Three New Phenolic Compounds from a Manipulated Plant Cell Culture, *Mirabilis jalapa*

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Bioassay-guided fractionation of an organic extract of the cell mass from a manipulated plant cell culture of *Mirabilis jalapa* led to the isolation and subsequent identification of three new phenolic compounds, **1**, **2**, and **3**. The isoflavone **1** and dehydrorotenoid **2** were identified as the principal antifungal principles from this plant cell culture with IC_{50}'s of 25 and 48 μg/mL, respectively, against the test organism, *Candida albicans* DSY1024. The rotenoid **3** was inactive at 200 μg/mL in this assay.

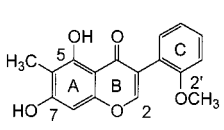
Introduction

Although natural products have provided a wide variety of complex and chemically diverse pharmaceuticals, modern natural product drug discovery encounters several obstacles. These include the problem of low abundance active metabolites, time-consuming and high cost of collection and re-collection, and variable recovery of active compounds. The yield of active compounds from wild plants is seasonally and environmentally dependent; moreover, large-scale plant collection results in destruction or damage to the producing plants and concomitant loss of natural resources. Plant cell culture offers a good alternative to whole plant collection and allows for the production of bioactive secondary metabolites under controlled and reproducible protocols.

We have developed techniques to manipulate plant cell culture by altering or stimulating their genome and/or the subsequent processes, which result in the enzymatic biosynthesis of secondary metabolites.[1] These techniques allow the production of secondary metabolites with a high degree of chemical diversity from our existing plant cell culture library. The manipulation techniques utilized include elicitation, hormone treatment, enzyme inhibition, epigenetic manipulation of the cell line, and precursor-directed biosynthesis resulting in the production of previously undiscovered plant metabolites.[1] The major advantages of these techniques are their reproducibility, fast growth, and ease of scale-up.

A whole cell growth inhibition assay of *Candida albicans* strain DSY1024 is the basis of one of several high-throughput screens used at Phytera. In this assay cell viability is determined by the reduction of the dye XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide).[2] DSY1024 is a strain of the pathogen *C. albicans* that has been rendered sensitive to a wide variety of chemotypes by deletion mutation of four genes encoding multiple-drug-resistance (MDR) pumps (Δcdr1, Δcdr2, Δflu, and Δben). Hence, this strain is supersensitive for the detection of anticandidal agents in the growth inhibition assay where the mode of action involves an intracellular target.

Cell cultures were grown under a number of manipulation protocols aimed at differential biosynthetic gene expression. Those analyzed here include unmanipulated production medium (T1), treatment of methyl jasmonate (T2), treatment of methyl jasmonate and cell wall preparation of *C. albicans* (T3), and treatment of 5-azacytidine, methyl jasmonate, and *C. albicans* (T4). Biomass derived from these protocols were harvested and extracted, and the extracts were assayed. In this paper, we have isolated active principles from the plant cell culture *Mirabilis jalapa* (Nyctaginaceae) subjected to T3 protocol. In this example, only one protocol (T3) yielded an extract active against *C. albicans* in our high-throughput screening assay. Followed by the bioassay-guided fractionation of this lead, three new phenolic compounds, **1**, **2**, and **3**, were discovered. Their structures were resolved unambiguously by extensive NMR analysis. The antifungal activity of these compounds has been determined. Literature reports of the chemistry of *M. jalapa* are sparse, with only a few yellow pigments being isolated from the flowers of the wild plant.[3]



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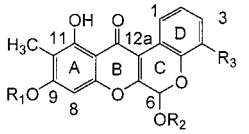
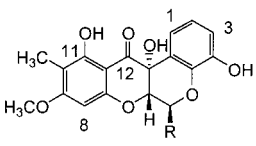
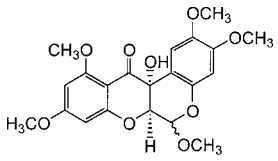


2 $R_1 = CH_3$; $R_2 = H$; $R_3 = OH$
4 $R_1 = H$; $R_2 = CH_3$; $R_3 = H$
5 $R_1 = H$; $R_2 = H$; $R_3 = H$



3 R = OCH_3
6 R = H



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Results and Discussion

In primary screening, the extract of a plant cell culture of *M. jalapa*, which had been treated with methyl jasmonate and a cell wall preparation from *C. albicans*, has shown an inhibitory effect on *C. albicans* DSY1024 with 100% growth inhibition at 50, 100, and 200 μg/mL. Similar extracts from three other protocols of this culture were inactive at 100 μg/mL. Therefore, the active extract culture was nominated as a lead and subjected to bioassay-guided fractionation. The dichloromethane−methanol extract A (2.7 g, see Extraction Procedures in Experimental Section)

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Table 1. NMR Spectral Data for Compound **1** in CD_3OD

C/H no.	^{1}H	^{13}C	HMBC	ROESY
2	7.96, s	155.9 d	C-3, C-4, C-9, C-1′	
3		122.2 s		
4		181.7 s		
5		160.3 s		
6		108.9 s		
7		163.6 s		
8	6.41, s	93.8 d	C-6, C-7, C-9, C-10	
9		157.2 s		
10		105.7 s		
1′		121.3 s		
2′		159.1 s		
3′	7.07, dd, J = 7.5, 0.9	112.1 d	C-1′, C-5′	2′-*O*-Me
4′	7.39, td, J = 7.5, 1.8	131.0 d	C-2′, C-6′	
5′	7.00, td, J = 7.5, 0.9	121.4 d	C-1′, C-3′	
6′	7.25, dd, J = 7.5, 1.8	132.6 d	C-3, C-2′, C-4′	
6-Me	2.07, s	7.4 q	C-5, C-6, C-7	
2′-*O*-Me	3.80, s	56.1 q	C-2′	H-3′

of the dry cell mass from the T3 culture of *M. jalapa* was applied to a ~200 mL polystyrene (Diaion HP-20) column (4 cm × 30 cm), and the column was stepwise eluted with a gradient of acetonitrile (ACN) in water (300 mL each of 20% (A1), 30% (A2), 50% (A3), and 80% (A4)). The column was then washed with MeOH (A5) and finally with acetone (A6). Fractions A3 to A5 were active in the *C. albicans* DSY1024 assay. Dried fraction A4 (344 mg) from the 80% ACN eluent was applied to a Sephadex LH-20 column (2 cm × 40 cm), and the column was eluted with dichloromethane−methanol (1:1) to generate fractions B1 through B10. The precipitate (ppt) of fraction B5 from the LH-20 column was identified as compound **2** (15 mg). Fraction B4 in methanol was allowed to stand at −20 °C for 3 days, whereupon a precipitate formed. This was filtered off, dried, and identified as compound **3** (8 mg). The mother liquors from fraction B4 gave, on standing at −20 °C for a week, a second precipitate, which was collected, washed with dichloromethane, and dried. This ppt was identified as isoflavone **1** (10 mg). ^{1}H NMR analysis indicated that the same compounds **1** and **2** were present in fractions A3, A5, and several B fractions, which were not further investigated. The total yields of compounds **1**, **2**, and **3** from dried biomass were estimated from proton NMR spectra of chromatographic fractions containing these compounds as 0.05, 0.04, and 0.05%, respectively.

From high-resolution FABMS (Experimental Section), the molecular formula of **1** was established as $C_{17}H_{14}O_5$ in keeping with the results of a DEPT experiment in the ^{13}C NMR, which showed 17 carbons with 12 attached hydrogens. From an HMQC experiment, a singlet proton signal at δ 7.82 ppm in the ^{1}H NMR spectrum was attached to an oxygenated olefinic carbon (δ 155.9, in ^{13}C NMR), characteristic of the H-2 and C-2 in a typical isoflavone. The isoflavone skeleton of **1** was supported by the long-range H−C heterocoupling of this proton to a carbonyl (C-4, δ 181.7), an oxygenated sp^2 (C-9, δ 157.2), and two sp^2 (C-1′, δ 121.3; C-3, δ 122.2) carbons. After accounting for the isoflavone functionalities, there were still one methyl, one methoxyl, and two hydroxyl groups remaining as substituents, based on the molecular formula and ^{13}C NMR data. From the coupling pattern (see Table 1) and ^{1}H−^{1}H COSY, H-3′ through H-6′ comprised a 1,2,3,4 system of an ortho-disubstituted aromatic ring. A lone methoxyl group gave rise to a singlet at δ 3.80 in the proton spectrum, and this was correlated in an HMBC experiment to the carbon C-2′ at δ 159.1. The HMBC experiment also established long-range coupling of this carbon with H-4′ (δ 7.39) and H-6′ (δ 7.25) and hence established the C ring as that with only two ortho substituents. All of the ^{1}H−^{13}C long-range correlations, including H-6′ to C-3 and C-2′, H-5′ to C-1′, and H-2 to C-1′ and C-3 in this portion of the molecule, support the structure of ring C as shown in **1**. One methyl and two hydroxyl groups remain to be positioned on ring A. From the HMBC experiment, the C-methyl group at δ 2.07 ppm was attached to a carbon flanked by the two oxygenated carbons at δ 160.3 and 163.6. The other oxygenated carbon in ring A at δ 157.2 is identified as C-9 by its long-range (HMBC) coupling to H-2. The chemical shifts for these six carbons on ring A were typical of the 1,3,5-trioxy substitution pattern, common in ring A of flavonoids.[4] The long-range coupling of H-8 also fit this substitution pattern and supports formulation of compound **1** as 5,7-dihydroxy-2′-methoxy-6-methylisoflavone. Abronisoflavone C, a 2′-*O*-demethyl analogue of compound **1**, has been isolated from *Abronia latifolia* (Nyctaginaceae).[5]

Compound **2** showed a molecular ion at *m*/*z* 343.0803 [M + H]$^+$ (cald 343.0818), suggesting the molecular formula $C_{18}H_{14}O_7$. This formula is supported by a ^{13}C NMR and DEPT experiments, which show 18 carbons with 11 attached protons. The ^{1}H, ^{13}C NMR, and HMBC data for compound **2** all suggest an A ring with substitution similar to that of compound **1** except for *O*-methylation of the C-9 phenol. From HMBC data, C-9 was substituted with an *O*-methyl group due to the correlation of 9-*O*-CH_3 (δ 3.91) and H-8 (δ 6.55) to C-9 (δ 165.0). The typical singlet in ring B for an isoflavone was not observed in the ^{1}H NMR spectrum of **2**, suggesting that ring B was fully substituted. The singlet proton signal at δ 6.19 was coupled (HMQC) to a carbon signal at δ 89.5 ppm, highly suggestive of an acetal. This proton showed long-range coupling to carbons at δ 158.0 (C-6a) and δ 110.8 (C-12a), in agreement with the formulation of compound **2** as a dehydrorotenoid. This accommodates the requirement of the molecular formula of **2**, which has one more unit of unsaturation than that of compound **1**. The compounds boeravinone A (**4**) and B (**5**), isolated previously from *Boerhaavia diffusa* Linn, have the same carbon skeleton.[6] *Mirabilis* and *Boehaavia* both belong to the family Nyctaginaceae. The protons H-1, H-2, and H-3 gave rise to a 1,2,3 aromatic coupling pattern, and their C−H long-range couplings indicated two ortho-substituted oxygens in ring D. Complete analysis of the HMQC and HMBC data for compound **2** resulted in its formulation as 9-*O*-methyl-4-hydroxyboeravinone B.

From high-resolution FABMS, the molecular formula of compound **3** was determined as $C_{19}H_{18}O_8$ (see Experimental Section). The proton signals on ring A of compound **3** resembled those of compound **2**. From HMBC data, C-9 was substituted with an *O*-methyl group, as evidenced by the three-bond correlation of the 9-*O*-CH_3 (δ 3.67) to C-9 (δ 166.8). The proton−proton, the proton−carbon, and multiple-bond couplings of the three remaining aromatic protons in compound **3** suggest the same substitution pattern as in ring D of compound **2**. The stoichiometry of compound **3** differs from that of compound **2** by the addition of the elements of methanol with concomitant loss of one degree of unsaturation. The two methoxyl groups in compound **3** have very similar carbon and proton chemical shifts but give rise to long-range coupling to C-6 and C-9. This leads to the formulation of compound **3** as the novel rotenoid shown.

The typical carbon signal for the carbonyl in ring B of the isoflavone and dehydrorotenoid, around δ 181 ppm (C-4 in **1** and C-12 in **2**), is replaced by a signal at δ 194.4 in compound **3**. This downfield shift is a direct result of the

Table 2. NMR Spectral Data for Compounds **2** and **3**

	2 (in methanol-d_4)			**3** (in pyridine-d_5)		
C/H no.	^{1}H	^{13}C	HMBC	^{1}H	^{13}C	HMBC
1	8.27, dd, J = 8.1, 1.5	118.7 d	C-3, C-4a	8.33, dd, J = 8.1, 1.5	122.1 d	C-3, C-4a, C-12a
1a		118.5 s			122.4 s	
2	6.94, t, J = 8.1	123.1 d	C-1a, C-4	7.05, t, J = 8.1	122.3 d	C-1a, C-4
3	6.85, dd, J = 8.1, 1.5	116.5 d	C-1, C-4a	7.28, dd, J = 8.1, 1.5	117.6 d	C-1, C-4a
4		147.0 s			146.7 s	
4a		137.6 s			142.3 s	
6	6.19, s	89.5 d	C-4a, C-6a, C-12a	6.06, d, J = 8.2	99.4 d	6-*O*-Me
6a		158.0 s		4.90, d, J = 8.2[b]	80.3 d	C-6, C-12, C-12a
7a		156.2 s			160.9 s	
8	6.55, s[a]	90.4 d	C-7a, C-9, C-10, C-11a	6.34, s[c]	91.4 d	C-7a, C-9, C-10, C-11a
9		165.0 s			166.8 s	
10		110.1 s			107.0 s	
11		160.0 s			162.2 s	
11a		106.7 s			102.4 s	
12		181.4 s			194.4 s	
12a		110.8 s			68.1 s	
10-Me	2.07, s	7.4 q	C-9, C-10, C-11	2.11, s	7.4 q	C-9, C-10, C-11
9-*O*-Me	3.91, s[a]	56.5 q	C-9	3.67, s[c]	56.4[d]q	C-9
6-*O*-Me				3.66, s[b]	56.0[d]q	C-6

[a−c] NOE observed in these pairs from ROESY experiments; NOE of compound **3** was summarized in Figure 1. [d] Interchangeable.

Table 3. IC_{50}'s (μg/mL) of Compounds **1**, **2**, and **3** and Several Reference Compounds against Wild-Type and MDR Knockout Strains

	1	**2**	**3**	genistein	daidzein	biochanin A	amphotericin B	fluconazole
DSY1024	25	48	>200	>100	>100	>100	0.19	6.7
SC5314 (wild type)	16	>200	>200	>100	>100	>100	0.15	0.18

loss of conjugation from the C-6a C-12a olefin. The saturation of this bond results in dramatic shifts for these carbons and significant shifts for C-1a and C-4a. The acetal proton (H-6, δ 6.06, d, J = 8.2) couples to an oxygenated proton (H-6a, δ 4.90, d, J = 8.2), giving rise to an isolated pair of doublets. From the HMBC spectrum, it was apparent that the oxygenated proton (H-6a) is coupled to carbons at δ 99.4 (C-6), 194.4 (C-12), and 68.1 (C-12a), confirming the rotenoid skeleton and positioning the quaternary oxygenated carbon at C-12a. This structure is supported by comparison with spectral data of the related known compound boeravinone C (**6**).[7]

A combination of ROESY, molecular modeling, and comparison of the proton coupling between H-6 and H-6a to the known model compounds determined the stereochemistry of compound **3**. The stereochemistry of rings B and C in **3** should be *trans* based on the following evidence. In the proton data of B−C *cis* compounds, H-6a had small couplings (1−3.5 Hz) to H-6 in compound **7**.[8] The dihedral angles of H-6 (α or β) and H-6a in compound **7** fall in the range 57−63°. Therefore, the B and C rings in **3** must be *trans*. The protons H-6 (δ 6.06, d, J = 8.2) and H-6a (δ 4.90, d, J = 8.2) in compound **3** were *trans* to each other, with a dihedral angel of ~174°; a *cis* configuration, with a dihedral angel around 65°, would cause a small coupling constant (<3 Hz). The *trans* configuration of the B and C rings in compound **3** is the same as that in boeravinone C (**6**).[6] Moreover, there is no significant γ effect observed on C-12a due to 6-*O*-CH_3 substitution in **3**. Therefore, 6-*O*-CH_3 should be *cis* to H-6a. This configuration was also confirmed by the NOE correlation between H-6a and 6-*O*-CH_3 in the ROESY spectrum (Figure 1).

The bioassay data are given in Table 3. The antifungal assay using the DSY1024 strain showed that the IC_{50}'s for compounds **1** and **2** were 25 and 48 μg/mL, respectively. Compound **1** had an IC_{50} of 16 μg/mL, and compound **2** was inactive at 200 μg/mL against the wild-type strain (SC5314). The dose response curves of compounds **1** and **2** in these assays are given in Figure 2. Compound **3** was inactive at 200 μg/mL against both wild-type and MDR knockout strains. Three known isoflavones, daidzein, genistein, and biochanin A, were inactive in both assays (>100 μg/mL). The dose response curves of two standards, amphotericin B and fluconazole, are given in Figure 3.

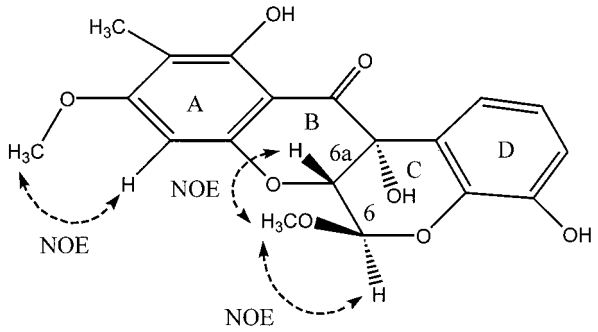

Figure 1. Stereostructure of **3** and the NOE correlations.

Experimental Section

General Experimental Procedures. NMR spectra were recorded in methanol-d_4 or pyridine-d_5 on a Varian Unity 300 NMR instrument at 300 MHz for ^{1}H and 75 MHz for ^{13}C, using standard Varian pulse sequence programs (VNMR Version 5.3 software). Electrospray MS was performed on a HP-59987A API electrospray LC/MS. Exact mass measurements (HRFAB) were obtained at the Nebraska Center for Mass Spectrometry (Chemistry Department, U. of Nebraska).

Plant Cell Culture. Culture Initiation. The seed of *M. jalapa* was originally supplied by Nanjing Botanic Garden (Nanjing, China). Leaves of *M. jalapa* seedlings were sterilized with 5% sodium hypochorite for 15 min, and sterile leaf explants were placed upon agar-solidified Gamborg's B5 medium[9] containing 2% sucrose, 2,4-dichlorophenoxyacetic acid (1 mg/L), and kinetin (0.1 mg/L). Callus tissue formed after incubation at 25 °C under continuous low light (ca. 60 lx) was dispersed in liquid Gamborg's B5 medium (components as above, lacking only agar) to yield a suspension culture. The culture was maintained by inoculating 100 mL of fresh B5

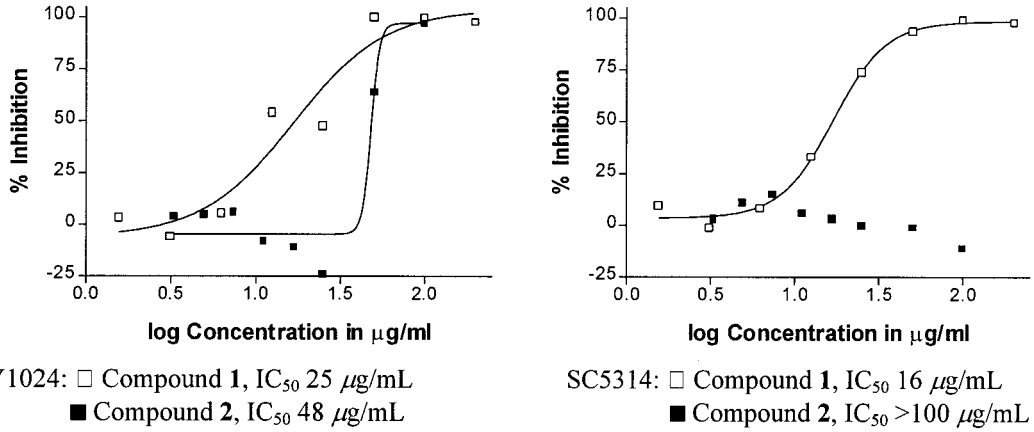

Figure 2. Dose response curves of compounds **1** and **2** in DSY1024 and SC5314 assays (curves were generated with PRISM).

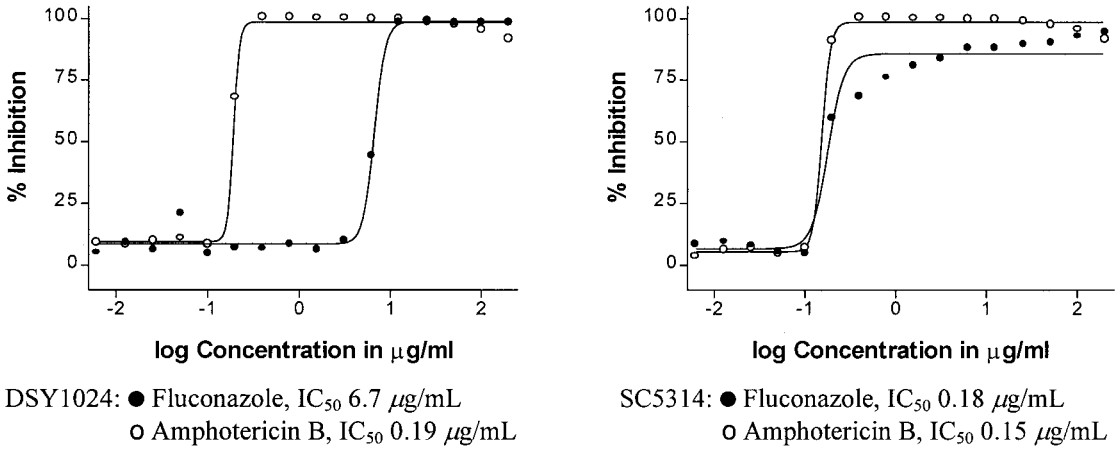

Figure 3. Dose response curves of amphotericin B and fluconazole in DSY1024 and SC5314 assays (curves were generated with PRISM).

medium with 20 mL of suspension every 14 days and incubated under continuous low light (ca. 60 lx) at 25 °C with shaking at 140 rpm. Suspension cultures were typically light yellow and finely dispersed at 25 °C with shaking at 140 rpm.

For production of metabolites **1**, **2**, and **3** the suspension culture was inoculated into a production medium comprising Gamborg's B5 salts and vitamins with 5% sucrose and no hormone additions. At 7 days after inoculation, autoclaved *C. albicans* (final concentration 50 mg/L) and methyl jasmonate (final concentration 250 μM) were added. After a further 7 days the culture was harvested.

Extraction Procedures. The biomass (dark brown in contrast to the nontreated culture) from 6 L of culture was washed with 250 mL of distilled water by filtration under vacuum. The biomass (560 g wet weight) was freeze-dried to yield a total of 52 g dry weight. The dried biomass was then extracted with 300 mL of dichloromethane and methanol (1:1) twice. The filtered organic solution was dried in a vacuum to generate the cell culture extract A (~5 g). The isolation of compounds **1**, **2**, and **3** was described in the text.

2′-*O*-Methylabronisoflavone (1): amorphous solid; UV (MeOH) λ_{max} (log ϵ) 213 (4.56), 262 (4.35) nm; IR ν_{max} (KBr) 3388 (OH), 1646, 1621, 1574, 1458, 1360, 1320, 1219, 1120, 1077 cm^{-1}; ^{1}H, ^{13}C NMR, HMBC, and ROESY (methanol-d_4), see Table 1; ESMS and FABMS *m/z* 321 [M + Na] $^+$; HR-FABMS *m/z* 321.07292 [M + Na]$^+$ (calcd for $C_{17}H_{14}O_5Na$, 321.07389).

9-*O*-Methyl-4-hydroxyboeravinone B (2): white amorphous powder; UV (MeOH) λ_{max} (log ϵ) 217 (4.67), 274 (4.58), 321 (3.87), 328 (3.86) nm; IR ν_{max} (KBr) 3382, 1653, 1624, 1595, 1473, 1355, 1288, 1208, 1140, 1015 cm^{-1}; ^{1}H, ^{13}C NMR, ROESY, and HMBC (methanol-d_4), see Table 2; ^{1}H NMR (pyridine-d_5, 300 MHz) δ 8.86 (1H, dd, J = 8.1 and 1.5, H-1), 7.44 (1H, dd, J = 8.1 and 1.5, H-3), 7.31 (1H, t, J = 8.1, H-2), 6.89 (1H, s, H-8), 6.71 (1H, s, H-6), 3.96 (3H, s, 9-*O*-CH$_3$), 2.39 (3H, s, 10-CH$_3$); ^{13}C NMR (pyridine-d_5, 75 MHz) δ 180.9, 163.9, 159.2, 158.3, 155.4, 148.1, 138.2, 122.8, 118.7, 118.1, 117.0, 110.4, 109.2, 106.3, 90.2, 89.5, 56.2, 7.7; FABMS *m/z* 343 [M + H] $^+$; HRFABMS *m/z* 343.0803 [M + H]$^+$ (calcd for $C_{18}H_{15}O_7$, 343.0818).

6-Methoxyboeravinone C (3): white amorphous powder; UV (MeOH) λ_{max} (log ϵ) 258 (4.67), 284 (4.37), 332 (4.13) nm; IR ν_{max} (KBr) 3383. 1647. 1612, 1511, 1467, 1432, 1357, 1278, 1199, 1146, 1072 cm^{-1}; ^{1}H, ^{13}C NMR, and HMBC (pyridine-d_5), see Table 2; ROESY, see Figure 1; ESMS and FABMS *m/z* 397 [M + Na] $^+$; HRFABMS *m/z* 397.0889 [M + Na]$^+$ (calcd for $C_{19}H_{18}O_8Na$, 397.0899).

Antifungal Assay. Fractions or pure compounds were tested at final concentrations of 200, 100, 50, 10, and 1 μg/mL in a 100 μL total volume in a 96-well plate. IC$_{50}$'s of pure compounds were calculated from 8-point dose response curves. To each well was added 5 μL of the test sample in DMSO. Amphotericin B was used as a positive control. *C. albicans* MDR knockout strain, DSY1024, or wild-type was cultivated on an agar plate for 18 h, transferred to a sterilized vial, and diluted to a final concentration of 5 × 10^3 cells/mL with RPMI medium. The cell suspension (95 μL) was added to wells containing test samples. The covered plate was shaken well and then incubated at 35 °C for 24 h. A 25 μL sample of XTT stock solution was added to each well, and the plate was incubated on a shaker for another 2 h at 35 °C. The plates were read using the dual wavelength set at 450/650 nm. Data were analyzed in Microsoft Excel.

Acknowledgment. HRFAB mass spectra were obtained at the Midwest Center for Mass Spectroscopy in the Department of Chemistry, University of Nebraska.

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