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Curvularides A–E: Antifungal Hybrid Peptide–Polyketides from the Endophytic Fungus *Curvularia geniculata*

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Abstract: Five new hybrid peptide–polyketides, curvularides A–E (**1**–**5**), were isolated from the endophytic fungus *Curvularia geniculata*, which was obtained from the limbs of *Catunaregam tomentosa*. Structure elucidation for curvularides A–E (**1**–**5**) was accomplished by analysis of spectroscopic data, as well as by single-crystal X-ray crystallography. Curvularide B (**2**) exhibited antifungal activity against *Candida albicans*, and it also showed synergistic activity with a fluconazole drug.

Keywords: antifungal agents · natural products · peptides · phytochemistry · polyketides

Introduction

At present, the incidence of severe invasive mycoses caused by *Candida albicans*, an opportunistic fungal pathogen, is remarkably increased, especially in HIV-infected patients. The number of available antifungal drugs is limited, and the development of drug resistance occurs frequently.[1,2] Research towards the discovery of new and effective antifungal drugs is thus urgently needed. Endophytic fungi grow symbiotically within plant living tissues and provide structurally novel and biologically active compounds,[3–6] some of which are anticancer drugs, for example, paclitaxel (Taxol) and camptothecin.[7,8] Thailand has a great diversity of flora and fauna; however, very little is known about the fungal endophytes. These hidden microbes are therefore an interesting source to search for new bioactive substances that may be therapeutically important for the treatment of various diseases. Herein, we report the isolation, structure elucidation, and antifungal activity of the hybrid peptide–polyketides curvularides A–E (**1**–**5**); these compounds were obtained from a culture broth of the endophytic fungus *Curvularia geniculata* CTOM11, which was isolated from the limbs of a plant, *Catunaregam tomentosa* (Blume ex DC.) Tirveng. Interestingly, curvularide B (**2**) showed antifungal activity against *Candida albicans*, and it also exhibited synergistic activity with a fluconazole drug. Natural compounds possessing a 12-carbon polyketide conjugated with L-isoleucine (or derivatives) are rare in nature, and to our knowledge, only coronatine and jasmonoyl–L-isoleucine (JA-Ile) have had such a skeleton. Curvularides A–E (**1**–**5**) are additional representatives of

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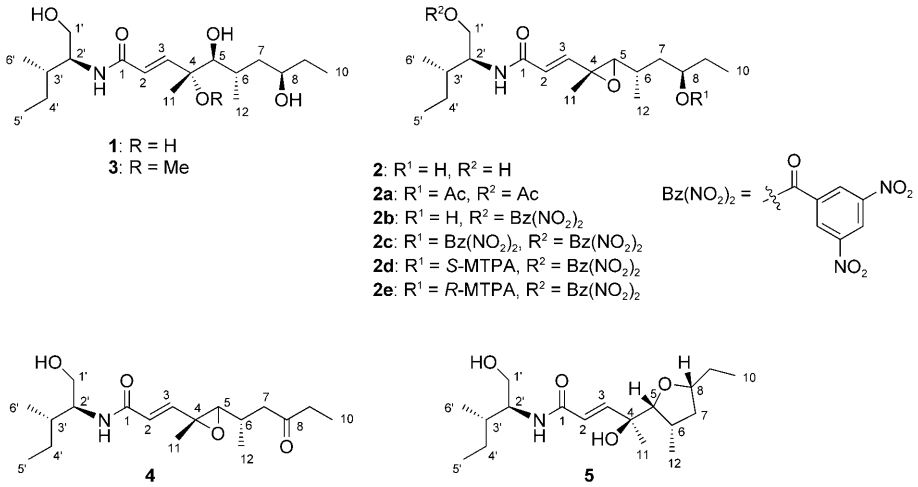
this compound class. The ecological role of the antifungal curvularides A–E (**1**–**5**) is also discussed in this report.

Results and Discussion

Curvularide A (**1**; $[\alpha]_D^{28} = -9.3$ ($c = 0.26$, $CHCl_3$); Scheme 1) has the molecular formula $C_{18}H_{35}NO_5$, as deduced from the ESI-TOF MS results. The IR spectrum of **1** showed absorption bands for hydroxy ($\tilde{\nu} = 3307\ cm^{-1}$) and carbonyl ($\tilde{\nu} = 1667\ cm^{-1}$) groups. The ^1H NMR spectrum of **1** indicated the exchangeable NH moiety of an amide ($\delta_H = 7.07$ ppm, d, $J = 8.9$ Hz), two *trans* olefinic protons (H2 and H3), three downfield methines (H5, H8, and H2′), an oxygenated methylene ($H_2$1′), five methyls (a singlet, two doublets, and two triplets), and a number of upfield methines and methylenes. The ^{13}C NMR spectrum of **1** showed 18 lines attributable to two quaternary, seven methine, four methylene, and five methyl carbon atoms, as revealed by the DEPT technique. The ^1H–^1H COSY spectrum of **1** established connectivity from the H5 atom along the chain through to the H10 atom and from the $H_2$1′ atoms through to the $H_3$5′ atoms, and it also showed correlations between the H2/H3, H6/$H_3$12, and H3′/H6′ protons. Analyses of the HMBC spectrum allowed the structure elucidation of **1** with the following key correlations between atoms: from both H2 and H3 to the C1 amide carbonyl atom; from H3 to C4, C5, and C11; from H5 to C11; from H6 to C4; from the $H_3$11 atoms to C3, C4, and C5; from the NH proton to C1, C1′, and C2′; and from H2′ to C1. On the basis of these spectroscopic data, a gross structure of curvularide A (**1**) was established, and the ^1H and ^{13}C resonances in **1** were assigned (Table 1). However, the configuration of the six stereogenic centers in **1** could not be clarified by analysis of the NMR data because **1** is a linear molecule. Curvularide A (**1**) was subsequently analyzed by single-crystal X-ray crystallography, which proved the relative configuration shown in Figure 1. Interestingly, in the solid state, curvularide A (**1**) exists as an antiparallel dimer stabilized by intermolecular hydrogen bonds (Figure 1). Fortunately, in the present study, curvularide B (**2**) was also isolated, and the absolute configuration at the C8 atom was defined as *R* by the Mosher method. The structure of curvularide A (**1**) is closely related to that of curvularide B (**2**), so they may have the same biosynthetic pathways; accordingly, the absolute configuration at the C8 atom of **1** should also be *R*. Therefore, the absolute configurations of curvularide A (**1**) are 4*R*, 5*S*, 6*S*, 8*R*, 2′*S*, and 3′*S*. It should be noted that the C1′–C6′ skeleton is derived from the amino acid L-isoleucine, with the carboxylic functionality reduced to a hydroxy group, and that the C1–C12 structure is a polyketide. Curvularide A (**1**) is therefore a hybrid peptide–polyketide, the structure of which contains both polyketide and L-isoleucine derivative units.



Scheme 1. Structures of curvularides A–E (**1**–**5**). Bz: benzoyl; MTPA: α-methoxy-α-(trifluoromethyl)phenylacetyl.

Curvularide B (**2**; Scheme 1) possesses the molecular formula

Table 1. ^1H and ^{13}C NMR data (in $CDCl_3$) for curvularides A (**1**) and B (**2**).

Atom no.	Curvularide A (**1**)		Curvularide B (**2**)	
	δ_H [ppm] (multiplicity; *J* [Hz])	δ_C [ppm]	δ_H [ppm] (multiplicity; *J* [Hz])	δ_C [ppm]
1	–	167.3	–	166.0
2	6.14 (d; 15.3)	122.0	6.06 (d; 15.2)	123.2
3	6.82 (d; 15.3)	149.8	6.76 (d; 15.2)	145.0
4	–	76.7	–	60.2
5	3.36 (d; 3.1)	80.6	2.60 (d; 9.0)	71.6
6	2.10 (m)	31.4	1.65 (m)	31.0
7	1.23 (m); 1.86 (ddd; 4.2, 10.5, 14.6)	38.9	1.51 (m); 1.65 (m)	42.6
8	3.55 (m)	72.6	3.64 (m)	71.3
9	1.47 (m)	31.3	1.51 (m); 1.65 (m)	30.7
10	0.93 (t; 7.2)	10.0	0.96 (t, 7.5)	9.9
11	1.30 (s)	24.1	1.47 (s)	15.4
12	1.05 (d; 6.6)	21.4	0.99 (d; 6.6)	16.6
1′	3.55 (m); 3.76 (dd; 2.6, 11.3)	62.6	3.65 (m); 3.71 (m)	63.2
2′	3.88 (m)	56.0	3.87 (m)	55.9
3′	1.60 (m)	36.0	1.65 (m)	35.6
4′	1.14 (m); 1.47 (m)	25.7	1.16 (m); 1.51 (m)	25.6
5′	0.89 (t; 7.8)	11.4	0.90 (t; 7.4)	11.3
6′	0.91 (d; 6.6)	15.4	0.94 (d; 6.8)	15.7
NH	7.07 (d; 8.9)	–	6.39 (d; 7.7)	–

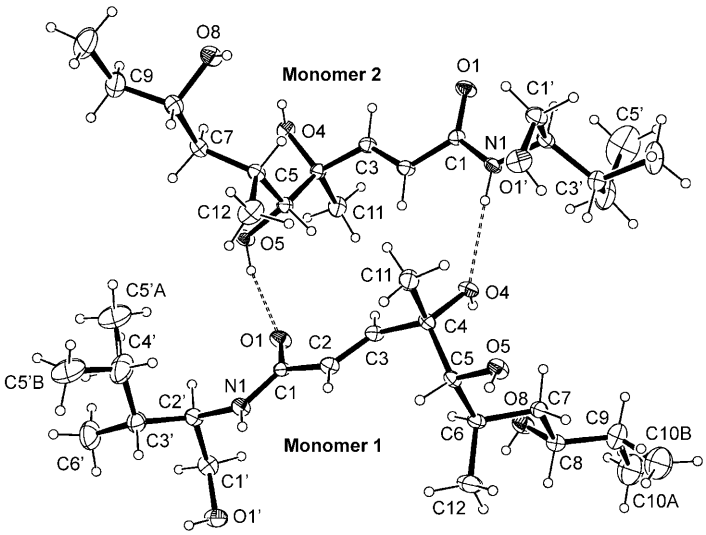
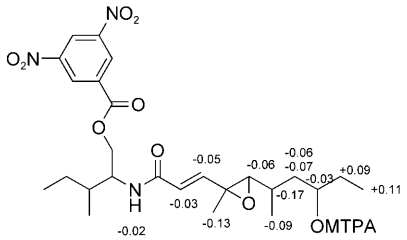


Figure 1. ORTEP plot of curvularide A (**1**). The methyl C10–H_3 and C5′–H_3 groups of monomer 1 are doubly disordered (labeled with letters A and B). In the asymmetric unit, curvularide A (**1**) forms an antiparallel dimer stabilized by intermolecular hydrogen bonds (indicated by dashed lines).

$C_{18}H_{33}NO_4$ (as determined by ESI-TOF MS), and its ^{1}H and ^{13}C NMR spectra shared many similarities to those of curvularide A (**1**). Careful analysis of the ^{1}H and ^{13}C NMR and ^{1}H–^{1}H COSY spectra indicated that a downfield oxygenated sp^3 methine (H5) in **1** ($\delta_H = 3.36$ ppm, d, $J = 3.1$ Hz; $\delta_C = 80.6$ ppm) was shifted upfield in **2** ($\delta_H = 2.60$ ppm, d, $J = 9.0$ Hz; $\delta_C = 71.6$ ppm). Moreover, the ESI-TOF mass spectrum of **2** revealed the loss of H_2O, which implied the condensation of hydroxy groups in **2**. Acetylation of **2** yielded a diacetate, **2a**; the hydroxy groups at the C1′ and C8 positions were esterified but not the hydroxy group at the C5 position, which suggests condensation of the C5 and C4 hydroxy groups to form an epoxide unit. Based upon these spectroscopic data, curvularide B (**2**) was determined to be an epoxide derivative of **1**. Although the epoxide orientation could not be conclusively defined, it was tentatively assigned as *trans* because of the absence of a NOESY correlation between the H5 and $H_3$11 atoms. The assignment of protons and carbon atoms in **2** was achieved by analysis of the 2D NMR spectra (Table 1). The absolute configuration of the C8 secondary alcohol in **2** was addressed by the Mosher method.[9] Prior to the application of the Mosher technique, the primary alcohol at the C1′ position in **2** was protected with 3,5-dinitrobenzoic acid to give the desired benzoylated product **2b**, as well as a dibenzoylated derivative, **2c**. Subsequently, the benzoylated product **2b** was esterified with both (*S*)- and (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) to yield esters **2d** and **2e**. The $\Delta\delta$ values ($\delta_{(S)} - \delta_{(R)}$) are shown in Scheme 2; these values indicated that the absolute configuration of the C8 atom is *R*. The structures of curvularide B (**2**) and curvularide A (**1**) are closely related, so they should share the same biogenesis and, thus, stereochemistry.



Scheme 2. $\Delta\delta$ values [$\delta_{(S)} - \delta_{(R)}$] for the MTPA esters **2d** and **2e**.

Curvularide C (**3**; Scheme 1) exhibited a molecular formula of $C_{19}H_{37}NO_5$ (as determined by ESI-TOF MS). The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of curvularide A (**1**), except for the additional signal of a methoxy group ($\delta_H = 3.22$ ppm, s; $\delta_C = 50.6$) in **3**. These NMR data, as well as the molecular formula obtained by MS, suggested that curvularide C (**3**) was an *O*-methyl derivative of curvularide A (**1**). All proton and carbon atom resonances in **3** were assigned by analysis of the NMR data (Table 2).

Table 2. ^{1}H and ^{13}C NMR data (in $CDCl_3$) for curvularides C (**3**) and D (**4**).

Atom no.	Curvularide C (**3**)		Curvularide D (**4**)	
	δ_H [ppm] (multiplicity; *J* [Hz])	δ_C [ppm]	δ_H [ppm] (multiplicity; *J* [Hz])	δ_C [ppm]
1	–	166.8	–	165.9
2	5.99 (d; 15.9)	125.6	6.01 (d; 15.2)	123.2
3	6.71 (d; 15.9)	144.3	6.73 (d; 15.2)	145.6
4	–	80.6	–	59.6
5	3.37 (br d; 2.2)	81.8	2.61 (d; 9.5)	70.1
6	1.89 (m)	31.6	2.04 (m)	29.6
7	1.21 (m); 1.71 (m)	39.3	2.40 (t; 8.5); 2.69 (dd; 4.6, 16.3)	46.6
8	3.45 (quin; 4.8)	72.2	–	210.1
9	1.43 (m)	31.4	2.43 (m); 2.47 (m)	36.6
10	0.93 (t; 6.9)	10.0	1.07 (t; 7.3)	7.7
11	1.34 (s)	18.1	1.47 (s)	15.5
12	1.04 (d; 6.9)	21.2	0.98 (d; 6.8)	16.4
1′	3.64 (dd; 6.4, 11.5); 3.75 (dd; 2.6, 11.5)	63.1	3.69 (dd; 5.9, 11.1); 3.74 (dd; 2.9, 11.1)	63.7
2′	3.87 (m)	56.0	3.88 (m)	56.1
3′	1.66 (m)	35.8	1.67 (m)	35.8
4′	1.16 (sept; 6.0); 1.51 (m)	25.7	1.16 (sxt; 7.5); 1.51 (m)	25.6
5′	0.91 (t; 7.4)	11.3	0.91 (t; 7.4)	11.3
6′	0.94 (d; 6.9)	15.4	0.95 (d; 6.8)	15.5
NH	6.53–6.60 (m)	–	5.95 (br d; 8.3)	–
OMe	3.22 (s)	50.6	–	–

A molecular formula of $C_{18}H_{31}NO_4$ was deduced from the ESI-TOF MS results for curvularide D (**4**; Scheme 1). In general, the ^{1}H spectrum of curvularide D (**4**) was similar to that of curvularide B (**2**), except that the signal for the oxygenated H8 methine ($\delta_H = 3.64$ ppm) was missing in **4**. Careful analysis of the ^{13}C NMR spectra revealed that the oxygenated carbon atom (C8; $\delta_C = 71.3$ ppm) in curvularide B (**2**) was replaced by a ketone carbon atom ($\delta_C = 210.1$ ppm) in curvularide D (**4**). These NMR data, in combination with

the MS data, indicated that the C8 hydroxy group of curvularide B (**2**) was oxidized to a ketone in curvularide D (**4**). On the basis of these data, curvularide D (**4**) was a ketone derivative of curvularide B (**2**); therefore, all stereogenic centers in **4** should be the same as those in **2**. The ^{1}H and ^{13}C resonances in **4** were completely assigned by analysis of the 2D NMR data, as well as by data correlation with those of **2** (Table 2).

Curvularide E (**5**; Scheme 1) possessed the molecular formula $C_{18}H_{33}NO_4$, as revealed by ESI-TOF MS. Analysis of the ^{1}H and ^{13}C NMR spectra revealed that curvularide E (**5**) possessed polyketide (C1–C12) and L-isoleucine derivative (C1′–C6′) units like those of **1**–**4**. However, a downfield shift of the signals for the H5 ($\delta_H = 3.71$ ppm) and H8 ($\delta_H = 3.76$ ppm) atoms in curvularide E (**5**) was observed in comparison with those of **1** and **3** (H5: $\delta_H = 3.36$–3.37 ppm; H8: $\delta_H = 3.45$–3.55 ppm), which suggested the presence of an ester or ether linkage at these positions. The mass spectrum indicated that curvularide E (**5**) had two protons less than curvularide A (**1**; $C_{18}H_{35}NO_5$); therefore, the downfield shift of the signals for the H5 and H8 atoms in **5** originated from the formation of an ether linkage of the H5/H8 hydroxy groups in **1**, which led to the formation of a furan ring in **5**. Key HMBC atom correlations were observed from H2, H3, H2′, and NH to C1; from H2, H3, $H_3$11, and 4OH to C4; from H6 to C8; from $H_3$11 to C3, C4, and C5; and from $H_3$12 to C5, C6, and C7. Based upon these spectral data, a gross structure of curvularide E (**5**) was successfully established. There was no NOESY correlation between the H5 and $H_3$12 atoms, which implied a *trans* orientation between these two protons. However, the relative configuration of the furan ring in curvularide E (**5**) could not be conclusively defined by analysis of the NOESY spectrum due to the overlapping signals of the H5, H8, and $H_2$1′ protons. Fortunately, an appropriate crystal of curvularide E (**5**) was obtained for single-crystal X-ray crystallographic analysis, and the configurations of all stereogenic centers in **5** were revealed (Figure 2). Curvularide E (**5**) is a derivative of curvularides A–D (**1**–**4**), and they should all share the same biosynthetic pathway; therefore, the absolute configuration of the C8 atom in **5** should be *R*, like those in **1**–**4**. Accordingly, the absolute configurations of curvularide E (**5**) are 4*S*, 5*R*, 6*S*, 8*R*, 2′*S*,and 3′*S*. The ^{1}H and ^{13}C resonances in **5** were assigned as shown in Table 3.

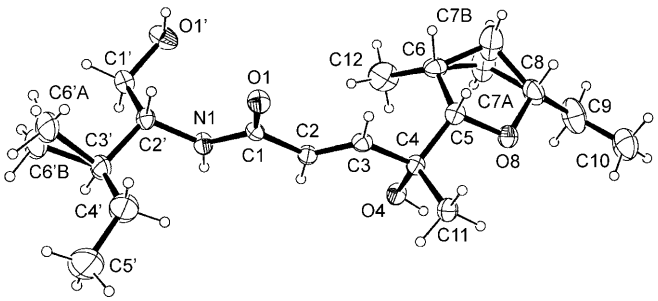


Figure 2. ORTEP plot of curvularide E (**5**). The methylene C7–H_2 and methyl C6′–H_3 groups are doubly disordered (labeled with letters A and B).

In a disk diffusion assay, curvularide B (**2**), the major metabolite, exhibited activity against *Candida albicans* with an inhibition zone diameter of 12.1 mm. Diacetate **2a** showed no activity. In the presence of a subinhibitory concentration of ketoconazole, curvularide B (**2**) produced an inhibition zone of 23.6 mm. This suggested that the curvularide B (**2**) and azole drug combination might be synergistic against *C. albicans*. Therefore, the chequerboard technique was used to analyze the interaction of curvularide B (**2**) and the azole drug. Fluconazole was selected for the study. The minimum inhibitory concentration (MIC) of fluconazole was found to be 1 $\mu g\,mL^{-1}$ (MIC-2) according to the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines.[10] For complete inhibition of growth, the MIC-0 value of fluconazole was $>8\ \mu g\,mL^{-1}$ due to the fungistatic nature of the drug. With fluconazole and curvularide B (**2**) in combination, the MIC values that produced no visible growth (MIC-0) were found to be 1 and 16 $\mu g\,mL^{-1}$, respectively. These gave a fractional inhibitory concentration (FIC) index of 0.1875 (1/8+16/256), which suggests synergistic activity of the fluconazole and curvularide B (**2**).

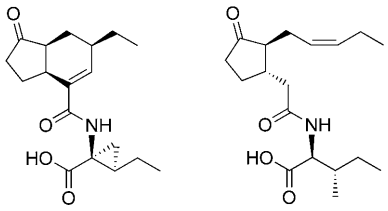
Curvularides A–E (**1**–**5**) were inactive (at 50 $\mu g\,mL^{-1}$) toward a panel of ten cell lines including the HeLa (cervical adenocarcinoma cell line), HuCCA-1 (human lung cholangiocarcinoma cell line), HepG2 (human hepatocellular liver carcinoma cell line), T47-D (human mammary adenocarcinoma cell line), MDA-MB-231 (human breast cell line), S102 (human liver cancer cell line), A549 (human lung carcinoma cell line), HL-60 (human promyelocytic leukemia cell line), MOLT-3 (acute lymphoblastic leukemia cell line), and MRC-5 (normal embryonic lung cell) cell lines (see the Supporting Information). The noncytotoxic properties of

Table 3. ^{1}H and ^{13}C NMR data (in $CDCl_3$) for curvularide E (**5**).

Atom no.	Curvularide E (**5**)	
	δ_H [ppm] (multiplicity; *J* [Hz])	δ_C [ppm]
1	–	166.7
2	6.16 (d; 15.2)	121.5
3	6.92 (d; 15.2)	146.8
4	–	74.4
5	3.71 (d; 6.6)	85.4
6	2.34 (sept; 7.1)	35.6
7	1.24 (m); 2.13 (m)	40.0
8	3.76 (m)	79.4
9	1.50 (m); 1.67 (m)	29.2
10	0.91 (t; 7.3)	10.5
11	1.37 (s)	27.8
12	1.04 (d; 7.2)	16.8
1′	3.70 (m); 3.74 (m)	63.7
2′	3.88 (m)	56.2
3′	1.68 (m)	35.8
4′	1.16 (m); 1.51 (m)	25.6
5′	0.94 (t; 7.4)	11.4
6′	0.95 (d; 6.8)	15.5
NH	6.16 (d; 11.0)	–
4-OH	2.83 (brs)	–

curvularide B (**2**) may therefore provide a therapeutic advantage for **2** as an antifungal agent.

Curvularides A–E (**1**–**5**) are hybrid peptide–polyketides; their structures contain a 12-carbon polyketide unit linked, through an amide bond, with a derivative of L-isoleucine. To our knowledge, there have been two natural compounds, coronatine and JA-Ile (Scheme 3), with a 12-carbon polyketide conjugated with L-isoleucine or its derivative. It should be noted that both coronatine and JA-Ile are important phytohormones, which act as signaling molecules in plants.[11,12] Interestingly, coronatine is not produced by plants but by bacteria and it acts as a molecular mimic of JA-Ile.[12,13] Similarly, we speculate that the curvularides might mimic the plant hormone JA-Ile. It is known that there are mutualistic relationships between endophytes and their plant hosts.[14,15] The endophytic fungi may produce antifungal agents to protect their plant hosts from phytopathogens.[16] For example, the fungus *Serratia marcescens*, isolated from an aquatic plant, *Rhyncholacis pedicillata*, produced a potent antifungal agent, oocydin A, which protected its plant host from phytopathogenic infection.[15,17] The ecological role of the antifungal curvularides A–E (**1**–**5**) might also be to prevent phytopathogenic fungi from growing in the plant hosts.



Scheme 3. Structures of coronatine (left) and (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile; right).

Conclusion

Five new hybrid peptide–polyketides, curvularides A–E (**1**–**5**), were isolated from the endophytic fungus, *C. geniculata* CTOM11. Curvularides A–E (**1**–**5**) contain a 12-carbon polyketide conjugated with an L-isoleucine derivative; this compound class is rare in nature. The major metabolite, curvularide B (**2**), exhibited activity against *C. albicans*, and it also had synergistic effect with a fluconazole drug. Curvularide B (**2**) showed no cytotoxicity toward ten cell lines. The protection of the plant hosts from fungal phytopathogens may be the ecological role of the antifungal curvularides, which are produced by an endophytic fungus.

Experimental Section

General: Melting points were measured on a digital Electrothermal 9100 melting point apparatus and reported without correction. Optical rotations were measured with the sodium D line (590 nm) on a JASCO DIP-370 digital polarimeter. UV/Vis spectra were obtained from a Shimadzu UV-1700 PharmaSpec spectrophotometer. FTIR data were obtained by using a universal attenuated total reflectance (UATR) attachment on a Perkin–Elmer Spectrum One spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM400 NMR instrument (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C) and a Bruker AVANCE 600 NMR spectrometer (operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C). Single-crystal X-ray crystallographic analysis was performed on a Bruker X8 APEXII Kappa CCD area-detector diffractometer. ESI-TOF MS were determined by using a Bruker MicroTOF$_{LC}$ instrument.

Cultivation, extraction, and isolation of a broth extract of *C. geniculata*: The fungus *C. geniculata* CTOM11 was cultured in potato dextrose broth (PDB) for 21 days at 25 °C (static conditions). Fungal cells and broth (6 L) were separated by filtration, and the filtrate was extracted with an equal volume of EtOAc (three times) to obtain a crude broth extract (1.8 g). Fungal cells were macerated sequentially in MeOH (500 mL) and CH_2Cl_2 (500 mL), each for two days. The crude cell extract was a mixture of triglycerides and fatty acids, as revealed by ^{1}H NMR spectroscopy.

The crude broth extract (1.8 g) was subjected to Sephadex LH-20 column chromatography (CC; 45×4 cm), eluted with MeOH/CH_2Cl_2 (1:1), to yield eight fractions (A1–A8). Fraction A3 was further purified by Sephadex LH-20 CC (56×3 cm), eluted with MeOH, to afford ten fractions (B1–B10). Fractions B5 and B6 were combined and subjected to silica gel CC (2.5×40 cm), eluted with EtOAc/CH_2Cl_2 (2:8) to yield 12 fractions (C1–C12). Fraction C3 contained curvularide D (**4**; 32.7 mg), whereas fractions C7 and C8 gave curvularide B (**2**; 900 mg). Fraction C5 was further purified by preparative TLC, eluted with hexane/CH_2Cl_2/acetone (1:1:1), to yield curvularide E (**5**; 85 mg). Fraction C9 was separated by preparative HPLC (C_{18} reversed-phase column), eluted with MeCN/H_2O (25:75; flow rate of 20.0 mL min^{-1}), to yield curvularide C (**3**; 20.7 mg; t_R = 18 min). Fraction C11 was subjected to preparative HPLC (C_{18} reversed-phase column; MeCN/H_2O (2:8); flow rate of 20.0 mL min^{-1}) to furnish curvularide A (**1**; 10.8 mg; t_R = 19 min).

Curvularide A (1): Colorless crystals (from CH_2Cl_2/acetone, 1:1); m.p. 123–124 °C; $[\alpha]_D^{28} = -9.3$ (c = 0.26, $CHCl_3$); UV (MeOH): λ_{max} (log ε) = 218 nm (4.12); FTIR (UATR): $\tilde{\nu}_{max}$ = 3307, 2960, 2926, 2876, 1667, 1619, 1542, 1459, 1376, 1351, 1283, 1058, 982 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 1; ESI-TOF MS: m/z calcd for $C_{18}H_{35}NNaO_5$: 368.2407 $[M+Na]^+$; found: 368.2405.

Curvularide B (2): Pale brown oil; $[\alpha]_D^{28} = +10.2$ (c = 0.43, $CHCl_3$); UV (MeOH): λ_{max} (log ε) = 220 nm (4.41); FTIR (UATR): $\tilde{\nu}_{max}$ = 3296, 2962, 2931, 2877, 1668, 1628, 1542, 1460, 1381, 1349, 1286, 1068, 975 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 1; ESI-TOF MS: m/z calcd for $C_{18}H_{33}NNaO_4$ $[M+Na]^+$: 350.2302; found: 350.2297.

Curvularide C (3): Yellowish oil; $[\alpha]_D^{28} = -14.2$ (c = 0.32, $CHCl_3$); UV (MeOH): λ_{max} (log ε) = 213 nm (4.06); FTIR (UATR): $\tilde{\nu}_{max}$ = 3302, 2961, 2931, 2876, 1666, 1626, 1542, 1459, 1377, 1349, 1282, 1068, 990 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 2; ESI-TOF MS: m/z calcd for $C_{19}H_{37}NNaO_5$: 382.2563 $[M+Na]^+$; found: 382.2558.

Curvularide D (4): Colorless oil; $[\alpha]_D^{28} = -1.5$ (c = 0.35, $CHCl_3$); UV (MeOH): λ_{max} (log ε) = 220 nm (4.39); FTIR (UATR): $\tilde{\nu}_{max}$ = 3299, 2964, 2934, 2878, 1712, 1669, 1629, 1540, 1459, 1378, 1351, 1287, 1068, 977 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 2; ESI-TOF MS: m/z calcd for $C_{18}H_{31}NNaO_4$: 348.2145 $[M+Na]^+$; found: 348.2145.

Curvularide E (5): Colorless crystals (from CH_2Cl_2/acetone, 1:1); m.p. 118–119 °C; $[\alpha]_D^{28} = -7.8$ (c = 0.33, $CHCl_3$); UV (MeOH): λ_{max} (log ε) = 216 nm (4.20); FTIR (UATR): $\tilde{\nu}_{max}$ = 3342, 3240, 3065, 2962, 2929, 2876, 1670, 1626, 1575, 1455, 1380, 1354, 1279, 1068, 980, 698 cm^{-1}; ^{1}H and ^{13}C NMR data in Table 3; ESI-TOF MS: m/z calcd for $C_{18}H_{34}NO_4$: 328.2482 $[M+H]^+$; found: 328.2491.

Acetylation of curvularide B (2): A solution containing curvularide B (**2**; 55.2 mg), pyridine (1 mL), and Ac_2O (3 mL) was stirred at room temperature for 2 h. H_2O (6 mL) was added, and the reaction mixture was extracted with $CHCl_3$. The organic layer was evaporated to give an oily mixture (79.5 mg), which was purified by preparative TLC, eluted with CH_2Cl_2/EtOAc (9:1), to afford the diacetate **2a** (44.0 mg) as a colorless

oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H=0.90 (3H, t, J=3.7 Hz, H10), 0.92 (3H, t, J=4.1 Hz, H5′), 0.93 (3H, d, J=6.8, H6′), 0.97 (3H, d, J=6.3 Hz, H12), 1.16 (2H, m, H4′), 1.43 (3H, s, H11), 1.51 (2×1H, m, overlapping signals of H6, and H_a7), 1.63 (2×1H, m, overlapping signals of H9 and H3′), 1.88 (1H, quin, J=8.7 Hz, H_b7), 2.05 (3H, s, CH_3 of acetate), 2.06 (3H, s, CH_3 of acetate), 2.56 (1H, d, J=8.7 Hz, H5), 4.09 (1H, dd, J= 3.5, 11.2 Hz, H_a1′), 4.15 (1H, m, H2′), 4.25 (1H, dd, J=6.0, 11.2 Hz, H_b1′), 5.01 (1H, m, H8), 5.76 (1H, d, J=9.1 Hz, NH), 5.97 (1H, d, J= 15.2 Hz, H2), 6.74 ppm (1H, d, J=15.2 Hz, H3); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C=9.4 (C10), 11.3 (C5′), 15.3 (C6′), 15.7 (C11), 16.1 (C12), 20.9 (CH_3 of acetate), 21.2 (CH_3 of acetate), 25.4 (C4′), 27.4 (C9), 30.2 (C6), 36.1 (C3′), 38.9 (C7), 52.3 (C2′), 58.7 (C4), 64.3 (C1′), 70.8 (C5), 72.8 (C8), 123.0 (C2), 145.7 (C3), 164.9 (C1), 171.0 (COO of acetate), 171.1 ppm (COO of acetate); ESI-TOF MS: m/z calcd for $C_{22}H_{38}NO_6$: 412.2699 $[M+H]^+$; found: 412.2703.

Benzoylation of curvularide B (2): A reaction mixture containing curvularide B (**2**; 20.0 mg), 3,5-dinitrobenzoic acid (25.9 mg), *N*-[3-(dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (23.4 mg), a catalytic amount of *N*,*N*-(dimethylamino)pyridine, and CH_2Cl_2 (3 mL) was stirred for 2 h. The reaction mixture was added to H_2O (10 mL) and extracted with $CHCl_3$ (10 mL). The crude reaction mixture was purified by preparative TLC, with EtOAc/CH_2Cl_2/hexane (1:1:2) as the eluent, to give compounds **2b** (20.4 mg) and **2c** (8.9 mg).

Benzoylated ester **2b**: white amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ_H=0.95 (3H, t, J=7.5 Hz, H10), 0.96 (3H, t, J=7.5 Hz, H5′), 0.97 (3H, d, J=5.0 Hz, H12), 1.04 (3H, d, J=15.5 Hz, H6′), 1.45 (3H, s, H11), 1.50 (2×1H, m, overlapping signals of H_a7 and H_a9), 1.61 (3×1H, m, overlapping signals of H6, H_b7, and H_b9), 1.71 (1H, m, H3′), 2.55 (1H, d, J=8.8 Hz, H5), 3.63 (1H, m, H8), 4.39 (1H, m, H2′), 4.39 (1H, m, H_a1′), 4.63 (1H, q, J=7.6 Hz, H_b1′), 5.79 (1H, m, NH), 6.00 (1H, d, J=15.1 Hz, H2), 6.77 (1H, d, J=15.1 Hz, H3), 9.10 (2×1H, dd, J=1.0, 2.1 Hz, H3″ and H7″), 9.22 ppm (1H, q, J=2.1 Hz, H5″); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C=9.9 (C10), 11.2 (C5′), 15.5 (C6′), 15.7 (C11), 16.7 (C12), 25.5 (C4′), 30.7 (C9), 31.1 (C6), 36.2 (C3′), 42.7 (C7), 52.1 (C2′), 60.1 (C4), 66.9 (C1′), 71.4 (C8), 71.6 (C5), 122.5 (2×C, C2, C5″), 129.5 (2×C, C3″, C7″), 133.6 (C2″), 146.1 (C3), 148.6 (2×C, C4″, C6″), 162.6 (C1″), 165.3 ppm (C1); ESI-TOF MS: m/z calcd for $C_{25}H_{36}N_3O_9$: 522.2452 $[M+H]^+$; found: 522.2451.

Dibenzoylated ester **2c**: white amorphous solid; ^{1}H NMR (600 MHz, $CDCl_3$): δ_H=0.96 (3H, t, J=8.1 Hz, H5′), 0.98 (3H, t, J=7.4 Hz, H10), 1.03 (3H, d, J=3.1 Hz, H12), 1.04 (3H, d, J=3.0 Hz, H6′), 1.35 (3H, s, H11), 1.21 (1H, m, H_a4′), 1.56 (3×1H, m, overlapping signals of H_b4′, H6, and H_a7), 1.71 (H, m, H3′), 1.80 (2H, m, H9), 2.04 (1H, m, H_b7), 2.60 (1H, d, J=9.0 Hz, H5), 4.37 (2×1H, m, overlapping signals of H_a1′ and H2′), 4.44 (1H, dd, J=7.7, 11.2 Hz, H_b1′), 5.31 (1H, m, H8), 5.73 (1H, d, J=9.3 Hz, NH), 5.88 (1H, d, J=15.1 Hz, H2), 6.77 (1H, d, J= 15.1 Hz, H3), 9.13 (2×1H, d, J=2.1 Hz, H3″ and H7″), 9.19 (2×1H, d, J=2.1 Hz, H3‴ and H7‴), 9.22 ppm (2×1H, q, J=2.3 Hz, H5″ and H5‴); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C=9.5 (C10), 11.2 (C5′), 15.5 (C6′), 17.2 (C12), 25.5 (C4′), 27.6 (C9), 31.6 (C6), 36.2 (C3′), 39.6 (C7), 52.1 (C2′), 53.8 (C4), 67.0 (C1′), 71.6 (C5), 77.5 (C8), 122.0 (C5″), 122.1 (C5‴), 122.4 (C2), 129.6 (2×C, C3″, C7″), 129.7 (2×C, C3‴, C7‴), 133.7 (C2″), 134.7 (C2‴), 146.1 (C3), 148.5 (2×C, C4″, C6″), 148.7 (2×C, C4‴, C6‴), 162.5 (C1″), 162.6 (C1‴), 165.1 ppm (C1); ESI-TOF MS: m/z calcd for $C_{32}H_{38}N_5O_{14}$; 716.2415 $[M+H]^+$; found: 716.2423.

Preparation of the (*S*)- and (*R*)-MTPA esters 2d and 2e: Compound **2b** (7.2 mg), (*R*)-(+)-MTPA (10.3 mg), *N*-[3-(dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (8.5 mg), and a catalytic amount of *N*,*N*-(dimethylamino)pyridine were dissolved in CH_2Cl_2 (4 mL), and this reaction mixture was stirred for 2 h. The reaction mixture was added to H_2O (5 mL) and extracted with $CHCl_3$ (5 mL). The mixture was purified by preparative TLC, with EtOAc/hexane (1:4) as the eluent, to give the (*R*)-MTPA ester **2e** (5.0 mg). The (*S*)-MTPA ester was prepared in the same manner as that described above: compound **2b** was treated with (*S*)-(−)-MTPA to give the (*S*)-MTPA ester **2d** (6.2 mg).

(*S*)-MTPA ester **2d**: ^{1}H NMR (600 MHz, $CDCl_3$): δ_H=0.88 (3H, d, J= 6.8 Hz, H12), 0.94 (3H, t, J=7.5 Hz, H10), 0.97 (3H, t, J=7.4 Hz, H5′), 1.03 (3H, J=6.8 Hz, H6′), 1.22 (3H, s, H11), 1.25 (1H, m, H_a4′), 1.30 (1H, m, H6), 1.47 (1H, m, H_a7), 1.58 (1H, m, H_b4′), 1.68 (1H, m, H3′), 1.72 (2H, m, H9), 1.92 (1H, m, H_b7), 2.47 (1H, d, J=9.3 Hz, H5), 3.55 (3H, brs, OCH_3 of MTPA), 4.37 (1H, t, J=8.3 Hz, H_a1′), 4.39 (1H, m, H2′), 4.64 (1H, dd, J=2.5, 10.3 Hz, H_b1′), 5.21 (1H, m, H8), 5.53 (1H, d, J=8.9 Hz, NH), 5.95 (1H, d, J=15.1 Hz, H2), 6.73 (1H, d, J=15.1 Hz, H3), 7.38 (3H, m, aromatic signals of MTPA), 7.53 (2H, m, aromatic signals of MTPA), 9.10 (2×1H, d, J=2.1 Hz, H3″ and H7″ of Bz$(NO_2)_2$), 9.22 ppm (1H, t, J=2.1 Hz, H5″ of Bz$(NO_2)_2$); ESI-TOF MS: m/z calcd for $C_{35}H_{43}F_3N_3O_{11}$: 738.2850 $[M+H]^+$; found: 738.2837.

(*R*)-MTPA ester **2e**: ^{1}H NMR (600 MHz, $CDCl_3$): δ_H=0.83 (3H, t, J= 9.9 Hz, H10), 0.97 (3H, d, J=6.6 Hz, H12), 0.97 (3H, t, J=7.2 Hz, H5′), 1.04 (3H, J=6.8 Hz, H6′), 1.23 (1H, m, H_a4′), 1.35 (3H, s, H11), 1.47 (1H, quin, J=8.3 Hz, H6), 1.54 (1H, m, H_a7), 1.59 (1H, m, H_b4′), 1.63 (2H, m, H9), 1.70 (1H, m, H3′), 1.98 (1H, quin, J=7.1 Hz, H_b7), 2.53 (1H, d, J=9.2 Hz, H5), 3.53 (3H, brs, OCH_3 of MTPA), 4.36 (1H, t, J= 8.4 Hz, H_a1′), 4.40 (1H, m, H2′), 4.65 (1H, dd, J=3.1, 10.8 Hz, H_b1′), 5.24 (1H, m, H8), 5.55 (1H, d, J=9.1 Hz, NH), 5.98 (1H, d, J=15.1 Hz, H2), 6.78 (1H, d, J=15.1 Hz, H3), 7.40 (3H, m, aromatic signals of MTPA), 7.54 (2H, m, aromatic signals of MTPA), 9.10 (2×1H, d, J=2.1 Hz, H3″ and H7″ of Bz$(NO_2)_2$), 9.22 ppm (1H, t, J=2.1 Hz, H5″ of Bz$(NO_2)_2$); ESI-TOF MS: m/z calcd for $C_{35}H_{43}F_3N_3O_{11}$: 738.2850 $[M+H]^+$; found: 738.2825.

Crystal structure determination of curvularide A (1): Plate-like, colorless crystals of **1** were recrystallized from CH_2Cl_2/acetone (1:1) and mounted with epoxy glue on the tip of glass fibers. Crystal data: M=690.94; triclinic; space group $P1$ (no. 1); a=6.4466(2), b=9.8941(4), c= 16.4164(7) Å; α=99.619(2), β=100.660(2), γ=90.330(2)°; V= 1013.83(7) Å^3; T=298(2) K; Z=1; 5277 reflections measured; 2799 unique reflections (R_{int}=0.021), which were used in all calculations. Final $R_1(F^2)$=0.040 and $wR(F^2)$=0.080 (all data).

Crystal structure determination of curvularide E (5): Block-like, colorless crystals of **5** were obtained by recrystallization from CH_2Cl_2/acetone (1:1) and mounted with epoxy glue on the tip of glass fibers. Crystal data: M= 326.45; monoclinic; space group $P2_1$ (no. 4); a=5.2320(3), b=11.1666(6), c=16.4686(8) Å; β=91.882(2)°; V=961.64(9) Å^3; T=298(2) K; Z=2; 4056 reflections measured; 1232 unique reflections (R_{int}=0.025), which were used in all calculations. Final $R_1(F^2)$=0.051 and $wR(F^2)$=0.120 (all data).

CCDC-768608 (**1**) and CCDC-768609 (**5**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Cytotoxicity: Cytotoxic activity for HeLa, HuCCA-1, HepG2, T47-D, MDA-MB-231, S102, A549, HL-60, MOLT-3, and MRC-5 cell lines was evaluated with MTT[18] and XTT[19] assays. Etoposide and doxorubicin were used as the reference drugs (see the Supporting Information).

Determination of antimicrobial activity: The antimicrobial activities of the major metabolite, curvularide B (**2**) and its acetate derivative **2a** were determined by the disk diffusion method. The test microorganisms were *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 90028. They were grown on tryptic soy agar and Sabouraud's dextrose agar (SDA), respectively, at 37°C for 24 h. Each culture was suspended in 0.85% NaCl to a turbidity of 50% at 580 nm. The *S. aureus* suspension was added at 1% inoculum into molten Mueller Hinton agar and the *C. albicans* suspension was added at 1% inoculum into molten SDA, with and without 0.125 μg mL^{-1} ketoconazole, which was a subinhibitory concentration. Each seeded medium (9 mL) was pipetted into a Petri dish (9 cm). Test compound (10 μL, 160 μg) in dimethylsulfoxide (DMSO) and pure DMSO (as a control) were applied to paper disks placed on the inoculated agar surface. After being kept at room temperature for 1 h, the test plates were incubated at 37°C for 24 h. The clear zone of inhibition around the disk, indicating antimicrobial activity of the compound, was measured.

Determination of the minimum inhibitory concentration of active compound: Curvularide B (**2**) was tested against *C. albicans* ATCC 90028 over a final concentration range of 256–0.5 μg mL^{-1} with serial two-fold dilution by the broth microdilution method, as described in the CLSI M27-A2 method.[10] Fluconazole, at a final concentration of 8–

0.031 μg mL^{-1}, was used as a positive control. The experiments were done in triplicate. The MIC value of curvularide B (**2**) was defined as the lowest concentration that gave rise to no visible growth (MIC-0). For fluconazole, the MIC value was the lowest concentration in which a prominent decrease in turbidity, as compared with the growth control, was observed (MIC-2).[10]

Determination of synergistic activity by the microdilution chequerboard technique: The synergistic activity of curvularide B (**2**) and fluconazole against *C. albicans* was determined by the broth microdilution chequerboard method.[20,21] The final drug concentrations after addition of inoculum (100 μL) ranged from 2 to 512 μg mL^{-1} for curvularide B (**2**) and from 0.031 to 2 μg mL^{-1} for fluconazole. Plates were incubated at 37 °C for 48 h. The experiments were done in triplicate.

The fractional inhibitory concentration index was used to indicate the effect of the combination. The FIC index is the sum of the MIC value of each drug when used in combination divided by the MIC value of the drug when used alone. The MIC value of the drug in combination was defined as the lowest concentration that rendered no visible growth (MIC-0). Synergy, indifference, and antagonism were defined as FIC indices of ≤ 0.5, >0.5–4, and >4, respectively, as previously described.[22]

Isolation and taxonomy of the fungus: A fresh, apparently healthy twig of *Catunaregam tomentosa* (Blume ex DC.) Tirveng (family Rubiaceae) was collected from Phitsanulok Province, Thailand. The plant specimen was cleaned under running tap water, and the cleaned twig was surface sterilized according to the previously described method of Schulz et al.[23] The surface-sterilized leaves were cut into small pieces with a sterile blade and placed on water agar plates for further incubation at 30 °C. The hyphal tip of the endophytic fungus growing out from the plant tissue was cut by a sterile Pasteur pipette and transferred onto a potato dextrose agar plate. After incubation of the cultures at 30 °C for 7 days, the culture purity was determined from the colony morphology. The endophytic fungus CTOM11 was identified based on the morphology on malt extract agar (MEA) and on analyses of the DNA sequence of the ITS1-5.8S-ITS2 section of the ribosomal RNA gene region and the DNA sequence of the 1,3,8-trihydroxynaphthalene reductase gene (*Brn1*).

On MEA, the CTOM11 fungus grew as an effused, brown, velvety colony. Microscopically, the mycelium was composed of branched, septate, brown hyphae. Conidiophores arising singly, terminally, and laterally on the hyphae were often simple, rarely branched, straight to flexuous, occasionally geniculate, brown, paler towards the apex, up to 420 μm long, and 2.5–4.2 μm wide. Conidia were acropleurogenous, usually curved, often clearly geniculate, and mostly four-septate; the end cells were subhyaline or very pale brown, the intermediate cells were brown to dark brown, and the central cells were usually the darkest and swollen, 20–27 × 7–10 μm in size. Both macroscopic and microscopic morphologies agree with the characteristics of fungi in the genus *Curvularia*. Identification to species level cannot be made based solely on fungal morphology. Thus, phylogenetic analyses based on DNA sequence comparison were attempted.

The CTOM11 fungus was cultured in potato dextrose broth, and the total cellular DNA was extracted from the fungal mycelia by using an FTA Plant Kit (Whatman, USA) according to the manufacturers' instructions. Primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC)[24] were used to amplify the ITS1-5.8S-ITS2 region from the extracted DNA, and the PCR product was subjected to DNA sequencing as previously described.[25] A BLAST 2.2.22+ search[26] through the GenBank database for DNA sequence similarity suggested that the CTOM11 strain is a fungus in the genus *Curvularia*. However, the ITS1-5.8S-ITS2 sequences of reference fungal type strains were not available in the GenBank database, which made reliable species assignment for the CTOM11 strain not possible by this method. Alternatively, the DNA sequence of the *Brn1* gene encoding 1,3,8-trihydroxynaphthalene reductase, an enzyme in the melanin biosynthetic pathway, was investigated.[27] PCR primers 5′-GCCAACATCGAGCAAACATGG-3′ and 5′GCAAGCAGCACCGTCAATACCAAT-3′ were used to amplify the *Brn1* gene from the CTOM11 genomic DNA, and the product was subjected to DNA sequencing on both strands. The DNA sequence obtained was BLAST searched against the GenBank database and was found to be similar to those of some 4-distoseptate conidia *Curvularia*. The 688-nucleotide partial *Brn1* gene of the CTOM11 strain was aligned with similar nucleotide sequences from the GenBank database and analyzed by using the MUSCLE program[28] in the CLC Main Workbench 5.1 Software package (CLC Bio, Denmark). Phylogenetic analysis was performed by using the neighbor-joining method[29] with 1000 replicate-sampling bootstrap analyses to determine the confidence levels of the inferred phylogeny. The *Brn1* gene of *Bipolaris maydis* (AB001564) was used as an outgroup. The resultant phylogenetic tree showed the CTOM11 fungus to be closely affiliated with the *C. geniculata* cluster.[27] This fungus is thus tentatively identified as *Curvularia geniculata* CTOM11.

The culture of *C. geniculata* CTOM11 has been deposited at the MIM Laboratory, Department of Microbiology, Mahidol University, Thailand. The DNA sequences of the ITS1-5.8S-ITS2 section and the *Brn1* gene have been submitted to the GenBank database with the accession numbers of GU073454 and GU073455, respectively.

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