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Morphology Regulatory Metabolites from *Arthrobotrys oligospora*

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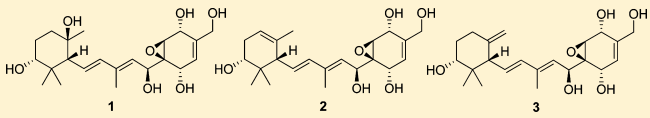
Supporting Information

ABSTRACT: Novel autoregulatory metabolites, arthrosporols A−C (**1**−**3**), involved in regulating the morphological switch in fungi, were purified and characterized from the carnivorous fungus *Arthrobotrys oligospora*. These compounds possess a novel hybrid carbon skeleton consisting of an epoxycyclohexenol combined with a rare monocyclic sesquiterpenol substructure. This is the first report of a monocyclic sesquiterpenol of this type of fungal origin. Compounds **1**−**3** displayed significant inhibitory activities toward the formation of conidiophores, while compounds **1** and **3** showed the opposite effects on the formation of a two-dimensional network with increasing rates of 40−90% and inhibiting rates of 30−90%, respectively.



Fungi produce a diverse array of secondary metabolites and undergo morphological transitions in response to environmental conditions.[1] Since the production of secondary metabolites is commonly associated with fungal sporulation processes and presumably costly to maintain, one view regarding the relationship of secondary metabolites with morphological differentiation in fungi is that secondary metabolites may stimulate sporulation. This may influence the development of the producing organism and neighboring members of the same species, perhaps enhancing the fitness of a community of related species.[2] For example, *Fusarium graminearum* produces an estrogenic mycotoxin called zearalenone that enhances perithecial production in *F. graminearum*.[3] Butyrolactone I produced by *Aspergillus terreus* increases hyphal branching and sporulation in this fungus.[4] The first evidence that secondary metabolites could play a central role in complex morphogenesis in the producing fungus comes from studies of the human pathogen *Candida albicans* that showed the fungus could produce farnesol and farnesoic acid, which blocked the morphological transition from budding yeast to the invasive filament form,[5] and tyrosol as a quorum-sensing molecule to stimulate the formation of filamentous protrusions under conditions permissive for germ-tube formation.[6] Thus far, this kind of chemical phenomena in other fungi has not been reported. In addition, although it has generally been assumed that fungal secondary metabolites may provide protection against other inhabitants in an ecological niche, the role of such metabolites in the fungal defensive morphological transition remains unexplored.

Arthrobotry oligospora is by far the most abundant nematode-trapping fungus in the environment. It has been assumed to be among the biggest contributors in controlling the population of nematodes and extensively studied as a potential biological agent for nematode control.[7] *A. oligospora* is also the model system for identifying and characterizing the ecology and biology of nematode-trapping fungi.[8] *A. oligospora* can develop two-dimensional networks under pressure. This switch from hyphal growth form to a two-dimensional network form correlates with defense.[9] The underlying mechanisms remained unknown.

A unique group of oligosporol metabolites of mixed biosynthetic origins have been characterized from *A. oligospora*.[10] The structural features of these secondary metabolites are formed by a polyketide-derived epoxy nucleus with a terpenoid-derived linear farnesyl unit. Previous studies suggested that the oligosporol metabolites are produced by the fungus regardless of strain or environment conditions,[10c] and some of them showed moderate antinematodal and antibacterial activities in vitro.

In this paper, we describe the purification of novel signal molecules for regulating the defensive and reproductive morphological transitions in *A. oligospora*. The structures of these compounds possessing a novel skeleton of 4-methyl-1-(3-methyl-5-(2,2,6-trimethylcyclohexyl)pentyl)-7-oxabicyclo[4.1.0]hept-3-ene were determined by NMR and mass spectrometry.

Compounds **1**−**3** were isolated from *A. oligospora* as amorphous solids. In contrast to all other known oligosporon compounds, **1**−**3** all displayed a quaternary carbon signal at δ_C = 38−41 in the high-field region of their ^{13}C NMR spectra.

Compound **1** was assigned to a molecular formula of $C_{22}H_{34}O_7$, confirmed by HRESIMS and NMR spectral data. The ^1H NMR spectrum (Table 1) of **1** displayed four tertiary methyls at δ_H 1.87, 1.20, 0.93, and 0.87 (each 3H of singlet) in

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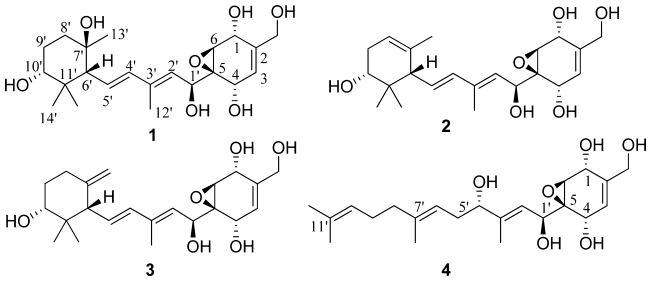

Figure 1. Compounds **1**–**4** from *A. oligospora*.

the high-field region. In the middle to low-field region, two singlets (δ_H 4.30 and 3.05), a pair of AB doublets (δ_H 4.12 and 4.08, J = 13.6 Hz), and six doublets (δ_H 6.11, 5.78, 5.36, 5.20, 4.61, and 3.28) were also observed. Other signals were between 1.5 and 2.0 ppm. The ^{13}C NMR spectrum (Table 1) of **1** demonstrated only 18 carbon resonances possibly due to long relaxation times or dynamic effects. These carbon signals were further classified by DEPT-90 and DEPT-135 experiments as two methyls, one methylene, one methine, one quaternary carbon, one oxymethylene, five oxymethines, two oxygen-bearing quaternary carbons, three olefinic methines, and two olefinic quaternary carbons. Through analysis of the HSQC spectrum, the remaining four carbons including two methyls, one methylene, and one olefinic methine, corresponding to the signals at δ_H 0.87 (3H), 1.20 (3H), 1.76 (1H), 1.52 (1H), and 5.78 (1H), respectively, were readily recognized. Comparison of the NMR data of **1** with those of arthrobotrisin B (**4**),[10c] which was obtained as the major oligosporol from this culture, demonstrated that **1** contained an identical epoxy-cyclohexenol moiety to that of **4**, and the difference between these two compounds occurred in the C_{15} side chains.

The presence of a quaternary carbon at δ_C 40.2 in **1** indicated that the C_{15} side chain in **1** was not a normal farnesyl substructure. Three spin systems, H-1′/H-2′, H-4′/H-5′/H-6′, and H-8′/H-9′/H-10′, were identified through their coupling

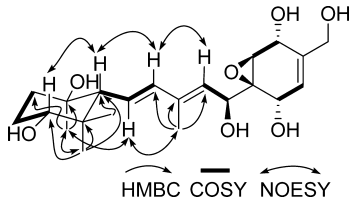

Figure 2. HMBC, $^1H-^1H$ COSY, and NOESY correlations of compound **1** (indicated by arrows).

relationships in the $^1H-^1H$ COSY experiment (Figure 2). The $^1H-^{13}C$ long-range correlations of one methyl at δ_H 1.87 with C-2′/C-3′/C-4′ and of one methyl at δ_H 1.20 with C-7′/C-8′ in the HMBC spectrum (Figure 2) led to the establishment of the connections of these three spin systems. The $^1H-^{13}C$ long-range correlations of two geminal methyls at δ_H 0.87 and 0.93 with C-11′/C-10′/C-6′ indicated a direct linkage between the quaternary carbon at δ_C 40.2 (s, C-11′) and C-6′. Thus, a six-membered carbocyclic ring with one methyl group at C-7′ and

Table 1. NMR Spectroscopic Data for Compounds 1–3 (400 MHz)

	1[a]		**2**[b]		**3**[b]	
position	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)
1	63.9, CH	4.30, brs	64.2, CH	4.28, s	64.5, CH	4.26, s
2	137.8, C		138.0, C		138.3, C	
3	123.3, CH	5.78, overlap	122.6, CH	5.71, d (4.7)	123.0, CH	5.70, d (4.8)
4	63.6, CH	4.61, d (5.0)	63.9, CH	4.59, d (4.2)	64.2, CH	4.58, d (5.0)
5	62.6, C		62.0, C		62.5, C	
6	58.8, CH	3.05, brs	58.8, CH	3.04, s	59.2, CH	3.03, s
1′	67.3, CH	5.20, d (8.8)	68.3, CH	5.04, d (8.7)	68.7, CH	5.04, d (8.8)
2′	128.7, CH	5.36, d (8.8)	129.3, CH	5.43, d (8.8)	130.3, CH	5.41, d (8.8)
3′	138.2, C		136.8, C		137.2, C	
4′	139.9, CH	6.11, d (15.5)	137.6, CH	6.11, d (15.6)	137.9, CH	6.06, d (15.6)
5′	127.5, CH	5.78, overlap	130.8, CH	5.68, dd (10.4, 15.2)	128.9, CH	5.87, dd (10.3, 15.6)
6′	61.8, CH	1.90, d (10.2)	56.1, CH	2.41, d (9.9)	57.3, CH	2.49, d (10.3)
7′	73.0, C		135.0, C		150.7, C	
8′	40.2, CH_2	1.76, m	120.1, CH	5.35, brs	34.5, CH_2	2.34, ddd (14.0, 4.2, 3.8)
		1.52, m				2.14, m
9′	29.2, CH_2	1.74, m	32.7, CH_2	2.20, d (17.1)	32.8, CH_2	1.76, m
		1.55, m		2.00, d (17.1)		1.51, m
10′	78.6, CH	3.28, d (3.7)	74.1, CH	3.46, t (6.2)	77.4, CH	3.42, dd (11.4, 3.6)
11′	40.2, C		38.4, C		41.5, C	
2-CH_2OH	63.8, CH_2	4.12, d (13.6)	64.0, CH_2	4.14, d (13.5)	64.4, CH_2	4.13, d (13.5)
		4.08, d (13.6)		4.07, d (13.5)		4.06, d (13.5)
12′	13.4, CH_3	1.87, s	13.4, CH_3	1.80, s	13.9, CH_3	1.84, s
13′	24.2, CH_3	1.20, s	23.1, CH_3	1.53, s	109.5, CH_2	4.75, s
						4.56, s
14′	16.7, CH_3	0.87, s	17.5, CH_3	0.81, s	15.7, CH_3	0.78, s
15′	29.5, CH_3	0.93, s	26.2, CH_3	0.92, s	27.5, CH_3	0.95, s

[a]Recorded in CD_3OD. [b]Recorded in acetone-d_6.

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two geminal methyl groups at C-11′ was determined in the C_{15} side chain of **1**.

The stereochemistry of the epoxy-cyclohexendiol moiety and C-1′ in the monocyclic farnesyl substructure was determined to be the same as those of **4**,[10c] according to their similar chemical shifts and the corresponding NOE correlations (Figure 2). The NOEs of H-4′ with H-2′, and of H-5′ with Me-12′, in combination with the large vicinal coupling constant between H-4′ and H-5′ (J = 15.5 Hz), indicated an E configuration of the double bond between C-4′ and C-5′. The strong 1,3-diaxial NOE correlations of H-6′ and H-10′ illustrated the preferable chair conformation of the trimethylcyclohexane moiety where H-6′ and H-10′ were both in axial position. The 1,3-diaxial NOE correlation of Me-13′ and Me-14′ suggested these two methyl groups were axial. This was confirmed by the NOE cross-peaks of H-4′/H-6′ and H-5′/Me-13′. The above evidence indicated that the alkyl chain at C-6′ and the hydroxyls at C-7′ and C-10′ were equatorial. Finally, compound **1** was characterized as shown in Figure 2 and was named arthrosporol A.

Compounds **2** and **3** were assigned to a molecular formula of $C_{22}H_{32}O_6$ by their HRESIMS and NMR spectral data. The ^{1}H, ^{13}C NMR and DEPT spectra (Table 1) of **2** and **3** revealed that both compounds closely resembled **1**. Detailed comparison of their NMR data revealed that an oxygen-bearing quaternary carbon (δ_C 73.0) and a methyl group (δ_C 24.2, δ_H 1.20) in **1** were replaced by an olefinic quaternary carbon (δ_C 135.0) and an olefinic methine group (δ_C 120.1, δ_H 5.35) in **2** and by an olefinic quaternary carbon (δ_C 150.7) and an olefinic methylene (δ_C 109.5, δ_H 4.75 and 4.56) in **3**, respectively. The ^{1}H−^{13}C long-range correlations of the methyl at δ_H 1.53 with δ_C 135.0/120.1 and H-10′ at δ_H 3.46 with δ_C 120.1 in the HMBC spectrum of **2** indicated that the olefinic quaternary carbon (δ_C 135.0) and the olefinic methine (δ_C 120.1) were assignable to C-7′ and C-8′, respectively. In the HMBC spectrum of **3**, the olefinic quaternary carbon (δ_C 150.7) and the olefinic methylene (δ_C 109.5) displayed long-range correlations with H-6′ at δ_H 2.46, which further demonstrated long-range correlations with δ_C 137.9 (d, C-4′), 128.9 (d, C-5′), 15.7 (q, C-14′), 27.5 (q, C-15′), 34.5 (t, C-8′), and 77.4 (d, C-10′). Thus, the olefinic quaternary carbon (δ_C 150.7) and the olefinic methylene (δ_C 109.5) in **3** were established as C-7′ and C-13′, respectively. Unambiguous assignments of the NMR data of **2** and **3** were achieved through 2D NMR experiments. All the physicochemical data are in full agreement with the proposed structures of **2** and **3** as shown, which were named arthrosporols B and C, respectively.

Compounds **1**−**3** possessed a monocyclohexyl sesquiterpenol unit with a 1,3,3-trimethylcyclohexyl ring instead of a linear farnesyl unit in all other known oligosporols. Among the known fungal metabolites,[11] only trisporic acids,[12] C-18 derivatives of β-carotene, showed similarity to the monocyclohexyl sesquiterpenol units in **1**−**3**. Trisporic acids B and C were shown to stimulate zygophore formation in water mold *Mucor mucedo* as fungal sex hormones.[12]

To evaluate the activities of arthrosporols A−C, morphology bioassays using *A. oligospora* were performed.[5,6] At concentrations of 0.05−5.0 μg/mL, compounds **1**−**3** showed obvious inhibitory activities toward the formation of conidiophores in the fungus. **3** displayed the strongest activity, with inhibitory rates of 50−90% (Table 2). In the morphological bioassay of hypha transition to a two-dimensional network, it was interesting to note that compounds **1** and **3** showed opposite effects on the formation of a two-dimensional network with increasing rates of 40−90% and inhibiting rates of 30−90%, respectively. In addition, compound **3** inhibited spore germination with a rate of 43% within 4 h at a concentration of 5 μg/mL.

Table 2. Influence of Compounds 1−3 on the Morphology of *A. oligospora*

		morphological results (mean % + SD)	
	concn (μg/mL)	conidiophore	2D network[b]
control[a]	0	38.9 ± 5.5	22.2 ± 2.7
1	5.00	15.6 ± 7.8	32.4 ± 4.9
	0.50	9.9 ± 1.4	34.6 ± 4.0
	0.05	11.7 ± 5.8	42.4 ± 3.5
2	5.00	6.7 ± 5.2	26.6 ± 0.9
	0.50	10.0 ± 1.6	22.4 ± 2.5
	0.05	20.0 ± 9.7	23.4 ± 3.1
3	5.00	4.4 ± 2.5	14.0 ± 4.1
	0.50	8.0 ± 5.2	15.6 ± 5.6
	0.05	14.4 ± 8.2	6.4 ± 1.5

[a]For the control, no compound was added. [b]2D network: two-dimensional network.

The results indicated that compounds **1** and **3** displayed significant autoregulatory effects on the formation of conidiophores and the transition of hypha to a two-dimensional network in *A. oligospora*, which correlate with fungal reproductive and defensive capabilities, respectively. These findings have implications for secondary metabolites by the fungus and might have agrochemical value in the development of biological control for nematodes.

EXPERIMENTAL SECTION

General Experimental Procedures. Silica gel 60 (Merck, 230−400 mesh) was used for column chromatography. Column chromatography was performed on 200−300 mesh silica gel (Qingdao Marine Chemical Factory, P. R. China). Optical rotations were measured on a Horiba-SEAP-300 spectropolarimeter. UV spectral data were obtained on a Shimadzu-210A double-beam spectrophotometer. IR spectra were recorded on a Bruker-Tensor-27 spectrometer with KBr pellets. NMR experiments were carried out on either a Bruker AV-400 or a DRX-500 spectrometer with TMS as internal standard. MS were recorded on a VG-Auto-Spec-3000 spectrometer. High-resolution ESIMS data were measured on a Bruker Bio-TOF III electrospray ionization mass spectrometer. Compounds on plates were detected by spraying with 20% (w/v) H_2SO_4 and heating on a hot plate. The UPLC-MS/MS analysis for the identification of the arthrosporols was carried out on samples using a Waters Acquity Ultra Performance LC (Waters, Milford, MA, USA), coupled to an API 4000 MS/MS System (Applied Biosystems, Foster City, CA, USA), equipped with a 4.6 × 150 mm, 3.0 μm Atiantis dC_{18} column (Waters, Milford, MA, USA).

Fungal Material. The strain *A. oligospora* YMF 1.3170 was isolated in Jiuquan, Gansu, People's Republic of China, and identified as *A. oligospora* by morphological features of the conidiophores and the submerged hyphae and rates of growth.[8] The isolate was deposited in the strain collection of Laboratory for Conservation and Utilization of Bio-Resources & Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University.[10c] DNA extraction of *A. oligospora* YMF 1.3170 and PCR amplification of the ITS rRNA gene were carried out according to the literature.[8] A characteristic fragment (GenBank, accession no. JX244893) was amplified by PCR using the species-specific primers ITS4 and ITS5, and the genome DNA of YMF 1.3170 as template. The BLAST searches in the GenBank showed that DNA fragments of 31 randomly selected strains were most similar to

the published ITS sequences of *A. oligospora*, consistent with morphological identifications.

After the conidia had developed on PDA slants in test tubes at 25 °C, the strains were kept at −30 °C as stock cultures. The strain was cultured in three types of culture media including corn medium (corn 20%), PDL medium (potato 200 g L^{-1}, dextrose 20 g L^{-1}), and YPB medium (yeast extract 1 g L^{-1}, multipeptone 2 g L^{-1}, beef extract 1 g L^{-1}, glucose 10 g L^{-1}). The culture broth of a 25-day-old liquid fermentation (1 L) was filtered to separate the mycelia from the culture. The culture filtrate was concentrated in vacuo to 50 mL and was exhaustively extracted overnight with ethyl acetate (1:1 v/v). The organic phase was concentrated under reduced pressure. The dried organic residue was then dissolved in 2 mL of methanol, filtered through a 0.22 μm membrane, and further analyzed using LC-MS/MS.

Extraction and Isolation. The strain *A. oligospora* YMF 1.3170 cultured on PDA medium at 28 °C for 7 days was inoculated into 4 × 500 mL flasks containing 250 mL of the seed media (PDB: potato 200 g, glucose 20 g, and H_2O 1000 mL). The pH of the medium was unadjusted. Cultivation was performed in a 70 L fermentor containing 40 L of production medium (yeast extract 1 g L^{-1}, multipeptone 2 g L^{-1}, beef extract 1 g L^{-1}, glucose 10 g L^{-1}). The strain was cultured at 28 °C, with 50 L/min aeration and stirring at 200 rpm for 4 days. A total of 80 L of culture broth of the strain *A. oligospora* was separated by filtration into the mycelia and filtrate. The filtrate was concentrated to 2.5 L and extracted five times with equal volumes of ethyl acetate. The EtOAc layer was combined and evaporated under reduced pressure to give a brown gum (25.5110 g). This gum was loaded onto a macroporous resin column and eluted with H_2O−MeOH with decreasing polarity to yield four fractions (A−D) based on TLC behavior. Fraction C (10.5371 g), obtained on elution with 50−90% MeOH−H_2O, was further subjected to a Sephadex LH-20 gel column eluting with MeOH to yield six subfractions. Subfraction 4 was subjected to a silica gel column (40 g, 200−300 mesh) eluting with $CHCl_3$−MeOH (20:1) and further separated on a Sephadex LH-20 gel column eluting with acetone to yield five fractions. Fraction 4 (0.5653 g) was loaded onto a C_{18} column and eluted with H_2O−MeOH (30−50%) with decreasing polarity to give **4** (0.2512 g). The remaining fraction 4 was subjected to repeated silica gel column chromatography eluting with $CHCl_3$−MeOH (25:1) and a C_{18} column eluting with H_2O−MeOH (30−40%) with decreasing polarity to obtain **3** (30 mg). Fraction 3 (0.4603 g) was repeatedly subjected to a Sephadex LH-20 gel column eluting with acetone, a C_{18} column eluting with H_2O−MeOH (35−45%) with decreasing polarity, and a silica gel column eluting with $CHCl_3$−MeOH (35:1) with increasing polarity to obtain **1** (10 mg) and **2** (18 mg).

*Arthrosporol A (**1**):* amorphous solid; $[\alpha]_D^{23.5}$ −3.47 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 235.8 (4.62); IR (KBr) ν_{max} 3475, 3041, 3005, 2963, 2926, 2874, 1729, 1628, 1601, 1582, 1462, 1444, 1413, 1391, 1371, 1304, 1288, 1261, 1205, 1139, 1121, 1082, 1030, 1006, 981, 963, 943, 918, 869, 837, 818, 744, 652 cm^{-1}; ^1H NMR and ^{13}C NMR see Tables 1 and 2; negative HRESIMS *m/z* 409.2232 $[M - H]^-$, calculated 409.2231 for $C_{22}H_{33}O_7$; positive HRESIMS *m/z* 433.2198 $[M + Na]^+$, calculated 433.2196 for $C_{22}H_{34}O_7Na$.

*Arthrosporol B (**2**):* amorphous solid; $[\alpha]_D^{23.5}$ −102.66 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 236.0 (4.73); IR (KBr) ν_{max} 3418, 3029, 2963, 2927, 2873, 2855, 1726, 1657, 1640, 1629, 1442, 1412, 1382, 1286, 1202, 1171, 1117, 1077, 1025, 977, 948, 892, 871, 745 cm^{-1}; ^1H NMR and ^{13}C NMR see Tables 1 and 2; negative HRESIMS *m/z* 391.2205 $[M - H]^-$, calculated 391.2207 for $C_{22}H_{31}O_6$.

*Arthrosporol C (**3**):* amorphous solid; $[\alpha]_D^{23.5}$ −14.80 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 234.4 (4.50); IR (KBr) ν_{max} 3424, 2959, 2927, 2855, 2872, 1729, 1641, 1629, 1463, 1452, 1422, 1380, 1286, 1174, 1124, 1074, 1024, 982, 947, 893, 744 cm^{-1}; ^1H NMR and ^{13}C NMR see Tables 1 and 2; negative HRESIMS *m/z* 391.2210 $[M - H]^-$, calculated 391.2207 for $C_{22}H_{31}O_6$.

Bioassay. *A. oligospora* strain was maintained on a potato dextrose agar (PDA) slant and transferred at regular intervals. To prepare spores for the experiments, *A. oligospora* was cultured at 28 °C on PDA medium for a week. Sterile water (5 mL) was used to harvest *A. oligospores* from one-week-old cultures on PDA. The spores were washed three times with sterile distilled water before use. A 5 mm diameter disk of *A. oligospora* was placed on the PDA medium in a 9 cm Petri dish and incubated in darkness at a constant temperature of 28 °C for a week. For the bioassays, fungal spore suspensions were prepared by washing the cultures with 5 mL of sterile water. The suspension was concentrated to 1 mL by vacuum centrifuge at 6000 rpm. An aqueous suspension (1×10^7 spores per milliliter) was prepared for inoculation. Test samples were dissolved in 500 μL of acetone respectively and then diluted with sterile water to prepare a stock solution 200 μg/mL. The same amount of acetone dissolved in water was established as a control. A 100 μL amount of 200, 20, and 2 μg/mL sample solutions prepared from the stock solution was added to a clean 5 cm Petri dish that contained 4 mL of agar medium (2 g of agar in 100 mL of water). A 10 μL sample of the fungal spore suspension was then transferred to each Petri dish and gently mixed. All dishes were incubated at 28 °C. At time zero, the inoculated spores are 98% undifferentiated with 0% germ tubes. Spores in control experiments usually developed germ tubes after about 1−2 h, and the germination of spores was assessed at 2, 4, 6, and 8 h using a normal binocular microscope. At each time point during the experiment, percentage spore germination was determined microscopically by counting 200 spores from triplicate dishes. After 72 h, the differentiation assay was performed, and the bioassay of the morphological transitions of hypha to two-dimensional nets and conidiophores was assessed. Seven fields in each Petri dish were picked at random for counting. All the tests were repeated three times, and data obtained were statistically analyzed.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spectroscopic data (1D and 2D NMR spectra) of compounds **1**−**3** are 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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