	21.8(q)	2.06 (s)	21.8 (q)	2.07 (s)
1′	105.8(d)	4.37 (d, 7.8)	105.8 (d)	4.37 (d, 7.8)
2′	75.9(d)	3.12 (dd, 9.1, 7.8)	75.9 (d)	3.12 (dd, 9.1, 7.8)
3′	78.2(d)	3.32 (dd, 9.2, 7.8)	78.2 (d)	3.32 (d, 9.1)
4′	72.0(d)	3.26 (dd, 9.2, 9.1)	72.0 (d)	3.26 (m)
5′	77.8(d)	3.22 (m)	77.8 (d)	3.22 (m)
6′	63.0(t)	a 3.85 (dd, 11.5, 2.2) b 3.66 (dd, 11.5, 5.4)	63.0 (t)	a 3.85 (dd, 11.5, 2.2) b 3.66 (dd, 11.5, 5.4)

[a] ^{1}H NMR spectra were run at 500 MHz and were referenced to the solvent signal at 3.30 ppm. ^{13}C NMR spectra were run at 125 MHz and were referenced to the solvent signal at 49.0 ppm. HMQC was used to correlate carbon signals with their attached protons. HMBC spectra were run at 500 MHz (^{1}H) and 125 MHz (^{13}C) and were referenced to the ^{1}H solvent signal at 3.30 ppm and the ^{13}C solvent signal at 49.0 ppm.

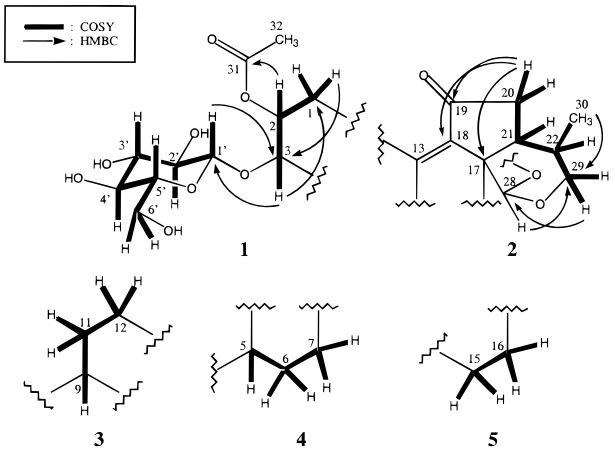

Figure 2. Substructures of hyalodendroside A (**1**).

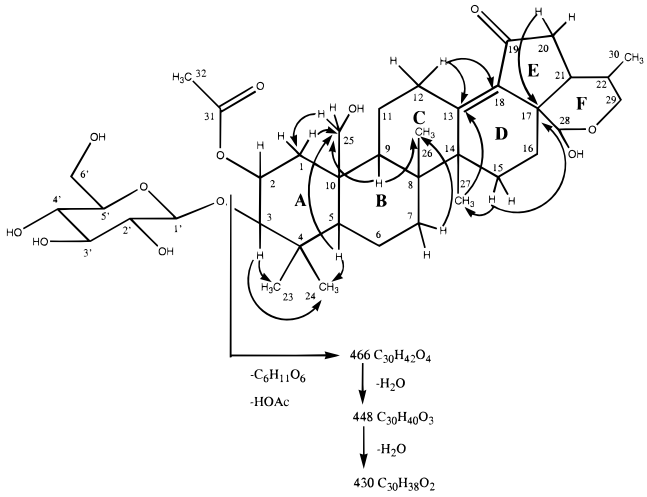

Figure 3. Selected HMBC correlations and MS fragmentations (*m*/*z*) for hyalodendroside A (**1**).

able protons in **1** indicated by the APT experiment. MS data (Figure 3) were consistent with the proposed structure **1** for hyalodendroside A.

The relative stereochemistry of **1** (Figure 4) was determined using NOESY. Rings A, B, C, and F are in the chair configuration, while rings D and E are in the half-chair and five-membered envelope configurations, respectively.

The UV spectrum of hyalodendroside B (**2**) possessed a strong absorption at 264 nm, similar to that observed for **1**; the IR spectrum indicated the presence of carbonyl and hydroxyl functionalities. The HRFABMS suggested a molecular formula of $C_{38}H_{60}O_{10}$ for **2** (found *m*/*z* 699.4031 [M + Na], corrected 676.4134, calcd 676.4186), indicating nine degrees of unsaturation. MS fragmentation implied the presence of a hexose sugar ($C_6H_{11}O_6$) and an acetate

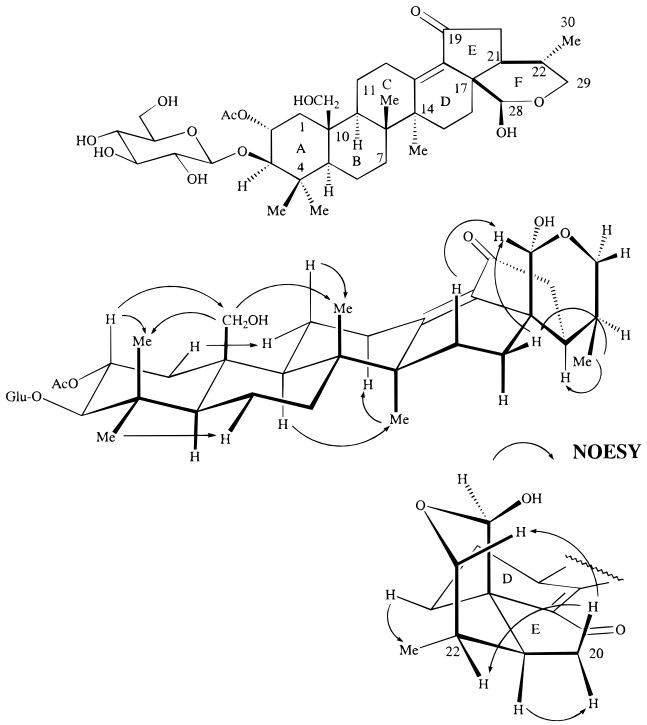

Figure 4. NOE correlations and the relative stereochemistry of hyalodendroside A (**1**).

group. The ^{13}C NMR/APT spectra revealed all 38 carbon atoms, namely eight methyls, 10 methylenes, 11 methines, and nine quaternaries. The APT experiment gave an attached proton count of 55, suggesting five exchangeable protons.

A preliminary examination of the spectral data for **2** suggested that it was very similar in structure to **1**. In fact, the ^1H and ^{13}C NMR chemical shifts of atoms associated with the A, B, and C rings (Table 1) and HMBC correlations for those rings were virtually identical. However, there were significant differences between the functionalities present on the E rings of the two molecules. Hyalodendroside B (**2**) clearly lacked the hemiacetal moiety present in hyalodendroside A (**1**). In addition, it had two fewer oxygen atoms, two more methyl groups, and one less degree of unsaturation in its structure. Based upon analysis of spectral data generated for **2**, and by comparison to the structure of **1**, the structure of hyalodendroside B was determined to be that shown in Figures 1 and 5. MS data (Figure 5) agreed with this proposed structure.

Hyalodendroside B (**2**) was moderately antifungal toward *Candida albicans*, *Saccharomyces cerevisiae*, and *Cryptococcus neoformans*. Hyalodendroside A (**1**) had little activity against *C. neoformans* and *Aspergillus fumigatus* at the levels tested. The antifungal effects of compounds **1** and **2** were counteracted in the presence of serum at 24 h (data not shown), but by 48 h, the serum antagonism was not easily seen because fungal cells were fully grown (Table 2).

Triterpenoids are ubiquitous in filamentous fungi, but terpenoid glycosides have rarely been reported.[6] WF11605, a tetratracyclic triterpene glucoside from an unidentified fungus, was found to be an antagonist of leucotriene B4-induced chemotaxis and degranulation of rabbit polymorphonuclear leukocytes.[7] Recently, an antifungal triterpenoid glycoside, ascosteroside, has been reported to be produced by *Ascotricha amphitricha*[5,8] and *Mycoleptodiscus atromaculans*.[4]

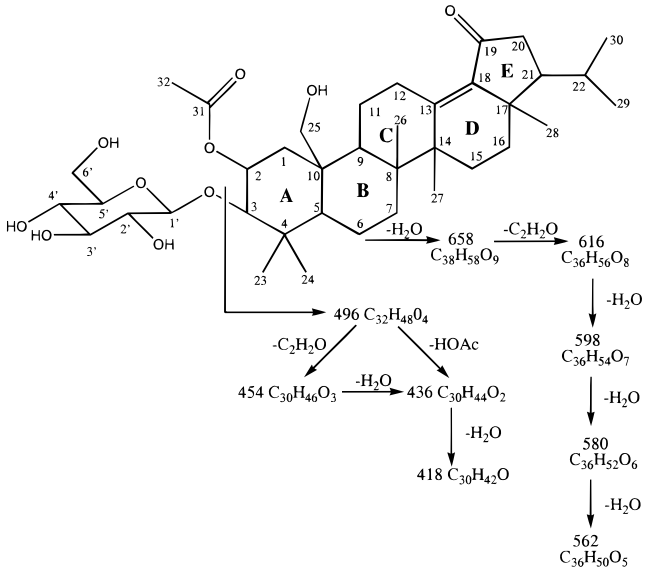

Figure 5. Structure and MS fragmentations (*m*/*z*) of hyalodendroside B (**2**).

Table 2. Antifungal Activity after 48 h of Hyalodendrosides A (**1**) and B (**2**) Compared with Amphotericin B

		MIC (μg/mL)		
species	strain	**1**	**2**	amphotericin B
Candida albicans	MY1055	128	16	0.5
Candida albicans in serum[a]	MY1055	128	128	0.25
Cryptococcus neoformans	MY2062	>256	4	0.25
Saccharomyces cerevisiae	MY2214	128	32	0.25
Aspergillus fumigatus	MF5668	>256	64	1

[a] Cells grown in 10% bovine serum albumin for 48 h.

Experimental Section

General Experimental Procedures. IR absorption spectra were obtained with a Nicolet Nexus model 670 Infrared Fourier-transform spectrophotometer using an attenuated total reflectance cell on neat 2-μg samples. UV absorption spectra were measured with a Beckman DU-70 spectrophotometer.

NMR spectra were recorded at 300 MHz on a Varian XL-300 spectrometer, at 400 MHz on a Varian Unity 400 spectrometer, or at 500 MHz on a Varian Unity 500 spectrometer (499.98 MHz proton and 125 MHz carbon frequencies) at 25 °C. Proton and carbon pw90's were 4.5 and 11.0 μs, respectively. ^1H NMR spectra were recorded in CD_3OD at 25 °C using the solvent peaks at δ 3.30 as internal references downfield of tetramethylsilane (TMS) at 0 ppm. ^{13}C NMR spectra were recorded at 100 MHz in CD_3OD at 25 °C, where chemical shifts are given in parts per million downfield of TMS using the solvent peak at 49.0 ppm as internal reference. Proton−proton chemical shift correlation spectra (COSY) were recorded in CD_3OD using the standard pulse sequence.[9] Proton−carbon chemical shift correlations were obtained using inverse detection via HMQC[10] and HMBC[11] pulse sequences. The HMBC spectra (CD_3OD, 400 MHz) were optimized for a long-range $^nJ_{CH}$ of 7 Hz and utilized either a 6.2 or 12.4 ms Gaussian pulse for the selective 90 degree ^{13}C pulse. ROESY [12] spectra were obtained using the pulse sequence described by Kessler et al.[13] with a mixing time of 200 ms.

Gradient strengths for gradient-enhanced 2D NMR experiments were nominally 10 g/cm. No significant decomposition was observed in any of the two samples.

HMQC: gradients for phase-sensitive coherence selection 2:2:1:±1,[14] ^{13}C GARP decoupling at 24 000 Hz bandwidth; relaxation delay 0.75 s; 16 scans of 352 complex points in *F*2 over 3066 Hz zero-filled to 1K complex points; 320 complex increments over 14 329 Hz in *F*1 were zero-filled to 512

complex points. A shifted Gaussian function was applied in both dimensions prior to Fourier transform.

HMBC: gradients for coherence selection 2:2:1,[14] relaxation delay 1.0; long-range C−H correlation optimized for 5 Hz (3.6 ms); 128 scans of 512 complex points in *F*2 over 3066 Hz zero-filled to 1k complex points; 512 complex increments over 25 343 Hz in *F*1 were zero-filled to 1K complex points; squared sinebell function was applied in both dimensions prior to Fourier transform.

COSY: gradients for coherence selection 1:1:2,[15] 16 scans of 1088 points in *F*2 over 3066 Hz zero-filled to 1K complex points; 512 real increments over 3500 Hz in *F*1 were zero-filled to 1k points; squared sinebell function was applied in both dimensions prior to Fourier transform.

NOESY: mix 300 ms; relaxation delay 1.2 s;[16] 32 scans of 1024 complex points in *F*2 over 3500 Hz zero-filled to 2K complex points; 320 complex increments over 3066 Hz in *F*1 were zero-filled to 1K points; shifted Gaussian function was applied in both dimensions prior to Fourier transform.

ROESY: mix 300 ms;[17] mix pw90 108 μs providing an effective field of 3700 Hz, relaxation delay 2 s; 32 scans of 768 points in *F*2 over 3894 Hz zero-filled to 1K complex points; 388 complex increments over 3894 Hz in *F*1 were zero-filled to 1K complex points; Gaussian function was applied in both dimensions prior to Fourier transform.

Nominal LRMS data were obtained on a Finnigan-MAT TSQ700 instrument. HRMS measurements were performed on a JEOL SX102A instrument using perfluorokerosene as internal standard. Trimethylsilyl (TMSi) derivatives were prepared by reaction with a 1:1 mixture of *bis*-trimethylsilyltrifluoroacetamide and pyridine at 50 °C for 30 min. The ESI (electrospray ionization) MS data were obtained on a Finnigan NewStar Fourier transform mass spectrometer (FT−MS). The sample was introduced as a liquid at a concentration of 1 μg/μL with an eluent of 90/10 acetronitrile−water with 0.01% trifluoroacetic acid (1.3 mM) plus 1.3 mM ammonium formate. Ions were generated by ESI (positive ion mode). The exact mass data were obtained using an internal standard of poly(methyl methacrylate) 620 (PMMA-620).

Fungal Material, Fermentation, and Extraction. *Hyalodendron* sp. (MF6209, GB4224) was collected on decorticated wood at Scott's Run, Fairfax Co., VA, in May 1996. A culture of the fungus was initiated from germinating conidia. Frozen vegetative mycelium (−80 °C) of GB4224 and cultures of the antifungal assay strains listed below were maintained in the Merck Microbial Resources Culture Collection. Only the asexual stage of the fungus has been observed. This stage of the life cycle has hyaline to pale grayish pink blastosporic conidia produced in dry, branched, chains. We have tentatively assigned it to the hyphomycetous anamorph genus *Hyalodendron*,[18] but it does not conform to any of the known species.[3]

The culture was inoculated into seed flasks by transferring a 1-mL aliquot of the frozen vegetative mycelium into a 250-mL Erlenmeyer flask containing 50 mL of seed medium of the following composition (g/L H_2O): yeast extract, 4.0; malt extract, 8.0; glucose, 4.0; and Junlon [a poly(acrylic acid) used to prevent mycelial clumping], 1.5. The seed medium was adjusted to pH 7.0, dispensed into 250-mL Erlenmeyer flasks and capped with cotton plugs, and autoclaved at 121 °C for 20 min. The seed cultures were incubated at 25 °C on a gyratory shaker (220 rpm), at 85% relative humidity, for 2 days prior to the inoculation of the production medium.

Fermentations were performed on a solid production medium grown in 2-L roller bottles containing approximately 675 mL of large-particle vermiculite (sterilized separately from the liquid), with 220 mL of a liquid nutrient solution poured over it. The nutrient solution contained (g/L H_2O): glucose, 150.0; glycerol, 20.0; yeast extract, 4.0; $NaNO_3$, 1.0; monosodium glutamate, 3.0; Na_2HPO_4, 0.5; $MgSO_4{+}7H_2O$, 1.0; $CaCO_3$, 8.0; trace elements solution, 1 mL/L (consisting of, in g/L, $FeCl_3{+}6H_2O$, 5.8; $MnSO_4{+}H_2O$, 0.1; $CoCl_2{+}6H_2O$, 0.02; $CuSO_4{+}5H_2O$, 0.015; $Na_2MoO_4{+}2H_2O$, 0.012; $ZnCl_2$, 0.02; $SnCl_2{+}2H_2O$, 0.005; H_3BO_3, 0.01; KCl, 0.02; concentrated HCl, 2 mL/L), with the pH adjusted to 7.0 before the addition of $CaCO_3$. The glucose was autoclaved separately to prevent caramelization. The production medium was dispensed as 220-mL aliquots in 500-mL bottles and autoclaved 15 min at 121 °C. The solid and liquid portions of the production medium were combined at the time of inoculation. Each bottle was inoculated with 12 mL of vegetative seed and shaken to coat the vermiculite with the seed growth and nutrient solution. Inoculated bottles were incubated on a Wheaton rolling machine (4 rpm), at 22 °C and 70% relative humidity, for 19 days. Fungal growth in each bottle was extracted with 250 mL of methyl ethyl ketone.

Antifungal Activity. MIC determinations were based on a microtiter broth dilution assay previously described by Bartizal et al.[19] Test organisms were grown in Synthetic Complete medium (SC), a defined medium optimized for growth of the test organisms. SC consists of 6.7 g/L yeast nitrogen base without amino acids (Difco, Detroit, MI), 20 g/L dextrose, and 0.87 g/L complete supplement mixture (BIO 101, Vista, CA). The strains tested were *Candida albicans* (MY1055), *Cryptococcus neoformans* (MY2062), *Aspergillus fumigatus* (MF5668), and *Saccharomyces cerevisiae* (MY2214 = W303-1A). Inhibition of growth was measured at 24 and 48 h. The starting concentrations for titration of these compounds was 256 μg/mL. Culture density was determined on log-phase cells by spectrophotometer readings at 600 nm and diluted to the initial inoculum absorbance of 0.0014. Endpoints were determined visually; the MIC reported was the lowest concentration that produces no visible growth.

Isolation of Hyalodendrosides A (1) and B (2). Methyl ethyl ketone extract of the fermentation of *Hyalodendron* sp. (30 mL) was dried under vacuum and partitioned between the two phases formed from 3:1:4 MeOH−0.1% aqueous trifluoroacetic acid (TFA)−hexanes. The *Saccharomyces* activity partitioned into the lower aqueous MeOH−0.1% TFA layer. Whole broth equivalents (29 mL) of the lower layer were dried and resuspended in 1:1 CH_3CN−0.1% TFA. This was chromatographed on reversed-phase HPLC [Zorbax RX-C_8; 250 × 9.4 mm; 40 °C; 2 mL/min] utilizing the following gradient for elution: 0−20 min, 50:50 (v/v) CH_3CN−0.1% TFA; 35 min 90:10 CH_3CN−0.1% TFA; 50 min 90:10 CH_3CN−0.1% TFA. Two *Saccharomyces*-active components eluted, the first at 6.59 min (7.75 mg; **1**) and the second at 23.04 min (1.32 mg; **2**). Structure determination was accomplished using these samples.

Hyalodendroside A (1): obtained as a white amorphous solid; dec 233−244 °C; $[\alpha]_D$ +36° (MeOH); UV (MeOH) λ_{max} 263 nm; IR ν_{max} 3388 cm^{-1}, 1701 cm^{-1}.

Hyalodendroside B (2): obtained as a white amorphous solid; mp 178−184 °C; $[\alpha]_D$ +10° (MeOH); UV (MeOH) λ_{max} 264 nm; IR ν_{max} 3413 cm^{-1}, 1701 cm^{-1}.

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