Clavariopsins C–I, Antifungal Cyclic Depsipeptides from the Aquatic Hyphomycete *Clavariopsis aquatica*

Thin Wut Soe,[†,‡] Chunguang Han,[§] Ryosuke Fudou,[⊥] Kenichi Kaida,[∥] Yuki Sawaki,[†] TomohikoTomura[†] and Makoto Ojika[,†]*

[†]Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan

[‡]Department of Chemistry, University of Yangon, Yangon 11041, Myanmar

[§]Research Center for Materials Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan

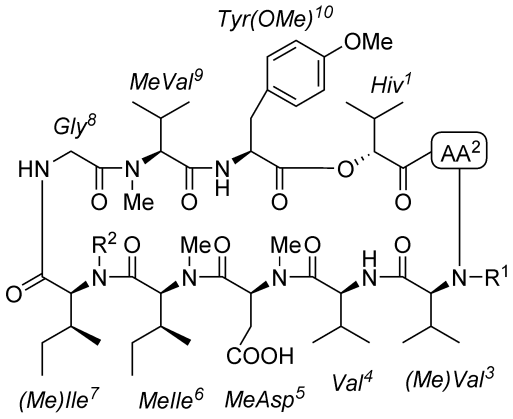
[⊥]R&D Planning Department, Ajinomoto Co., Inc., Chuo-ku, Tokyo 104-8315, Japan

[∥]Institute of Life Science, Ajinomoto Co., Inc., Kawasaki, Kanagawa 210-8681, Japan

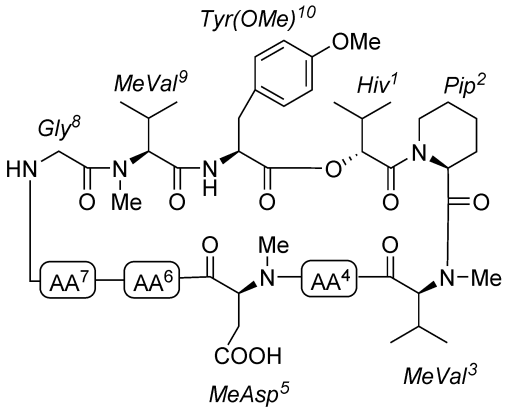
ABSTRACT: Seven new cyclic depsipeptides, clavariopsins C–I (**3**–**9**), together with two known congeners, clavariopsins A and B (**1** and **2**), were isolated from the aquatic hyphomycete *Clavariopsis aquatica*. Their planar structures, which consist of nine amino acids and one α-hydroxy acid, were elucidated by NMR spectroscopy and HRESIMS. The absolute configurations were established by the advanced Marfey's method and chiral-phase HPLC analysis. Their antifungal and cytotoxic activities were evaluated against six plant pathogenic fungi (*Botrytis cinerea, Magnaporthe oryzae, Colletotrichum orbiculare, Fusarium oxysporum, Alternaria alternata, and Aspergillus niger*) and a cancer cell line (HeLa-S3), respectively. Majority of the compounds exhibited potent antifungal activity against the fungi tested (minimum inhibition dose = 0.01–10 μg/disk) and induced hyphal swelling in *A. niger* (minimum effective dose = 0.3–3 μg/disk), whereas the compounds exhibited no cytotoxicity towards the cancer cell line. The results suggest that the clavariopsins could be a promising class of antifungal agents.

A variety of plant diseases are caused by fungi and fungus-like organisms in agricultural and horticultural fields. The vast majority of pathogenic fungi can attack specific or nonspecific hosts, leading to the annual crop losses exceeding $200 billion in the field and postharvest each year.[1] To control these fungal diseases, farmers use fungicides, most of which are associated with environmental damage and the development of fungicide resistance.[2] To overcome these problems, researchers and cultivators have been searching for new natural fungicides that preferably have reduced toxicity, short degradation periods, and potent activity.

To search for such natural fungicides, we have been examining ecologically unique fungi such as freshwater aquatic hyphomycetes (or Ingoldian fungi). They, unlike actinomycetes and other terrestrial fungi, are usually abundant in leaf litter in streams and stream foams and release a vast amount of asexual spores with characteristic shapes such as sigmoid or multiradiate forms.[3] Due to the difficulty of isolating and culturing them, chemical investigations of this group of fungi are limited; some examples of fungal products with a pesticidal properties include tricladolides,[4] tricladic acids,[4] anguillosporal,[5] heliconols,[6] and tenellic acids.[7] Thus, there may be a number of unexplored metabolites that possess new skeletons and attractive biological properties in this class of fungi. Our previous investigation of the aquatic hyphomycete *Clavariopsis aquatica* resulted in the discovery of two antifungal cyclic depsipeptides, clavariopsins A and B (**1** and **2**).[8] In this study, we investigated further related constituents from a large scale extract of *C. aquatica* and isolated seven new congeners, clavariopsins C–I (**3**–**9**). We herein describe the isolation, structure elucidation, and biological evaluation of these clavariopsins.



1 AA^2 = Pip, R^1 = Me, R^2 = Me
2 AA^2 = Pip, R^1 = H, R^2 = Me
3 AA^2 = Pip, R^1 = Me, R^2 = H
8 AA^2 = L-Pro, R^1 = Me, R^2 = Me



4 AA^7 = L-MeIle, AA^6 = L-MeVal, AA^4 = L-Val
5 AA^7 = L-MeVal, AA^6 = L-MeIle, AA^4 = L-Val
6 AA^7 = L-MeIle, AA^6 = L-MeLeu, AA^4 = L-Val
7 AA^7 = L-MeLeu, AA^6 = L-MeIle, AA^4 = L-Val
9 AA^7 = L-MeIle, AA^6 = L-MeIle, AA^4 = L-Leu

RESULTS AND DISCUSSION

The aquatic hyphomycete *C. aquatica* was cultured on a 15-L scale at 25 ˚C for 18 days. The culture broth was treated with acetone and, after removal of the fungal cells and concentration, the resulting extract was partitioned between EtOAc and H_2O. The organic fraction was separated by using silica gel column chromatography followed by repeated reversed-phase HPLC to afford seven new clavariopsins C–I (**3**–**9**) together with the known clavariopsins A and B (**1** and **2**).[8] The planar structures of the new compounds were mainly elucidated by HRMS and 2D NMR analyses. The ^1H and ^{13}C NMR data are summarized in Tables 1 and 2.

Clavariopsin C (**3**) was obtained as a colorless powder with the molecular formula of $C_{58}H_{93}N_9O_{14}$, which was determined by the ion peaks at *m/z* 1140.6920 $[M+H]^+$ and *m/z* 1162.6741 $[M+Na]^+$ observed by HRESIMS. The absorption bands at 1736 and 1647 cm^{-1} in the IR spectrum suggested the presence of ester and amide functionalities, respectively. A comparison of the NMR data (Table 1) with those for clavariopsin A (**1**)[8] revealed that the

majority of the signals closely resembled each other, with the exception of the missing NMe signal of the MeIle[7] residue observed at δ_H 3.11/δ_C 41.1 in **1** and the unique NH signal at δ_H 7.08 in **3**. Therefore, **3** was assumed to be the congener that is demethylated at the MeIle[7] residue of **1**. This idea was supported by the COSY correlations of NH (δ_H 7.08)–α-CH (δ_H 3.11)–β-CH (δ_H 2.96)–[γ-CH_3 (δ_H 1.06)]–γ-CH_2 (δ_H 1.58, 1.01)–δ-CH_3 (δ_H 0.85) within the Ile[7] residue and the interresidual HMBC correlation of NH (δ_H 7.08, Ile[7])/CO (δ_C 170.8, MeIle[6]) (Figure 1). The identities of the remaining amino acids and their order were finally established by COSY, HMBC and ROESY correlations (Figure 1). Only two small fragments, MeVal[3]-Val[4] and MeIle[6]-Ile[7], were confirmed by the HMBC correlations of NH (Val[4])/CO (MeVal[3]) and NH (Ile[7])/CO (MeIle[6]) due to many indistinguishable carbonyl signals. The connectivities of the other residues were finally determined by the ROESY correlations prsented in Figure 1, which confirmed the planar structure of **3** to be *cyclo*[-Hiv-Pip-MeVal-Val-MeAsp-MeIle-Ile-Gly-MeVal-Tyr(OMe)-] (Hiv = 2-hydroxyisovaleryl, Pip = pipecolyl).

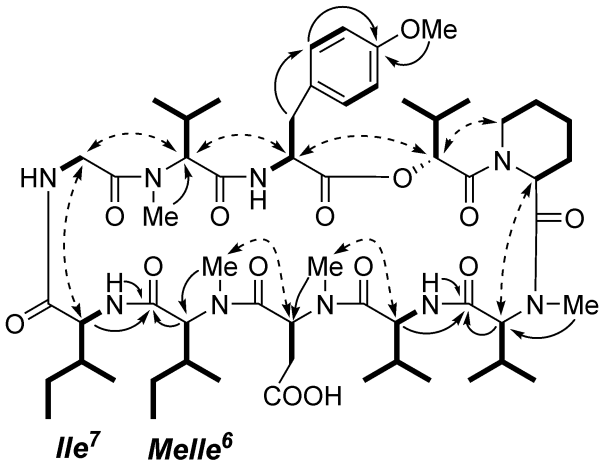


Figure 1. Key COSY (bold bonds), HMBC (arrows) and ROESY (dotted arrow) correlations of **3**.

Clavariopsin D (**4**) was isolated as a colorless powder with the molecular formula of $C_{58}H_{93}N_9O_{14}$ as determined by HRESIMS. The NMR spectroscopic data acquired in benzene-d_6 (Table 1) revealed that **4** was an isomer of **3** and a congener of **1**. This compound was found to exist as a mixture of two conformers in the ratio of 4:1 in benzene-d_6 because the ratio changed to 3:2 in pyridine-d_5. The NMR data for the major conformer of **4** (Table 1) were similar to those of **1** except for the absence of the γ-methylene signals (δ_H 1.27, 1.01, and δ_C 26.1) of the MeIle[6] residue in **1**. Interpretation of the HSQC, COSY, HMBC and NOESY spectra of **4** revealed that MeIle[6] in **1** is replaced by MeVal[6] in **4**. The identities of the amino acids and their order in **4** were then confirmed by the interresidual HMBC cross-peaks of α-CH (Val[4])/CO (MeVal[3]), NMe (MeVal[6])/CO (MeAsp[5]), NMe (MeIle[7])/CO (MeVal[6]), α-CH (Gly[8])/CO (MeIle[7]), NMe (MeVal[9])/CO (Gly[8]), and NH (Tyr(OMe)[10])/CO (MeVal[9]) and the NOESY correlations of α-CH (Tyr(OMe)[10])/α-CH (Hiv[1]), α-CH (Hiv[1])/ε-CH (Pip[2]), α-CH (Pip[2])/α-CH (MeVal[3]), and α-CH (Val[4])/NMe (MeAsp[5]). Based on these results, clavariopsin D (**4**) was identified as *cyclo*[-Hiv-Pip-MeVal-Val-MeAsp-MeVal-MeIle-Gly-MeVal-Tyr(OMe)-].

Table 1. NMR Spectroscopic Data (^{1}H 600 MHz, ^{13}C 150 MHz, benzene-d_6) for Clavariopsins C and D (3, 4)

	Clavariopsin C (**3**)		Clavariopsin D (**4**)[b]	
position[a]	δ_C, type	δ_H, mult. (*J* in Hz)	δ_C, type	δ_H, mult. (*J* in Hz)
Hiv[1]				
CO	172.1[c], C		171.9, C	
α	75.1, CH	5.29, d (3.6)	75.0, CH	5.28, d (3.6)

β	30.5, CH	1.76, m	30.5, CH	1.73, m
γ	19.0[d], CH_3	0.95, d (7.2)	19.1, CH_3	0.96, d (6.6)
γ	16.2, CH_3	0.93, d (6.6)	16.3, CH_3	0.94, d (6.6)
Pip[2]				
CO	171.6[e], C		171.4, C	
α	46.8, CH	5.77, d (6.6)	46.6, CH	5.78, d (6.0)
β	28.2, CH_2	1.65, m; 1.22, m	28.1, CH_2	1.64, m; 1.19, m
γ	18.8, CH_2	2.22, m; 1.18, m	18.9, CH_2	2.22, m; 1.17, m
δ	25.3, CH_2	1.27, m; 0.88, m	25.3, CH_2	1.25, m; 0.85, m
ε	43.4, CH_2	4.25, m; 3.08, m	43.3, CH_2	4.27, m; 3.06, m
MeVal[3]				
CO	168.2, C		168.0, C	
α	67.4, CH	4.56, d (10.8)	67.4, CH	4.56 d (10.8)
β	26.4, CH	2.54 m	26.3, CH	2.55 m
γ	19.8, CH_3	1.32, d (6.0)	19.9, CH_3	1.31, d (6.0)
γ	19.1[d], CH_3	0.75, d (6.6)	19.1, CH_3	0.74, d (6.6)
NMe	28.8, CH_3	2.93, s	28.8, CH_3	2.94, s
Val[4]				
CO	171.3, C		171.4, C	
α	54.9, CH	4.80, t (10.2)	55.1, CH	4.79, t (10.2)
β	29.5, CH	2.43, m	29.5, CH	2.44, m
γ	18.2, CH_3	0.91, d (7.2)	18.2, CH_3	0.93, m
γ	20.3, CH_3	0.87, d (6.0)	20.3, CH_3	0.89, d (6.0)
NH		7.33, d (10.2)		7.30, d (10.2)
MeAsp[5]				
CO	171.5[e], C		169.7, C	

α	53.0, CH	6.76, m	52.6, CH	6.60, dd (11.4, 5.4)
β	35.7, CH_2	3.44, m; 3.21 m	35.6, CH_2	3.60, m; 3.23 m
COOH	172.0[c], C		172.1, C	
NMe	30.9, CH_3	3.12, s	31.0, CH_3	3.14, s
MeIle[6]/MeVal[6]				
CO	170.8, C		170.6, C	
α	61.2, CH	4.73, d (11.4)	58.9, CH	5.30, d (10.8)
β	30.6, CH	2.24, m	27.5, CH	2.35, m
γ	24.7, CH_2	1.24, m; 0.97, m	19.3, CH_3	0.88, d (6.6)
γ	15.1, CH_3	0.89, d (6.6)	18.7, CH_3	0.72, d (6.6)
δ	9.5, CH_3	0.74, t (7.5)		
NMe	30.5, CH_3	3.10, s	30.1, CH_3	3.17, s
Ile[7]/MeIle[7]				
CO	171.3, C		170.3, C	
α	64.9, CH	3.11, m	74.6, CH	2.89, d (10.2)
β	33.2, CH	2.96, m	34.0, CH	3.06, m
γ	26.1, CH_2	1.58, m; 1.01, m	26.0, CH_2	1.48, m; 0.85, m
γ	16.4, CH_3	1.06, d (6.6)	18.1, CH_3	1.22, d (6.6)
δ	10.5, CH_3	0.85, t (7.5)	11.1, CH_3	0.82, m
NH/NMe		7.08, m	41.0, CH_3	3.09, s
Gly[8]				
CO	169.2, C		169.2, C	
α	41.2, CH_2	4.20, dd (17.4, 6.6); 3.37, m	41.6, CH_2	4.10, dd (17.4, 5.4); 3.65, dd (17.4, 3.6)
NH		8.06, br s		7.30, m
MeVal[9]				
CO	169.2, C		169.0, C	

α	62.5, CH	4.95, d (10.8)	63.3, CH	5.02, d (10.8)
β	26.9, CH	2.27, m	27.0, CH	2.26, m
γ	19.8, CH_3	1.12, d (6.6)	20.0, CH_3	1.10, d (6.0)
γ	18.8, CH_3	0.81, d (6.6)	18.7, CH_3	0.67, d (6.6)
NMe	28.4, CH_3	2.34, s	28.3, CH_3	2.22, s
Tyr(OMe)[10]				
CO	171.9[c], C		171.9, C	
α	51.2, CH	5.63, ddd (12.0, 9.6, 4.8)	51.2, CH	5.64, ddd (12.0, 10.2, 4.8)
β	35.1, CH_2	3.68, m; 3.54, dd (13.8, 4.8)	35.0, CH_2	3.70, dd (13.8, 12.0); 3.53, dd (13.8, 4.8)
1'	130.6, C		130.3, C	
2', 6'	131.0, CH	7.13, d (8.4)	130.9, CH	7.10, d (8.4)
3', 5'	113.6, CH	6.76, d (8.4)	113.7, CH	6.80, d (8.4)
4'	158.5, C		158.7, C	
OMe	54.9, CH_3	3.42, s	55.1, CH_3	3.60, s
NH		7.73, d (9.6)		7.68, d (10.2)

[a]Hiv = 2-hydroxyisovaleric acid, Pip = pipecolic acid; [b]The data for the major conformer of **4** are listed. See Supporting Information for the data for the minor conformer; [c-e]Interchangeable within the same letters.

Clavariopsin E (**5**), isolated as a colorless powder, was found to be an additional isomer of **3** and **4** based on the molecular formula of $C_{58}H_{93}N_9O_{14}$ deduced by HRESIMS and the similar NMR spectra (Table 2). A COSY experiment indicated that the amino acid composition was identical to that of **4**, indicating that **5** possessed an amino acid sequence distinct from that of **4**. The amino acid sequence of **5** was then determined by the relevant HMBC correlation of NMe (δ_H 3.08, MeVal[7])/CO (δ_C 170.2, MeIle[6]). These data indicated that the MeVal[6] and MeIle[7]

residues in **4** were exchanged in **5**. The linkages of the remaining amino acids in **5** were established by the HMBC correlations of NH (Tyr(OMe)^{10})/CO (MeVal^{9}), NMe (MeVal^{9})/CO (Gly^{8}), NMe (MeVal^{7})/CO (MeIle^{6}), and NH (Val^{4})/CO (MeVal^{3}) and the NOESY correlations of α-CH (Tyr(OMe)^{10})/α-CH (Hiv^{1}), α-CH (Hiv^{1})/ε-CH (Pip^{2}), α-CH (Pip^{2})/α-CH (MeVal^{3}), α-CH (Val^{4})/NMe (MeAsp^{5}), α-CH (MeAsp^{5})/NMe (MeIle^{6}) and α-CH (MeVal^{7})/NH (Gly^{8}). Based on these results, clavariopsin E (**5**) was identified as *cyclo*[-Hiv -Pip-MeVal-Val-MeAsp-MeIle-MeVal-Gly-MeVal-Tyr(OMe)-].

Clavariopsin F (**6**), obtained as a colorless powder, was found to be a new isomer of **1** based on its identical molecular formula, $C_{59}H_{95}N_9O_{14}$, which was determined by the HRESIMS analysis. The NMR spectra measured in benzene-d_6 suggested that **6** was an isomeric or conformational mixture in the ratio of 3:2, which made the NMR analysis complicated. Fortunately, the spectra measured in pyridine-d_5 appeared to have one main compound, indicating that this compound exists as a conformational mixture in benzene. The NMR data for the major conformer of **6** (Table 2) were similar to those for **1** with the exception of the MeIle^{6} residue, which was replaced by MeLeu^{6} in **6**. This was confirmed by the COSY correlations of α-CH (δ_H 5.91)–β-CH_2 (δ_H 2.63, 1.29)–γ-CH (δ_H 1.70)–δ-$(CH_3)_2$ (δ_H 1.06 and 1.07) within MeLeu^{6} and the relevant HMBC correlations of NMe (δ_H 3.05, MeLeu^{6})/CO (δ_C 170.1, MeAsp^{5}) and NMe (δ_H 3.21, MeIle^{7})/CO (δ_C 169.5 MeLeu^{6}). The identities of the amino acids and their order in **6** were then confirmed by the interresidual HMBC cross-peaks of NH (Val^{4})/CO (MeVal^{3}), NMe (MeAsp^{5})/CO (Val^{4}), NMe (MeLeu^{6})/CO (MeAsp^{5}), NMe (MeIle^{7})/CO (MeLeu^{6}), and NMe (MeVal^{9})/CO (Gly^{8}) and the NOESY correlations of α-CH (Tyr(OMe)^{10})/α-CH (Hiv^{1}), α-CH (Pip^{2})/α-CH (MeVal^{3}), α-CH (MeIle^{7})/α-CH (Gly^{8}) and α-CH (MeVal^{9})/α-CH (Tyr(OMe)^{10}).

Based on these results, clavariopsin F (**6**) was identified as *cyclo*[-Hiv-Pip-MeVal-Val-MeAsp-MeLeu-MeIle-Gly-MeVal-Tyr(OMe)-].

Table 2. NMR Spectroscopic Data (^{1}H 600 MHz, ^{13}C 150 MHz, benzene-d_6) for Clavariopsins E and F (5, 6)

	Clavariopsin E (**5**)		Clavariopsin F (**6**)[b]	
position[a]	δ_C, type	δ_H, mult. (*J* in Hz)	δ_C, type	δ_H, mult. (*J* in Hz)
Hiv[1]				
CO	171.9[c], C		171.7, C	
α	74.9, CH	5.28, d (3.0)	74.7, CH	5.38, d (3.0)
β	30.5, CH	1.74, m	30.6, CH	1.74, m
γ	19.1, CH_3	0.96, d (6.6)	19.0, CH_3	0.91, d (6.6)
γ	16.3, CH_3	0.92, d (6.6)	16.0, CH_3	0.91, d (6.6)
Pip[2]				
CO	171.4, C		171.5, C	
α	46.6, CH	5.78, d (6.0)	46.7, CH	5.76, m
β	28.2, CH_2	1.63, m, 1.18 m	28.3, CH_2	1.63, m; 1.19, m
γ	18.9, CH_2	2.22, m, 1.17 m	18.8, CH_2	2.19, m; 1.17, m
δ	25.3, CH_2	1.24, m, 0.85 m	25.2, CH_2	1.23, m; 0.85, m
ε	43.4, CH_2	4.26, m, 3.04 m	43.3, CH_2	4.24, m; 3.00, m
MeVal[3]				
CO	168.0, C		167.9, C	
α	67.4, CH	4.55, d (10.8)	67.4, CH	4.49, d (10.8)
β	26.4, CH	2.55, m	26.3, CH	2.53, m
γ	19.9, CH_3	1.30, d (6.0)	19.8, CH_3	1.26, d (6.6)

γ	19.1, CH_3	0.73, d (6.6)	19.1, CH_3	0.73, d (6.6)
NMe	28.8, CH_3	2.93, s	28.8, CH_3	2.94, s
Val[4]				
CO	171.4, C		171.4, C	
α	55.2, CH	4.81, t (10.2)	54.7, CH	4.72, t (10.2)
β	29.5, CH	2.44, m	29.7, CH	2.30, m
γ	18.2, CH_3	0.89, d (6.0)	18.2, CH_3	0.88, d (6.6)
γ	20.3, CH_3	0.92, d (6.0)	20.0, CH_3	0.73, d (6.6)
NH		7.31, d (10.2)		7.22, d (10.2)
MeAsp[5]				
CO	169.9[d], C		170.1, C	
α	52.7, CH	6.60, dd (11.4, 5.4)	53.3, CH	6.77, dd (11.4, 5.4)
β	35.7, CH_2	3.60, m; 3.28, m	35.5, CH_2	3.66, m; 3.33, m
COOH	172.1, C		172.6, C	
NMe	31.0, CH_3	3.21, s	30.8, CH_3	3.08, s
MeIle[6]/MeLeu[6]				
CO	170.2, C		169.5, C	
α	56.4, CH	5.45, d (10.8)	53.6, CH	5.91, dd (10.2, 3.6)
β	32.7, CH	2.33, m	39.9, CH_2	2.63, m; 1.29, m
γ	24.6, CH_2	1.25, m; 1.00 m	25.7, CH	1.70, m
γ/δ	15.4, CH_3	0.83, d (6.6)	23.8, CH_3	1.06, d (6.6)
δ	9.7, CH_3	0.76, t (7.5)	22.1, CH_3	1.07, d (6.6)
NMe	30.3, CH_3	3.19, s	30.8, CH_3	3.05, s
MeVal[7]/MeIle[7]				
CO	169.8[d], C		168.8, C	
α	74.8, CH	2.76, d (10.2)	65.3, CH	4.23, d (10.8)

β	28.1, CH	3.28, m	33.8, CH	2.18, m
γ	22.6, CH_3	1.29, d (6.6)	25.1, CH_2	1.44, m; 1.01, m
γ	20.2, CH_3	0.80, d (6.6)	15.5, CH_3	0.79, d (6.6)
δ			11.8, CH_3	0.78, t (7.2)
NMe	41.2, CH_3	3.08, s	29.7, CH_3	3.21, s
Gly[8]				
CO	169.1, C		168.4, C	
α	41.8, CH_2	4.01, dd (17.4, 5.4); 3.74, dd (17.4, 3.0)	41.6, CH_2	4.11, dd (17.4, 5.4); 3.73 m
NH		7.17 br s		6.89 br s
MeVal[9]				
CO	168.9, C		168.8, C	
α	62.4, CH	5.06, d (11.4)	62.3, CH	5.05, d (11.4)
β	26.9, CH	2.26, m	25.9, CH	2.21, m
γ	20.0, CH_3	1.10, d (6.6)	20.3, CH_3	1.07, d (6.6)
γ	18.6, CH_3	0.64, d (6.6)	18.2, CH_3	0.49, d (6.6)
NMe	28.3, CH_3	2.18, s	27.8, CH_3	2.01, s
Tyr(OMe)[10]				
CO	171.8[c], C		171.5, C	
α	51.2, CH	5.63, ddd (12.6, 10.2, 4.8)	51.2, CH	5.67, ddd (12.6, 10.2, 4.8)
β	34.9, CH_2	3.71, dd (13.8, 12.6); 3.53, dd (13.8, 4.8)	34.8, CH_2	3.87, dd (13.8, 12.6); 3.53, m
1'	130.3, C		130.6, C	
2', 6'	130.9, CH	7.10, d (8.4)	130.8, CH	7.14, d (8.4)
3', 5'	113.8, CH	6.83, d (8.4)	113.5, CH	6.69, d (8.4)
4'	158.8, C		158.6, C	

OMe	55.2, CH_3	3.63, s	54.9, CH_3	3.52, s
NH		7.66, d (10.2)		7.25, d (10.2)

[a]Hiv = 2-hydroxyisovaleric acid, Pip = pipecolic acid; [b]The data for the major conformer of **6** are listed. See Supporting Information for the data for the minor conformer; [c,d]Interchangeable within the same letters.

Clavariopsin G (**7**), obtained as a colorless powder, was found to possess an identical molecular formula ($C_{59}H_{95}N_9O_{14}$) to those of **1** and **6** based on the HRESIMS ion peaks at *m/z* 1154.7072 $[M+H]^+$ and 1176.6895 $[M+Na]^+$. The NMR data (Table S1) were almost identical to those of **6**. 2D NMR analyses revealed that the MeLeu^6 and MeIle^7 residues in **6** were replaced by MeIle^6 and MeLeu^7 in **7** respectively; the relevant HMBC correlation is NH (δ_H 7.05, Gly^8)/CO (δ_C 169.8, MeLeu^7). The identities of the remaining amino acids and their order were established by HMBC and NOESY correlations. Two fragments, Tyr(OMe)^{10}-MeVal^9-Gly^8-MeLeu^7 and Val^4-MeVal^3, were confirmed by the HMBC correlations of NH (Tyr(OMe)^{10})/CO (MeVal^9), NMe (MeVal^9)/CO (Gly^8), NH (Gly^8)/CO (MeLeu^7), and NH (Val^4)/CO (MeVal^3). Other residual connectivities were determined by the NOESY correlations of α-CH (Tyr(OMe)^{10})/α-CH (Hiv^1), α-CH (Hiv^1)/ε-CH (Pip^2), α-CH (Pip^2)/α-CH (MeVal^3), α-CH (Val^4)/NMe (MeAsp^5), α-CH (MeAsp^5)/NMe (MeIle^6) and α-CH (MeIle^6)/NMe (MeLeu^7). Based on these results, clavariopsin G (**7**) was identified as *cyclo*[-Hiv-Pip-MeVal-Val-MeAsp-MeIle-MeLeu-Gly-MeVal-Tyr(OMe)-].

Clavariopsin H (**8**) was isolated as a colorless powder, and its molecular formula was determined to be $C_{58}H_{93}N_9O_{14}$ by the HRESIMS ion peaks at *m/z* 1140.6916 $[M+H]^+$ and 1162.6741 $[M+Na]^+$. The NMR data for **8** (Table S1) closely resembled those for **1**, with the exception of the absence of a pair of signals for one methylene of the Pip^2 residue of **1**, which was supported by the molecular formula. 2D NMR analyses revealed that this residue was

replaced by a proline (Pro^2) in **8**, as supported by the COSY correlations of α-CH (δ_H 5.09)–β-CH_2 (δ_H 1.68, 1.36)–γ-CH_2 (δ_H 2.42, 1.35)–δ-CH_2 (δ_H 3.49, 2.87) within Pro^2 and the relevant HMBC correlation of NMe (δ_H 2.88, $MeVal^3$)/CO (δ_C 173.1, Pro^2). The identities of amino acids and their order were established by HMBC and NOESY correlations. The interresidual HMBC correlations of NMe ($MeIle^6$)/CO ($MeAsp^5$), NMe ($MeAsp^5$)/CO (Val^4), NH (Val^4)/CO ($MeVal^3$), NMe ($MeVal^3$)/CO (Pro^2), and α-CH (Hiv^1)/CO ($Tyr(OMe)^{10}$), NH ($Tyr(OMe)^{10}$)/CO ($MeVal^9$), NMe ($MeVal^9$)/CO (Gly^8) indicated the presence of two fragments $MeIle^6$-$MeAsp^5$-Val^4-$MeVal^3$-Pro^2 and Hiv^1-$Tyr(OMe)^{10}$-$MeVal^9$-Gly^8. The NOESY correlations of α-CH (Hiv^1)/δ-CH (Pro^2), α-CH ($MeIle^6$)/NMe ($MeIle^7$) and α-CH ($MeIle^7$)/α-CH (Gly^8) confirmed the remaining connectivities. Based on these results, **8** was identified as *cyclo*[-Hiv-Pro-MeVal-Val-MeAsp-MeIle-MeIle-Gly-MeVal-Tyr(OMe)-].

Clavariopsin I (**9**) was acquired as a colorless powder with the molecular formula of $C_{60}H_{97}N_9O_{14}$ as determined by HRESIMS, which is larger than **1** by one CH_2 unit. The NMR spectra in benzene-d_6 suggested that **9** was a mixture of two conformers in the ratio of 7:3, which was confirmed by single compound-like spectra obtained in pyridine-d_5. The NMR data for the major conformer (Table S1) were similar to those for **1** except for the additional CH_2 signals at δ_H 2.02 and 1.48/δ_C 41.0. The 2D NMR analyses confirmed that the Val^4 residue in **1** was replaced by Leu^4 in **9** as supported by the Leu^4 COSY correlations of NH (δ_H 7.37)–α-CH (δ_H 5.38)–β-CH_2 (δ_H 2.02, 1.48)–γ-CH (δ_H 1.68)–δ-$(CH_3)_2$ (δ_H 0.96 and 0.97) and the relevant HMBC correlations of NH (δ_H 7.37, Leu^4)/CO (δ_C 167.4, $MeVal^3$) and NMe (δ_H 3.22, $MeAsp^5$)/CO (δ_C 171.5, Leu^4). The remaining connectivities were determined by HMBC correlations, and **9** was identidied as *cyclo*[-Hiv-Pip-MeVal-Leu-MeAsp-MeIle-MeIle-Gly-MeVal-Tyr(OMe)-].

The absolute configurations of the amino acid residues in the clavariopsins were determined by the advanced Marfey's method[9-11] with L- and D-1-fluoro-2,4-dinitrophenyl-5-leucinamides (FDLA). Free amino acids were obtained by the acid hydrolysis of each compound and converted to the L- and D-DLA derivatives, which were then analyzed by LC/MS. The retention times of the derivatives detected by extracted ion chromatograms were compared to those of the standard amino acid derivatives, and all of the amino acids were determined to possess the L-configuration. On the other hand, the configuration of 2-hydroxyisovaleric acid (Hiv[1]) was determined as *R* by chiral-phase HPLC analysis following purification of the hydroxy acid from the acid hydrolysate by using reversed-phase HPLC. It was found to be difficult to explain the reason why some of the clavariopsins exist as a mixture of two conformers in benzene-d_6; 4:1 ratio for **4**, 3:2 ratio for **6**, and 7:3 ratio for **9**. Although the common structural feature of these compounds is the presence of the MeIle[7] residue, no significant participation of this residue was found in the ROESY analyses.

A previous study reported that clavariopsin A (**1**) possesses antifungal activity.[8] In this study, the antifungal activities of all of the clavariopsins were comprehensively evaluated by a paper disk diffusion method against six plant pathogenic fungi: *Botrytis cinerea*, *Magnaporthe oryzae*, *Colletotrichum orbiculare*, *Fusarium oxysporum*, *Alternaria alternata*, and *Aspergillus niger*. The results are summarized in Figure 2, in which the activities were expressed as the minimum inhibitory dose (MID, μg/disk). These results demonstrate that all of the clavariopsins (**1**–**9**) had significant or moderate antifungal activity against all of the tested fungi. For example, clavariopsins A (**1**), C (**3**) and D (**4**) showed the highest activity (MID = 0.01 μg/disk) against *A. alternata*, and the remaining clavariopsins exhibited MIDs of 0.03–0.3 μg/disk. Although no clear structure-activity relationship (SAR) was observed, all of the clavariopsins displayed

significant or moderate inhibitory activity (MID = 0.01–0.3 μg/disk) against two important plant pathogens, *B. cinerea* and *A. alternata*, which suggest that this class of compounds contain potential natural fungicides. Because **1** was reported to induce hyphal malformation such as swelling in *A. niger*,[8] all of the isolated compounds were evaluated for this activity. After incubation at 30 °C for 16 h, the hyphae were observed to be partially deformed into bulb-blunt shapes at the minimum effective dose of 0.3 μg/disk for **1** and **3**–**8** and at 3 μg/disk for **2** and **9** (Figure 3 and Table 3).

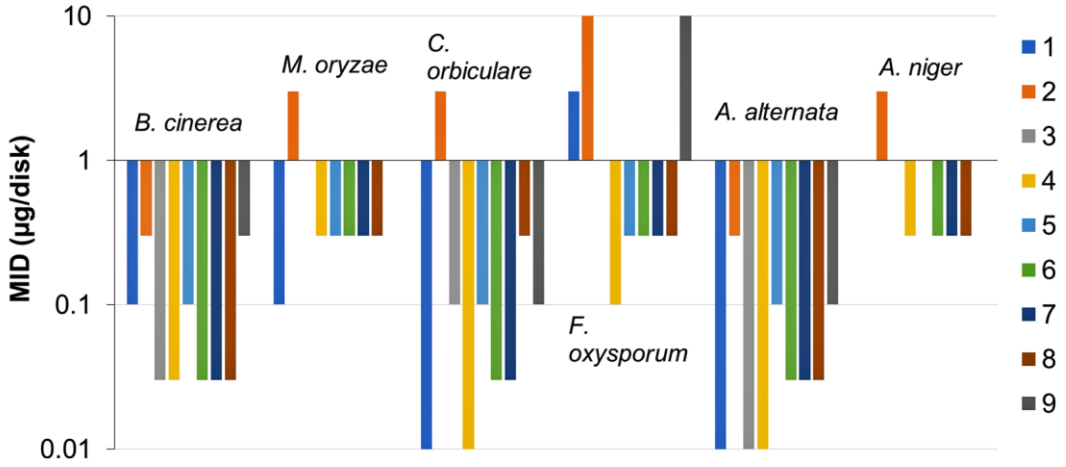


Figure 2. Antifungal activity of the clavariopsins against six plant pathogenic fungi. The values indicate the minimum inhibitory dose (MID, μg/disk) that induced a visually distinct inhibition zone between the paper disk and the colony front.

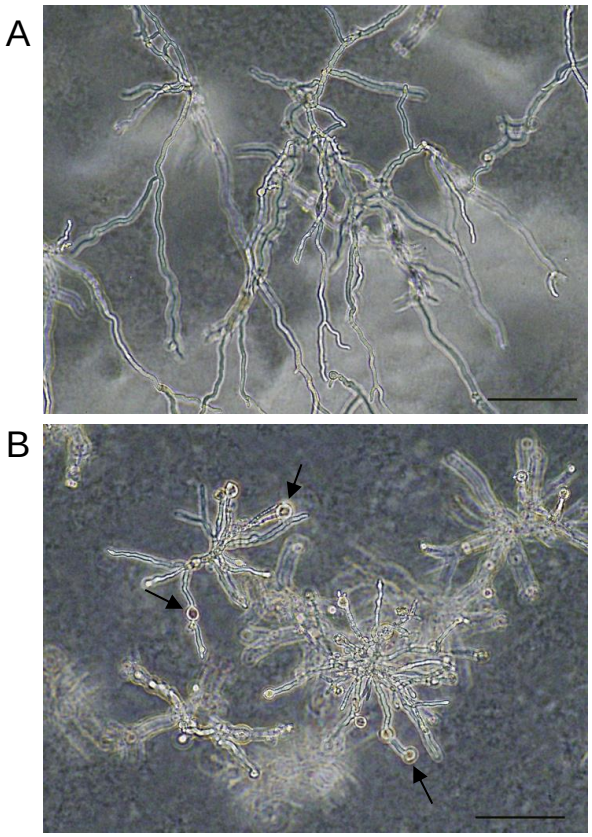


Figure 3. Hyphal malformation of *Aspergillus niger* treated with clavariopsin H (**8**). Conidia of *A. niger* were cultured in PDA medium with a paper disk that contained DMSO (A) or **8** at 0.3 μg/disk (B). Germinated mycelia were observed under a phase contrast microscope 16 h after inoculation. The arrows show some of swelling malformation of the mycelia. The scale bar represents 100 μm.

Table 3. Induction of Hyphal Malformation in *A. niger* by Clavariopsins

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Minimum effective dose[a] (μg/disk)	0.3	3	0.3	0.3	0.3	0.3	0.3	0.3	3

[a]The minimum dose that induces obvious swelling on hyphae.

Because the anti-proliferative activities of the previously identified clavariopsins A and B (**1** and **2)** had not been reported, all of the clavariopsins were next tested against HeLa-S3 cells by an MTT assay. The clavariopsins were found not to be cytotoxic (IC_{50} > 10 μM; Table S3).

In summary, seven new clavariopsin congeners **3**–**9** have been isolated from the aquatic hyphomycete *C. aquatica* and chemically characterized. They exhibited significant antifungal activity against agriculturally important pathogenic fungi, especially multihost plant pathogens such as the leaf-spot disease pathogen *A. alternata* and the gray mold fungus *B. cinerea*. The fact that they exhibited low cytotoxicity against human cancer cells suggest that they are potentially safe pesticides. Overall, the present study suggests that aquatic hyphomycetes are an attractive resource for valuable bioactive natural products.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded on a DIP-370 digital polarimeter (JASCO). UV and IR spectra were measured on a V-730 BIO spectrophotometer and a FT/IR-4100 (JASCO), respectively. NMR spectra were obtained on an Avance ARX400 (400 MHz for ^{1}H) or Avance III HD 600 MHz Cryo-probe spectrometer (600 MHz for ^{1}H) (Bruker). The chemical shifts (ppm) were referenced to the solvent peak of benzene-d_6 (δ_H 7.16/δ_C 128.0) or pyridine-d_5 (δ_H 7.22) as an internal standard. LC/MS data for amino acid analyses were obtained using a 1260 HPLC system (Agilent Technologies) coupled with an HCTplus-N Ion Trap Mass Spectrometer (Bruker) in the negative ion mode. High-resolution electrospray ionization time-of-flight mass spectra (HRESITOFMS) were recorded on a 6220 TOF LC/MS system (Agilent Technologies) in the positive ion mode. Preparative HPLC was performed on a

high pressure gradient system equipped with PU-2087 plus pumps, a DG-2080-53 degasser, an MX-2080-32 mixer and a UV-2075 plus detector (JASCO).

Fungal Materials. The clavariopsin-producing fungus *Clavariopsis aquatica* AJ117363 was isolated and cultured by the method described previously.[8] The species was identified by using the 18S rDNA sequence (DDBJ accession number: LC471408) and the ITS region (LC472491), indicating high similarity to the reference strain *C. aquatica* (MH047194) with the identity values of 100% and 97.2%, respectively. *Botrytis cinerea* NBc1, *Fusarium oxysporum* f. sp. *lycopersici* CK3-1, and *Alternaria alternata* M-71 were provided by Dr. Takashi Tsuge (Chubu University, Kasugai, Japan), *Magnaporthe oryzae* Ken53-35 was from Dr. Yukio Tosa (Kobe University, Kobe, Japan), *Colletotrichum orbiculare* 104-T was provided by Dr. Yasuyuki Kubo (Kyoto Prefectural University, Kyoto, Japan), and *Aspergillus niger* AJ117065 was provided by Ajinomoto Co. (Kawasaki, Japan). These pathogenic fungi were cultured on potato-dextrose-agar (PDA) medium at 25 °C for an appropriate length of time.

Extraction and Isolation. The culture broth (15 L in total, 100 flasks, 15 °C, 18 d) of *C. aquatica* was extracted with acetone (10 L). After filtration, the filtrate was concentrated to give an aqueous concentrate (2.2 L), which was extracted with EtOAc (1.2 L, twice). The combined organic layers were concentrated to obtain an EtOAc extract (11.2 g), which was subjected to pretreatment on a silica gel (240 mL) open column eluting with $CHCl_3$-MeOH (100:0, 550 mL; 90:10, 750 mL). The eluates were combined, concentrated, and suspended in 60% MeCN-0.1% TFA (300 mL) to obtain a supernatant (340 mL), an intermediate layer (0.57 g), and an insoluble tar (18 mL). The supernatant was applied to an ODS column (Mega Bond Elute C18, 10 g x 4 columns, Agilent Technologies) eluting with 60% (500 mL), 80% (200 mL), and 100% (200 mL) MeCN-0.1% TFA to give three fractions (Frs. A-1–3). Fraction A-2 (5.17 g) that eluted

with 80% MeCN-0.1% TFA was separated by flash chromatography [Hi-Flash Column (SI-40W-3L, silica gel 110 g, i.d. 46 × 130 mm) (Yamazen), 2–27% solvent A in $CHCl_3$ (A = MeOH-acetone 1:1), 50 min linear gradient, flow rate 20 mL/min], yielding six fractions (Frs. B-1–6). Fraction B-3 that eluted with $CHCl_3$ was subjected to flash chromatography [Hi-Flash Column 2L (Silica gel 40 μm, 45 g, i.d. 26 × 150 mm, Yamazen), 2–12% solvent B in $CHCl_3$ (B = MeOH-conc. NH_3 20:1), 50 min linear gradient, 20 mL/min flow rate] to obtain three fractions (Frs. C-1–3). Fraction C-1 (3.2 g) that eluted with $CHCl_3$ was separated into three aliquots and each aliquot (ca. 1 g) was subjected to HPLC [Develosil ODS-UG-5 (28 × 250 mm), 85–100% MeOH in H_2O (150 min linear gradient), 15 mL/min flow rate, three injections] to give four fractions (Frs. D-1–4). The compounds in Frs. D-2 and D-3 were identified as **7** (316 mg, t_R = 41.0 min) and **1**[8] (1.33 g, t_R = 49.8 min), respectively. Fraction D-1 (221 mg) that eluted from 12.8–39.5 min was subjected to preparative HPLC [CAPCELL PAK C18 UG80 (20 × 250 mm, Shiseido), 70–85% MeCN-0.1% TFA (90 min linear gradient), 8 mL/min, monitored at 230 nm] to obtain seven fractions (Frs. E-1–7). Among them, the compound in Fr. E-5 was identified as **3** (37.8 mg, t_R = 53.6 min). Fraction E-2 (76.7 mg) that eluted from 34–40 min was purified by HPLC (same column, 80% MeOH-0.1% TFA, 8 mL/min, detected at 230 nm) to yield **2** (26.1 mg, t_R = 60.8 min). Fraction E-4 (55.4 mg) that eluted from 42–49 min was separated by HPLC (same column, 70% MeCN-0.1% TFA, 8 mL/min, monitored at 230 nm, recycled 5 times) to afford **8** (14.2 mg). Fraction D-4 (40.0 mg) that eluted from 57–63.4 min was purified by HPLC [same column, 80–95% MeCN-0.1% TFA (90 min linear gradient), 8 mL/min, monitored at 230 nm] to yield **9** (3.3 mg, t_R = 51.6 min). The insoluble tar (18 mL) mentioned above was suspended in 60% MeCN-0.1% TFA (800 mL) and the supernatant was applied to an ODS column (Mega Bond Elute C18, 10g x 8 columns) eluting with 60% (1200 mL), 80% (400 mL),

and 100% (400 mL) MeCN-0.1% TFA. The fraction that eluted with 80% MeCN-0.1% TFA was concentrated and the resulting aqueous solution was extracted with EtOAc (100 mL, three times). The organic layers were combined and concentrated to give an oily material (4.59 g), which was chromatographed on silica gel (240 mL, 0, 2, 5, 10, 50% MeOH in $CHCl_3$, 200 mL for each solvent). The fractions that eluted with 5% and 10% MeOH in $CHCl_3$ was combined and concentrated. The resulting reddish-brown oil (1.03 g) was separated by HPLC [CAPCELL PAK C18 UG80 (20 x 250 mm), 95% MeOH-0.1% TFA, 8 mL/min, monitored at 277 nm]. The fractions that eluted from 19–25 min were combined and concentrated to give **1** (724 mg). The fraction that eluted from 18–18.5 min (57 mg) was separated by HPLC [Develosil ODS-HG-5 (20 x 250 mm) (Nomura Chemical), 75–90% MeCN-0.1% TFA (120 min linear gradient), 8 mL/min, monitored at 245 nm] to give a mixture of **4** and **5** (30.8 mg, t_R = 47.5 min), **6** (17 mg, t_R = 53.4 min), and **7** (7.7 mg, t_R = 57.1 min). The mixture was then separated by HPLC [same column, 70% MeCN-50 mM CH_3COONH_4, 8 mL/min, monitored at 240 nm, recycled 7 times] to afford **4** (7.5 mg, t_R = 209.4 min) and **5** (5.5 mg, t_R = 221.8 min).

Clavariopsin C (**3**): white amorphous powder; $[\alpha]^{26}_D$ –172 (*c* 0.14, $CHC1_3$); UV (MeCN) λ_{max} (log ε) 224 (4.25), 277 (3.23), 285 (3.15) nm; IR (film) ν_{max} 3376, 1736, 1647, 1611, 1515, 1471, 1247 cm^{-1}; NMR data, Table 1; HRESIMS *m/z* 1140.6920 $[M + H]^+$ (calcd for $C_{58}H_{94}N_9O_{14}$, 1140.6915) and *m/z* 1162.6741 $[M + Na]^+$ (calcd for $C_{58}H_{93}N_9O_{14}Na$, 1162.6735).

Clavariopsin D (**4**): white amorphous powder; $[\alpha]^{26}_D$ –217 (*c* 0.13, $CHC1_3$); UV (MeCN) λ_{max} (log ε) 224 (4.25), 278 (3.18), 285 (3.09) nm; IR (film) ν_{max} 3384, 1739, 1647, 1613, 1514, 1465, 1244 cm^{-1}; NMR data, Table 1; HRESITOFMS *m/z* 1140.6943 $[M + H]^+$ (calcd for $C_{58}H_{94}N_9O_{14}$, 1140.6915) and *m/z* 1162.6756 $[M + Na]^+$ (calcd for $C_{58}H_{93}N_9O_{14}Na$, 1162.6735).

Clavariopsin E (**5**): white amorphous powder; $[\alpha]^{26}_{D}$ –202 (*c* 0.13, $CHC1_3$); UV (MeCN) λ_{max} (log ε) 225 (4.24), 277 (3.20), 284 (3.14) nm; IR (film) ν_{max} 3384, 1739, 1643, 1613, 1514, 1472, 1244 cm^{-1}; NMR data, Table 2; HRESITOFMS *m/z* 1140.6900 $[M + H]^+$ (calcd for $C_{58}H_{94}N_9O_{14}$, 1140.6915) and *m/z* 1162.6713 $[M + Na]^+$ (calcd for $C_{58}H_{93}N_9O_{14}Na$, 1162.6735).

Clavariopsin F (**6**): white amorphous powder; $[\alpha]^{26}_{D}$ –163 (*c* 0.09, $CHC1_3$); UV (MeCN) λ_{max} (log ε) 225 (4.20), 277 (3.26), 285 (3.19) nm; IR (film) ν_{max} 3384, 1738, 1680, 1645, 1612, 1515, 1469, 1248 cm^{-1}; NMR data, Table 2; HRESIMS *m/z* 1154.7067 $[M + H]^+$ (calcd for $C_{59}H_{96}N_9O_{14}$, 1154.7071) and *m/z* 1176.6894 $[M + Na]^+$ (calcd for $C_{59}H_{95}N_9O_{14}Na$, 1176.6891).

Clavariopsin G (**7**): white amorphous powder; $[\alpha]^{26}_{D}$ –164 (*c* 0.11, $CHC1_3$); UV (MeCN) λ_{max} (log ε) 227 (4.15), 277 (3.26), 285 (3.20) nm; IR (film) ν_{max} 3381, 1738, 1679, 1644, 1611, 1515, 1468, 1248 cm^{-1}; NMR data, Table S1; HRESIMS *m/z* 1154.7072 $[M + H]^+$ (calcd for $C_{59}H_{96}N_9O_{14}$, 1154.7071) and *m/z* 1176.6895 $[M + Na]^+$ (calcd for $C_{59}H_{95}N_9O_{14}Na$, 1176.6891).

Clavariopsin H (**8**): white amorphous powder; $[\alpha]^{26}_{D}$ –192 (*c* 0.12, $CHC1_3$); UV (MeCN) λ_{max} (log ε) 224 (4.28), 277 (3.32), 284 (3.25) nm; IR (film) ν_{max} 3382, 1737, 1680, 1646, 1515, 1471, 1246 cm^{-1}; NMR data, Table S1; HRESIMS *m/z* 1140.6916 $[M + H]^+$ (calcd for $C_{58}H_{94}N_9O_{14}$, 1140.6915) and *m/z* 1162.6741 $[M + Na]^+$ (calcd for $C_{58}H_{93}N_9O_{14}Na$, 1162.6735).

Clavariopsin I (**9**): white amorphous powder; $[\alpha]^{26}_{D}$ –175 (*c* 0.06, $CHC1_3$); UV (MeCN) λ_{max} (log ε) 225 (4.22), 277 (3.29), 285 (3.21) nm; IR (film) ν_{max} 3380, 1737, 1680, 1645, 1611, 1515, 1468, 1247 cm^{-1}; NMR data, Table S1; HRESIMS *m/z* 1168.7225 $[M + H]^+$ (calcd for $C_{60}H_{98}N_9O_{14}$, 1168.7228) and *m/z* 1190.7047 $[M + H]^+$ (calcd for $C_{60}H_{97}N_9O_{14}Na$, 1190.7048).

Advanced Marfey's Analysis. Each clavariopsin (0.43–0.48 mg, 0.38–0.42 μmol) was hydrolyzed with 6 N HCl (200 μL) at 110 °C for 2 h in a sealed tube. The reaction mixture was concentrated to dryness under N_2 stream and dissolved in 100 μL of H_2O to give an amino acid

solution (theoretical concentration: 4.0 mM). The solution was divided into two portions (50 μL each), which were treated with 40 μL of 1 M $NaHCO_3$ and 60 μL of 1% L- and D-1-fluoro-2,4-dinitrophenyl-5-leucinamide (FDLA) in acetone, respectively. After being vortexed, the reaction mixtures were incubated at 37 °C for 1 h. Then, 20 μL of 1 N HCl was added and diluted up to 500 μL with MeCN. A portion (1 μL) of the resulting mixture was diluted with 1000 μL of 70% MeCN, and a 5 μL aliquot was used for LC/MS analysis with a Cadenza CD-C18 column (2 × 75 mm, Imtakt) [50–100% A solvent-0.1% formic acid over 40 min, A = MeOH-MeCN (1:1), 0.15 mL/min flow rate]. For **4** and **5**, a mixture (0.43 mg, 0.38 μmol) was used for the analysis. The DLA derivatives of the amino acids were observed by extracted ion chromatograms.

Chiral-phase HPLC Analysis. Each compound (0.26–0.56 mg, 0.22–0.49 μmol) was treated with 6 N HCl (200 μL) at 110 °C for 6 h. The hydrolysate was dried under N_2 stream and separated by HPLC [Develosil ODS-HG-5 column (10 × 250 mm) (Nomura Chemical), 10% MeCN-0.05% TFA in H_2O, 3.3 mL/min flow rate, UV detection at 205 nm] to afford the fraction containing 2-hydroxyisovaleric acid (t_R = 12−13 min), which was concentrated and redissolved in 50 μL of H_2O. A portion (14 μL, theoretical amount 0.06–0.14 μmol) of the solution was analyzed by chiral-phase HPLC [CHIRALPAK WH column (4.6 × 250 mm), 10% MeOH in 0.5 mM $CuSO_4$, 1 mL/min flow rate, UV detection at 270 nm]. For **4** and **5**, a mixture (0.51 mg, 0.45 μmol) was used for the analysis. The (*R*)- and (*S*)-hydroxy acids eluted at 16.7, and 20.4 min, respectively.

Antifungal Activity. The fungi (*B. cinerea*, *M. oryzae*, *C. orbiculare, F. oxysporum*, and *A. alternata*) were cultured on PDA medium (0.4% potato extract, 2% glucose, and 1.5% agar) in a 9-cm petri dish at 25 °C for 1–12 d until colonies grew to approximately 3–4 cm in diameter. Paper disks (6 mm in diameter) soaked with 5 μL of a compound solution in DMSO were placed

1 cm (5 mm in the case for *C. orbiculare*) away from the colony front. The inhibition zone (distance between the paper disk edge and the colony front, mm) was measured to evaluate the activity. The minimum inhibitory dose (MID, μg/disk) is defined as the minimum dose that induced a weak but obvious inhibition zone (1–1.5 mm).

The antifungal test against *A. niger* was carried out using a different method due to the tendency for spore formation. A loopful of conidia was inoculated on PDA medium and incubated at 25 °C for 4 d. The colony surface was gently washed with 5 mL of 0.1% Tween 20, and 0.5 mL (approximately 5×10^6 conidia) was suspended in 100 mL of PDA (0.4% potato extract, 2% glucose, and 1.5% agar) at 43 °C to prepare conidia-containing agar plates. Paper disks soaked with a sample solution in DMSO were placed on the agar plates, which were incubated for an additional 2 and the diameter of the halo was measured. The MID for *A. niger* was defined as the minimum dose that showed a weak but obvious halo around the paper disk.

Effect on the Hyphal Morphology of *A. niger*. The test was performed according to the previous report.[8,12] After culturing *A. niger* at 27 °C for 4 according to the method described above, conidia were harvested with 5 mL of sterile H_2O supplemented with 0.1% Tween 20. The conidia were carefully rubbed with a sterile cotton swab and transferred into a 15-mL centrifuge tube. The suspension was homogenized with a vortex mixer for 15 s, and the mixture was filtered using a sterile filter paper (47 mm in diameter). Then, the filtrate was collected in a 15-mL centrifuge tube. The conidia were counted by using a haemocytometer under a microscope. The suspension (0.5 mL, approximately 5×10^6 conidia) was diluted with 100 mL of PDA medium (0.2% potato extract, 1% glucose and 1% agar) at 43 °C, and 3 mL of the conidia-containing medium was laid in a 9-cm petri dish. Paper disks soaked with a compound in DMSO were applied to the agar plates and incubated at 30 °C for 16 h. The hyphal morphology of the fungus

in the inhibition zone around the paper disk was observed under a phase contrast microscope. The activity was expressed as the minimum effective dose that results in obvious morphological changes (swelling, etc.) in the fungal hyphae.

Cytotoxicity Assay. The HeLa-S3 cell line was used according to the reported procedure.[13] Briefly, the cells (1×10^4 cells per well) in 99 μL of Eagle's Minimal Essential Medium (EMEM) (FUJIFILM Wako Pure Chemical Corporation) were incubated in the wells of a 96-well plate at 37 °C for 24 h in a 5% CO_2 incubator. Then, 1 μL of a compound in DMSO (0.15, 0.5, 1.5, 5 mM), paclitaxel in DMSO (0.3, 1, 3 μM) as a positive control, or DMSO as negative control was added to each well. After incubating for 48 h, the cells were treated with 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate buffer saline (5 mg/mL) and incubated for an additional 3 h. The medium was removed and the cells were dissolved in 100 μL of DMSO. Finally, the absorbance values were measured at 595 nm by using a Multiscan FC microplate reader (Thermo Fisher Scientific).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge via the Internet at http://pubs.acs.org. 1D and 2D NMR, UV, IR and HRESIMS spectra of new compounds; LC-MS chromatographs of hydrolysates and chiral HPLC of hydroxy acid of new compounds; photographs of growth inhibition of tested fungi and hyphae of *A. niger*; NMR chemical shifts for **7**–**9** and minor conformers of **4**, **6** and **9**; cytotoxicity data (PDF).

AUTHOR INFORMATION

Corresponding Author

*Tel: +81-52-789-4116. Fax: +81-52-789-4118. E-mail: ojika@ agr.nagoya-u.ac.jp.

Author Contributions

T.W.S. purified and elucidated the structures, conducted the antifungal and the cytotoxic activities, and prepared the manuscript. C.H. isolated and analyzed the compounds. R.F. and K.K. isolated, cultured and extracted *C. aquatica*. T.T. and Y.S. performed the cytotoxic activity assay. M.O. designed the project and revised the manuscript.

Notes

The authors declare no competing financial interest**.**

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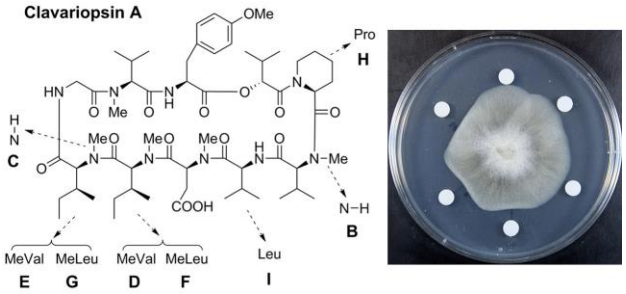
TOC Graphic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