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Note

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Pyridone Alkaloids from a Marine-Derived Fungus, *Stagonosporopsis cucurbitacearum*, and Their Activities against Azole-Resistant *Candida albicans*

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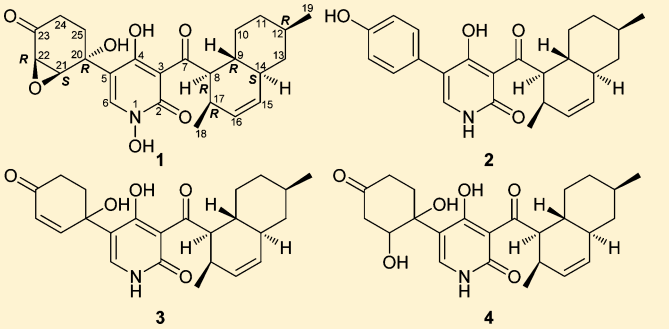
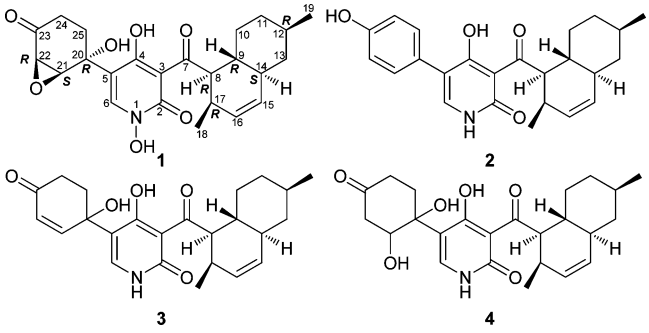
Supporting Information

ABSTRACT: Four new 4-hydroxy-2-pyridone alkaloids, didymellamides A–D (**1**–**4**), were isolated from the marine-derived fungus *Stagonosporopsis cucurbitacearum*. The structures of **1**–**4** were elucidated from spectroscopic data (NMR, MS, and IR), and the absolute configuration of **1** was determined by X-ray diffraction analysis. Didymellamide A (**1**) exhibited antifungal activity against azole-resistant *Candida albicans*.



Azole compounds are the most widely used antifungal agents to treat deep-seated candidiasis; however, the number of azole-resistant *Candida* strains is increasing.[1] Therefore, the development of new antifungal drugs is required. We investigated the antifungal activities of marine-derived fungal metabolites. Marine fungi have great potential as a source of drugs because they produce a diversity of natural products.[2] We previously reported several novel compounds from marine-derived fungi including phomactins H, I, J, K, L, and M, 13-*epi*-phomactin I,[3−5] and myrocin D.[6]

In this paper, we report new 4-hydroxy-2-pyridone alkaloids didymellamides A (**1**), B (**2**), C (**3**), and D (**4**). The isolated compounds (**1**–**4**) were evaluated for their antifungal activities. Didymellamide A (**1**) showed antifungal activity against azole-resistant *C. albicans* (MIC = 3.1 μg/mL).



Stagonosporopsis cucurbitacearum was grown on wheat medium, then extracted with $CHCl_3$. The $CHCl_3$ extract showed antifungal activity against azole-resistant *C. albicans*. The $CHCl_3$ extract was subjected to silica gel column chromatography (CC), HPLC, and Sephadex LH-20 CC. Four new compounds were obtained.

The molecular formula of compound **1** was determined to be $C_{24}H_{29}NO_7$ by HRFABMS and NMR data, which indicated 11 degrees of unsaturation. The IR spectrum exhibited absorptions at 3500 cm^{-1} (hydroxy) and 1710 and 1640 cm^{-1} (carbonyl). The ^1H and ^{13}C NMR (Table 1), DEPT, and HMQC spectra revealed the presence of two methyls, five methylenes, seven methines, one oxygenated quaternary carbon, three olefinic methines, three carbonyls, and three other sp^2 quaternary carbons, which accounted for six of the 11 degrees of unsaturation. The COSY correlations from H-21 to H-22 and from H-24 to H-25 and the HMBC correlations from H-21 to C-20 and C-25, H-22 to C-23, H-24 to C-23 and C-25, and H-25 to C-20, C-21, and C-23 suggested the presence of a 2,3-epoxycyclohexanone. The presence of an epoxide and oxygenated carbon was indicated by chemical shifts of C-21 (δ_C 58.6, δ_H 3.79), C-22 (δ_C 55.7, δ_H 3.43), and C-20 (δ_C 71.5), respectively. The COSY correlations from H-9 to H-8, H-10, and H-14, from H-17 to H-8, H_3-18, and H-16, and from H-14 to H-15 and the HMBC correlations from H-8 to C-7, C-17, C-

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Table 1. NMR Spectroscopic Data (δ in ppm) for Compounds 1−4

	didymellamide A (**1**)[a]		didymellamide B (**2**)[b]		didymellamide C (**3**)[a]		didymellamide D (**4**)[a]	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1				11.68, brd (6.4)		11.23, brs		10.88, brs
2	156.5		161.1		163.2		162.6	
3	106.3		106.7		107.7		107.0	
4	172.7		175.7		177.7		178.2	
5	112.9		112.3		114.7		114.7	
6	132.9	7.98, s	140.7	7.55, d (6.4)	137.7	7.38, s	139.3	7.39, brs
7	210.9		209.4		211.2		211.3	
8	53.5	4.31, dd (11.2, 5.6)	51.7	4.38, dd (11.4, 5.7)	52.9	4.31, dd (11.4, 5.8)	52.9	4.29, dd (11.3, 5.9)
9	36.1	1.61, m	35.8	1.47, m	36.2	1.60, m	35.8	1.56, m
10 α	29.9	0.88, m	29.3	0.86, m	29.9	0.88, m	30.1	0.84, m
10 β		1.87, m		1.80, m		1.88, m		1.90, m
11	35.3	1.06, m	35.0	0.97, m	35.3	1.06, m	35.3	1.06, m
		1.74, m		1.70, m		1.73, m		1.73, m
12	33.1	1.49, m	32.5	1.47, m	33.1	1.48, m	33.2	1.47, m
13	41.7	0.82, m	41.3	0.74, m	41.7	0.82, m	41.7	0.87, m
		1.74, m		1.70, m		1.73, m		1.73, m
14	41.8	1.87, m	41.2	1.80, m	41.8	1.88, m	41.9	1.78, m
15	130.8	5.43, brd (9.8)	130.2	5.39, brd (9.7)	130.8	5.42, brd (9.8)	130.8	5.39, brd (9.8)
16	130.9	5.59, m	131.4	5.60, m	131.1	5.57, m	131.1	5.53, m
17	31.2	2.82, m	30.5	2.78, m	31.2	2.81, m	31.6	2.69, m
18	18.0	0.84, d (7.3)	17.8	0.76, d (7.1)	18.1	0.85, d (7.1)	18.0	0.79, d (7.1)
19	22.5	0.91, d (6.6)	22.4	0.88, d (6.4)	22.5	0.91, d (6.6)	22.5	0.92, d (6.3)
20	71.5		123.2		70.4		73.0	
21	58.6	3.79, d (3.9)	130.0	7.25, d (8.5)	150.1	6.90, d (10.1)	73.3	4.59, s
22	55.7	3.43, d (3.9)	114.9	6.78, d (8.5)	130.0	6.13, d (10.1)	45.0	2.42, m
								3.22, dd (14.6, 3.1)
23	202.6		156.6		198.4		211.2	
24	32.3	2.42, m	114.9	6.78, d (8.5)	34.4[c]	2.72, m	36.7	2.28, m
		2.65, m						2.94, m
25	28.0	2.21, m	130.0	7.25, d (8.5)	35.0[c]	2.32, brd (11.7)	30.5	2.10, m
								2.49, m
4-OH		17.36, brs		16.88, s		18.02, s		18.22, s
20-OH		4.33, brs		9.48, s		4.05, s		4.62, s
21-OH								4.19, brs

[a]In $CDCl_3$. [b]In DMSO-d_6. [c]May be interchanged.

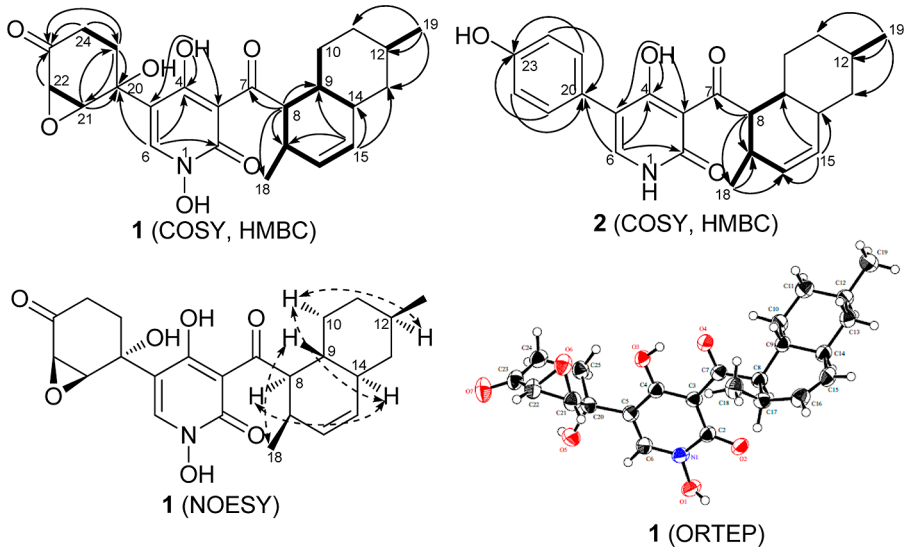

Figure 1. Key correlations for **1** and **2**: COSY (bold) and HMBC (arrows), key NOESY correlations of **1**, and an ORTEP drawing of **1** obtained by X-ray analysis [Flack parameter: x = −0.11(16)].

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18, and C-9, H-15 to C-9, C-13, C-14, and C-17, and H_3-19 to C-11, C-12, and C-13 indicated the presence of a decalin moiety. The remaining ^1H and ^{13}C NMR signals were attributable to an sp^2 methine, three quaternary carbons, one carbonyl, and a hydroxy group. In addition, the existence of one nitrogen and one hydroxy group could be suggested on the basis of the HRFABMS data. The HMBC correlations from H-6 to C-2, C-4, and C-20 and from OH-4 to C-3, C-4, and C-5 suggested the presence of a highly substituted six-membered heterocyclic ring system, in particular an α-1,4-dihydroxypyridone moiety. The spectroscopic data of the α-1,4-dihydroxypyridone moiety of compound **1** were indeed in good agreement with those of fungal metabolites such as fischerin.[7] However, the α-1,4-dihydroxypyridone moiety of compound **1** did not match with data for the 4-hydroxypyridone moiety of YM-215343.[8] The ^1H NMR chemical shifts for H-6 differed significantly (**1** δ 7.98; fischerin δ 7.93,[7] YM-215343 δ 7.48[8]), supporting an *N*-hydroxy group in **1**. The attachment position of the cyclohexanone at C-5 was established via the three-bond correlation between H-6 and C-20 (Figure 1). The *trans* orientations of H_3-18/H-8, H-8/H-9, and H-9/H-14 were deduced on the basis of the NOESY spectrum and $^3J_{HH}$ values. The coupling constant for H-8/H-9 (11.2 Hz) indicated a *trans*-diaxial coupling. The coupling constant for H-8/H-17 (5.6 Hz) suggested that the orientation of H-17 was equatorial. The NOESY correlations of H_3-18/H-9 and H-8/H-14 indicated diaxial-like relationships of H_3-18/H-8, H-8/H-9, and H-9/H-14. Additional NOESY correlations of Hα-10/H-12 and H-14 located H-8, H-12, H-14, and H-17 on the same face, which positioned H-9, H_3-18, and H_3-19 on the opposite face (Figure 1). Compound **1** has the general skeleton as reported for YM-215343,[8] but differs by having an additional *N*-hydroxy (hydroxamic acid group). However compound **1** has the hydroxamic acid group. The proposed structure was confirmed by X-ray diffraction analysis of suitable single crystals of compound **1**. The absolute configuration of 8*R*, 9*R*, 12*R*, 14*S*, 17*R*, 20*R*, 21*S*, and 22*R* was deduced from the Flack parameter,[9] −0.11(16), refined using 3569 Friedel pairs (Figure 1). Williams et al. revealed the absolute configuration of YM-215343 (isolated from *Phoma* sp.) by total synthesis in 2005.[10] Although the absolute configurations for the decalin ring systems are identical, the absolute configurations for the cyclohexanone epoxides are opposite (20*R*,21*S*,22*R* for **1** and 20*S*,21*R*,22*S* for YM-215343). Hence, the structure of **1** was determined at the level of its absolute configuration, and the compound was named didymellamide A.

The molecular formula of compound **2** was determined to be $C_{24}H_{27}NO_4$ by HREIMS and NMR data, which indicated 12 degrees of unsaturation. The spectroscopic data indicated that **2** was very similar to **1**, except that the 2,3-epoxycyclohexanone was no longer present in **2** (Table 1). The ^1H NMR spectrum of compound **2** (Table 1) showed signals of a typical AA′BB′ spin system attributable to H-21, H-25 and H-22, H-24. This spin system indicated a 1,4-substituted benzene. The phenolic group at C-23 was deduced from the HMBC correlations of H-21 and H-25 (δ_H 7.25) to C-23 (δ_C 156.6) and corroborated by the upfield shifts of proton and carbon signals at the adjacent 22- and 24-positions. The quaternary C-20 carbon resonance (δ_C 123.2) was assigned via its distinct HMBC correlations with H-22 and H-24 (δ_H 6.78). The attachment position of the *p*-hydroxyphenyl at C-5 was established via the three-bond correlation between H-6 and C-20 (Figure 1). The key NOESY correlations and $^3J_{HH}$ values for the decalin ring were in good agreement with the data for **1**. This new 4-hydroxy-2-pyridone was named didymellamide B.

The molecular formula of compound **3** was determined to be $C_{24}H_{29}NO_5$ by HRFABMS and NMR data, which indicated 11 degrees of unsaturation. The spectroscopic data indicated that **3** was very similar to **1**, except that the 2,3-epoxycyclohexanone was no longer present in **3** (Table 1). The COSY correlations from H-21 to H-22 and H-24 to H-25 and the HMBC correlations from H-22 to C-20 and C-23, H-24 to C-23 and C-25, and H-25 to C-23 suggested the presence of a 2-cyclohexen-1-one moiety. The *cis* geometry of the olefinic protons was deduced by the coupling constant for H-21/H-22 ($J_{H\text{-}21/H\text{-}22}$ = 10.1 Hz). The attachment position of the cyclohexene at C-5 was established via the three-bond correlations between H-25 and C-5 and H-6 and C-20 (Figure 1). The key NOESY correlations and $^3J_{HH}$ values for the decalin ring were in good agreement with the data for **1** and **2**. This new 4-hydroxy-2-pyridone was named didymellamide C.

The molecular formula of compound **4** was determined to be $C_{24}H_{31}NO_6$ by HREIMS and NMR data, which indicated 10 degrees of unsaturation. The spectroscopic data indicated that **4** was very similar to **1**, **2**, and **3**, except that the cyclohexenone was no longer present in **4** (Table 1). The COSY correlations from H-21 to H-22 and H-24 to H-25 and the HMBC correlations from H-21 to C-23, H-22 to C-20 and C-23, H-24 to C-20, C-23, and C-25, H-25 to C-20 and C-23, and OH-21 to C-20 suggested the presence of a 1,2-dihydroxycyclohexanone ring moiety. The presence of two oxygenated carbons was indicated by chemical shifts of C-20 (δ_C 73.0) and C-21 (δ_C 73.3, δ_H 4.59). The attachment position of the cyclohexene at C-5 was established via the three-bond correlation between OH-20 and C-5 (Figure 1). The key NOESY correlations and $^3J_{HH}$ values for the decalin ring were in good agreement with the data for **1**, **2**, and **3**. This new 4-hydroxy-2-pyridone was named didymellamide D.

The relative configurations of the decalin ring systems of compounds **2**−**4** were clarified by NOESY data. Because **2**−**4** were isolated from the same strain as compound **1**, the absolute configurations of the decalin parts of compounds **2**−**4** must be the same as those of compound **1**. Because the information for the relative configurations at C-20 in **3** and C-20 and C-21 in **4** could not be obtained from NOESY data, these configurations could not be established.

We investigated the antifungal activities of compounds **1**−**4** against azole-resistant *C. albicans* strains and other pathogenic fungi. Compound **1** inhibited the growth of azole-resistant and -sensitive *C. albicans*, *C. glabrata*, and *Cryptococcus neoformans* at concentrations of 1.6 or 3.1 μg/mL (Table 2). Compound **2** inhibited only *C. neoformans* with an MIC of 6.3 μg/mL.

Table 2. Antifungal Activities of Compounds from *S. cucubitacearum*

	Candida albicans	*C. albicans*	*C. glabrata*	*Cryptococcus neoformans*
compound	azole-resistant J2-36	azole-sensitive J1-97	J 92	MPU-B
1	3.1[a]	3.1	3.1	1.6
2	>25	>25	>25	6.3
3	>25	>25	>25	>25
4	>25	>25	>25	>25

[a]MIC, minimum inhibitory concentration (μg/mL).

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Compounds **3** and **4** did not exhibit any antifungal activity at a concentration of 25 μg/mL. On the basis of these results, we propose that the hydroxamic acid moiety plays an important role in antifungal activity. YM-215343 has previously shown antifungal activities against *C. albicans*, *C. neoformans*, and *Aspergillus fumigatus*.[8] Perhaps the epoxycyclohexanone may also be important for antifungal activity.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Yanaco MP apparatus. Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were recorded with a Shimadzu UV-240 spectrophotometer. IR spectra were recorded with a Jasco IR Report-100 spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured with a JEOL JNM-AL400 MHz spectrometer using tetramethylsilane as the internal standard. Low- and high-resolution EIMS and FABMS spectra were measured with a JEOL JMS-700 spectrometer. Column chromatography was performed using silica gel 60N (Kanto Chemical) and Sephadex LH-20 (GE Healthcare). HPLC was performed on a Senshu flow system SSC-3461 equipped with a SSC-5410 UV/vis detector at 254 nm, with an ODS [PEGASIL ODS (10 ⌀ × 250 mm No. 340096)] column. X-ray analysis was conducted using a Rigaku RAXIS RAPID imaging plate area detector with graphite-monochromated Cu Kα radiation.

Examination of the Fungus. The fungus was isolated from the surface of an unidentified sponge collected off the coast of Atami-shi, Shizuoka Prefecture, Japan, in April 2006. The isolate was identified by DNA sequence analyses of the genes encoding beta-tubulin and the rRNA internal transcribed spacer (ITS) region. The DNA sequences of these fungal genes were identical to those of *Stagonosporopsis cucurbitacearum*. The beta-tubulin and ITS DNA sequences of the fungus have been deposited in the DNA Data Bank of Japan under accession numbers AB762766 and AB762767, respectively.

Fermentation. *S. cucurbitacearum* was inoculated into 500 mL Roux flasks (20 flasks) containing wheat (150 g per flask) and artificial seawater (50 mL per flask). The flasks were incubated at 25 °C in the dark for 11 days.

Extraction and Isolation. The fermented wheat substrate was extracted with $CHCl_3$ and EtOAc. The $CHCl_3$ extract (48.95 g) was fractionated by silica gel column chromatography (9.0 × 13 cm, $CHCl_3$/MeOH, step gradient elution from 1:0 to 1:1) to obtain four fractions, A–D. Fraction B (10.21 g) was subjected to silica gel column chromatography (6.0 × 11 cm, $CHCl_3$/MeOH, step gradient elution from 1:0 to 0:1) to obtain seven fractions, B1–B7. B2 (4.34 g) was chromatographed on silica gel (5.5 × 10 cm, $CHCl_3$/acetone, step gradient elution from 50:1 to 0:1) to obtain three fractions, B2-1–B2-3. B5 (0.82 g) was fractionated by Sephadex LH-20 column chromatography (3.0 × 38 cm, MeOH) to obtain four fractions, B5-1–B5-4. B5-2 (49.4 mg) was purified by semipreparative HPLC (1.0 × 25 cm, 5 μm, 30:1 $CHCl_3$/MeOH, 2.5 mL/min) to afford compound **4** (36.5 mg, t_R 10.0 min). B5-3 (13.9 mg) was purified by semipreparative HPLC (1.0 × 25 cm, 5 μm, 100:1 $CHCl_3$/MeOH, 2.5 mL/min) to afford compound **1** (6.7 mg, t_R 40.0 min). B6 (0.47 g) was fractionated by Sephadex LH-20 column chromatography (3.0 × 38 cm, MeOH) to obtain four fractions, B6-1–B6-4. B6-2 (81.6 mg) was purified by semipreparative HPLC (1.0 × 25 cm, 5 μm, 30:1 $CHCl_3$/MeOH, 2.5 mL/min) to afford compounds **2** (22.9 mg, t_R 18.0 min) and **3** (24.0 mg, t_R 28.0 min). B6-3 (41.7 mg) was purified by semipreparative HPLC (1.0 × 25 cm, 5 μm, 30:1 $CHCl_3$/MeOH, 2.5 mL/min) to afford compounds **4** (5.1 mg, t_R 10.0 min) and **2** (10.0 mg, t_R 18.0 min). Fraction C (0.54 g) was subjected to silica gel column chromatography (3.2 × 13 cm, $CHCl_3$/MeOH, step gradient elution from 100:1 to 0:1) to obtain three fractions, C1–C3. C2 (42.6 mg) was fractionated by Sephadex LH-20 column chromatography (3.0 × 44 cm, 3:4 $CHCl_3$/MeOH) to obtain three fractions, C2-1–C2-3. C2-2 (18.6 mg) was purified by semipreparative HPLC (1.0 × 25 cm, 5 μm, 30:1 $CHCl_3$/MeOH, 2.5 mL/min) to afford compound **3** (5.5 mg, t_R 28.0 min).

*Didymellamide A (**1**):* orange solid; mp 124–127 °C; $[\alpha]^{27}_D$ +9.9 (*c* 0.50, MeOH); UV (CH_3OH) λ_{max} (log ε) 342 (3.65), 286 (3.72), 231 sh (3.93), 209 (4.16) nm; IR (KBr) ν_{max} 3500, 2920, 2850, 1710, 1640, 1600, 1540, 1430, 1370, 1320, 1210 cm^{-1}; ^{1}H NMR ($CDCl_3$) see Table 1; ^{13}C NMR ($CDCl_3$) see Table 1; FABMS *m/z* 444 $[M + H]^+$ (32), 155 (79), 118 (100), 102 (41), 84 (57); HRFABMS *m/z* 444.2018 $[M + H]^+$ (calcd for $C_{24}H_{30}NO_7$, 444.2023).

X-ray Crystallographic Data of 1. Didymellamide A (**1**) was crystallized from *n*-hexane/acetone to give colorless platelets. Crystal data: $C_{24}H_{29}NO_7$, space group $P2_1$ (#4), a = 7.5649(7) Å, b = 9.785(1) Å, c = 29.716(3) Å, V = 2199.5(4) Å^3, Z = 4, D_{calc} = 1.339 g/cm^3, R = 0.0597, wR_2 = 0.1218. The absolute configuration was determined on the basis of a Flack parameter of −0.11(16), refined using 3569 Friedel pairs. Crystallographic data for **1** reported in this paper have been deposited at the Cambridge Crystallographic Data Centre, under reference number CCDC 909198. The data can be obtained free of charge at http://www.ccdc.cam.ac.uk/cgi-bin/catreq.cgi or from the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, fax: +44-1223-336-033; e-mail: data_request@ccdc.cam.ac.uk.

*Didymellamide B (**2**):* yellow powder; mp 157–161 °C; $[\alpha]^{25}_D$ −425 (*c* 0.14, MeOH); UV (CH_3OH) λ_{max} (log ε) 347 (3.68), 247 (4.30), 211 (4.27) nm; IR (KBr) ν_{max} 3400, 2920, 2910, 2360, 1650, 1600, 1540, 1450, 1370, 1260, 1230, 1210 cm^{-1}; ^{1}H NMR (DMSO-d_6) see Table 1; ^{13}C NMR (DMSO-d_6) see Table 1; EIMS *m/z* 393 $[M]^+$ (38), 230 (100), 203 (23); HREIMS *m/z* 393.1945 $[M]^+$ (calcd for $C_{24}H_{27}NO_4$, 393.1941).

*Didymellamide C (**3**):* pale yellow solid; mp 123–126 °C; $[\alpha]^{24}_D$ −7.4 (*c* 0.10, MeOH); UV (CH_3OH) λ_{max} (log ε) 333 (4.02), 272 (3.46), 230 (4.02), 210 (4.04) nm; IR (KBr) ν_{max} 3400, 2950, 2910, 1650, 1600, 1540, 1450, 1390, 1210 cm^{-1}; ^{1}H NMR ($CDCl_3$) see Table 1; ^{13}C NMR ($CDCl_3$) see Table 1; FABMS *m/z* 412 $[M + H]^+$ (38), 185 (72), 93 (100); HRFABMS *m/z* 412.2123 $[M + H]^+$ (calcd for $C_{24}H_{30}NO_5$, 412.2125).

*Didymellamide D (**4**):* pale yellow solid; mp 151–154 °C; $[\alpha]^{22}_D$ −38.5 (*c* 0.10, MeOH); UV (CH_3OH) λ_{max} (log ε) 333 (4.02), 276 (3.81), 232 (4.18), 212 (4.28) nm; IR (KBr) ν_{max} 3400, 2950, 2910, 1700, 1650, 1600, 1540, 1450, 1390, 1320, 1210 cm^{-1}; ^{1}H NMR ($CDCl_3$) see Table 1; ^{13}C NMR ($CDCl_3$) see Table 1; EIMS *m/z* 429 $[M]^+$ (3), 411 (17), 393 (40), 248 (25), 230 (100), 203 (23); HREIMS *m/z* 429.2156 $[M]^+$ (calcd for $C_{24}H_{31}NO_6$, 429.2152).

Compound Susceptibility Testing. Azole-resistant *C. albicans* J2-36 (resistant to fluconazole, itraconazole, and voriconazole), azole-sensitive *C. albicans* J1-97, *C. glabrata* J-92, and *Cryptococcus neoformans* MPU-B were examined in this study. Susceptibility testing was performed in triplicate according to the CLSI M27-A2 microdilution method.[11] Quality control was performed on each day of testing by using the reference strains *Candida albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258.

ASSOCIATED CONTENT

Supporting Information

^{1}H, ^{13}C, DIFNOE, COSY, HMBC, HMQC, and NOESY spectra of compounds **1**–**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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