Scytonemides A and B, Cyclic Peptides with 20S Proteasome Inhibitory Activity from the Cultured Cyanobacterium *Scytonema hofmanii*

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Received August 25, 2010

Two cyclic peptides, scytonemides A (**1**) and B (**2**), were isolated from the cultured fresh water cyanobacterium *Scytonema hofmannii* (UTEX 1834) by bioassay-guided fractionation using a proteasome inhibition assay. The planar structures of the compounds were determined by a combination of MS and 1D and 2D NMR spectroscopy. The advanced Marfey's method was used to determine the absolute configuration of both peptides. Scytonemide A possesses an unusual imino linkage, while scytonemide B is a depsipeptide containing 3-hydroxyoctanoic acid in the macrocycle. Both isolates were evaluated for their inhibition of the 20S proteasome, and scytonemide A displayed an IC_{50} value of 96 nM, while scytonemide B was inactive at 50 μM.

Cyanobacteria have been found to be an abundant source of secondary metabolites with diverse biological activities and chemical structures. Numerous biologically active natural products have been reported from cyanobacteria, including alkaloids, polyketides, and linear as well as cyclic peptides.[1–3] These cyanobacterial peptides often contain modified amino acid residues and the addition of a lipid component. Many cyanobacterial peptides have been shown to be inhibitors of various proteases.[4] As part of our ongoing effort to find novel bioactive natural products from cultured freshwater and terrestrial cyanobacteria, we have evaluated cyanobacterial extracts for the inhibition of the 20S proteasome. The 20S proteasome is the catalytic core of the proteasome complex, the major proteolytic system in the cytosol of eukaryotic cells, and catalyzes the degradation of regulatory proteins.[5,6] The proteasome complex plays a central role in maintaining cellular homeostasis and controlling the cell cycle.[7–9] Accordingly, proteasome inhibitors are of interest as potential anticancer agents.[10–15] Currently, there is one FDA-approved proteasome inhibitor on the market (bortezomib, Velcade),[16,17] and there is an ongoing research effort to identify additional inhibitors.

In our studies, the organic extract of the cultured cyanobacterium *Scytonema hofmannii* (UTEX 1834) showed significant inhibitory activity against the human 20S proteasome. Bioassay-guided fractionation resulted in the identification of two new cyclic peptides, scytonemides A (**1**) and B (**2**). Scytonemide A, which contained an unusual imine linkage in the cyclic peptide core, was found to be a potent inhibitor of the 20S proteasome. Scytonemide B, while inactive in the 20S proteasome assay, was identified as a cyclic lipopetide with the unusual amino acid D-citrulline and a hydroxylated fatty acid (3-hydroxyoctanoic acid). Herein, we report the isolation, structure elucidation, and biological activity of these compounds.

Results and Discussion

Lyophilized cell material of *S. hofmannii* was extracted with $MeOH-CH_2Cl_2$ (1:1), and the concentrated extract was fractionated by flash chromatography on silica gel using mixtures of organic solvents of increased polarity, concluding with 100% MeOH. Reversed-phase HPLC of the 100% MeOH fraction led to isolation of **1** and **2**.

Scytonemide A was obtained as a white, amorphous powder. The molecular formula was determined as $C_{36}H_{56}N_8O_9$, on the basis of the HRMS data (*m/z* 745.4186 $[M + H]^+$). Inspection of the DEPTQ and ^1H NMR spectra indicated nine quaternary, 12 methine, seven methylene, and six methyl carbons, corresponding to 34 carbons and 44 protons. The signal intensity at δ_C 131.4 and 115.2 suggested two additional methine carbons were present as part of a *para*-substituted phenyl ring, thus bringing the total number of carbon signals to 36 and protons to 46. On the basis of the molecular formula, eight nitrogens, nine oxygens, and 10 exchangeable protons were also present in **1**.

The ^1H and DEPTQ NMR spectra of **1** (Table 1) displayed signals characteristics of a peptide: six exchangeable amide NH protons (δ_H 8.55, 8.40, 8.17, 7.90, 7.78, and 7.36) as well as one NH_2 group (δ_H 7.33 and 6.78) of a glutamine were evident. The amide protons correlated with α-hydrogen signals (δ_H 4.93, 4.37, 4.33, 4.32, 4.17, 4.01, and 3.53) in the COSY spectrum. In the upfield region of the spectra, six doublet or triplet methyl groups appeared with chemical shifts at δ_H 0.93, 0.90, 0.86, 0.83, 0.80, and 0.72. The two downfield doublets at δ_H 6.86 (d, H-5/6, J = 8.5 Hz) and 6.61 (d, H-8/9, J = 8.5 Hz) were assigned to a *para*-substituted phenyl ring, also apparent in the DEPTQ spectrum. The presence of seven amide carbonyl resonances (δ_C 173.68, 172.93, 172.58, 172.56, 171.28, 170.15, and 170.31) in the DEPTQ NMR spectrum further implied the peptidic nature of this compound. An unusual low-field resonance at δ_C 166.0 connected to a proton at 6.80 ppm was also observed (Table 1). This resonance was assigned as the carbon of an imino double bond in a modified Leu residue. This accounted for all eight nitrogens required by the molecular formula as well as eight of the exchangeable hydrogens and seven of the oxygens. The two remaining protons and oxygens were assigned to hydroxy functional groups.

The presence of Gly, Val, Gln, Ile, Ser, Tyr, and a modified Leu residue was determined by interpretation of ^1H, DEPTQ, and 2D COSY, TOCSY, HSQC, HMBC, and NOESY spectra (Table 1).

The amino acid sequence of the macrocycle was confirmed by analysis of the HMBC correlations. However, resonance overlap between the Gln/Ser and Ile/Val carbonyls could not be resolved by standard gradient HMBC. Correlations to these carbons in the HMBC spectrum were at first ambiguous due to insufficient digital resolution (33557 Hz/128 points − 262 Hz/pt) in the ^{13}C dimension, even at the highest field available to us. Digital resolution in the ^{13}C dimension can be increased significantly through selective excitation of a narrow region (20 ppm) of the ^{13}C spectrum using

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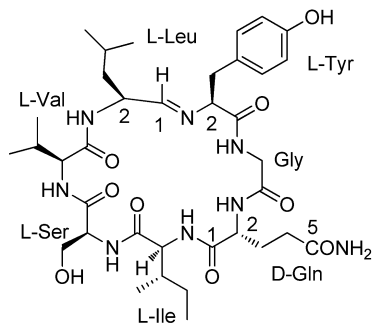
10.1021/np100600z
Published on Web 11/08/2010



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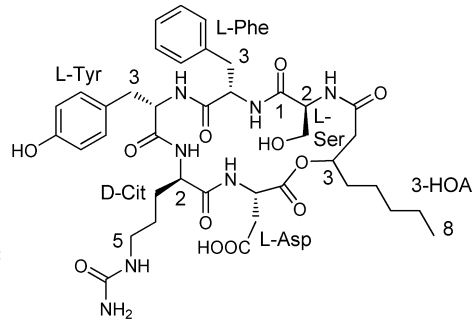

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shaped pulses. These so-called "band-selective" or "semi-selective" HMBC experiments incorporate selective pulses on the H or X channel to excite only a subset of all carbon resonances and so focus the 2D correlation spectrum on these alone.[18,19]

The "band-selective" HMBC experiment was applied to the carbonyl region of **1** (centered at 172 ppm). Selective excitation with a 1.2 ms custom Sinc pulse yielded digital resolution of 3018/256 points (12 Hz/pt), in about one-third the instrument time compared to regular HMBC. The resulting 20-fold increase in digital resolution allowed for unambiguous assignment of the carbonyl carbon signals (Table 1 and Figure 1). The connectivity of the modified Leu to Val was established by HMBC correlation from Leu-NH (δ_H 7.90) to C-1 of Val (δ_C 170.31). Correlations between Val-2 (δ_H 4.01) and C-1 of Ser (δ_C 172.56) connected these two amino acids. The adjacent position of Ser and Ile was established by HMBC correlation of Ser-NH (δ_H 7.36) to Ile-1 (δ_C 170.15).

Table 1. NMR Data for Scytonemide A (**1**) in DMSO-d_6[a]

	position	δ_C, mult.	δ_H, mult., J(Hz)	COSY	HMBC[b]	NOESY/ROESY
Tyr	1	172.93, C				
	2	74.0, CH	3.53, dd (11.3, 2.8)	H-3a,3b	4, Leu 1	H-5/9, H-1 Leu
	3a	40.1, CH_2	3.04, dd (13.5, 2.4)	H-2	1, 4	H-5/9
	3b		2.48, ddd (13.5, 13.5, 2.4)			
	4	127.6, C				
	5/9	131.4, CH	6.86, d (8.5)	H-6/8	3, 7, 9/5	H-3, 3′, H-6/8
	6/8	115.3, CH	6.61, d (8.5)	H-5/9	4, 8/6	H-5/9
	7	156.4, C				
	OH					
Gly	1	171.28, C				
	2a	40.6, CH_2	4.32, m	NH	1, Tyr 1	H-2b
	2b		3.42, m		1, Tyr 1	H-2a, NH
	NH		7.78, dd (10.0, 2.6)	H-2	2, Tyr 1	H-2a, H-2 Gln, Gln-NH_2, H-3b Ser, H-2 Tyr, H-2 Val
Gln	1	172.58, C				
	2	53.8, CH	4.33	H-3a,3b, NH	1	H-3a, 3b, H-4b, NH, Ile-NH
	3a	25.7, CH_2	1.85, m	H-2, H-4a,4b	1, 4	H-2
	3b		1.76, m		1, 5	
	4a	31.8, CH_2	2.11	H-3a,3b	1, 5	H-2
	4b		1.99			
	5	173.68, C				
	NH		8.55, d (6.3)	H-2	1, 2, 3, Gly 1	H-2, H-2a, 2b Gly
	NH_2		7.33, br s		5	H-4
			6.78, br s		4	
Ile	1	170.15, C				
	2	58.0, CH	4.17, dd (9.2, 3.9)	H-3, NH	1, 3, 4, Gln 1	H-3, NH, Ser-NH
	3	35.7, CH	2.06, m	H-4, 3-Me	2	H-2, 3-Me, NH
	3-Me	16.4, CH_3	0.86, d (7.0)	H-3	2, 3, 4	H-3, NH
	4	24.1, CH_2	1.28, m	H-3, 5	2, 3-Me	
	5	12.5, CH_3	0.83, m	H-4	3, 4	
	NH		8.17, d (9.3)	H-2	2, 3, Gln 1	H-2, Gln-2, Ser-NH
Ser	1	172.56, C				
	2	53.0, CH	4.93, m	H-3a, NH	1, 3	H-3a, 3b, NH, Val-NH
	3a	62.8, CH_2	3.87, dd (10.0, 10.0)	H-2	1, 2	H-2, H-3b
	3b		3.60, dd (9.8, 6.0)		1	H-2, NH, Gly-NH, H-3b Tyr, H-5/9 Tyr
	OH					
	NH		7.36, d (9.0)	H-2	Ile 1	H-2, 3, Ile-NH, Ile-2, Gln-2, Val-2
Val	1	170.31, C				
	2	59.7, CH	4.01, dd (7.7, 4.2)	H-3, NH	1, 3, 3-Me, Ser 1	H-3, Leu-NH
	3	29.0, CH	2.22, m	H-4, 3-Me	1	H-2, H-4
	3-Me	17.4, CH_3	0.90d (7.0)	H-3	2, 3, 4	H-3
	4	19.7, CH_3	0.93, d (7.0)	H-3	2, 3, 3-Me	H-3
	NH		8.40, brs	H-2		H-Ser-2
Leu	1	166.0, CH	6.80, d (1.8)	H-2	3, Tyr 2	H-Tyr-2
	2	49.6, CH	4.37, m	H-1, 3, NH	1, 3, 4	H-1, H-3a, 3b, H-4, NH
	3a	40.6, CH_2	1.05	H-2, 4	1, 4-Me, 5	H-2, 4-Me
	3b		0.81		1	
	4	24.8, CH	1.29, m	H-3a, 4-Me	2, 3	
	4-Me	23.4, CH_3	0.80, d (6.7)	H-4	3, 4, 5	H-3a
	5	21.7, CH_3	0.72, d (6.5)	H-4	3, 4, 4-Me	
	NH		7.90, d (9.4)	H-2	2, 3, Val 1	H-2, Val-2, Tyr-5/9

[a] Frequency: 900 MHz for ^{1}H and 226 MHz for ^{13}C. [b] HMBC correlations are from the proton(s) stated to the indicated carbon.

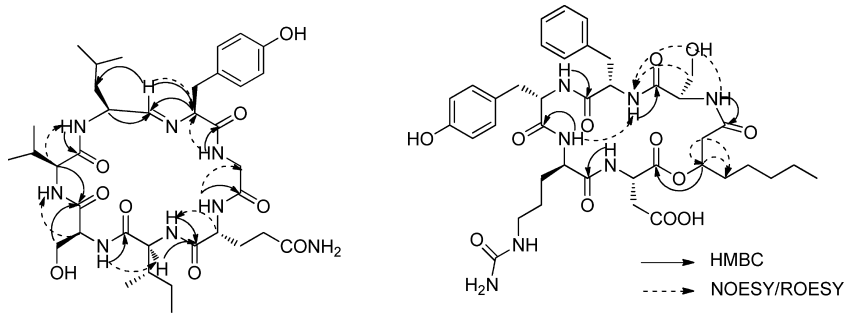

Figure 1. Key HMBC and NOESY correlations in **1** and **2**.

The Gln residue was determined to be adjacent to Ile by the HMBC correlation from H-2 of Ile (δ_H 4.17) and C-1 of Gln (δ_C 172.58). The signal at lowest field (δ_C 173.68) was assigned as the C-5 carbonyl of the Gln residue, based on the HMBC correlations from Gln protons 3a/b (δ_H 2.11 and 1.99) and 4a/b (δ_H 1.85 and 1.76) to C5. Gln was connected to Gly on the basis of the HMBC correlation from Gln-NH (δ_H 8.55) to Gly-1 (δ_C 171.28). Correlations from both H-2a (δ_H 4.32) and NH of Gly (δ_H 7.78) to C-1 of Tyr (δ_C 172.93) confirmed the linkage of these two residues. The structure of **1** was completed by the HMBC correlation from H-2 of Tyr (δ_H 3.53) to the C-1 methine (δ_C 166.0) of the modified Leu, supporting the imino linkage and closing the heptapeptide macrocycle. The amino acid sequence was also confirmed by NOE correlations between alpha protons and amide protons on the neighboring amino acid residue (Table 1 and Figure 1).

The determination of the absolute configuration of the amino acid units in **1** was carried out by chemical degradation. Acid hydrolysis of **1** followed by advanced Marfey's analysis[20,21] of the resulting hydrolysate showed the presence of six amino acids: Gly, L-Tyr, L-Ser, L-Ile, D-Gln (as D-Glu), and L-Val. Peroxidation of **1** with *meta*-chloroperbenzoic acid (*m*-CPBA) and subsequent hydrolysis indicated the presence of an additional amino acid, L-Leu, in the acid hydrolysate (see Experimental Section). On the basis of these results, the structure of scytonemide A was concluded as shown.

Scytonemide B was obtained as a yellow, amorphous solid. The molecular formula was determined as $C_{39}H_{53}N_7O_{12}$, on the basis of the HRESIMS of $[M + H]^+$ and pseudomolecular ion $[M + Na]^+$ peaks at *m*/*z* 812.3825 and 834.3640, respectively. The NMR data set for **2** was collected in a 3 mm Shigemi microcell assembly on a 900 MHz spectrometer equipped with a carbon-sensitive cryoprobe. The initial structure elucidation of **2** was carried out on the first 247 μg of sample.[22] Even this small amount of sample allowed for rapid collection and assignment of all ^{13}C resonances through HSQC/HMBC experiments. Subsequent direct ^{13}C data collection with a DEPTQ experiment[23,24] was performed with material reisolated from a larger culture. The peptidic nature of **2** was evident from the ^1H NMR spectrum in DMSO-d_6 (Table 2), where four amide proton signals were observed. Two of the amide protons were found to be isochronous and remained unresolved at δ_H 8.50 even at 900 MHz. Two additional broad signals corresponding to three protons were found at δ_H 5.75 and 5.35. These protons were determined to be a part of the ureido moiety of citrulline (Cit), based on the number of nitrogens implied by the molecular formula. Two additional singlets, located further downfield (above δ_H 9.0 ppm), were assigned as the OH protons of Tyr and Ser. The exchangeable nature of these protons was confirmed by the HSQC spectra.

Detailed analysis of the ^1H NMR spectrum, combined with the 2D COSY, TOCSY, HSQC, HMBC, and NOESY NMR data for **2** (Table 2), revealed the presence of the amino acids Phe, Tyr, Asp, Ser, and Cit in the molecule (Figure 1). In the α-proton region, a multiplet observed at δ_H 4.88 did not display correlations to any amide proton. The COSY pattern of this multiplet was sufficiently resolved at 900 MHz to allow mapping of its correlations to the remaining protons in the spin system. This, combined with the HMBC correlations from δ_H 4.88 to the ester carbonyl resonance at δ_C 168.3, identified the fragment as 3-hydroxyoctanoic acid (3-HOA). Together, these moieties accounted for 16 (two aromatic rings and eight ester/amide carbonyls) of the 17 degrees of unsaturation required by the molecular formula, and the macrocyclic ring represented the remaining degree of saturation. The subsequent DEPTQ spectrum confirmed the number of carbon signals ascertained through indirect methods.

The amino acid sequence was determined by a combination of HMBC and ROESY data (Table 2). The ^{13}C NMR chemical shifts for the amide bond carbonyls belonging to amino acids Cit (δ_C 171.34), Phe (δ_C 171.25), and Asp (δ_C 171.48) were virtually indiscernible in a standard HMBC spectrum and were resolved by application of a band-selective HMBC experiment. The Tyr-Cit-Asp sequence was established by HMBC correlations from Asp-NH (δ_H 8.17) to C-1 of Cit (δ_C 171.34) and from Cit-NH (δ_H 7.58) to C-1 of Tyr (δ_C 172.0). A second sequence, Phe-Ser-3-HOA, was identified by the HMBC correlations from Phe-NH (δ_H 8.54) to C-1 of Ser (δ_C 172.6) and from Ser-NH (δ_H 8.50) to C-1 of 3-HOA (δ_C 168.3). HMBC correlations from Tyr-NH (δ_H 8.49) to C-1 of Phe (δ_C 171.25) and from H-3 of 3-HOA (δ_H 4.88) to C-1 of Asp (δ_C 171.48) connected these two fragments, thus completing the planar structure of **2**. The amino acid sequence was also confirmed by NOE correlations between alpha protons and amide protons on the neighboring amino acid residue (Table 2 and Figure 1).

The determination of the absolute configuration of the amino acid units in **2** was carried out by chemical degradation. Acid hydrolysis of **2** followed by advanced Marfey's analysis[20,21] of the resulting hydrolysate confirmed the presence of D-Cit and showed the presence of four other amino acids: L-Asp, L-Ser, L-Tyr, and L-Phe. Attempts to selectively hydrolyze **2** at the site of the ester bond for Mosher's analysis were not successful under various reaction conditions, and the configuration at C-3 of the 3-HOA moiety was not determined. The final structure of scytonemide B is as shown.

Both isolates were evaluated to determine their potential inhibition of the 20S proteasome. Scytonemide A was found to be a potent inhibitor of the 20S proteasome chymotrypsin catalytic activity (IC_{50} = 96 nM, with bortezomib as positive control), while scytonemide B was not active ($IC_{50} > 50$ μM). Scytonemide A possesses a rare imine linkage, which has been encountered previously only as minor constituents from one species of *Nostoc*.[25] The major structural difference between the reported nostocyclopetides A1/A2[25] and **1** is the replacement of 4-MePro found in the nostocyclopeptides with Val in **1**. The possible mechanistic explanation for the action of noncovalent inhibitors of the 20S proteasome has been recently explored by a combination of solution NMR and X-ray crystallography.[26,27] Nonpolar amino acid residues bind to the auxiliary hydrophobic binding site of the 20S proteasome, while Tyr and Asn (or Gln as seen in **1**) side chains appear to be crucial for optimal fit in the main binding pocket. The presence of an imine in scytonemide A also opens up the possibility of a nucleophilic attack at the

Table 2. NMR Data for Scytonemide B (**2**) in DMSO-d_6[a]

unit	position	δ_C, mult.	δ_H, mult., *J*(Hz)	COSY	HMBC[b]	ROESY
Ser	1	172.6, C				
	2	55.3, CH	4.41 dd (7.8, 4.6)	H-3a, 3b′, NH	1, 3	NH, Phe NH
	3a	62.9, CH_2	4.37 dd (11.4, 4.6)	H-1, OH	1, 2	Phe NH
	3b		3.84 dd (12.3, 3.6)			OH, NH
	OH		3.84 d (11.2)	H-2, H-2′		H-3b, H-3bTyr
	NH		8.50 d (7.6)	H-1	2, 3, 1 HOA	H-2, H-3b, Phe NH, H-2 3-HOA
Phe	1	171.25, C				
	2	56.5, CH	4.20 ddd (9.1, 4.1, 4.0)	H-3a, NH	1, 3, NH	H-3b, NH
	3a	35.3, CH_2	3.02, dd (14.8, 3.4)	H-2, H-3b	2, 5	H-2, NH, H-5/9
	3b		2.89, dd (14.7, 9.8)	H-2, H-3a		
	4	137.7, C				
	5,6,8,9	128.6, CH	7.23, m	H-7	3, 7	
	7	126.4, CH	7.20, dt (7.1, 7.6)	H-6, H-8	5, 6, 7, 8	
	NH		8.54, d (4.8)	H-1	Ser 1	H-2, H-3a, H-3b, Ser NH, Ser H-2, Ser H-3a, Cit NH
Tyr	1	172.0, C				
	2	57.2, CH	4.10, ddd (12.0, 7.5, 3.0)	H-3a, NH	3, 4, NH	H-3a, H-3b, NH
	3a	35.4, CH_2	2.96, dd (14.1, 12.4)	H-2, H-3b	2, 5/9, NH	H-3a, H-5/9
	3b		2.75, dd (14.2, 3.2)	H-2, H-3aNH, Ser OH		
	4	128.3, C				
	5,9	129.7, CH	7.10, d (8.5)	H-6, H-8	3	H-2, H-3
	6,8	115.0, CH	6.67, d (8.5)	H-5, H-9	5/9	
	7	155.7, C				
	OH		9.24, s		6, 7	
	NH		8.49, d (7.6)	H-2	2, 3, Phe 1	
Asp	1	171.48, C				
	2	49.4, CH	4.32, ddd (5.7, 2.5, 2.5)	NH	1, 3, NH	H-3b
	3a	39.5, CH_2	2.54,	H-3b	4, NH	H-2, H-3b
	3b		2.27, dd (13.7, 6.0)	H-3a	2, 4, NH	H-2, H-3a, NH
	4	173.3, C				
	NH		8.17, d (8.4)	H-2	Cit 1	H-2, H-3b
Cit	1	171.34, C				
	2	52.7, CH	4.14, ddd (11.0, 7.0, 3.0)	H-3b, NH	1, 3	NH
	3a	28.5, CH_2	1.90, m	H-3a, H-4a, H-4b	NH	NH
	3b		1.66, m	H-2, H-3b, H-4bNH		
	4a	25.8, CH_2	1.47, m	H-4b, H-3b, H-5		
	4b		1.28, m	H-3b, H-3a, H-4b, H-5		
	5a	39.1, CH_2	2.89, m	H-4a, NH (guan.)	3	
	5b		2.83, m	H-4′		
	NH (amino)		7.58, d (8.6)	H-2	Tyr 1	
	NH (guan.)		5.76, s	H-5		
	NH2 (guan.)		5.35, s			
	6	158.6, C			5	
3-HOA	1	168.3, C				
	2a	40.3, CH_2	2.59, dd (13.5, 3.3)	H-2b	1, 3	H-3, Ser NH
	2b		2.10, dd (13.4, 3.5)	H-2a, H-3		H-3
	3	71.4	4.88, ddd (8.0, 3.4, 2.0)	H-2a, H-2b, H-4	Asp 1	H-2a, H-2b, H-4a. H-4b, H-5a
	4a	31.4, CH_2	1.54, m	H-3, H-4′, H-5	5	H-3
	4b		1.44, m	H-4, H-5		H-3
	5a	31.0, CH_2	1.08, m	H-4, H-4′, H-5′, H-6	4, 6, 7, 8	H-3
	5b		1.14, m	H-5		
	6a	25.1, CH_2	1.26, m	H-5	8	
	6b		1.09, m	H-5		
	7/7′	22.2, CH_2	1.23, m	H-8	5	
						H-6a
	8	13.9, CH_3	0.83, t (7.4)	H-7	6, 7	H-7

[a] Frequency: 900 MHz for ^1H and 226 MHz for ^{13}C. [b] HMBC correlations are from the proton(s) stated to the indicated carbon.

N-terminal Thr1OH and formation of a covalent linkage, similar to what has been described for other irreversible proteasome inhibitors.

Scytonemide A, while a potent 20S proteasome inhibitor in vitro, lacks activity in the HT-29 cell line ($ED_{50} > 20\ \mu g/mL$), even though the HT-29 cell line has been reported to be sensitive to the potent proteasome inhibitor bortezomib.[28] This difference may be explained by a lack of cell wall penetration or metabolic instability of scytonemide A. To evaluate if scytonemide A penetrates the cell wall, we performed a luminescence-based cellular 20S proteasome assay. Scytonemide A showed 80% inhibition at 6.7 μM, a concentration where no cytotoxic effect was observed in the same cell line. This indicates that chemical or metabolic instability in the longer HT-29 cytotoxic assay (3 days vs 2 h for cellular 20S proteasome assay) may be the reason for the lack of cytotoxic response. This aspect may be improved by chemical modifications of this compound.

Scytonemide B is a depsipeptide containing D-Cit and a short-chain β-hydroxy fatty acid in the macrocycle. Substituted and functionalized 3-hydroxyoctanoic acid derivatives have previously been found in cyanobacterial depsipeptides, such as yanucamide A, lyngbyabellin B, and trungapeptines A−C, but not the unsubstituted 3-hydroxyoctanoic acid.[29−31] 3-Hydroxyoctanoic acid has been previously identified as a minor constituent in cyanobacterial mats from Antarctic lake sediments.[32] Similarly, the presence of D-Cit has not been previously reported in freshwater cyanobacteria. There are a few examples in the literature of incorporation of L-Cit in the peptide macrocycle, including nostopeptins E−K and the recently reported symplocamide A, from a marine *Symploca* sp.[33,34] Metabolic selection and incorporation of Cit over Arg or metabolic

conversion of Arg to Cit via a deamination process and subsequent epimerization are associated with nonribosomal peptide synthase complexes.[4]

Structurally diverse cyclic peptides **1** and **2** represent the metabolic "fingerprint" of *S. hofmanii*. It would be of great interest to establish the functional roles that the unique building blocks play in both **1** and **2**.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. UV spectra were obtained on a Varian Cary 50 UV−vis spectrophotometer equipped with a Xenon flash lamp. IR spectra were obtained on a JASCO FTIR-410 Fourier transform infrared spectrometer. NMR spectra were obtained on a Bruker AV900 MHz NMR spectrometer with a 5 mm ATM CPTCI Z-gradient probe, referenced to the corresponding solvent peaks. Low-resolution ESI mass spectra were obtained on a ThermoFinnigan TSQuantum triple quadrupole mass spectrometer and an Agilent 1946A LC-MSD single quadrupole LC-mass spectrometer. High-resolution ESI mass spectra were obtained on a Thermo Electron LTQ FT ICR mass spectrometer. LC-MS data for the advanced Marfey's reaction were collected on an Agilent 1100 LC/MSD instrument.

Biological Material. *Scytonema hofmannii* was acquired from the Culture Collection of Algae at the University of Texas at Austin (UTEX 1834). The cyanobacterium was grown in a 2.8 L Fernbach flask containing 1 L of inorganic media (Z-45) without aeration.[35] Cultures were illuminated with fluorescent lamps at 2 klx with an 18/6 h light/dark cycle. The temperature of the culture room was maintained at 20 °C. After 6−8 weeks, the biomass of cyanobacteria was harvested by centrifugation and then freeze-dried.

Extraction and Isolation. The combined biomass from two 1 L cultures was extracted by repeated maceration with CH_2Cl_2−MeOH (1:1) to yield 270.5 mg of extract, subsequently fractionated on silica gel using a gradient with increasing polarity [hexane−EtOAc (4:1), EtOAc−2-PrOH (5:2), 2-PrOH, MeOH] to afford eight fractions. Fraction 8 (4 mg) was subjected to reversed-phase HPLC (Alltima C_8, 5 μm, 250 × 10 mm, 3 mL/min) with a solvent gradient of 5% MeCN−95% H_2O to 100% MeCN over 35 min to afford scytonemide B (**2**) (t_R = 14.2 min, 0.247 mg).

A second harvest from eight 1 L of cultures was extracted by repeated maceration with CH_2Cl_2−MeOH (1:1) to yield 257 mg of organic extract. The extract was fractionated using silica gel with the same method as described above. Fraction 8 (102.3 mg) was subjected to reversed-phase HPLC (Alltima C_8, 5 μm, 250 × 10 mm, 3 mL/min) with a solvent gradient of H_2O−MeCN (95:5) to 100% MeCN over 35 min to afford scytonemide A (**1**, t_R = 19.2 min, 1.2 mg) and scytonemide B (**2**, t_R = 14.2 min, 0.9 mg).

Scytonemide A (1): $[\alpha]^{25}_D$ −15 (*c* 0.0013, MeOH); UV λ_{max} (log ε) 201 (3.45), 224 (3.42), 277 (2.70) nm; IR (neat) ν_{max} 3734, 3296, 2932, 2360, 1650, 1540, 1418, 1257, 1025, 800, 746 cm^{-1}; ^1H NMR and ^{13}C NMR data shown in Table 1; ESIMS *m/z* 745 ($M + H^+$, 30), 763 (100), 767 (40), 783 (55); FTMS *m/z* $[M + H]^+$ 745.4186 (calcd for $C_{36}H_{57}N_8O_9$, 745.4165).

Scytonemide B (2): $[\alpha]^{25}_D$ −10 (*c* 0.0014, CH_3OH); UV λ_{max} (log ε) 209 (4.15), 226 (3.87), 277 (3.20), 365 (2.30) nm; IR (neat) ν_{max} 3285, 2925, 1651, 1540, 1516, 1396, 1253, 1026, 827, 745 cm^{-1}; ^1H NMR and ^{13}C NMR data shown in Table 2; ESIMS *m/z* 812 ($M + H^+$, 19), 834 (100); FTMS *m/z* $[M + H]^+$ 812.38247 (calcd for $C_{39}H_{53}N_7O_{12}$, 812.38250).

Peptide Hydrolysis. Samples of 80 μg each of **1** and **2** were hydrolyzed with 6 N HCl over the course of 18 h at 110 °C. Each hydrolysate was evaporated to dryness under a stream of air and redissolved in 100 μL of deionized H_2O.

Advanced Marfey's Analysis[20,21] of 1 and 2. To a 10% acetone solution (50 μL) of l-fluoro-2,4-dinitrophenyl-5-alanine amide (L-FDAA) and 1 M $NaHCO_3$ (40 μL) was added 100 μL of the acid hydrolysate of each peptide, and the mixture was kept at 40 °C for 1 h. After cooling to room temperature, 40 μL of 2 N HCl was added, and the resulting solution dried completely and then dissolved in 250 μL of MeCN. A 5 μL aliquot of each FDAA derivative was analyzed by reversed-phase LC-UV and LC-MS in the ESI positive-ion mode. A MeCN gradient (20−50% in H_2O) was used to separate the FDAA derivatives of **1** with UV detection at 340 nm on a 4.6 × 250 mm C_{18} column. The peaks were identified by co-injection with a D/L-mixture (L and DL for Cit in **2**) of the amino acid derivatives. For the FDAA derivatives of **2**, a MeCN (containing 0.1% formic acid) gradient (18−55% in H_2O) was used on a 2.1 × 50 mm C_{18} column. The following ions were found for **1**, corresponding to the Marfey's derivatives: Tyr *m/z* 434 $[M + H]^+$, Gly *m/z* 328 $[M + H]^+$, Glu (for Gln) *m/z* 400 $[M + H]^+$, Ile *m/z* 384 $[M + H]^+$, Ser *m/z* 358 $[M + H]^+$, Val *m/z* 370 $[M + H]^+$. The following ions were found for **2**, corresponding to the Marfey's derivatives: Phe *m/z* 418 $[M + H]^+$, Ser *m/z* 358 $[M + H]^+$, Asp *m/z* 368 $[M + H]^+$, Cit *m/z* 428 $[M + H]^+$, Tyr *m/z* 434 $[M + H]^+$. Retention times of the FDAA derivatives of **1** indicated the presence of L-Tyr, D-Glu, L-Ile, L-Ser, and L-Val. Retention times of the FDAA derivatives of **2** revealed L-Phe, L-Ser, L-Asp, D-Cit, and L-Tyr (see Supporting Information).

Peracid Oxidation of 1. A 0.2 mg aliquot of the peptide was oxidized by *m*-CPBA (0.1 mg) in 0.5 mL of EtOH−CH_2Cl_2 (1:1) for 6 h. The reaction was quenched with an excess of DMSO, and after 2 h, the solvent was removed and the oxidized product was subjected to acid hydrolysis and advanced Marfey's method analysis.[20,21] L-Leu was identified in the acid hydrolysate in addition to the six amino acids found in the acid hydrolysate of the unmodified peptide; Leu *m/z* 384 $[M + H]^+$.

20S Proteasome Bioassay. Evaluation of 20S proteasome inhibition was performed as reported previously.[36]

Cytotoxicity Bioassay. Cytotoxicity against HT-29 human cancer cell lines was conducted as reported previously.[37]

Acknowledgment. This research was supported by NIH grants R01 GM0758556 and P01 CA125066. The 900 MHz NMR spectrometer was funded by NIH grant P41 GM68944. We thank C. Pennington and C. A. Crot of the Mass Spectrometry Laboratory at the University of Illinois at Chicago for acquiring low-resolution MS and FT-MS data.

Supporting Information Available: 1D and 2D NMR data and results of Marfey's analyses of **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP100600Z