Kempopeptins A and B, Serine Protease Inhibitors with Different Selectivity Profiles from a Marine Cyanobacterium, *Lyngbya* sp.

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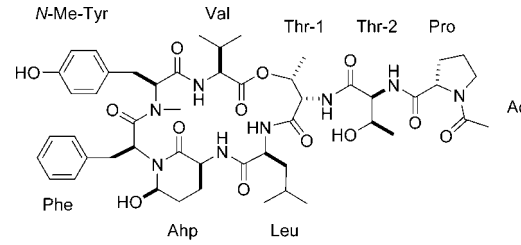
Two cyclodepsipeptides named kempopeptins A (**1**) and B (**2**) were isolated from a collection of a Floridian marine cyanobacterium, *Lyngbya* sp., that had previously afforded the structurally related potent elastase inhibitors lyngbyastatin 7 and somamide B. The structures of **1** and **2** were elucidated mainly by 1D and 2D NMR spectroscopy, and the absolute configuration was established by chiral HPLC and Marfey's analysis of the degradation products. Kempopeptin A (**1**) exhibited an IC_{50} against elastase of 0.32 μM and against chymotrypsin of 2.6 μM, while kempopeptin B (**2**) inhibited trypsin with an IC_{50} of 8.4 μM.

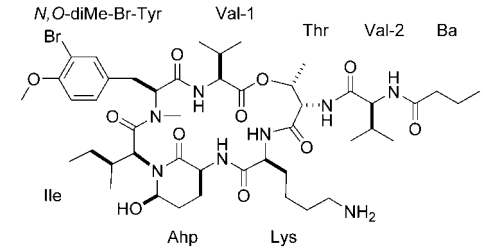
Marine cyanobacteria biosynthesize a plethora of structurally distinct bioactive secondary metabolites.[1] Several of these natural products have been shown to be protease inhibitors.[2] Since proteases are involved in a variety of biological processes, and many proteases are validated drug targets,[3] the discovery of new protease modulators is important to the development of pharmacological tools as well as potential therapeutics. We recently reported the isolation of potent elastase inhibitors, lyngbyastatins 4−7, from Floridian cyanobacteria.[4,5] The lyngbyastatin 7 producer *Lyngbya* sp., collected at Kemp Channel in the Florida Keys, which also produces another elastase inhibitor, somamide B,[5] now afforded two more serine protease inhibitors, to which we assigned the trivial names kempopeptin A (**1**) and kempopeptin B (**2**). Their discovery proves the potential of a single cyanobacterium to exhibit an array of serine protease inhibitory activities, but also raises questions about the role of protease inhibitors in the marine environment. Protease inhibitors are known to be co-biosynthesized with the microcystin cyanobacterial toxins;[6] in fact, metabolites other than microcystins have been shown to enhance microcystin activity.[7] The biosynthesis of protease inhibitors in nontoxic cyanobacterial strains has precedence as well.[8] Here we report the isolation, structure elucidation, and protease-inhibitory activity of compounds **1** and **2** from a nontoxic cyanobacterium.

The cyanobacterium *Lyngbya* sp. was collected from Kemp Channel, a mangrove channel southwest of Summerland Key in the Florida Keys. The sample was freeze-dried and extracted with CH_2Cl_2−MeOH (1:1). This extract was partitioned with organic solvents followed by various chromatographic steps using silica and C_{18} and ultimately reversed-phase HPLC to yield compounds **1** and **2**.

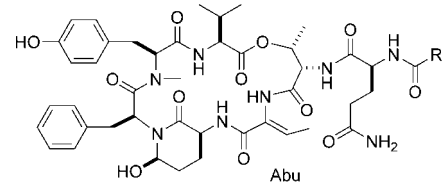
Kempopeptin A (**1**) was obtained as a colorless, amorphous solid and shown to have the molecular formula of $C_{50}H_{70}N_8O_{13}$ as determined by HRESI/APCIMS based on a $[M + Na]^+$ peak at *m/z* of 1013.4965 (calcd for $C_{50}H_{70}N_8O_{13}Na$, 1013.4960). The presence of a peptide backbone was evident from the ^1H NMR spectrum recorded in DMSO-d_6 due to a tertiary amide *N*-Me 3H singlet at δ 2.75 and characteristic secondary amide NH resonances occurring as one 1H doublet at δ 7.06 and eight 0.5H doublets at δ 7.42−8.40. The differential integration was suggestive of conformers in only one part of the molecule (Table 1). The combination of ^1H and ^{13}C NMR, COSY, HMQC, HMBC, and TOCSY data revealed the presence of valine, *N*-methyltyrosine,



Kempopeptin A (**1**)



Kempopeptin B (**2**)



Lyngbyastatin 7 R = $(CH_2)_4CH_3$
Somamide B R = $(CH_2)_2CH_3$

phenylalanine, leucine, proline, and two threonine residues, the modified amino acid 3-amino-6-hydroxy-2-piperidone (Ahp), and an acetyl group, with signal doubling for the two threonine moieties, proline, the acetyl group, and exchangeable protons of valine and leucine residues. HMBC analysis established the sequence including the planar structure depicted for **1**. The doubling of the ^1H NMR signals in the side chain was attributed to restricted rotation around the *N*-acetyl prolyl amide bond on the basis of ROESY cross-peaks between H-2 of proline (δ_H 4.52) and the acetyl protons (δ_H 1.83) for the *cis* isomer and between H-5b of proline (δ_H 3.47) and the acetyl protons (δ_H 1.95) in the *trans* isomer. A 1:1 ratio of c*is* and *trans* isomers in DMSO-d_6 around the *N*-acetyl−prolyl bond was also reported for the most closely related metabolite, oscillapeptilide 97-B,[9] which contains an isoleucine instead of the valine in the

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Table 1. NMR Data for Both Conformers of Kempopeptin A (**1**) in DMSO-d_6 (ratio 1:1) at 500 MHz (^{1}H) and 150 MHz (^{13}C)

		trans conformer[a]		*cis* conformer[a]			
unit	C/H no.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	HMBC[b,c]	key ROESY[c]
Val	1		172.1, qC		172.1, qC		
	2	4.65, dd (9.2, 4.5)	55.8, CH	4.64, dd (9.5, 4.5)	55.8, CH	1, 3, 4, 5, 1 (*N*-Me-Tyr)	
	3	2.04, m	31.8, CH	2.04, m	30.8, CH	2, 4, 5	
	4	0.85, d (6.5)	19.5, CH_3	0.84, d (6.5)	19.3, CH_3	2, 3, 5	*N*-Me (*N*-Me-Tyr)
	5	0.71, d (6.5)	17.2, CH_3	0.70, d (6.5)	17.2, CH_3	2, 3, 4	*N*-Me (*N*-Me-Tyr)
	NH	7.43, d (9.2)		7.42, d (9.5)		1 (*N*-Me-Tyr)	H-2 (*N*-Me-Tyr), *N*-Me (*N*-Me-Tyr), 6-OH (Ahp)
N-Me-Tyr	1		169.1, qC		169.1, qC		
	2	4.89, dd (10.6, 1.5)	60.9, CH	4.89, dd (10.6, 1.5)	60.9, CH		H-3a, *N*-Me, H-2 (Phe), H-5/9 (Phe), NH (Val)
	3a	3.10, dd (−13, 10.6)	32.8, CH_2	3.10, dd (−13, 10.6)	32.8, CH_2	4, 5/9	H-2
	3b	2.69, dd (−13, 1.5)		2.69, dd (−13, 1.5)		4, 5/9	
	4		127.5, qC		127.5, qC		
	5/9	6.99, d (8.5)	130.4, CH	6.99, d (8.5)	130.4, CH	3, 5/9, 7	*N*-Me, H-2 (Phe)
	6/8	6.77, d (8.5)	115.3, CH	6.77, d (8.5)	115.3, CH	4, 6/8, 7	
	7		156.2, qC		156.2, qC		
	7-OH	9.35, s				6/8, 7	
	N-Me	2.75, s	30.3, CH_3	2.75, s	30.3, CH_3	2, 1 (Phe)	H-2, H-5/9, H_3-4 (Val), H_3-5 (Val), NH (Val)
Phe	1		170.4, qC		170.4, qC		
	2	4.73, dd (11.5, 4.3)	50.3, CH	4.73, dd (11.5, 4.3)	50.3, CH	1, 2 (Ahp), 6 (Ahp)	H-3b, H-5/9, H-2 (*N*-Me-Tyr), H-5/9 (*N*-Me-Tyr), H-6 (Ahp)
	3a	2.85, dd (−13.8, 11.5)	35.3, CH_2	2.85, dd (−13.8, 11.5)	35.3, CH_2	2, 4	
	3b	1.77, dd (−13.8, 4.3)		1.77, dd (−13.8, 4.3)		2, 4	H-2
	4		136.7, qC		136.7, qC		
	5/9	6.82, d (7.0)	129.4, CH	6.82, d (7.0)	129.4, CH	3, 5/9, 6/8	H-2, H-2 (*N*-Me-Tyr)
	6/8	7.15, m	127.7, CH	7.15, m	127.7, CH	4, 7	
	7	7.12, m	126.2, CH	7.12, m	126.2, CH	5/9, 6/8	
Ahp	2		168.9, qC		168.9, qC		
	3	3.61, m	48.6, CH	3.61, m	48.6, CH	2	H-4b, H-5a, NH
	4a	2.37, m	21.7, CH_2	2.37, m	21.7, CH_2		H-4b, 6-OH, NH
	4b	1.55, m		1.55, m			H-3, H-4a
	5a	1.66, m	29.0, CH_2	1.66, m	29.3, CH_2		H-3, H-5b, H-6, 6-OH
	5b	1.54, m		1.54, m			H-5a, H-6, 6-OH
	6	5.05, br s	73.7, CH	5.05, br s	73.7, CH		H-5a, H-5b, 6-OH, H-2 (Phe)
	6-OH	6.02, br s		6.02, br s			H-4a, H-5a, H-5b, H-6, NH (Val)
	NH	7.06, d (9.1)		7.06, d (9.1)		1 (Leu)	H-3, H-4a, H-2 (Leu)
Leu	1		170.1, qC		170.1, qC		
	2	4.19, m	50.3, CH	4.19, m	50.3, CH		H-3a, H_3-5, NH, NH (Ahp)
	3a	1.70, m	40.0, CH_2	1.70, m	40.0, CH_2	5, 6	H-2
	3b	1.28, m		1.28, m			NH
	4	1.44, m	23.3, CH	1.44, m	23.3, CH		NH
	5	0.70, d (6.4)	20.9, CH_3	0.70, d (6.4)	20.9, CH_3	3, 4, 6	H-2
	6	0.83, d (6.4)	21.5, CH_3	0.83, d (6.4)	21.5, CH_3	3, 4, 5	
	NH	8.40, d (8.7)		8.39, d (8.8)		1 (Thr-1)	H-2, H-3b, H-4, H-2 (Thr-1), H-3 (Thr-1)
Thr-1	1		169.2, qC		169.2, qC		
	2	4.58, dd (9.1, 2.1)	54.6, CH	4.60, dd (9.1, 2.0)	54.6, CH	1, 1 (Thr-2)	H_3-4, NH (Leu)
	3	5.38, br q (6.4)	72.0, CH	5.39, br q (6.4)	72.0, CH	1 (Val)	NH (Leu)
	4	1.18, d (6.4)	17.7, CH_3	1.17, d (6.4)	17.7, CH_3		
	NH	7.62, d (9.1)		7.75, d (9.1)		1 (Thr-2)	H-2 (Thr-2), H-3 (Thr-2), H_3-4 (Thr-2)
Thr-2	1		170.7, qC		170.6, qC		
	2	4.30, dd (8.2, 4.1)	58.1, CH	4.39, dd (8.4, 4.2)	58.0, CH	1	NH (Thr-1)
	3	4.03, m	66.5, CH	4.03, m	66.5, CH		NH (Thr-1)
	4	1.02, t (6.7)	19.2, CH_3	1.04, t (6.8)	19.3, CH_3		NH (Thr-1)
	OH	4.86, d (5.1)		4.96, d (5.4)			
	NH	7.91, d (8.2)		8.12, d (8.4)		1 (Pro)	H-2 (Pro)
Pro	1		172.3, qC		172.1, qC		
	2	4.44, dd (8.4, 2.8)	58.9, CH	4.52, dd (8.6, 2.9)	60.1, CH		NH (Thr-2)
	3a	2.05, m	29.3, CH_2	2.23, m	29.5, CH_2		
	3b	1.90, m		1.93, m			
	4	1.89, m	24.3, CH_2	1.76, m	24.1, CH_2		
	5a	3.51, m	47.6, CH_2	3.40, m	46.3, CH_2		
	5b	3.47, m		3.38, m			
Ac	1		168.7, qC		168.5, qC		
	2	1.95, s	22.0, CH_3	1.83, s	22.2, CH_3	1	H-2[d] (Pro)^{cis} or H_2-5[e] (Pro)^{trans}

[a] Refers to restricted rotation around the *N*-acyl−prolyl amide bond. [b] Protons showing HMBC correlations to the indicated carbon. [c] Refers to nuclei within the same unit unless indicated otherwise. [d] Refers to *cis* isomer. [e] Refers to *trans* isomer.

cyclic core and a glutamine rather than the threonine-2 residue in the side chain.

Acid hydrolysis followed by modified Marfey's analysis[10] established the L-configuration of all amino acid residues, while deciphering the Ahp configuration (3*S*,6*R*) required prior CrO_3 oxidation and additionally ROESY analysis of the intact molecule (Table 1) as previously described.[4,5] The assignment was also consistent with the nearly identical NMR data for kempopeptin A (**1**) and oscillapeptilide 97-B,[9] suggesting that the relative configurations including the conformation of the cyclic core are the same for both compounds.

Kempopeptin B (**2**) was obtained as a colorless, amorphous powder. The HRESI/APCIMS data showed a $[M + H]^+$ peak at *m/z* 993.4663 and an isotope peak of approximately equal intensity at *m/z* 995.4656, indicating the presence of one bromine atom and a molecular formula of $C_{46}H_{73}BrN_8O_{11}$ (calcd for $C_{46}H_{74}{}^{79}BrN_8O_{11}$,

Table 2. NMR Data for Kempopeptin B (**2**) in DMSO-d_6 at 600 MHz (^{1}H) and 150 MHz (^{13}C)

unit	C/H no.	δ_H (J in Hz)	δ_C, mult.	COSY[a]	HMBC[b,c]	key ROESY[a,c]
Val-1	1		172.3, qC			
	2	4.63, dd (9.4, 5.5)	54.8, CH	H-3, NH	1, 1 (*N,O*-diMe-Br-Tyr)	
	3	2.04, m	30.5, CH	H_3-4, H_3-5	1, 2, 4, 5	
	4	0.85, d (6.8)	19.3, CH_3	H-3	2, 3, 5	*N*-Me (*N,O*-diMe-Br-Tyr)
	5	0.73, d (6.8)	17.6, CH_3	H-3	2, 3, 4	*N*-Me (*N,O*-diMe-Br-Tyr)
	NH	7.68, d (9.4)		H-2	1 (*N,O*-diMe-Br-Tyr)	H-2 (*N,O*-diMe-Br-Tyr), *N*-Me (*N,O*-diMe-Br-Tyr), 6-OH (Ahp)
N,O-diMe-Br-Tyr	1		169.4, qC			
	2	5.03, dd (11.3, 2.6)	60.6, CH	H-3a, H-3b	3	H-3a, H-5, H-9, *N*-Me, H-2 (Ile), NH (Val-1)
	3a	3.20, dd (−12.0, 2.6)	32.9, CH_2	H-2, H-3b		H-2, H-5
	3b	2.78, dd (−12.0, 11.3)		H-2, H-3a		
	4		131.3, qC			
	5	7.39, d (1.8)	133.5, CH	H-9	3, 6, 7, 9	H-2, H-3a, *N*-Me, H-2 (Ile)
	6		111.0, CH			
	7		154.6, qC			
	8	7.01, d (8.4)	113.0, CH	H-9	4, 6, 7	
	9	7.17, dd (8.4, 1.8)	130.2, CH	H-5, H-8	3, 5, 7	H-2
	O-Me	3.74, s	56.1, CH_3		7	
	N-Me	2.72, s	30.2, CH_3		2, 1 (Ile)	H-2, H-5, H_3-4 (Val-1), H_3-5 (Val-1), NH (Val-1)
Ile	1		169.7, qC			
	2	4.35, br d (10.7)	54.2, CH	H-3	1, 3, 6 (Ahp)	H-3, H-5, H_3-6, H-2 (*N*-Me-Br-Tyr), H-5 (*N,O*-diMe-Br-Tyr)
	3	1.79, m	32.8, CH	H-2, H-4a, H-4b, H_3-6		H-2
	4a	1.0, m	23.7, CH_2	H-3, H-4b, H_3-5		
	4b	0.629, m		H-3, H-4a, H_3-5		
	5	0.630, br t (6.9)	10.3, CH_3	H-4a, H-4b	3, 4	H-2
	6	−0.15, d (6.4)	13.8, CH_3	H-3	2, 3, 4	H-2
Ahp	2		170.4,[d] qC			
	3	4.42, m	48.8, CH	H-4a, H-4b, NH		H-4b, H-5, NH
	4a	2.55, m	21.7, CH_2	H-3, H-4b, H-5		H-4b, 6-OH, NH
	4b	1.71, m		H-3, H-4a, H-5		H-3, H-4a
	5	1.73, m (2H)	29.7, CH_2	H-4a, H-4b, H-6		H-3, H-6
	6	4.92, d (2.9)	74.1, CH	H-5, 6-OH		H-5, 6-OH
	6-OH	6.15, d (2.9)		H-6		H-4a, H-6, NH (Val-1)
	NH	7.34, d (9.3)		H-3		H-3, H-4a, H-2 (Lys)
Lys	1		169.3,[d] qC			
	2	4.26, br	52.1, qC	H-3a, H-3b, NH		H-3a, H-4, NH, NH (Ahp)
	3a	2.00, m	29.0, CH_2	H-2, H-3b, H_2-4		H-2
	3b	1.41, m		H-2, H-3a, H_2-4		NH
	4	1.23, m (2H)	22.1, CH_2	H-3a, H-3b, H_2-5		H-2
	5	1.47, m (2H)	26.3, CH_2	H_2-4, H_2-6		
	6	2.71, m (2H)	38.6, CH_2	H_2-5		
	NH	8.44, d (8.4)		H-2	1 (Thr)	H-2, H-3b, H-2 (Thr), H-3 (Thr), NH (Thr)
	NH_2	7.60, br s (2H)				
Thr	1		169.3, qC			
	2	4.59, dd (10.2, 6.5)	56.3, CH	H-3, NH	1 (Val-2)	H-3, H-4, NH (Lys), NH (Val-2)
	3	5.49, br q (6.5)	71.7, CH	H-2, H_3-4	4, 1 (Val-1)	H-2, NH (Lys)
	4	1.20, d (6.5)	17.7, CH_3	H-3	2, 3	H-2
	NH	7.80, d (10.2)		H-2	1	NH (Lys)
Val-2	1		172.4, qC			
	2	4.31, dd (9, 7.1)	57.6, CH	H-3, NH	1	H-3, NH, H_3-4 (Ba)
	3	2.01, m	30.0, CH	H_3-4, H_3-5		H-2
	4	0.83, d (7.6)	19.3, CH_3	H-3	2	
	5	0.82, d (7.6)	18.1, CH_3	H-3	2	
	NH	7.82, d (9)		H-2	1 (Ba)	H-2, H-2 (Thr), H_2-2 (Ba)
Ba	1		172.5, qC			
	2	2.16, m (2H)	37.1, CH_2	H_2-3	1, 3, 4	H_3-4, NH (Val-2)
	3	1.49, m (2H)	18.9, CH_2	H_2-2, H_3-4	1, 2, 4	
	4	0.84, t (7.5)	13.6, CH_3	H_2-3	2, 3	H_2-2, H-2 (Val-2)

[a] Recorded at 500 MHz. [b] Protons showing HMBC correlations to the indicated carbon (600 MHz). [c] Refers to nuclei within the same unit unless indicated otherwise. [d] Interchangeable. No HMBC correlations observed. Carbons assigned on the basis of remaining unassigned signals in the ^{13}C NMR spectrum.

993.4660). Five doublet NH proton signals in the amide range (δ_H 7.34, 7.68, 7.80, 7.82, 8.44) and one broad singlet for two primary amide protons (δ_H 7.60) in the ^{1}H NMR spectrum suggested that **2** was a peptide. ^{1}H NMR, ^{13}C NMR, HSQC, COSY, and TOCSY analysis revealed seven amino acid spin systems, one carboxylic acid unit, one *N*-Me (δ_H 2.72 s, δ_C 30.2), an *O*-Me group (δ_H 3.74 s, δ_C 56.1), and a 1,3,4-trisubstituted phenyl ring (Table 2). Further NMR including HMBC and ROESY analysis confirmed the presence of two valine units, threonine, isoleucine, lysine, *N*,*O*-dimethyl-3′-bromotyrosine, Ahp, and butanoic acid (Ba) moieties and provided the planar structure for **2** (Table 2). The most unusual structural feature of **2** is arguably the brominated tyrosine residue, which was recently also found in largamides D, F, and G,[11] symplocamide A,[12] and pompanopeptin A,[13] while other compounds such as scyptolin A[14] and cyanopeptolin 954[15] are chlorinated at this position.

Table 3. Protease Inhibitory Activity (IC_{50}) from Metabolites Isolated from the *Lyngbya* sp. from Kemp Channel

	elastase	chymotrypsin	trypsin
kempopeptin A (**1**)	320 ± 70 nM	2600 ± 100 nM	>67 000 nM
kempopeptin B (**2**)	>67 000 nM	>67 000 nM	8400 ± 200 nM
lyngbyastatin 7[a]	8.3 ± 5.4 nM	2500 ± 200 nM	>30 000 nM
somamide B[a]	9.5 ± 5.2 nM	4200 ± 500 nM	>30 000 nM

[a] Taken from ref 5.

A combination of UV-based Marfey's[10] (Lys, Thr, Val), LC-MS-based advanced Marfey's[16] (*N*,*O*-diMe-Br-Tyr), and chiral HPLC (Ile) analysis established the L-configuration of these amino acids, while the 3*S*,6*R* configuration of the Ahp unit was ascertained as described for **1**.

Many Ahp-containing cyclodepsipeptides isolated from cyanobacteria are known inhibitors of serine proteases such as elastase, chymotrypsin, and trypsin.[2,12] Thus, we determined the inhibitory activity of compounds **1** and **2** against these three enzymes. Kempopeptin A (**1**) inhibited elastase with a slight selectivity over chymotrypsin; conversely, kempopeptin B (**2**) inhibited only trypsin activity (Table 3). These results are in accordance with previous crystallographic and structure−activity relationship data, suggesting that the amino acid residue between Thr and Ahp binds to the enzyme's specificity pocket and thus plays an important role in determining the selectivity toward serine proteases.[12] A hydrophobic amino acid at this position commonly confers preference for chymotrypsin and elastase inhibition (Leu in **1**), while a basic amino acid such as lysine or arginine is necessary for trypsin inhibition (Lys in **2**). Our IC_{50} value for **2** against trypsin closely corresponds to data reported for the related lysine-containing metabolite micropeptin SD944 (8.0 μg/mL).[8]

The activity of **1** was comparable to those observed for oscillapeptin G,[9] scyptolin A,[17] and planktopeptins BL1125 and BL1061,[18] all of which contain Leu in the cyclic core at this position; however, the Phe residue is replaced by Thr. The different degrees of Tyr modification (chlorination or *O*-methylation) in these related compounds and substitution of Val for Ile in the planktopeptins likely do not affect protease-inhibitory activity significantly. In lyngbyastatin 7 and somamide B, a 2-amino-2-butenoic acid (Abu) unit presumably occupies the specificity pocket, while all other core residues are the same as in **1**.[5] This allows direct comparison of their activities. Since the side-chain composition is less important for activity as long as at least two residues flank the cyclic core,[5] the Leu → Abu change within the core structure seems to increase elastase-inhibitory activity but does not enhance chymotrypsin-inhibitory activity (Table 3). A postulated stabilization of the ethylidene moiety by CH/π interaction may be responsible for the potent elastase activity,[19] leading to more pronounced selectivity of lyngbyastatin 7 and somamide B for both proteases compared with **1** (Table 3).

Because a minority of related Ahp-containing compounds such as symplocamide A[12] has been described to also possess cytotoxic activity, we assessed the effects of compounds **1** and **2** on the proliferation of cancer cells. Both compounds did not significantly affect the growth of HT29 colon adenocarcinoma cells at the highest concentration tested (50 μM) by MTT-based cell viability assessment.

The discovery of four different protease inhibitors with three distinct selectivity profiles from a single homogeneous cyanobacterial collection illustrates the potential of cyanobacteria to execute their own combinatorial biosynthesis and structure optimization. While the role of these protease inhibitors in Nature is not fully understood, a defensive function against other microorganisms or consumers is probable.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a SpectraMax M5 (Molecular Devices). ^{1}H, ^{13}C, and 2D NMR spectra were recorded in DMSO-d_6 on either a Bruker Avance 500 MHz (for **1**, ^{1}H, 2D; for **2**, COSY, TOCSY, ROESY) or 600 MHz (for **1**, ^{13}C) or Bruker Avance II 600 MHz spectrometer equipped with a 1 mm triple-resonance high-temperature superconducting cryogenic probe (for **2**, ^{1}H, ^{13}C, HSQC, HMBC) using residual solvent signals (δ_H 2.49, δ_C 39.5) as internal standards. HRMS data were obtained using an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector. LC-MS data were obtained using an Agilent 1100 equipped with a ThermoFinnigan LCQ by ESI (positive mode).

Biological Material. *Lyngbya* sp. was collected from a mangrove channel at the northern end of Kemp Channel near Summerland Keys (Florida Keys) in May 2006. A morphological characterization including cell measurements was provided with our report of the isolation of lyngbyastatin 7 and somamide B from the same organism.[5] A specimen preserved in formalin has been retained at the Smithsonian Marine Station.

Extraction and Isolation. The freeze-dried organism was extracted with CH_2Cl_2−MeOH (1:1). The resulting lipophilic extract (24.1 g) was partitioned between hexanes and 20% aqueous MeOH, the methanolic phase was evaporated to dryness, and the residue was further partitioned between *n*-BuOH and H_2O. The *n*-BuOH layer was concentrated and subjected to chromatography over silica gel using CH_2Cl_2 and increasing gradients of *i*-PrOH (2, 5, 10, 20, 50 to100% *i*-PrOH) followed by 100% MeOH. The fraction that eluted with 50% *i*-PrOH was then applied to a C_{18} SPE cartridge and elution initiated with H_2O followed by aqueous solutions containing 25, 50, 75, and 100% MeOH. The fractions eluting with 75% aqueous MeOH were then subjected to semipreparative reversed-phase HPLC (YMC-pack ODS-AQ, 250 × 10 mm, 2.0 mL/min; UV detection at 220 and 254 nm) using a MeOH−H_2O linear gradient (50−100% for 60 min and then 100% MeOH for 10 min), yielding compound **1**, t_R 30.2 min (1.0 mg).

The fraction that eluted with 100% MeOH from silica gel was subjected to Si SPE cartridge and elution started with CH_2Cl_2 followed by CH_2Cl_2 mixtures containing 20, 40, 60, and 80% MeOH and then 100% MeOH. The fraction eluting with 20% methanolic CH_2Cl_2 was then applied to a semipreparative reversed-phase HPLC column (YMC-Pack ODS-AQ, 250 × 10 mm, 2.0 mL/min; UV detection at 220 and 254 nm) using a MeOH−H_2O (0.05% TFA) linear gradient (60−100% for 40 min and then 100% MeOH for 15 min). The fraction that had eluted with 100% MeOH from silica gel yielded compound **2**, t_R 25.2 min (1.6 mg).

Kempopeptin A (1): colorless, amorphous powder; $[\alpha]^{20}_D$ −45 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.66), 280 (sh) (2.67); IR (film) 3374 (br), 2958, 2924, 1735, 1655 (br), 1541, 1449, 1257, 1203, 1139 cm^{-1}; ^{1}H NMR, ^{13}C NMR, HMBC, and ROESY data, see Table 1; HRESI/APCIMS *m*/*z* [M + Na]$^+$ 1013.4965 (calcd for $C_{50}H_{70}N_8O_{13}Na$, 1013.4960).

Kempopeptin B (2): colorless, amorphous powder; $[\alpha]^{20}_D$ −18 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.80), 280 (sh) (3.12); IR (film) 3356 (br), 2926, 1738, 1736, 1658 (br), 1530, 1442, 1257, 1205, 1139 cm^{-1}; ^{1}H NMR, ^{13}C NMR, COSY, HMBC, and ROESY data, see Table 2; HRESI/APCIMS *m*/*z* [M + H]+ 993.4663 (calcd for $C_{46}H_{74}{}^{79}BrN_8O_{11}$, 993.4660), 995.4656 (calcd for $C_{46}H_{74}{}^{81}BrN_8O_{11}$, 995.4640), 1:1 ion cluster.

Acid Hydrolysis and Amino Acid Analysis by Modified Marfey's Method. Samples (~100 μg) of compounds **1** and **2** were subjected to acid hydrolysis at 110 °C for 24 h and analyzed using an L-FDLA-based Marfey's[10] procedure as described.[5] The retention times (t_R, min) of the L-FDLA-derivatized amino acids in the hydrolysate of compound **1** matched those of L-Thr (14.4), L-Val (24.7), L-Phe (29.6), L-Pro (19.7), L-Leu (28.8), and *N*-Me-L-Tyr (42.1). Conversely, the L-FDLA derivatives (t_R, min) of L-*allo*-Thr (15.6), D-Thr (20.5), D-*allo*-Thr (17.1), D-Val (33.9), D-Phe (36.7), D-Pro (23.1), D-Leu (39.5), and *N*-Me-D-Tyr (43.8) were not detected in the hydrolysate (retention times given for standard amino acids). The retention times (t_R, min) of the derivatized amino acids in the hydrolysate of compound **2** corresponded to those of L-Thr (14.4), L-Val (24.7), L-Lys (40.5), and L-Ile/L-*allo*-Ile (27.0); the latter had the same retention times, requiring chiral HPLC analysis of the acid hydrolysate (see below). Peaks for L-FDLA derivatives of the corresponding isomers were not detected (t_R, min): L-*allo*-Thr (15.6), D-Thr (20.5), D-*allo*-Thr (17.1), D-Val (33.9), D-Lys (42.5), D-Ile/D-*allo*-Ile (37.2). *N,O*-diMe-Br-Tyr adducts could not be reliably detected using this UV-based method, so LC-MS was used instead (see below).

Oxidation−Acid Hydrolysis−Marfey's Analysis Sequence for 1 and 2. CrO_3 oxidation of **1** and **2** followed by acid hydrolysis was carried out as described.[4,5] The resulting hydrolysates were derivatized with L-FDLA and aliquots subjected to reversed-phase HPLC using UV detection as above. When compared to the Marfey profiles without prior oxidation, the HPLC profiles derived from both compounds **1** and **2** showed one new peak for L-Glu (t_R 16.8 min), but not D-Glu (t_R 17.8 min).

Chiral HPLC Analysis for 2. Due to overlap of L-FDLA adducts of L-Ile and L-*allo*-Ile during Marfey's analysis, the acid hydrolysate derived from **2** was subjected to chiral HPLC analysis (column, Phenomenex Chirex phase 3126 *N,S*-dioctyl-(D)-penicillamine, 4.60 × 250 mm, 5 μm; solvents, 2 mM $CuSO_4$ in H_2O−MeCN (95:5) or 2 mM $CuSO_4$; flow rate 1.0 mL/min; detection at 254 nm). The absolute configuration of Ile in the hydrolysate of **2** was determined to be L-Ile by direct comparison with the retention times of authentic standards, while the configurations of the other amino acids obtained from Marfey's analysis were confirmed. The retention times (t_R, min) for standard amino acids were as follows: L-Val (16.6), D-Val (21.8), L-Ile (40.8), D-Ile (52.0), L-*allo*-Ile (34.6), D-*allo*-Ile (43.1) (solvent mixture 95:5); L-Lys (5.2), D-Lys (6.4), L-Thr (10.8), D-Thr (13.6), L-*allo*-Thr (15.1), and D-*allo*-Thr (17.8) (solvent 2 mM $CuSO_4$).

Advanced Marfey's Analysis of 2. The hydrolysate of compound **2** was derivatized with L-FDLA and analyzed by LC-MS according to the advanced Marfey's method[16] to reveal the L-configuration of *N*,*O*-diMe-Br-Tyr in **2** as described.[13]

Protease Inhibition Assays. Inhibitory activity against bovine pancreatic α-chymotrypsin and porcine pancreatic elastase and trypsin was determined as reported earlier.[5]

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