

N-Methyl-4-hydroxy-2-pyridinone Analogues from *Fusarium oxysporum*¹

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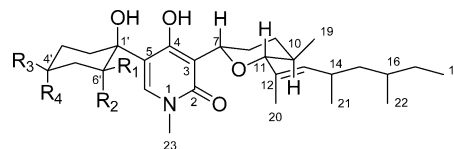
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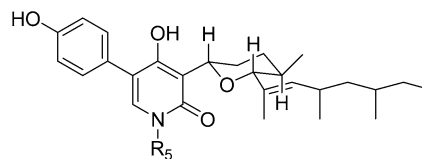
Three new *N*-methyl-4-hydroxy-2-pyridinone analogues, 6-*epi*-oxysporidinone (**3**), the dimethyl ketal of oxysporidinone (**4**), and *N*-demethylsambutoxin (**5**), along with the known compounds (–)-oxysporidinone (**1**), (–)-sambutoxin (**2**), wortmannin (**6**), enniatin A (**7**), enniatin A1 (**8**), and enniatin B1 (**9**) were isolated from *Fusarium oxysporum* (N17B) by bioassay-guided fractionation. Compounds **1** and **3** showed selective fungistatic activity against *Aspergillus fumigatus*, and wortmannin had selective potent activity against *Candida albicans*. Moderate activity was observed with the enniatins **7–9** against *C. albicans*, *Cryptococcus neoformans*, and *Mycobacterium intracellulare*. Compounds **1–5** had no activity against the agriculturally important fungi *Fusarium verticillioides* (syn. *F. moniliforme*) and *Aspergillus flavus*.

Opportunistic fungal infections constitute a major cause of morbidity and mortality in AIDS patients.¹ The drugs available for the treatment of these infections are of limited utility due to their toxicity, adverse side reactions, and the frequent emergence of resistant strains.² As a part of a program to identify new drug candidates for the treatment of opportunistic fungal infections, we have screened a number of extracts from various natural sources against the following common opportunistic fungal pathogens: *Candida albicans*, *Cryptococcus neoformans*, *Mycobacterium intracellulare*, and *Aspergillus fumigatus*. The ethyl acetate extract of the fungus *Fusarium oxysporum* (N17B) showed broad-spectrum antifungal activity. Previous studies on *F. oxysporum* (N17B) have shown that it produces a toxin that causes hemorrhaging and death in mice,³ and wortmannin has been identified as the compound responsible for this toxicity.^{4,5} Wortmannin, a powerful inhibitor of phosphatidylinositol 3-kinase,⁶ was shown to have antifungal properties.⁷

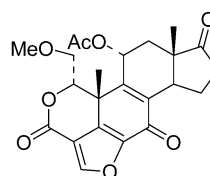
Bioassay-guided fractionation of the ethyl acetate extract of *F. oxysporum* (N17B) grown on rice medium gave two fractions with selective activity against *A. fumigatus* and *C. albicans*, respectively, and another with broad activity against *C. albicans*, *C. neoformans*, and *M. intracellulare*. Further purification of the fraction with selective activity against *A. fumigatus* led to the isolation of compounds **1** and **3** as the constituents responsible for the activity. Compound **1** had spectroscopic data including ¹H–¹³C NMR correlations identical to those reported for oxysporidinone, which was previously isolated from a different strain of *F. oxysporum*.⁸ However, the optical rotation observed for compound **1** ([α]_D –68.8) had the opposite sign of that reported for the previously reported compound ([α]_D +97).⁸ (+)-Oxysporidine was shown to be active against several agriculturally important pathogenic fungi including *A. niger*.⁸ Compound **3** was identified as the 6'-hydroxy epimer of oxysporidinone. From the inactive fractions, (–)-sambutoxin (**2**) and two further 4-hydroxy-2-pyridinone analogues (**4** and **5**) were isolated. Sambutoxin, a hemorrhagic mycotoxin, has been isolated from *F. sambucinum*.⁹ This is the first report of compounds **3–5** in nature. From the fraction with selective activity against *C. albicans*, wortmannin (**6**) was isolated as the active constituent. Separation of the fraction with broad activity against



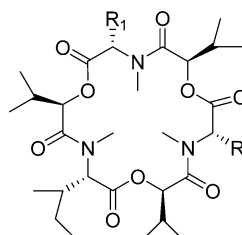
- 1** R₁ = OH R₂ = H R₃, R₄ = =O
3 R₁ = H R₂ = OH R₃, R₄ = =O
4 R₁ = OH R₂ = H R₃ = R₄ = OCH₃



- 2** R₅ = CH₃
5 R₅ = H



6



- 7** R₁ = R₂ = *s*-Bu
8 R₁ = *i*-Pr, R₂ = *s*-Bu
9 R₁ = R₂ = *i*-Pr

C. albicans, *C. neoformans*, and *M. intracellulare* led to the isolation of enniatins A (**7**), A1 (**8**), and B1 (**9**) as the compounds responsible

¹ Dedicated to Dr. Norman R. Farnsworth of the University of Illinois at Chicago for his pioneering work on bioactive natural products.

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Table 1. ^1H NMR and ^1H – ^{13}C NMR Correlation Data (600 MHz, CDCl_3) of Compounds **3**–**5**

3			4		5	
	position		δ (mult. J in Hz) [HMBC correlations]			
6		7.24 (s) [1',2,4,5,23]	7.33 (s) [1',2,5,6,23]		6.61 (s) [1',2,4,5]	
7	ax	4.61 (brd, 11.4) [2,3,4]	4.94 (brd, 11.2) [2,4,8]		5.07 (dd, 11.4, 2.4) [3,4]	
8	ax	2.12 (qd, 12.6, 3.0)	2.02 (brd, 12.0)		2.14 (dd, 12.6, 1.8)	
	eq	1.42 (brd, 12.6)	1.54 (brq, 12.6)		1.64 ^a	
9	ax	1.25 (qd, 12.3, 3.0)	1.44 (qd, 12.4, 3.0)		1.48 (qd, 12.6, 3.0)	
	eq	1.84 (dd, 12.6, 3.0)	1.87 ^a		1.94 (dd, 13.2, 3.0)	
10	ax	1.60 (m)	1.66 (m)		1.69 (m)	
11	ax	3.34 (d, 10.2) [9,12,13,19]	3.48 (d, 10.0) [10,12,13]		3.57 (d, 10.0) [9,12,13,20]	
13		5.12 (d, 9.0) [11,21]	5.18 (d, 9.6) [11]		5.23 (d, 9.0) [11,14,20,21]	
14		2.46 (heptet, 7.8)	2.47 (heptet, 7.6)		2.48 (heptet, 7.2) [13]	
15		1.03 ^a [16,22]	1.04 ^a [13,14,16,17,22]		1.06 ^a [13,16,17,22]	
		1.17 (pentet 6.0) [16,22]	1.19 ^a [13,14,16,17,22]		1.22 ^a [13,16,17,22]	
16		1.31 ^a	1.30 ^a [20,21]		1.31 ^a [17]	
17		1.37 ^a [18,22]	1.33 ^a [14,16]		1.37 ^a [18,22]	
		1.03 ^a [16,18,22]	1.02 ^a [15,16]		1.07 ^a [18,15]	
18		0.82 (t, 7.0) [16,17]	0.87 (t, 7.2) [17]		0.85 (t, 7.5) [17]	
19		0.69 (d, 6.6) [10,11]	0.78 (d, 6.4) [9,10,11]		0.76 (d, 6.6) [10,11]	
20		1.60 (s) [11,12,13]	1.62 (s) [11,12,13]		1.64 (s) [11,12,13]	
21		0.87 (d, 6.6) [13,14,15]	0.90 (d, 6.4) [13,14,15]		0.92 (d, 6.5) [13,14,15]	
22		0.81 (d, 6.6) [15,16,17]	0.87 (d, 6.4) [15,17]		0.84 (d, 6.5) [2',6',15,16]	
23		3.30 (s) [2,6]	3.43 (s) [2,6]			
2'					7.17 (d, 8.0) [4',5']	
	ax	2.32 (ddd, 15.0, 12.0, 4.2)	2.00 ^a			
	eq	2.19 (dt, 14.4, 4.2) [1',4',6']	1.80 ^a			
3'					6.98 (d, 8.0) [1',3',4',5']	
	ax	2.07 (ddd, 18.0, 12.0, 4.2) [4']	1.77 ^a			
	eq	2.40 (dt, 18.0, 4.2) [1',2']	1.89 ^a [2']			
5'					6.98 (d, 8.0) [1',3',4',5']	
	ax	2.78 (dd, 16.8, 4.8) [1',4',6']	1.79 ^a [3',4',5',6']			
	eq	2.91 (dd, 16.8, 4.8) [1',4',6']	2.22 (dd, 14.0, 4.4) [4',5',6']			
6'					7.17 (d, 8.0) [4',5']	
	ax		4.35 (dd, 11.0, 4.4)			
	eq	4.90 (t, 4.8) [1',4']				
OH			10.24 [3,4,5]		10.29	
OMe			3.17, 3.22, [4']			

^a Multiplicity cannot be determined due to overlapping.

for this activity. The current study thus led to the isolation of three classes of compounds with different activity profiles against human pathogenic fungi. However, various other activities⁴ or toxicities^{5,6,9,10} associated with these classes would preclude them as viable leads for the treatment of human fungal infections.

The ^1H NMR data of **3** were similar to those of oxysporidinone (**1**) except for the signals in the cyclohexanone moiety. The major difference was the appearance of the H-6' signal in **3** as a triplet (δ 4.90) with a coupling constant of 4.8 Hz. This is in contrast to the dd (J = 10.8, 5.4 Hz) that appeared at δ 4.65 for the same proton in oxysporidinone (**1**), suggesting an equatorial orientation for H-6' in **3**. The mass spectrum did not afford a molecular ion but gave the base peak at m/z 472.3067 [$\text{M} + \text{H} - \text{H}_2\text{O}$]⁺ (calcd for $\text{C}_{28}\text{H}_{42}\text{NO}_5$, 472.3063), indicating ready elimination of the 6'-hydroxyl group to generate a stable ion with an α,β -unsaturated carbonyl. This evidence suggested that compound **3** is the 6'-hydroxy epimer of oxysporidinone. COSY, HMBC, and HMQC NMR spectroscopic correlations (Table 1) further supported this structure.

The lack of NOESY correlations between the protons of the cyclohexanone ring and the rest of the molecule of **3** prevented the establishment of the relative configuration of the C-1' hydroxyl group. Both H-7 and H-11 were determined to be axial on the basis of coupling constants (11.4 and 10.2 Hz, respectively). The large coupling constant of H-11 showed that H-10 is also in an axial configuration, indicating that CH_3 -10 is in an equatorial position. The *E*-configuration for the 12,13 double bond was established on the basis of the chemical shift of CH_3 -20¹¹ and was further supported by the absence of a NOE correlation between CH_3 -20 and H-13. The difference in the chemical shifts of C-21 and C-22 (1.2 ppm) indicated that they are in an *anti* arrangement.¹² On the basis of this evidence, the chemical structure of compound **3** was established as 6-*epi*-oxysporidinone.

The molecular formula of compound **4** was determined to be $\text{C}_{30}\text{H}_{49}\text{NO}_7$ on the basis of HRMS data. The ^1H NMR spectrum of **4** was similar to that of compound **1**,⁸ except for the presence of two additional methoxy groups resonating at δ 3.22 and 3.17 ppm in the former. The additional methoxy group signals were also present in the ^{13}C NMR spectrum of compound **4**.⁸ Furthermore, the ^{13}C NMR spectrum of **4** showed a high-field signal at 100.7 ppm instead of a carbonyl signal in **1**. This information, in combination with the molecular formula, suggested that compound **4** is the dimethyl ketal of oxysporidinone (**1**). COSY, HMQC, and HMBC NMR spectroscopic correlations (Table 1) confirmed this structure for compound **4**. The possibility that compound **4** could be formed as an artifact during the chromatographic process was eliminated by confirming its presence in the original ethyl acetate extract.

The relative configuration of compound **4** was determined by ^1H NMR coupling constants and NOESY correlations. Large coupling constants observed for H-6' (11.0, 4.4) indicated that this proton is in an axial configuration. Strong NOE interactions between H-6' in the cyclohexane ring and H-6 in the pyridinone ring suggested that the pyridinone moiety is above the plane of the cyclohexane ring. This would be possible only if the pyridinone ring is in equatorial position. Both H-7 and H-11 exhibited large coupling constants (11.2 and 10.0 Hz, respectively), indicating that these two protons, as well as H-10, are in axial configurations. The configuration of the C12,13 double bond was assigned the *E*-configuration based on the chemical shift value of CH_3 -20¹⁰ and the lack of NOE interactions between CH_3 -20 and H-13. The small difference in the ^{13}C NMR chemical shifts suggested an *anti* arrangement for CH_3 -21 and CH_3 -22.¹¹ This combined evidence was used to establish the structure of compound **4** as the dimethyl ketal of oxysporidinone (**1**).

Table 2. ^{13}C NMR Chemical Shift Assignments (δ) (150 MHz, CDCl_3) of Compounds 3–5

position	3	4	5
2	163.3	161.5	163.2
3	109.0	111.0	110.1
4	166.1	162.4	164.2
5	116.7	115.1	116.6
6	132.8	136.1	132.7
7	72.1	78.2	77.0
8	29.2	30.9	31.7
9	33.1	32.3	32.3
10	32.1	32.6	32.7
11	91.9	92.7	92.8
12	133.6	130.2	129.9
13	136.5	138.3	138.1
14	29.7	29.8	30.0
15	45.1	44.9	45.0
16	31.8	32.2	32.3
17	29.1	29.2	29.2
18	11.5	11.4	12.0
19	17.8	17.8	18.0
20	11.4	11.8	11.6
21	21.0	20.8	21.1
22	19.8	19.8	20.0
23	38.0	37.4	
1'	76.4	74.2	125.1
2'	33.5	32.0	130.5
3'	35.1	27.2	115.6
4'	207.6	100.7	156.4
5'	42.1	36.5	115.6
6'	90.3	69.3	130.5

The ^1H and ^{13}C NMR data of compound 5 were very similar to those of sambutoxin.⁹ The major differences were the lack of a $N\text{-CH}_3$ signal and small changes in the chemical shifts of the protons and carbons in the pyridinone moiety. This evidence indicated that this compound is the N -demethyl analogue of sambutoxin. The molecular formula, $\text{C}_{27}\text{H}_{37}\text{NO}_4$, suggested by HRMS was in agreement with this observation. COSY, HMQC, and HMBC NMR spectroscopic correlations confirmed this structure.

The ^1H NMR coupling constants of 5 showed that H-7 ($J = 11.4, 2.4$ Hz) is in an axial configuration. The large coupling constant (10.0 Hz) of H-11 implied that both H-11 and H-10 are also in axial configurations. The ^{13}C chemical shift value of $\text{CH}_3\text{-20}^{11}$ and the lack of NOE correlations between $\text{CH}_3\text{-20}$ and H-13 were suggestive of an E -configuration for the 12,13 double bond. The ^{13}C NMR chemical shift difference of $\text{CH}_3\text{-21}$ and $\text{CH}_3\text{-22}$ (1.1 ppm) showed that they are in an *anti* arrangement, as in sambutoxin.¹² This evidence established compound 5 as the N -demethyl analogue of sambutoxin.

(–)-Oxysporidinone (1) suppressed the growth of *A. fumigatus* at low concentrations (Table 3); however, its inability to eliminate the fungus completely suggested fungistatic rather than fungicidal activity. Compound 3, the 6'-hydroxy epimer of oxysporidinone, had only marginal activity against *A. fumigatus*. Compounds 2, 4, and 5 were inactive against all test organisms. These results

indicated that the functional groups in the cyclohexyl ring are critical for the anti-*A. fumigatus* activity of this class of compounds. Change of configuration of the 6'-hydroxyl group resulted in reduction of activity, and the removal of the carbonyl or aromatization of the cyclohexyl ring led to complete loss of the antifungal activity. Wortmannin (6) exhibited potent selective activity toward *C. albicans*. Enniatins A (7), A1 (8), and B1 (9) showed moderate activity against *C. albicans*, *C. neoformans*, and *M. intracellulare*. Compounds 1–5 were also evaluated against agriculturally important fungi *F. verticillioides* and *A. flavus* using a disk assay but showed no activity up to a concentration of 1 mg/mL.

Experimental Section

General Experimental Procedures. Melting points (uncorrected) were recorded on an Electrothermal 9100 instrument. UV spectra were obtained in CHCl_3 , using a Hewlett-Packard 8452A spectrometer. ^1H NMR and ^{13}C NMR spectra were recorded on Varian Mercury-400BB (400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR), Bruker Avance DRX-500 (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR), or Varian Inova-600 (600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR) spectrometers, run in CDCl_3 with TMS as an internal standard. HRTOFMS were measured on an Agilent Series 1100 SL mass spectrometer equipped with an ESI source. Preparative TLC was carried out using silica gel F 254 plates (thickness 1 mm). Preparative HPLC was performed on an Agilent 1100 series instrument equipped with a photodiode array detector.

Organism and Fermentation. Isolation and identification of *Fusarium oxysporum* Schlecht. emend. Snyd. et Hans. N17B has previously been reported.^{4,5} The fungal isolate was grown on rice medium as previously described.⁵

Extraction and Isolation. Rice (2.3 kg) inoculated with *F. oxysporum* was ground and extracted three times with ethyl acetate at room temperature with sonication to give a thick gum (29 g). The hexane-soluble fraction (22 g) of this extract was chromatographed over silica gel and eluted with an increasing concentration of ethyl acetate in hexanes to give 19 fractions. Fractions 15 and 18 showed antifungal activity.

Fraction 15 was chromatographed over silica gel and eluted with CHCl_3 and $\text{CHCl}_3\text{-MeOH}$ (90:10) to give four fractions. The first fraction was purified by preparative TLC on silica gel using $\text{CHCl}_3\text{-MeOH}$ (98:2) to give wortmannin as white crystals (16 mg). The identity of this compound was confirmed by comparison of the reported physical and spectroscopic data.^{13,14} The second fraction yielded a mixture of enniatins. This fraction was separated by preparative HPLC using a Luna 10 C₁₈(2) (250 × 21 mm i.d., 10 μm particle size) column, with the mobile phase $\text{MeOH-H}_2\text{O}$ (80:20), to give enniatin A (7) (21 mg) and a fraction containing two compounds. This fraction was separated using the same column with the mobile phase $\text{CH}_3\text{CN-H}_2\text{O}$ (80:20) to give enniatins A1 (8) (19 mg) and B1 (9) (11 mg) as white amorphous residues. The identity of enniatins A (7), A1 (8), and B1 (9) was confirmed by comparison with reported physical and spectroscopic data.¹⁵

Fraction 18 from the first column was chromatographed over silica gel and eluted with $\text{CHCl}_3\text{-MeOH}$ (95:5) to yield three fractions. Fraction 1 was purified using a Luna 10 C₁₈(2) (250 × 21 mm i.d., 10 μm particle size) preparative column, with the mobile phase $\text{CH}_3\text{CN-}$

Table 3. Antifungal Activity of Compounds 1, 3, and 6–9^a

compound	<i>Candida albicans</i>		<i>Cryptococcus neoformans</i>		<i>Mycobacterium intracellulare</i>		<i>Aspergillus fumigatus</i>	
	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀	MIC
1	b		35				2.0	
3							35	
6	0.25	0.78						
7	2.0	3.13	3.5	12.5	5.0	50		
8	2.0	6.25	4.5	12.5	9.0	50		
9	2.0	6.25	9.0	25	15.0			
amphotericin B ^d	0.35	1.25	0.45	1.25	NT	NT	0.91	1.25
ciprofloxacin ^d	NT ^c	NT	NT	NT	0.30	0.63	NT	NT

^a IC₅₀ and MIC (minimum inhibitory concentration) values are in $\mu\text{g/mL}$. ^b Not active at the highest test concentration of 50 $\mu\text{g/mL}$. ^c NT: not tested. ^d Positive control.

H₂O (80:20), to give compound **4** as a white amorphous residue (16 mg). The second fraction was separated by HPLC using a Luna 10 C₁₈(2) (250 × 21 mm i.d., 10 μm particle size) preparative column, with the mobile phase MeOH–H₂O (80:20), to give **1** (21 mg) and **3** (17 mg). Sambutoxin was isolated from fraction 16 from the first column by preparative TLC on silica gel using CHCl₃–MeOH (92:8) as solvent (31 mg). The identity of this compound was confirmed by comparison with physical and spectroscopic data previously reported.⁹ Fraction 17 from the first column was chromatographed over silica gel, and elution with CHCl₃–MeOH (95:5) yielded three fractions. Fraction 2 was purified using a Luna 10 C₁₈(2) (250 × 21 mm i.d., 10 μm particle size) preparative column, with the mobile phase CH₃CN–H₂O (80:20), to give compound **5** as a white amorphous residue (19 mg).

Dimethyl ketal of oxysporidinone (4): [α]_D²⁶ –30.6 (c 0.1, CHCl₃); UV (CHCl₃) λ_{max} (log ε) 216 (3.62), 290 (3.45); ¹H NMR and ¹³C NMR data, Tables 1 and 2, respectively; HRESITOFMS *m/z* 536.3573 [M + H]⁺ (calcd for C₃₀H₅₀NO₇, 536.3587).

(–)-Oxysporidinone (1): [α]_D²⁶ –68.8 (c 0.15, EtOH); spectroscopic data and ¹H–¹³C NMR correlations were identical to those previously reported for (+)-oxysporidinone.⁸

6-epi-Oxysporidinone (3): white needles (CH₃OH); mp 176–78 °C; [α]_D²⁶ –86.9 (c 0.1, CHCl₃); UV (CHCl₃) λ_{max} (log ε) 220 (3.64), 296 (3.45); ¹H NMR and ¹³C NMR data, Tables 1 and 2, respectively; HRESITOFMS *m/z* 472.3067 [M + H – H₂O]⁺ (calcd for C₂₈H₄₂NO₅, 472.3063).

N-Demethylsambutoxin (5): [α]_D²⁶ –98.6 (c 0.1, CHCl₃); UV (CHCl₃) λ_{max} (log ε) 212 (4.32), 252 (4.03); ¹H NMR and ¹³C NMR data, Tables 1 and 2, respectively; HRESITOFMS *m/z* 440.2787 [M + H]⁺ (calcd for C₂₇H₃₈NO₄, 440.2800).

Antifungal Bioassay. All organisms were obtained from the American Type Culture Collection (Manassas, VA) and included *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, *Aspergillus fumigatus* ATCC 90906, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the NCCLS methods.^{16–18} *M. intracellulare* was tested using a modified method of Franzblau et al.¹⁹ Briefly, samples (dissolved in DMSO) were diluted serially using 20% DMSO–saline and transferred in duplicate (10 μL) to 96-well flat-bottom microplates. Inocula were prepared by diluting microbe suspensions with assay medium [RPMI 1640/2% dextrose–MOPS at pH 6.0 (Cellgro) for *C. albicans*, Sabouraud Dextrose (Difco) for *C. neoformans*, 5% Alamar Blue–RPMI 1640 broth (2% dextrose buffered with 0.165 M MOPS at pH 7.3) for *A. fumigatus*, and 5% Alamar Blue in Middlebrook 7H9 broth with OADC enrichment (Difco) pH = 7.3 for *M. intracellulare*] to afford the following colony-forming units/mL after addition to samples: *C. albicans*: 1.0 × 10⁴, *C. neoformans*: 1.0 × 10⁵, *A. fumigatus*: 3.0 × 10⁴, and *M. intracellulare*: 2.0 × 10⁶. The microbial inocula were added to the samples to achieve a final volume of 200 μL and final sample concentrations starting with 50 μg/mL. Drug controls [ciprofloxacin (ICN Biomedicals, Solon, OH) for *M. intracellulare* and amphotericin B (ICN Biomedicals) for fungi] were included. *C. albicans* and *C. neoformans* were read at 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Winooski, VT), and *M. intracellulare* and *A. fumigatus* were read at 544ex/590em using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Offenbourg, Germany) prior to and after incubation: *C. albicans* at 37 °C for 18–24 h, *C. neoformans* and *A. fumigatus* at 30 °C for 68–72 h, and *M. intracellulare* at 37 °C and 10% CO₂ for 68–72 h. Percent growth

was calculated and plotted versus test concentration to afford the IC₅₀ (sample concentration that affords 50% growth of the organism). The minimum inhibitory concentration (MIC) is defined as the lowest test concentration that allows no detectable growth.

Compounds **1–5** were assayed against *F. verticillioides* and *A. flavus* using a disk assay described by Alam et al.²⁰ using captan as the positive control.

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References and Notes

- (1) Ruhnke, M. *Drugs* **2004**, *64*, 1163–1180.
- (2) Polak, A. In *Antifungal Agents: Advances and Problems*; Jucker, E., Ed.; Birkhauser Verlag: Basel, 2003; Chapter 4, pp 59–190.
- (3) Abbas, H. K.; Mirocha, C. J.; Gunther, R. *Mycopathologia* **1989**, *105*, 143–151.
- (4) Abbas, H. K.; Mirocha, C. J. *Appl. Environ. Microbiol.* **1988**, *54*, 1268–1274.
- (5) Abbas, H. K.; Mirocha, C. J.; Shier, W. T.; Gunther, R. *JAOAC Int.* **1992**, *75*, 474–480.
- (6) Woscholski, R.; Kodaki, T.; McKinnon, M.; Waterfield, M. D.; Parker, P. J. *FEBS Lett.* **1994**, *342*, 109–114.
- (7) MacMillan, J.; Vanstone, A. E.; Yeboah, S. K. *Chem. Commun.* **1968**, 613–614.
- (8) Breinholt, J.; Ludvigsen, S.; Rassing, B. R.; Rosendahl, C. N.; Nielsen, S. E.; Olsen, C. E. *J. Nat. Prod.* **1997**, *60*, 33–35.
- (9) Kim, J.-C.; Lee, Y.-W.; Tamura, H.; Yoshizawa, T. *Tetrahedron Lett.* **1995**, *36*, 1047–1050.
- (10) Uhlig, S.; Gutleb, A. C.; Thrane, U.; Flaoyen, A. *Toxicon* **2005**, *46*, 150–159.
- (11) Couperus, P. A.; Clague, A. D. H.; Van Dongen, J. P. C. M. *Org. Magn. Reson.* **1976**, *8*, 426–431.
- (12) Organ, M. G.; Bilokin, Y. V.; Bratovanov, S. J. *Org. Chem.* **2002**, *67*, 5176–5183.
- (13) MacMillan, J.; Vanstone, A. E.; Yeboah, S. K. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2898–903.
- (14) Simpson, T. J.; Lunnion, M. W.; MacMillan, J. *J. Chem. Soc., Perkin Trans. 1* **1979**, 931–934.
- (15) Blais, L. A.; ApSimon, J. W.; Blackwell, B. A.; Greenhalgh, R.; Miller, J. D. *Can. J. Chem.* **1992**, *70*, 1281–1287.
- (16) NCCLS. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard*; NCCLS Document M27-A, 2nd ed.; National Committee on Clinical Laboratory Standards: Wayne, PA, 2002.
- (17) NCCLS. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard*; NCCLS Document M38-A; National Committee on Clinical Laboratory Standards: Wayne, PA, 2002.
- (18) NCCLS. *Susceptibility Testing of Mycobacteria, Nocardia, and Other Aerobic Actinomycetes; Tentative Standard*; NCCLS Document M24-T2, 2nd ed.; National Committee on Clinical Laboratory Standards: Wayne, PA, 2000.
- (19) Franzblau, S. G.; Witzig, R. S.; McLaughlin, J. C.; Torres, P.; Madico, G.; Hernandez, A.; Degnan, M. T.; Cook, M. B.; Quenzer, V. K.; Ferguson, R. M.; Gilman, R. H. *J. Clin. Microbiol.* **1998**, *36*, 362–366.
- (20) Alam, S.; Miah, M. A. J.; Islam, A. *J. Biol. Sci.* **2004**, *44*, 527–531.

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