Tolypocaibols: Antibacterial lipopeptaibols from a *Tolypocladium* sp. endophyte of the marine macroalga *Spongomorpha arcta*

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Abstract

Two new lipopeptaibols, tolypocaibols A (**1**) and B (**2**), and the mixed NRPS-polyketide-shikimate natural product maximiscin [(*P*/*M*)-**3**)] were isolated from a *Tolypocladium* sp. fungal endophyte of the marine alga *Spongomorpha arcta*. Analysis of NMR and mass spectrometry data revealed the amino acid sequences of the lipopeptaibols, which both comprise 11 residues with a valinol *C*-terminus and a decanoyl acyl chain at the *N*-terminus. The configuration of the amino acids was determined by Marfey's analysis. Tolypocaibols A (**1**) and B (**2**) showed moderate, selective inhibition against Gram-positive and acid-fast strains, while maximiscin [(*P*/*M*)-**3**)] showed moderate, broad-spectrum inhibition.

Natural products continue to be an important reservoir of structurally diverse and biologically relevant molecules.[1–3] Fungi, particularly those that are endophytic, represent a diverse biological source from which a growing number of new natural products are being discovered.[4–8] Fungi of the genus *Tolypocladium* are often isolated as endophytes[9–11] and are known to produce biologically relevant natural products, notably peptides derived from nonribosomal peptide synthetases (NRPS).[12–20] These include cyclic peptides such as the cyclosporins,[12,13] and peptaibiotics, such as the efrapeptins[14,15] and the peptaibols.[16–20]

In our on-going search for bioactive natural products from fungi,[21–25] an extract of an endophytic *Tolypocladium* sp., isolated from the marine alga *Spongomorpha arcta*, that exhibited broad spectrum bioactivity against Gram-positive bacteria and mycobacteria was investigated. Examination of the LC-MS profile of the extract and comparison of precursor masses with molecules in the Natural Products Atlas[26] microbial metabolite database allowed the tentative identification of peptaibiotics and highlighted two ions (*m/z* 1339.8354 $[M + H]^+$ and 1353.8550 $[M + H]^+$) as putatively new natural products. Bioassay- and LC-MS-guided fractionation of the extract by sequential application of C_{18} reversed-phase flash column chromatography and HPLC afforded two new lipopeptaibols, named tolypocaibols A (**1**) and B (**2**), and the known NRPS-polyketide-shikimate-hybrid metabolite, maximiscin [(*P*/*M*)-**3**)] as an inseparable mixture of interconverting atropisomers. Herein, we report the isolation and structure elucidation of the new compounds and summarize their antimicrobial activities.

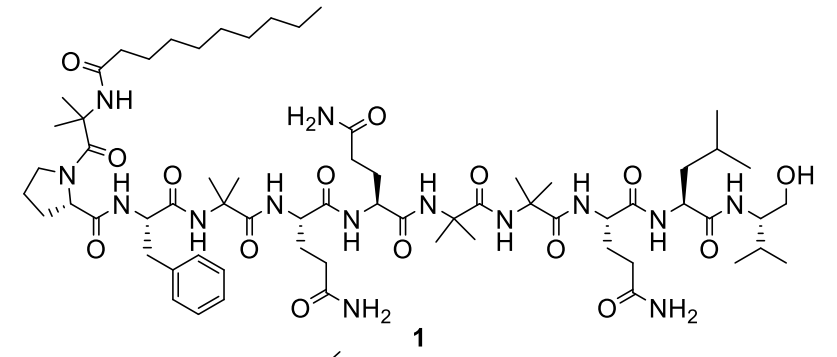
Table 1. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR of tolypocaibols A (**1**) and B (**2**) in DMSO-d_6

	Tolypocaibol A (**1**)			Tolypocaibol B (**2**)			
unit	position	δ_C, type	δ_H, mult (*J* in hz)	unit	position	δ_C, type	δ_H, mult (*J* in hz)
L-Valol[1]	1	55.6, CH	3.57, m	L-Valol[1]	1	55.6, CH	3.57, dq (9.3, 6.1)
	2	28.3, CH	1.82, dt (13.5, 6.8)		2	28.3, CH	1.81, dt (11.4, 6.8)
	3	19.6, CH_3	0.83, m		3	19.6, CH_3	0.83, m
	4	17.9, CH_3	0.80, m		4	17.9, CH_3	0.795, m
	5	61.7, CH_2	3.38, m		5	61.7, CH_2	3.38, m
	OH		4.20, bs		OH		4.20, bs
	NH		6.77, m		NH		6.773, m
L-Leu[2]	1	171.9,[a] C		L-Leu[2]	1	171.98,[c] C	
	2	52.2, CH	4.11, ddd (11.7, 8.0, 4.1)		2	52.2, CH	4.11, ddd (11.8, 8.2, 4.1)
	3	40.0, CH_2	1.64, m; 1.53, m		3	40.0, CH_2	1.63, m; 1.52, m
	4	24.1, CH	1.67, m		4	24.2, CH	1.66, m
	5	22.9, CH_3	0.81, m		5	22.9, CH_3	0.81, m
	6	20.7, CH_3	0.76, m		6	20.7, CH_3	0.77, d (6.3)
	NH		7.45, d (8.1)		NH		7.45, d (6.3)
L-Gln[3]	1	172.0,[a] C		L-Gln[3]	1	172.03,[c] C	
	2	54.8, CH	3.82, dt (10.5, 5.3)		2	54.75, CH	3.83, ddd (10.4, 5.9, 4.3)
	3	26.5, CH_2	1.96, m		3	26.5, CH_2	1.97, m
	4	31.7, CH_2	2.32, m; 2.21, m		4	31.7, CH_2	2.31, m; 2.22, m
	5	173.77,[b] C			5	173.8,[d] C	
	5-NH_2		7.20, m; 6.77, m		5-NH_2		7.31, s; 6.85, s
	NH		7.62, m		NH		7.63, d (6.0)
Aib[4]	1	175.6, C		Aib[4]	1	175.7, C	
	2	56.0, C			2	56.0, C	
	3	22.7, CH_3	1.338, s		3	22.7, CH_3	1.33, s
	4	26.6, CH_3	1.39, s		4	26.6, CH_3	1.39, s

	NH		7.55, s		NH		7.55, s
Aib[5]	1	175.2, C		Aib[5]	1	175.2, C	
	2	55.9, C			2	55.9, C	
	3	22.8, CH_3	1.328, s		3	22.86, CH_3	1.33, s
	4	25.7, CH_3	1.346, s		4	25.65, CH_3	1.346, s
	NH		7.93, s		NH		7.94, s
L-Gln[6]	1	173.3, C		L-Gln[6]	1	173.6, C	
	2	55.0, CH	3.98, dt (8.8, 6.4)		2	54.84, CH	4.00, m
	3	26.1, CH_2	2.00, m		3	26.1, CH_2	1.97, m
	4	31.47, CH_2	2.24, m; 2.12, m		4	31.52, CH_2	2.18, m; 2.13, m
	5	173.75,[b] C			5	173.4, C	
	5-NH_2		7.30, m; 6.83, m		5-NH_2		7.23, m; 6.78, m
	NH		7.847, m		NH		7.88, m
L-Gln[7]	1	173.2, C		L-Gln[7]	1	173.2, C	
	2	55.1, CH	3.93, dt (9.0, 5.6)		2	55.1, CH	3.95, m
	3	26.2, CH_2	2.01, m; 1.94, m		3	26.2, CH_2	1.97, m
	4	31.54, CH_2	2.20, m; 2.12, m		4	31.6, CH_2	2.22, m
	5	173.4, C			5	173.9,[d] C	
	5-NH_2		7.27, m; 6.81, m		5-NH_2		7.28, s; 6.81, s
	NH		7.820, m		NH		7.86, m
Aib[8]	1	175.4, C		Aib[8]	1	175.3, C	
	2	56.1, C			2	56.1, C	
	3	23.7, CH_3	1.38, s		3	23.8, CH_3	1.38, s
	4	25.66, CH_3	1.48, s		4	25.61, CH_3	1.48, s
	NH		7.60, s		NH		7.53, s
L-Phe[9]	1	172.4, C		L-Phe[9]	1	172.3, C	
	2	55.2, CH	4.36, ddd (11.4, 8.3, 4.8)		2	54.65, CH	4.38, ddd (11.4, 8.4, 4.6)
	3	35.9, CH_2	3.23, dd (14.0, 4.7); 2.90, dd (13.9, 11.2)		3	36.0, CH_2	3.26, dd (13.8, 4.5); 2.85, t (12.6)

	4	137.7, C			4	138.1, C	
	5	128.9, CH	7.24, m		5	129.0, CH	7.20, m
	6	128.1, CH	7.20, m		7	128.1, CH	7.24, m
	7	126.4, CH	7.18, m		9	126.7, CH	7.19, m
	8	128.1, CH	7.20, m		8	128.1, CH	7.24, m
	9	128.9, CH	7.24, m		6	129.0, CH	7.20, m
	NH		7.838, m		NH		7.85, m
L-Pro[10]	1	172.4, C		(2*S*,4*S*)-4-mPro[10]	1	171.9,[c] C	
	2	62.5, CH	4.21, t (7.7)		2	62.9, CH	4.17, dd (9.9, 7.6)
	3	28.1, CH_2	2.03, m; 1.20, m		3	36.3, CH_2	2.159, m; 0.57, q (12.7)
	4	25.3, CH_2	1.76, m; 1.64, m		4	33.1, CH	2.147, m
	5	48.1, CH_2	3.71, dt (11.6, 6.17); 3.29, m		5	55.2, CH_2	3.96, m; 2.59, m
					6	16.2, CH_3	0.87, d (5.8)
Aib[11]	1	172.9, C		Aib[11]	1	172.8, C	
	2	55.8, C			2	55.8, C	
	3	23.4, CH_3	1.36, s		3	23.5, CH_3	1.36, s
	4	26.3, CH_3	1.42, s		4	26.3, CH_3	1.42, s
	NH		8.80, s		NH		8.79, s
Dc	1	173.6, C		Dc	1	173.3, C	
	2	34.8, CH_2	2.28, m		2	34.8, CH_2	2.29, m
	3	25.1, CH_2	1.55, m		3	25.3, CH_2	1.61, m; 1.57, m
	4	28.6, CH_2	1.24, m		4	28.6, CH_2	1.25, m
	5	28.70, CH_2	1.24, m		5	28.72, CH_2	1.25, m
	6	28.85, CH_2	1.24, m		6	28.75, CH_2	1.25, m
	7	29.01, CH_2	1.24, m		7	28.84, CH_2	1.25, m
	8	31.2, CH_2	1.17, m		8	31.2, CH_2	1.18, m
	9	22.1, CH_2	1.22, m		9	22.1, CH_2	1.21, m
	10	13.9, CH_3	0.82, m		10	13.9, CH_3	0.816, m

[a,b,c,d]Note: Resonances designated with the same superscript letters may be interchanged.



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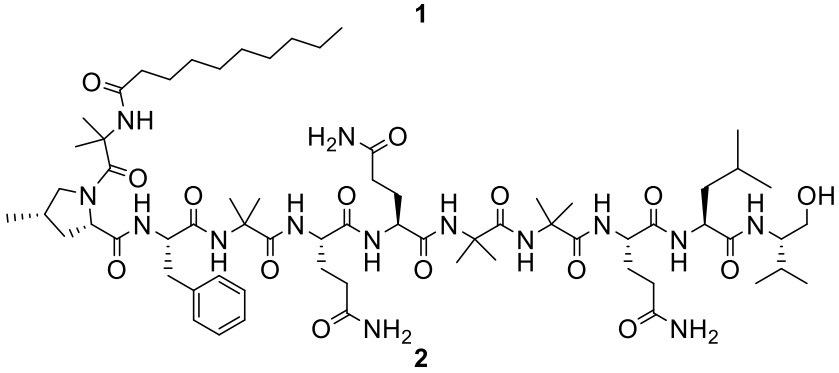

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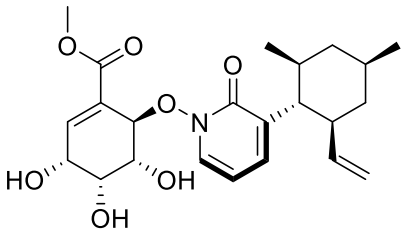
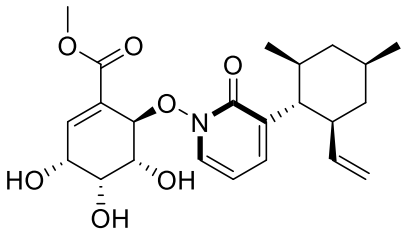


(*P*)-**3**



(*M*)-**3**

Results and Discussion

Tolypocaibol A (**1**) was obtained as a yellow oil. Its molecular formula was determined to be $C_{66}H_{110}N_{14}O_{15}$ by analysis of HR-MS and NMR, indicating 19 degrees of unsaturation. The ^{1}H and ^{13}C NMR data for **1** (Table 1) revealed 14 carbonyls (δ_C 175.6−171.9) and a monosubstituted benzene [δ_C 137.7, 128.9, 128.9, 128.1,128.1, 126.4 and δ_H 7.24 (m, 2H), 7.20 (m, 2H), 7.18 (m, 1H)], accounting for 18 degrees of unsaturation and indicated that **1** contained a second ring. The ^{1}H NMR data showed 16 amide hydrogens, suggesting **1** was a peptide, this was supported by MS/MS data of the $[M+H]^+$ ion showing fragmentation indicative of a peptide. One- and two-dimensional NMR experiments were employed to confirm the amino acid composition (Figure 1).

The existence of a valinol residue was confirmed by $^{1}H-^{1}H$ TOCSY and COSY correlations revealing the $-NH-CH(CH_2OH)-CH(CH_3)_2$ spin system and were further supported by the HMBC correlations from Valol[1]-H-1 (δ_H 3.57) to Leu[2]-C-1 (δ_C 171.9). The leucine residue was confirmed by the $-NH-CH-CH_2-CH(CH_3)_2$ spin system and HMBC correlations from Leu[2]-H-2 (δ_H 4.11) to Leu[2]-C-1 (δ_C 171.9). The existence of three glutamine residues was confirmed by the presence of three $-NH-CH-CH_2-CH_2-$ spin systems and HMBC correlations from Gln[3]-H-2 (δ_H 3.82) to Gln[3]-C-1 (δ_C 172.0) and Gln[3]-NH_2 (δ_H 6.77) to Gln[3]-C-4 (δ_C 31.7) and Gln[3]-C-5 (δ_C 173.77), Gln[6]-H-2 (δ_H 3.98) to Gln[6]-C-1 (δ_C 173.3) and Gln[6]-NH_2 (δ_H 6.83) to Gln[6]-C-4 (δ_C 31.47) and Gln[6]-C-5 (δ_C 173.75), and Gln[7]-H-2 (δ_H 3.93) to Gln[7]-C-1 (δ_C 173.2) and Gln[7]-NH_2 (δ_H 6.81) to Gln[7]-C-4 (δ_C 31.54) and Gln[7]-C-5 (δ_C 173.4). The monosubstituted benzene was determined to be a part of a phenylalanine residue by HMBC correlations from Phe[9]-H-2 (δ_H 4.36) to Phe[9]-C-1 (δ_C 172.4), Phe[9]-H-3 (δ_H 3.23 and 2.90) to Phe[9]-C-4 (δ_C 137.7), Phe[9]-C-5/9 (δ_C 128.9), and Phe[9]-C-1 (δ_C 172.4), and Phe[9]-H-5/9 (δ_H 7.24) to Phe[9]-C-3 (δ_C 35.9). The proline residue was confirmed by $^{1}H-^{1}H$ COSY correlations of Pro[10]-H-2 (δ_H 4.21)/ Pro[10]-H-3 (δ_H 2.03 and 1.20)/ Pro[10]-H-4 (δ_H 1.76 and 1.64)/ Pro[10]-H-5 (δ_H 3.71 and 3.29), along with HMBC correlations from Pro[10]-H-2 (δ_H 4.21) to Pro[10]-C-1 (δ_C 172.4) and Pro[10]-C-5 (δ_C 48.1). Four α-aminoisobutyrate (Aib) residues were revealed by HMBC correlations from Aib[4]-H-3/4 (δ_H 1.338 and 1.39) to Aib[4]-C-2 (δ_C 56.0) and Aib[4]-C-1 (δ_C 175.6), Aib[5]-H-3/4 (δ_H 1.328 and 1.346) to Aib[5]-C-2 (δ_C 55.9) and Aib[5]-C-1 (δ_C 175.2), Aib[8]-H-3/4 (δ_H 1.38 and 1.48) to Aib[8]-C-2 (δ_C 56.1) and Aib[8]-C-1 (δ_C 175.4), and Aib[11]-H-3/4 (δ_H 1.36 and 1.42) to Aib[11]-C-2 (δ_C 55.8) and Aib[11]-C-1 (δ_C 172.9). The remaining $C_{10}H_{19}O$ fragment was attributed to a C_{10} (Dc) lipid chain based on COSY correlations and an HMBC correlation from Dc-H-2 (δ_H 2.28) to Dc-C-1 (δ_C 173.6). The connection of amino acids was determined by ROESY correlations from Valol[1]-NH (δ_H 6.77) to Leu[2]-H-2 (δ_H 4.11), Leu[2]-NH (δ_H

7.45) to Gln^{3}-H-2 (δ_H 3.82), Gln^{3}-NH (δ_H 7.62) to Aib^{4}-NH (δ_H 7.55), Aib^{4}-NH (δ_H 7.55) to Aib^{5}-NH (δ_H 7.93), Aib^{5}-NH (δ_H 7.93) to Gln^{6}-H-2 (δ_H 3.98), Gln^{6}-NH (δ_H 7.847) to Gln^{7}-H-2 (δ_H 3.93), and Gln^{7}-NH (δ_H 7.820) to Aib^{8}-H-4 (δ_H 1.48) establishing the partial Valol^{1}–Leu^{2}–Gln^{3}–Aib^{4}–Aib^{5}–Gln^{6}–Gln^{7}–Aib^{8} as the C-terminus sequence. Similarly, ROESY correlations between Aib^{11}-NH (δ_H 8.80) to Dc-H-2 (δ_H 2.28) and Pro^{10}-H-5 (δ_H 3.71 and 3.29), Phe^{9}-NH (δ_H 7.838) to Pro^{10}-C-2 (δ_H 4.21), and Phe^{9}-H-2 (δ_H 4.36) to Aib^{8}-NH (δ_H 7.60) established the partial Dc–Aib^{11}–Pro^{10}–Phe^{9}–Aib^{8} as the *N*-terminus sequence. These connections were supported by examination of MS/MS fragmentation data through the observation of key b- and y-type fragments (Figure 2). Thus, the sequence of **1** was determined to be Dc–Aib^{11}–Pro^{10}–Phe^{9}–Aib^{8}–Gln^{7}–Gln^{6}–Aib^{5}–Aib^{4}–Gln^{3}–Leu^{2}–Valol^{1}.

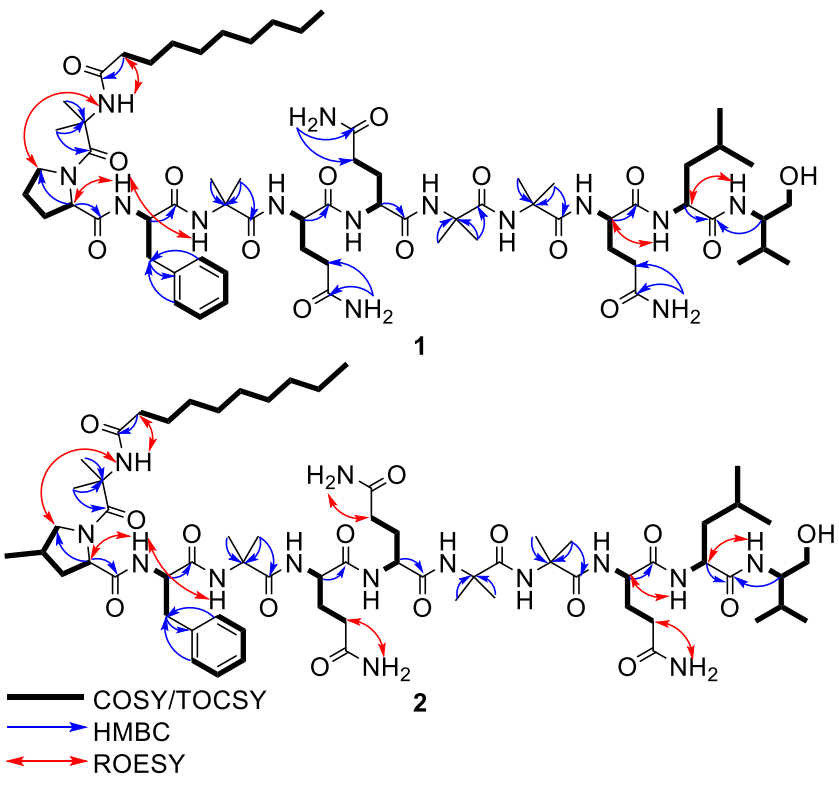


Figure 1. Key COSY/TOCSY, HMBC, and ROESY correlations of compounds **1** and **2**.

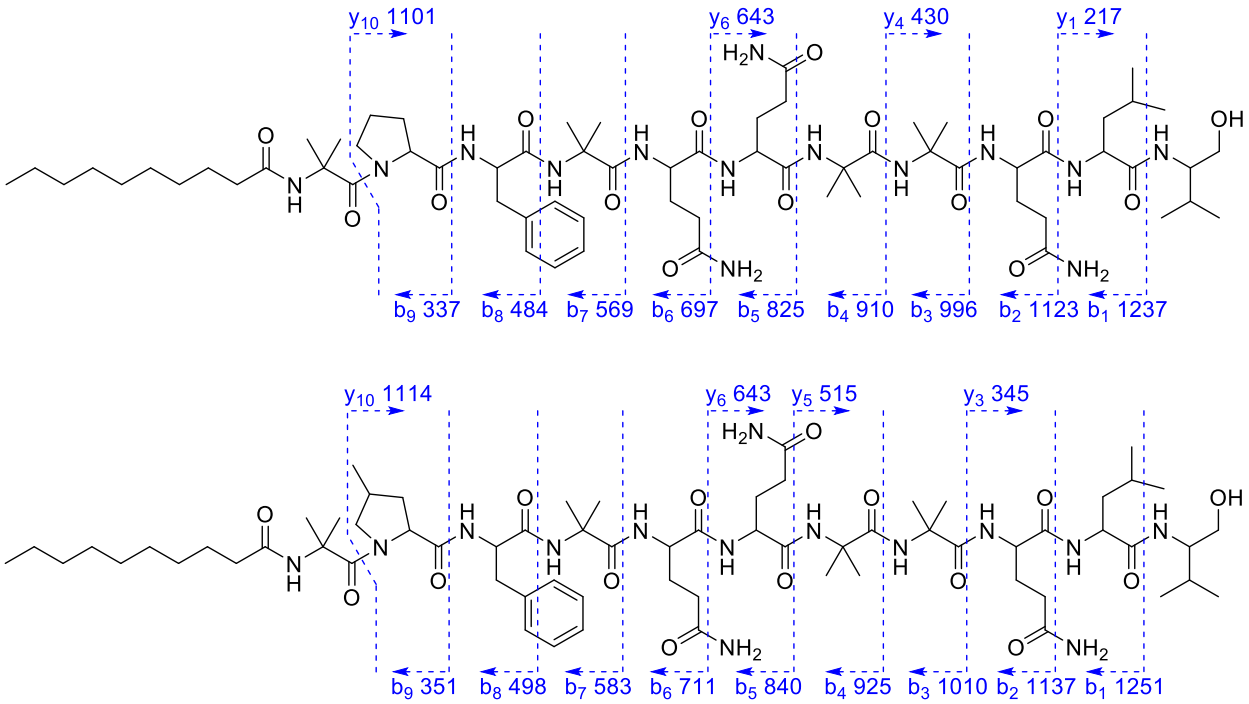


Figure 2. HR-MS/MS fragmentation patterns of compounds **1** and **2**.

Tolypocaibol B (**2**) was obtained as a yellow oil. Its molecular formula was determined to be $C_{67}H_{112}N_{14}O_{15}$ by analysis of HR-MS and NMR, indicating 19 degrees of unsaturation and that **2** differed to **1** by the presence of an additional carbon and two protons. Analysis of the 1D NMR data of **2** showed close similarities to **1** with the only difference being the replacement of the Pro^{10} in **1** with a 4-methyl-proline (4-$mPro^{10}$) residue in **2**. This was confirmed by TOCSY and COSY correlations of 4-$mPro^{10}$-H-2 (δ_H 4.17)/ 4-$mPro^{10}$-H-3 (δ_H 2.159 and 0.57)/ 4-$mPro^{10}$-H-4 (δ_H 2.147)/ 4-$mPro^{10}$-H-5 (δ_H 3.96 and 2.59), and 4-$mPro^{10}$-H-6 (δ_H 0.87). The sequence of residues was then confirmed in a similar fashion to **1**, using HMBC and ROESY correlations and MS/MS fragmentation (Figures 1 and 2). The relative stereochemistry of the 4-$mPro^{10}$ residue was determined to be *cis* by the presence of a ROESY correlation between 4-$mPro^{10}$-H-2 (δ_H 4.17) and 4-$mPro^{10}$-H-6 (δ_H 0.87).

The absolute configuration of the amino acid and amino alcohol residues in **1** and **2** were determined by hydrolysis and application of Marfey's method.[27] Hydrolysates of **1** and **2** were derivatized with L-FDAA, analyzed by C_{18} reversed-phase HPLC, and retention times were compared with those of enantiopure standard residues derivatized with L-FDAA (see Supporting Information Figures S20-S23). This revealed that all the chiral amino acids had an L configuration and the stereochemistry of the 4-mPro[10] was 2*S*,4*S*.

The antibacterial activities of tolypocaibols A (**1**), B (**2**), and maximiscin [(*P*/*M*)-**3**)] were assessed against a panel of ten Gram-negative, seven Gram-positive, and two acid-fast bacteria and their MIC values are given in Table 2. Tolypocaibols A and B both showed moderate to weak, selective inhibition against Gram-positive bacteria, and both acid-fast bacteria *Mycobacteria tuberculosis* H37Ra (ATCC 25177) and *M. smegmatis* (ATCC 70084) while being inactive (at 128 μM) against Gram-negative bacteria. This is consistent with previous observations of peptaibol-type natural products, which have been found to permeabilize lipid bilayers.[28] Maximiscin [(*P*/*M*)-**3**)] showed weak inhibitory activity against Gram-negative bacteria and moderate to weak activity against Gram-positive bacteria. This is the first report of antibacterial activity for **3**, although previous reports have shown antifungal[29] and antiproliferative activity[30] as well as cytotoxicity against several cancer cell lines.[31]

Table 2. Biological activity of tolypocaibols A (**1**), B (**2**), maximiscin [(*P*/*M*)-**3**)], and select antibiotic controls. MSSA = methicillin-susceptible *S. aureus*; MRSA = methicillin-resistant *S. aureus*; RIF = rifampicin; CIP = ciprofloxacin; NT = not tested.

	MIC (μM)				
Strain	**1**	**2**	**3**	**RIF**	**CIP**
E. coli	>128	>128	128	4	0.17
K. aerogenes	>128	>128	>128	8	2.83
K. pneumoniae	>128	>128	>128	16	2
S. enterica	>128	>128	128	2	0.05
S. sonnei	>128	>128	128	1.67	0.08
O. anthropi	>128	>128	128	4	0.83
P. alcalifaciens	>128	>128	128	8	0.13
P. aeruginosa	>128	>128	>128	53.3	0.5
V. cholerae	>128	>128	128	>128	0.02
Y. pseudotuberculosis	>128	>128	128	10.7	0.14
B. subtilis	8	4	128	0.19	0.38
S. epidermidis	>128	>128	128	0.02	1.33
MSSA	8	4	128	0.01	1.17
MRSA	16	16	128	2	64
L. ivanovii	8	8	16	0.02	8
E. faecium	32	16	128	21.3	0.67
E. faecalis	32	16	128	1	1
M. tuberculosis	20	40	250	0.0625	NT
M. smegmatis	80	80	>250	NT	3.8

Peptaibols are a structurally diverse subfamily of peptaibiotic natural products frequently isolated from Hypocreales fungi, particularly members of the genus *Trichoderma* (family Hypocreaceae).[32] While the peptaibiotics encompass *N*-acylated linear peptides of between five and twenty-two amino acid residues with a high occurrence of α-aminoisobutyrate (Aib) and other non-proteinogenic amino acids, the peptaibols are characterized by the additional presence of a *C*-terminal amino alcohol and represent the largest subclass of peptaibiotics.[32,33] Peptaibols that contain a lipophilic *N*-acyl moiety (typically octanoyl to pentadecenoyl chains) are further classified lipopeptaibols,[34] with 16 members of this class having been isolated from insect pathogens or soil/sediment-inhabiting fungal species congeneric with our *Tolypocadium* sp. isolate (order Hypocreales, family Ophiocordycipitaceae): LP237-F5, LP237-F7, LP237-F8,[17,18] the dakwaabakains A-E,[16] and the tolypocladamides A-H.[19,20] This is the first report of an endophytic *Tolypocladium* sp. producing lipopeptaibols and, although they are structurally similar to the previously isolated *Tolypocladium* lipopeptaibols, the tolypocaibols are the first examples of this group to contain a *C*-terminal valinol instead of a leucenol. Additionally, tolypocaibol B (**2**) is the first 11 residue peptaibol to contain the nonproteinogenic amino acid 4-methylproline.[35] The discovery of these natural products expands our knowledge of the potential sources of these molecules and provides additional examples of unusual amino acids being incorporated into natural products.

Experimental Section

General Experimental Procedures. Optical rotations were recorded in MeOH solution on an Optical Activity Ltd. AA-10 polarimeter at 589 nm. NMR spectra of **1** and **2** were recorded on a Bruker AVII 700 instrument equipped with a QNP cryoprobe in deuterated DMSO and were

calibrated to residual protonated solvent resonances (^{1}H, 2.50 ppm; ^{13}C, 39.52 ppm), while NMR spectra of **3** was recorded on an Agilent 400-MR DD2 instrument in deuterated DMSO. HR-MS data were recorded on a Thermo LTQ Exactive instrument with an electrospray ionization (ESI) source and HR-MS/MS were recorded on a Thermo Scientific Orbitrap ID-X Tribrid instrument. Solvents for extraction and isolation were purchased from Fisher Scientific (Ottawa, ON, Canada) and deuterated solvents for NMR spectroscopy were purchased from Sigma-Aldrich (Oakville, ON, Canada). Flash chromatography was performed using a Biotage Flash+ chromatography system fitted with C_{18} (reversed-phase) SiliaSep cartridges (40-63 μm, 60 Å, 25 g; SiliCycle, QC, Canada). Semi-preparative reversed-phase HPLC was performed on a Phenomenex Kinetex XB-C_{18} column (250 × 10 mm, 5 μm, 100 Å) using an Agilent 1100 HPLC system comprising a G1311A quaternary pump and a G1315C diode array detector.

Fungal Material. KP1-175E was isolated from the marine macroalga *Spongomorpha arcta* collected from the shores at Green's Point, L'Etete, NB, Canada (45° 02′ 22 N; 066° 53′ 32 W) in June, 2013. Portions (5 cm in length) of algal tissue were individually sterilized by sequential immersion in 6.0% sodium hypochlorite (5 seconds), sterile distilled water (10 seconds) and 70% ethanol (10 seconds). The tissue was then rinsed with sterile distilled water, blotted dry on an autoclaved paper towel, and cut into pieces (0.60 cm in diameter) that were placed onto 2.0% malt extract agar and incubated at room temperature under ambient light. Endophytic fungi were subcultured onto fresh 2.0% malt extract agar until pure cultures were obtained.

Isolate KP1-175E was identified as *Tolypocladium* sp. through the examination of spore morphology and colonies grown on cornmeal, Czapek- Dox, malt extract, and potato dextrose agar (see Supporting Information Figure S1). Attempts to identify the isolate through DNA sequencing were unsuccessful as DNA from the ITS region could not be isolated and amplified after repeated

attempts using published procedures.[23] On malt extract agar, the isolate grew as a flat, filamentous, white colony with a rough surface and filiform margin with white spore formation. On potato dextrose agar, the isolate grew as a crateriform, filamentous, white colony with a smooth surface, and undulating margin with white spore formation. On cornmeal agar, the isolate grew as a raised, filamentous, white colony with a rough surface and beige, entire margin that lacked spore formation. On Czapek–Dox agar, the isolate grew as a flat, filamentous, brown colony with white floccose growth, a rough surface, and undulating margin with brown spore formation. Microscopy revealed the isolate possessed branching, septate, and hyaline hyphae with phialides. Phialides were ovoid to obclavate and solitary. Conidia were single celled, hyaline, and subglobose to ellipsoidal.

Fermentation, Extraction, and Isolation. KP1-175E was fermented in 1.2% potato dextrose broth at room temperature, with ambient light, with shaking (150 rpm) for 2 weeks (10 L; 100 × 100 mL batches in 250 mL Erlenmeyer flasks stoppered with foam baffles). Fermentation cultures were then sonicated for 30 seconds. Next, fungal material was removed by filtration before the spent broth was extracted with EtOAc (3 × 3.3 L). The organic fractions were combined and concentrated *in vacuo* (673 mg). The extract was redissolved in methanol and loaded on 2 g of C_{18} silica and subjected to C_{18} reversed phase flash chromatography (stepwise gradient from 7:3 H_2O–CH_3CN to 100% CH_3CN in 10% increments to give 7 fractions. The fraction that eluted from the flash column in 3:7 H_2O–CH_3CN (34 mg) was subjected to C_{18} reverse-phase HPLC (3:2 H_2O–CH_3CN) to give **1** (7 mg) and **2** (5 mg). The fraction that eluted from the flash column in 3:2 H_2O–CH_3CN gave **3** (167 mg) as an inseparable mixture of atropisomers.

Tolypocaibol A (**1**). Amorphous solid; $[\alpha]_D^{20}$ −4.5 (c 0.01, MeOH); ^{1}H and ^{13}C NMR data, Table 1 and Supporting Information; HR-MS *m/z* 1339.8354 $[M + H]^+$ (calcd for $C_{66}H_{111}N_{14}O_{15}^+$ 1339.8348).

Tolypocaibol B (**2**). Amorphous solid; $[\alpha]_D^{20}$ −11 (c 0.01, MeOH); ^{1}H and ^{13}C NMR data, Table 1 and Supporting Information; HR-MS *m/z* 1353.8550 $[M + H]^+$ (calcd for $C_{67}H_{113}N_{14}O_{15}^+$ 1353.8504).

Maximiscin [(*P*/*M*)-**3**)]. Amorphous solid; $[\alpha]_D^{20}$ −64 (c 0.47, MeOH); ^{1}H and ^{13}C NMR data were consistent with literature values;[30] HR-MS *m/z* 450.2126 $[M + H]^+$ (calcd for $C_{23}H_{32}NO_8^+$ 450.2128).

Marfey's Analysis. The absolute configurations of amino acid and amino alcohol residues in **1** and **2** were determined using Marfey's method.[27] Each peptide (0.25 μmol) was separately dissolved in 6 N HCl (500 μL) and heated in a sealed glass vial at 110 °C for 16 h. The hydrolysates were evaporated to dryness *in vacuo* and redissolved in 200 μL of L-FDAA (1% solution in acetone) and 40 μL of 10% $NaHCO_3$ was added. The mixtures were heated at 40 °C for 1 h and then neutralized with 20 μL of 2M HCl and dried under nitrogen. The residues were then dissolved in 500 μL CH_3CN and 500 μL H_2O and analyzed by reversed-phase HPLC (Phenomenex Luna C_{18} column [250 × 4.6 mm, 5 μm, 100 Å], 1.0 mL/min, CH_3CN−H_2O gradient 20:80−40:60 containing 0.05% trifluoroacetic acid in 40 min and MeOH−H_2O isocratic 40:60 for 50 min). An Agilent G1315C diode array detector was used for detection. In the same manner, L-FDAA derivatives of enantiopure standard amino acids and amino alcohols were prepared and analyzed by HPLC. Due to the hydrolysis of the amide group in Gln residues to a carboxylic acid and ammonia,[36] glutamic acid was synthesized by the hydrolysis of glutamine in the same manner the peptides were hydrolyzed. The retention times (min) for the Marfey's reagent-derivatized amino

acid and amino alcohol standards using the $CH_3CN–H_2O$ gradient were as follows (see Supporting Information Table S1): L-Gln (16.3), D-Gln (16.2), L-Glu (18.9), D-Glu (19.7), L-Pro (22.3), D-Pro (23.4), L-Valol (26.2), D-Valol (30.1), (2*S*,4*S*)-4-mPro (26.5), (2*R*,4*R*)-4-mPro (27.9), L-Leu (32.3), D-Leu (36.0), L-Phe (32.3), and D-Phe (35.0). The retention times of L-phenylalanine and L-leucine using $MeOH–H_2O$ isocratic conditions were 30.81 and 38.89 mins respectively. The retention times for the L-FDAA derivatives of the acid hydrolysate of **1** (min) using the $CH_3CN–H_2O$ gradient were as follows: L-Glu (18.7), L-Pro (22.3), L-Valol (26.0), L-Leu (32.3), L-Phe (32.3). The retention times using $MeOH–H_2O$ isocratic conditions were L-Phe (30.62) and L-Leu (38.29). The retention times for the L-FDAA derivatives of the acid hydrolysate of **2** (min) using the $CH_3CN–H_2O$ gradient were as follows: L-Glu (19.0), L-Valol (26.2), (2*S*,4*S*)-4-mPro (26.5), L-Leu (32.4), and L-Phe (32.4). The retention times using $MeOH–H_2O$ isocratic conditions were L-Phe (30.68) and L-Leu (38.60).

Biological Activity Testing. Antimicrobial susceptibility tests for compounds **1**–**3** against the bacterial target panel were performed using a miniaturized high-throughput assay adapted from the broth microdilution method outlined by the Clinical and Laboratory Standards Institute (CLSI). Bacterial test strains were individually grown on fresh Nutrient Broth (NB, ATCC Medium 3) agar, Tryptic Soy Broth (TSB, ATCC Medium 18) agar or Brain Heart Infusion (BHI, ATCC Medium 44) agar, respectively (see Supporting Information Table S2), as recommended by the American Type Culture Collection (ATCC) cultivation protocol. Individual colonies were used to inoculate 3 mL of sterile NB, TSB or BHI media and grown overnight with shaking (200 RPM; 37 °C). *Listeria ivanovii* (ATCC BAA-139) was incubated overnight but not shaken (37 °C; 5% CO_2). Saturated overnight cultures were diluted in their respective media according to turbidity to achieve approximately 5 x 10^5 CFU/mL of final inoculum density and dispensed into sterile clear

polystyrene 384-well microplates (Thermo Scientific™ 265202) with a final screening volume of 30 µL. *L. ivanovii* was diluted with and grown in Haemophilus Test Medium (HTM; ATCC Medium 2167). DMSO solutions of test compounds and antibiotic controls were prepared as 1:1 dilution series and pinned into each assay plate (200 nL) using a high-throughput pinning robot (Tecan Freedom EVO 100) to achieve final screening concentrations ranging from 128 µM to 3.91 nM per compound. In each 384-well plate; lane 1 was reserved for DMSO vehicle and culture medium; lane 2 reserved for DMSO vehicle, culture medium, and target bacteria; lanes 23 and 24 reserved for antibiotic controls, DMSO vehicle, culture medium, and target bacteria. After compound pinning, assay plates were read as t_0 at OD_{600} using an automated plate reader (BioTek Synergy Neo2), sealed with a lid and placed in a humidity-controlled incubator at 37 °C for 18-20 hours before optical density is obtained for t_{20}. *L. ivanovii* was incubated in a separate incubator (37 °C; 5% CO_2). Resulting growth curves for each dilution series were used to determine the MIC values for all test compounds following standard procedures. Antimycobacterial activity against *M. tuberculosis* H37Ra (ATCC 25177) and *M. smegmatis* (ATCC 70084) was evaluated using the microplate resazurin assay, as previously described.[25,37]

Associated Content

Data Availability Statement: The NMR data for compounds **1** and **2** have been deposited to the Natural Products Magnetic Resonance Database (NP-MRD; www.np-mrd.org). Accession numbers will be generated when the manuscript is accepted for publication and included when galley proofs are reviewed.

Supporting Information: Bacterial strains and culture conditions; Marfey's analysis results, HRMS and NMR spectra for compounds **1** and **2**, and NMR spectra for **3**.

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