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Mortiamides A−D, Cyclic Heptapeptides from a Novel *Mortierella* sp. Obtained from Frobisher Bay

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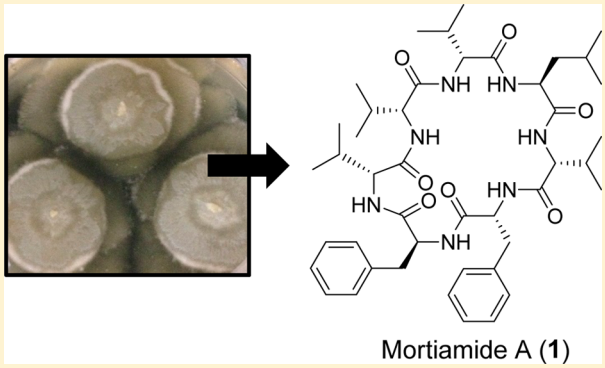
Supporting Information

ABSTRACT: Four new cyclic heptapeptides, mortiamides A−D (**1**−**4**), were obtained from a novel *Mortierella* sp. isolate obtained from marine sediment collected from the intertidal zone of Frobisher Bay, Nunavut, Canada. The structures of the compounds were elucidated by NMR spectroscopy and tandem mass spectrometry. The absolute configurations of the amino acids were determined using Marfey's method. Localization of L and D amino acids within each compound was ascertained by retention time comparison of the partial hydrosylate products of each compound to synthesized dipeptide standards using LC-HRMS. Compounds **1**−**4** did not exhibit any significant antimicrobial or cytotoxic activity.



Fungi are an excellent source of novel and bioactive natural products, but limited investigations of arctic fungi have been undertaken. Arctic fungi are diverse and abundant[1] and represent a vast untapped resource for natural product discovery. Unique stresses associated with arctic life including low temperature, high ultraviolet radiation, and low nutrient availability have led to unique adaptations within these fungi[2] and may lead to the potential for the discovery of new natural products.

Canada's arctic has been greatly underexplored in terms of fungal and associated natural product diversity. Frobisher Bay located within Canada's arctic is a 230 km inlet of the Labrador Sea located on the southeastern corner of Baffin Island, Nunavut. This inlet experiences some of the highest diurnal tidal variations in the world (7−11 m) and represents an extreme environment for microorganisms. Previous work conducted in our lab from this region has resulted in the isolation of two new natural products, iqalisetins A and B, from a *Tolypocladium* species.[3] As part of ongoing bioprospecting efforts within Frobisher Bay, a new member within the genus *Mortierella* was isolated from sediment samples from the intertidal zone. The genus *Mortierella* falls within the order Mortierellales, one of the largest orders within the phylum Mucoromycota, and is commonly isolated from cold environments.[4,5] Most notably, members within this genus are known for the production of polyunsaturated fatty acids[6,7] and for the ability to biotransform various organic compounds.[8] Very few natural products have been reported from this genus.[9−12]

The new *Mortierella* isolate presented an anamorphic state consistent with the genus and was found to be phylogenetically distinct from all known taxa of the genus *Mortierella* (using barcoding gene sequences derived from rDNA). Chemical investigation of this isolate led to the isolation of four new cyclic heptapeptides, mortiamides A−D (**1**−**4**), containing an unusually high proportion of D amino acids. LC-HRMS was used to identify partial hydrolysis products and aid in the localization of these L and D amino acids within each compound. The isolation, structure elucidation, and biological activity testing of these compounds are reported within.

RESULTS AND DISCUSSION

The fungal isolate *Mortierella* sp. RKAG 110 was obtained from sediment collected from Frobisher Bay, Nunavut, Canada. BlastN search results of several rDNA genes (ITS and nLSU) placed this isolate within the genus *Mortierella* and suggested that this isolate was unique to the genus as the closest match of the ITS rDNA nucleotide sequence to an accessioned strain was with that of *Mortierella antarctica* (NR_111580.1) with 96% sequence similarity. Production of a white rosette-like phenotype and microscopic observation of spherical sporangia in agar culture confirmed the isolate RKAG 110 to be a member of the genus *Mortierella*. As the organism originated from a rarely studied geographic location and was taxonomically novel, further chemical investigations were carried out.

The 1 L solid agar fermentation of isolate RKAG 110 was extracted with EtOAc, dried, and partitioned between 80%

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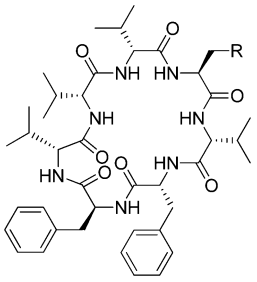
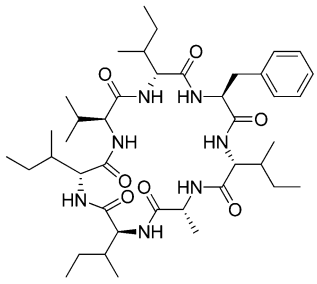


1 R = $CH(CH_3)_2$
2 R = C_6H_5



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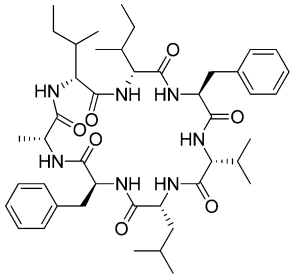

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CH_3CN and hexane. The CH_3CN-soluble portion was subjected to flash chromatography and reversed-phase HPLC, yielding four new cyclic heptapeptides, mortiamide A (**1**) (4.1 mg), mortiamide B (**2**) (1.5 mg), mortiamide C (**3**) (3.2 mg), and mortiamide D (**4**) (2.4 mg).

Mortiamide A (**1**) (Table 1) was obtained as a white powder, and HRESIMS supported a molecular formula of $C_{44}H_{65}N_7O_7$, requiring 16 degrees of unsaturation. The peptidic nature of the compound was determined by the presence of seven amide protons within the ^{1}H NMR spectrum, while the ^{13}C NMR spectrum revealed the presence of seven amide carbonyls between δ_C 170.5 and 173.3 ppm and seven α-amino acid carbon resonances between δ_C 53.4 and 60.3 (Table 1). Additionally TOCSY experiments revealed the presence of seven ^{1}H spin systems corresponding to seven amino acid side chains consisting of one leucine, two phenylalanine, and four valine residues. Amino acid side chains were assigned using COSY, HMBC, and TOCSY NMR spectra whereby connectivity was determined using HMBC, ROESY, and ESI-MS^n analysis. HMBC correlations from Val^1-NH (δ_H 8.20) to Phe^2-C=O (δ_C 171.4) and Val^2-NH (δ_H 7.86) to Val^1-C=O (δ_C 172.4) established the three amino acid sequence Phe^2-Val^1-Val^2. HMBC correlations between Val^4-NH (δ_H 7.64) and Leu-C=O (δ_C 171.6) established the two amino acid sequence Leu-Val^4. ROESY correlations from Val^2-Hα (δ_H 4.01) to Val^3-NH (δ_H 8.29) and Val^3-Hα (δ_H 4.03) to Leu-NH (δ_H 7.38) connected both sequences via a Val residue, resulting in the six amino acid sequence Phe^2-Val^1-Val^2-Val^3-Leu-Val^4. ROESY correlations from Val^4-Hα (δ_H 4.12) to Phe^1-NH (δ_H 7.72) and Phe^1-Hα (δ_H 4.63) to Phe^2-NH (δ_H 7.64) resulted in the final sequence of the heptapeptide being assigned as [cyclo-(Phe^1-Phe^2-Val^1-Val^2-Val^3-Leu-Val^4)] (Figure 1), which was subsequently confirmed using MS/MS experiments (Supporting Information, Figure S5).

The absolute configuration of **1** was determined using Marfey's method.[13] The peptide was found to contain L-Leu, D-Val, L-Phe, and D-Phe (Supporting Information, Figure S11). In order to localize L-Phe and D-Phe within the structure, a partial hydrolysis was undertaken to generate dipeptide fragments which were derivatized with *N*-(5-fluoro-2,4-dinitrophenyl-5)-L-alaninamide (FDAA) and analyzed by LC-HRMS.[14] The retention time of the partial hydrosylate product (FDAA-Phe-Phe) was compared to synthesized dipeptide standards that were derivitized in the same manner (FDAA-L-Phe-D-Phe and FDAA-D-Phe-L-Phe) and revealed the retention time of the partial hydrosylate fragment was consistent with the retention time of the synthesized FDAA-D-Phe-L-Phe standard (Figure 2). Therefore, the final structure of **1** was assigned as [cyclo-(D-Phe-L-Phe-D-Val-D-Val-D-Val-L-Leu-D-Val)].

Mortiamide B (**2**) (Table 1) was obtained as a white powder, and HRESIMS supported a molecular formula of $C_{47}H_{63}N_7O_7$ requiring 20 degrees of unsaturation. TOCSY experiments revealed the presence of seven ^{1}H spin systems corresponding to three Phe and four Val residues (Table 1). HMBC correlations from Phe^1-NH (δ_H 7.81) to Val^4-C=O (δ_C 170.7) and Val^4-NH (δ_H 7.56) to Phe^3-C=O (δ_C 170.5) established the three amino acid sequence Phe^3-Val^4-Phe^1. ROESY correlations from Phe^3-NH (δ_H 7.70) to Val^3-Hβ (δ_H 2.06), Val^3-NH (δ_H 8.00) to Val^2-Hβ (δ_H 2.29), Val^2-NH (δ_H 7.78) to Val^1-Hα (δ_H 4.07), Val^1-NH (δ_H 8.24) to Phe^2-Hα (δ_H 4.49), and Phe^2-NH (δ_H 8.19) to Phe^1-Hα (δ_H 4.61) established the final sequence as cyclo-Phe^1-Phe^2-Val^1-Val^2-Val^3-Phe^3-Val^4 (Figure 1). Peptide **2** differed from **1** by the replacement of the Leu residue with a Phe residue. Marfey's analysis[13] of the total hydrosylate of **2** revealed the presence of D-Val, L-Phe, and D-Phe in a 2:1 ratio, respectively. After partial hydrolysis and derivatization, the retention time of the FDAA-Phe-Phe dipeptide (t_R 48.55 min) was consistent with the retention time of the synthesized FDAA-D-Phe-L-Phe dipeptide (t_R 48.40 min) (Supporting Information, Figure S12). The remaining Phe residue was assigned as L, and the final structure of **2** was assigned as [cyclo-(D-Phe-L-Phe-D-Val-D-Val-D-Val-L-Phe-D-Val)].

Mortiamide C (**3**) (Table 2) was obtained as a white powder, and HRESIMS supported a molecular formula of $C_{41}H_{67}N_7O_7$ requiring 12 degrees of unsaturation. TOCSY experiments revealed the presence of seven ^{1}H spin systems corresponding to one Phe, one Ala, one Val, and four Ile residues (Table 2). HMBC correlations from Ala-NH (δ_H 7.54) to Ile^4-C=O (δ_C 170.8) and Ile^4-NH (δ_H 7.80) to Phe-C=O (δ_C 170.6) established the three amino acid sequence Phe-Ile^4-Ala. ROESY correlations from Phe-NH (δ_H 7.61) to Ile^3-Hα (δ_H 4.00), Ile^3-NH (δ_H 7.89) to Val-Hα (δ_H 4.03), Val-NH (δ_H 7.67) to Ile^2-Hα (δ_H 4.31), Ile^2-NH (δ_H 8.44) to Ile^1-Hα (δ_H 4.27), and Ile^1-NH (δ_H 7.93) to Ala-Hα (δ_H 4.42) established the final sequence as [cyclo-(Ala-Ile^1-Ile^2-Val-Ile^3-Phe-Ile^4)] (Figure 1), which was confirmed by MS/MS experiments (Supporting Information, Figure S7). The absolute configurations of the amino acid residues were determined to be D-Ala, D-Val, L-Phe, L-Ile, and D-Ile, whereby D-Ile and L-Ile were in a 3:1 ratio. Due to the coelution of Ile and *allo*-Ile by RP-HPLC, we were unable to definitively assign the absolute configuration of these residues. By comparison of the FDAA-Ile-Ala (t_R 37.41 min) and FDAA-Ile-Ile (t_R 42.56 min) partial hydrosylate products of **3** to synthesized dipeptide standards it was determined that the retention times by LC-HRMS corresponded to FDAA-D-Ile-D-Ala (t_R 37.03 min) and FDAA-L-Ile-D-Ile (t_R 41.85) (Supporting Information, Figure S13). Because L-Ile had been localized, the remaining Ile was assigned as D, resulting in the final structure being assigned as [cyclo-(D-Ala-L-Ile-D-Ile-D-Val-D-Ile-L-Phe-D-Ile)].

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Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of Mortiamides A (1) and B (2) in DMSO-d_6

1				2			
position	δ_C, type	δ_H (J, Hz)		position		δ_C, type	δ_H (J, Hz)
D-Phe[1]	CO	171.1, C		D-Phe[1]	CO	171.1, C	
	NH		7.72, d (8.1)		NH		7.81, d (7.8)
	α	53.4, CH	4.63, m		α	53.3, CH	4.61, m
	β_A	36.8, CH_2	2.98, dd (13.7, 7.3)		β_A	36.8, CH_2	2.97, dd (6.2, 13.5)
	β_B		2.76, dd (13.7, 7.5)		β_B		2.77, dd (8.1, 13.5)
	1′	137.5, C			1′	137.7, C	
	2′	129.1, CH	7.10, m		2′	128.9, CH	7.09, m
	3′	128.2, CH	7.21, m		3′	129.0, CH	7.21, m
	4′	126.4, CH	7.18, m		4′	126.2, CH	7.17, m
L-Phe[2]	CO	171.4, C		L-Phe[2]	CO	171.4, C	
	NH		8.29[a]		NH		8.19, d (5.7)
	α	55.2, CH	4.47, m		α	55.2, CH	4.49, m
	β_A	36.3, CH_2	2.88, dd (13.6, 7.6)		β_A	36.3, CH_2	2.91[a]
	β_B		2.81, dd (13.6, 8.0)		β_B		2.83, dd (13.6, 8.2)
	1′	137.2, C			1′	137.4, C	
	2′	129.1, CH	7.19, m		2′	129.0, CH	7.21, m
	3′	128.2, CH	7.23, m		3′	128.0, CH	7.24, m
	4′	126.5, CH	7.23, m		4′	126.2, CH	7.17, m
D-Val[1]	CO	172.4, C		D-Val[1]	CO	171.8, C	
	NH		8.20, d (7.0)		NH		8.24[b]
	α	58.4, CH	4.06, m		α	58.5, CH	4.07[a]
	β	29.3, CH	2.10, m		β	29.3, CH	2.10, m
	γ1	17.0, CH_3	0.73, d (6.9)		γ1	17.1, CH_3	0.75, d (6.9)
	γ2	19.1, CH_3	0.62, d (6.9)		γ2	18.8, CH_3	0.65, d (6.9)
D-Val[2]	CO	173.3, C		D-Val[2]	CO	171.4, C	
	NH		7.86, d (5.3)		NH		7.78, d (7.8)
	α	60.3, CH	4.01, m		α	59.7, CH	4.03[a]
	β	28.3, CH	2.34, m		β	28.3, CH	2.29, m
	γ1	19.4, CH_3	1.03, d (6.6)		γ1	19.0, CH_3	0.99, d (6.7)
	γ2	18.9, CH_3	0.91[a]		γ2	18.8, CH_3	0.89, d (6.7)
D-Val[3]	CO	170.5, C		D-Val[3]	CO	172.4, C	
	NH		8.29[a]		NH		8.00, d (5.2)
	α	59.0, CH	4.03, m		α	58.5, CH	4.03[a]
	β	29.1, CH	2.22, m		β	29.3, CH	2.06, m
	γ1	18.9, CH_3	0.91[a]		γ1	18.7, CH_3	0.76, d (6.9)
	γ2	16.8, CH_3	0.84, d (6.9)		γ2	16.5, CH_3	0.63, d (6.9)
L-Leu	CO	171.6, C		L-Phe[3]	CO	170.5, C	
	NH		7.38, d (4.4)		NH		7.70[b]
	α	58.8, CH	4.09, m		α	55.4, CH	4.39, m
	β	38.8, CH_2	1.55, m		β_A	35.8, CH_2	3.08, dd (13.8, 6.2)
	γ	24.0, CH_3	1.65, m		β_B		2.90, m
	δ1	22.7, CH_3	0.90[a]		1′	137.9, C	
	δ2	21.1, CH_3	0.82, d (6.6)		2′	128.9, CH	7.29, m
					3′	128.0, CH	7.24, m
					4′	126.2, CH	7.17, m
D-Val	CO	170.5, C		D-Val	CO	170.7, C	
	NH		7.64, d (9.3)		NH		7.56, d (7.8)
	α	58.8, CH	4.12, m		α	58.5, CH	4.06[a]
	β	30.7, CH	1.91, m		β	29.7, CH	1.88, m
	γ1	17.7, CH_3	0.69, d (6.8)		γ1	17.2, CH_3	0.51[a]
	γ2	19.0, CH_3	0.64, d (6.8)		γ2	18.7, CH_3	0.50[a]

[a]Overlapping signals. [b]Broad signals.

Mortiamide D (4) (Table 2) was obtained as a white powder, and HRESIMS supported a molecular formula of $C_{44}H_{65}N_7O_7$ requiring 16 degrees of unsaturation. TOCSY experiments revealed the presence of seven ^{1}H spin systems corresponding to one Ala, one Leu, one Val, two Phe, and two Ile residues (Table 2). HMBC correlations from Leu-NH (δ_H 8.32) to Val-C=O (δ_C 171.3) and Val-NH (δ_H 7.35) to Phe[2]-C=O (δ_C 170.9) established the three amino acid sequence Phe[2]-Val-Leu. HMBC correlations between Ile[1]-NH (δ_H 7.44) and Ala-C=O (δ_C 171.9) established a two amino acid sequence of Ala-Ile[1]. Both peptide segments were connected via an Ile residue supported by ROESY correlations from Phe[2]-NH

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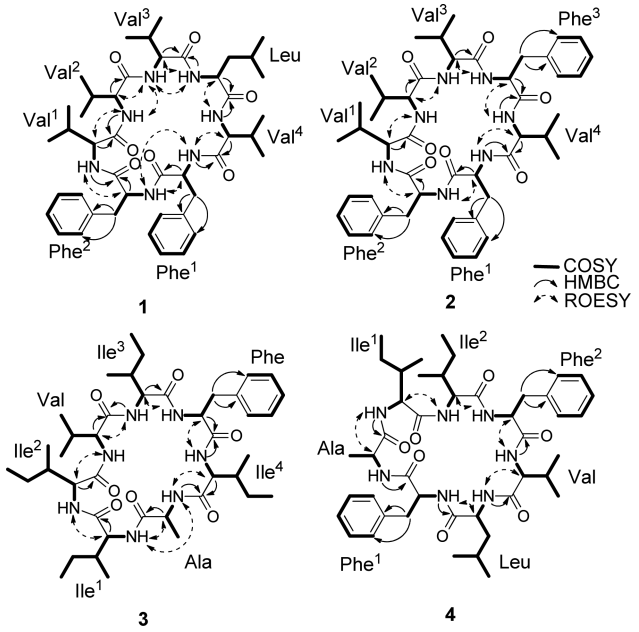

Figure 1. Key COSY, HMBC, and ROESY correlations of **1**−**4**.

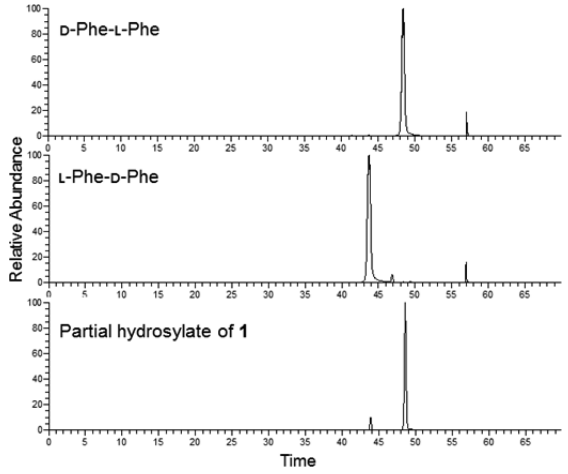

Figure 2. Comparison of the FDAA-derivatized partial hydrosylate of **1** compared to FDAA-derivatized D-Phe-L-Phe and L-Phe-D-Phe standards by LC-MS.

(δ_H 8.13) to Ile^2-Hα (δ_H 3.98) and Ile^2-NH (δ_H 8.38) to Ile^1-Hα (δ_H 4.28), resulting in the six amino acid sequence Ala-Ile^1-Ile^2-Phe^2-Val-Leu. Due to overlapping ^{1}H signals from Ala-NH (δ_H 8.22) and Phe^1-NH (δ_H 8.22), placement of Phe^1 could not be definitively assigned using ROESY or HMBC correlations. In order to support the molecular formula and requirement for unsaturation, the remaining phenylalanine residue was used to cyclize the peptide sequence by peptide bonds between the Ala and Leu residues, resulting in the final sequence as [cyclo-(Leu-Phe^1-Ala-Ile^1-Ile^2-Phe^1-Val-)] (Figure 1). The final sequence of the peptide was confirmed by MS/MS experiments (Supporting Information, Figure S8). The absolute configurations of the amino acids within the peptide were definitively assigned using the advanced Marfey's method and consisted of D-Ala, D-Val, L-Phe, D-Leu, and D-Ile, allowing the final structure to be assigned as [cyclo-(D-Leu-L-Phe D-Ala-D-Ile-D-Ile-L-Phe-D-Val)] (Supporting Information, Figure S14).

Compounds **1**−**4** were assayed for biological activity against methicillin-resistant *Staphyloccocus aureus* (ATCC 33591), vancomycin-resistant *Enterococcus faecium* (EF379), *Staphylococcus warneri* (ATCC 17917), *Pseudomonas aeruginosa* (ATCC 14210), and *Candida albicans* (ATCC 14035). No significant activity was observed for any compound tested at a concentration of 128 μg/mL. Compounds **1**−**4** were also tested for cytotoxicity against keratinocyte, fibroblast, HTB-26, and MCF-7 breast cancer cell lines. None of the tested compounds exhibited activity against these cell lines at 128 μg/mL.

Cyclic heptapeptides have previously been reported from many microorganisms including fungi and bacteria[15−17] as well as macroorganisms including tunicates, sponges, and plants[18−20] and exhibit a wide range of activities.[16,17,21,22] Mortiamides A−D are new members within the cyclic heptapeptide family. They are characterized by the presence of seven hydrophobic amino acids, whereby five of these residues are in the D-configuration. The asymmetry of these molecules is conserved across each of the peptides, whereby site 2 and 6 always contain amino acids in the L-configuration and sites 1, 3, 4, 5, and 7 contain amino acids in the D-configuration. The occurrence of a large number of D amino acids is relatively rare for cyclic peptides and makes the mortiamides intriguing members within the cyclic peptide family. Overall, this report adds to a growing body of evidence that Canada's arctic is a resource for the discovery of new taxa of microbes that have the ability to produce structurally novel natural products.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Autopol III polarimeter using a 50 mm microcell (1 mL). Infrared spectra were recorded using attenuated total reflectance, on a Thermo Nicolet 6700 FT-IR spectrometer. NMR spectra were obtained on a 600 MHz Bruker Avance III NMR spectrometer, where chemical shifts (δ) are reported in ppm and referenced to the DMSO-d_6 residual solvent signal (δ_H 2.50 and δ_C 39.51) or $CDCl_3$ residual solvent signal (δ_H 7.26 and δ_C 77.16). LC-HRMS using a Thermo Gold UPLC column were recorded on Accela Thermo equipment with hyphenated MS-ELSD-UV detection (Thermo LTQ Exactive fitted with an ESI source, PDA, and LT-ELSD Sedex 80). Tandem mass spectra were measured on a Thermo Velos Orbitrap mass spectrometer using a collision-induced dissociation energy of 35 eV at a rate of 2 μL/min. Automated flash chromatography was performed on a Teledyne Combiflash Rf200 using C_{18} RediSep columns or prepacked RediSep Rf Gold normal-phase silica flash columns (24 g). HPLC purifications were carried out on a Waters autopurification system coupled with an evaporative light-scattering detector and UV detector. Silica gel on TLC Al foil plates (Sigma-Aldrich) was used to monitor dipeptide synthesis reaction progress and assess purity of compounds. All reported R_f values were determined using the same plates. All protected dipeptides were visualized under 254 nm UV light or using potassium permanganate dip (1.5 g $KMnO_4$, 10 g K_2CO_3, 125 mg NaOH, 200 mL H_2O) followed by heating. All amino acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were purchased from commercial sources and used without further purification unless otherwise stated. ACS grade benzene and anhydrous ether were used for benzylation reactions and purifications. All solvents used for coupling reactions and chromatographic methods were HPLC grade.

Isolation and Cultivation of *Mortierella* sp. RKAG 110. A marine sediment core was collected using a sterile sediment sampler (LaMotte) at low tide at a depth of 30 cm in Frobisher Bay, Nunavut, Canada (63.72804° N 68.41989° W) in August 2011. The core was transferred to a sterile 50 mL conical tube, brought back to the lab on ice, and stored at −80 °C until processing. The sediment was passed through a series of sieves (104 and 51 μM) and separated based on

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Table 2. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of Mortiamides C (3) and D (4) in DMSO-d_6

	3				4		
	position	δ_C, type	δ_H (J, Hz)		position	δ_C, type	δ_H (J, Hz)
D-Ala	CO	172.9, C		D-Leu	CO	171.8, C	
	NH		7.54, d (7.4)		NH		8.32[b]
	α	47.9, CH	4.42, m		α	51.9, CH	4.01, m
	β	17.3, CH_3	1.18, d (6.8)		β	39.1, CH_2	1.42, m
					γ	23.9, CH	1.22, m
					δ1	22.6, CH_3	0.78, d (6.6)
					δ2	22.1, CH_3	0.72[a]
L-Ile[1]	CO	172.2, C		L-Phe[1]	CO	170.5, C	
	NH		7.93, d (7.6)		NH		8.22, d (7.0)
	α	57.4, CH	4.27, m		α	55.4, CH	4.26, m
	β	35.1, CH	1.71, m		β_A	36.4, CH_2	3.16, m
	γ1$_A$	24.3, CH_2	1.49, m		β_B		2.78, dd (13.7, 10.9)
	γ1$_B$		1.11, m		1′	138.2, C	
	γ2	14.7, CH_3	0.81[a]		2′	129.0, CH	7.24, m
	δ	10.6, CH_3	0.83[a]		3′	128.1, CH	7.23, m
					4′	126.2, CH	7.19, m
D-Ile[2]	CO	172.2, C		D-Ala	CO	171.9, C	
	NH		8.44[b]		NH		8.22, d (7.1)
	α	56.0, CH	4.31, m		α	48.5, CH	4.31, m
	β	35.5, C	2.00, m		β	17.5, CH_3	1.20, d (7.2)
	γ1$_A$	25.6, CH_2	1.28, m				
	γ1$_B$		1.22, m				
	γ2	14.3, CH_3	0.87, d (7.0)				
	δ	11.2, CH_3	0.81[a]				
				D-Ile[1]	CO	172.4, C	
D-Val	CO	173.5, C			NH		7.44, d (6.9)
	NH		7.67[b]		α	57.3, CH	4.28, m
	α	59.5, CH	4.03, m		β	36.1, CH	1.92, m
	β	28.2, CH_2	2.25, m		γ1$_A$	25.4, CH_2	1.34, m
	γ1	18.7	0.94, d (6.7)		γ1$_B$		1.09, m
	γ2	18.8, C	0.85, d (7.1)		γ2	15.0, CH_3	0.85[a]
					δ	11.4, CH_3	0.86[a]
D-Ile[3]	CO	170.7, C		D-Ile[2]	CO	171.5, C	
	NH		7.89, d (8.8)		NH		8.38[b]
	α	57.8, CH	4.00, m		α	58.6, CH	3.98, m
	β	35.9, C	1.78, m		β	35.8, C	1.72, m
	γ1$_A$	23.5, CH_2	1.10, m		γ1	23.6, CH_2	1.02, m
	γ1$_B$		1.02, m		γ2	15.3, CH_3	0.63, d (6.7)
	γ2	15.1, CH_3	0.72, m		δ	11.2, CH_3	0.66[a]
	δ	11.1, CH_3	0.68, t (7.4)				
L-Phe	CO	170.6, C		L-Phe[2]	CO	170.9, C	
	NH		7.61[b]		NH		8.13[b]
	α	55.4	4.40, m		α	55.2, CH	4.44, m
	β_A	35.9, CH_2	3.03, dd (13.8, 6.2)		β_A	36.5, CH_2	3.13, m
	β_B		2.85, dd (13.8, 9.2)		β_B		2.94, dd (10.1, 13.7)
	1′	137.5, C			1′	138.0, C	
	2′	129.0, CH	7.30, m		2′	129.0, CH	7.25, m
	3′	128.1, CH	7.25, m		3′	128.1, CH	7.27, m
	4′	126.3, CH	7.19, m		4′	126.2, CH	7.19, m
D-Ile[4]	CO	170.8		D-Val	CO	171.3, C	
	NH		7.80, d(8.5)		NH		7.35, d (6.7)
	α	56.0, CH	4.30, m		α	58.4, CH	4.00, m
	β	36.6, CH	1.81, m		β	29.5, CH	2.02, m
	γ1$_A$	25.1, CH_2	0.99, m		γ1	18.2, CH_3	0.73[a]
	γ1$_B$		0.86, m		γ2	18.8, CH_3	0.68[a]
	γ2	14.0, CH_3	0.64, d (6.9)				
	δ	11.3, CH_3	0.74[a]				

[a]overlapping signals. [b]Broad signals.

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particle size in order to increase the rate of isolation of fungi originating from vegetative propagules embedded within substrate particles as opposed to dormant spores. The separated particles were resuspended in sterile H_2O containing 0.2 g/L chloramphenicol and 18 g/L Instant Ocean and were serially diluted (100- and 1000-fold dilutions). A 10 μL aliquot from each dilution was pipetted into each well of a 48-well plate containing YM and cottonseed oil agar and incubated at 4 or 22 °C for three months. Emerging fungal colonies were purified to obtain axenic cultures of isolate (RKAG 110). Identification of the fungus was performed by observation of the culture phenotype (both macro- and micromorphology) and sequence homology of the rDNA genes, including the ITS1-5.8S-ITS2 region, and the nLSU as described previously[22] (sequence data are deposited in GenBank with the accession numbers KY886144 and KY886145).

Extraction and Purification. Isolate RKAG 110 was inoculated into 15 mL of YM liquid seed medium at 22 °C and agitated at 200 rpm for 5 days. The seed culture (200 μL) was used to inoculate the isolate onto 150, 100 × 15 mm Petri plates containing 20 mL of solid PDA agar and grown for 21 days at 22 °C. The solid agar cultures were roughly cut up, pooled, and extracted with EtOAc. The extract was evaporated to dryness and partitioned between 80% CH_3CN and 100% hexane. The CH_3CN layer was collected and evaporated to dryness *in vacuo* to give a CH_3CN extract (820 mg).

The extract was fractionated using automated reversed-phase flash chromatography with a linear gradient from 20% aqueous MeOH to 100% MeOH over 15 min on a 15.5 g C_{18} column (High Performance GOLD RediSep Rf) with a flow rate of 30 mL/min. The semipure fraction eluting at 9.5 min was subjected to reversed-phase HPLC using a Gemini 110A C18 column (5 μm 250 × 10 mm, Phenomenex) and 20 min isocratic elution with 65% aqueous CH_3CN (0.1% formic acid), resulting in the purification of **1** (4.1 mg), **2** (3.2 mg), **3** (2.4 mg), and **4** (2 mg).

*Mortiamide A (**1**):* white powder; $[\alpha]^{25}_D$ +26 (*c* 0.1, MeOH); IR (film) ν_{max} 3309, 2962, 2934, 2875, 1652, 1533, 1456, 1386, 1027 cm^{-1}; ^1H and ^{13}C NMR Table 1; (+) HRESIMS *m/z* 804.5024 $[M + H]^+$ (calcd for $C_{44}H_{65}N_7O_7$, 804.5019).

*Mortiamide B (**2**):* white powder; $[\alpha]^{25}_D$ +11 (*c* 0.1, MeOH); IR (film) ν_{max} 3329, 2963, 1651, 1592, 1531, 1456, 1385, 1352, 1026 cm^{-1}; ^1H and ^{13}C NMR Table 1; (+) HRESIMS *m/z* 838.4851 $[M + H]^+$ (calcd for $C_{47}H_{63}N_7O_7$, 838.4862).

*Mortiamide C (**3**):* white powder; $[\alpha]^{25}_D$ +11 (*c* 0.1, MeOH); IR (film) ν_{max} 3330, 2963, 2936, 2875, 1646, 1531, 1468, 1392, 1029 cm^{-1}; ^1H and ^{13}C NMR Table 2; (+) HRESIMS *m/z* 770.5189 $[M + H]^+$ (calcd for $C_{41}H_{67}N_7O_7$, 770.5175).

*Mortiamide D (**4**):* white powder; $[\alpha]^{25}_D$ +20 (*c* 0.1, MeOH); IR (film) ν_{max} 3329, 2962, 2931, 1652, 1603, 1531, 1465, 1392, 1027 cm^{-1}; ^1H and ^{13}C NMR Table 2; (+) HRESIMS *m/z* 804.5021 $[M + H]^+$ (calcd for $C_{44}H_{65}N_7O_7$, 804.5019).

Synthesis of Dipeptide Standards. Benzylation of amino acids was accomplished according to literature precedent.[23,24] All dipeptide coupling reactions were carried out using benzyl ester protected amino acids and purchased *N*-Boc-protected amino acids according to modified literature precedent.[25]

Deprotection of all dipeptides was accomplished first by hydrogenolysis with ∼20% w/w of 10% Pd/C in CH_2Cl_2 under a H_2 atmosphere for 24−48 h at room temperature, to produce the corresponding free carboxylic acid. Reactions were filtered through a Celite plug, dried *in vacuo*, and used without further purification. Boc removal was accomplished by treatment with a 5 mL mixture of 1:1 CH_2Cl_2/trifluoroacetic acid (TFA) for 1 h at room temperature with stirring. Residual CH_2Cl_2 and TFA were removed *in vacuo*. To ensure TFA removal, samples were dried, reconstituted in CH_2Cl_2, and dried again. This process was repeated several times (4×) to ensure complete TFA removal, yielding the fully deprotected trifluoroacetic acid salt of the corresponding dipeptide. Reaction progress was monitored by TLC analysis. Deprotected dipeptides were used without further purification or extensive characterization.

Absolute Configuration Assignments. Mortiamides A−D (0.25 mg each) were hydrolyzed with stirring in 6 N HCl (60 μL) at 80 °C for 6 h and neutralized with 1 M $NaHCO_3$ solution. *N*-(5-Fluoro-2,4-dinitrophenyl-5)-L-alaninamide (0.4 mg in 380 μL of acetone) was added to the reaction mixture and stirred at 37 °C for 2 h. The reaction was quenched with 1 N aqueous HCl (80 μL).[13] MeOH was added, and the sample was analyzed by LC-HRMS using a Hypersil Gold 100 Å column (Thermo, 1.9 μm C_{18} 50 mm × 2.1 mm) and a flow rate of 400 μL/min. The following method was used: 0−55 min 95% H_2O/0.1% formic acid (solvent A) and 5% CH_3CN/0.1% formic acid (solvent B) to 60% solvent A and 40% solvent B, 55−57 min 60% solvent A:40% solvent B to 100% solvent B, 57−60 min 100% solvent B. Retention times were compared to derivatized amino acid standards to determine the amino acid configurations. Partial hydrolysis of the mortiamides occurred with 6 N HCl at 80 °C for 2 h. The partial hydrolysis of the compound was monitored by LC-MS for the mass of the required hydrosylate products. Retention times for the derivatized amino acid standards are as follows L-Ala 23.07 min, D-Ala 27.71 min, L-Val 32.24 min, D-Val 38.33 min, L-Phe 39.09 min, D-Phe 43.87 min, L-Leu 38.64 min, D-Leu 44.17 min, L-Ile 37.42 min, D-Ile 43.49 min, L-*allo*-Ile 37.58 min, D-*allo*-Ile 43.52 min, L-Ile-D-Ala 31.20 min, D-Ile-D-Ala 37.03 min, L-Phe-D-Phe 43.71 min, D-Phe-L-Phe 48.40 min, L-Ile-D-Ile 41.85 min, D-Ile-L-Ile 47.69 min, and D-Ile-D-Ile 46.76 min.

Antimicrobial Assays. Compounds **1**−**4** were tested for antimicrobial activity according to Clinical Laboratory Standards Institute testing standards in a 96-well plate microbroth dilution assay in triplicate against the human microbial pathogens methicillin-resistant *Staphyloccocus aureus* ATCC 33591 (MRSA), vancomycin-resistant *Enterococcus faecium* EF379 (VRE), *Staphylococcus warneri* ATCC 17917, *Pseudomonas aeruginosa* ATCC 14210, and *Candida albicans* ATCC 14035 as previously described.[26] Optical density was recorded at T_{zero} and T_{final} using a Thermo Scientific Varioskan Flash plate reader to determine growth inhibition after incubation for 22 h.

Cytotoxicity Assays. Compounds **1**−**4** were tested for cytotoxicity using human foreskin BJ fibroblast cells (ATCC CRL-2522), adult human epidermal keratinocytes (HEKa), Vero kidney cells (ATCC CL-81), human breast adenocarcinoma cells (ER−) (ATCC HTB-26), and human breast adenocarcinoma cells (ER+) (ATCC MCF-7) in triplicate in a 96-well cell culture plate as described previously.[26] Cell viability was determined 24 h after treatment using the redox dye Alamar Blue to extrapolate cell viability. Fluorescence was monitored using a Thermo Scientific Varioskan Flash plate reader at 560/12 excitation, 590 nm emission at both time zero and 4 h after Alamar Blue addition.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00383.

Dipeptide synthesis methodology, 1D and 2D NMR spectra, HRMS and MS/MS data for mortiamides A−D (PDF)

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Notes

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