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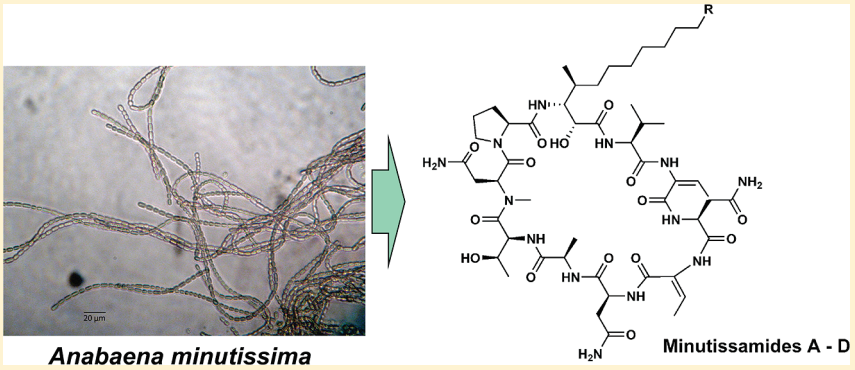
Minutissamides A−D, Antiproliferative Cyclic Decapeptides from the Cultured Cyanobacterium *Anabaena minutissima*

Hahk-Soo Kang, Aleksej Krunic, Qi Shen, Steven M. Swanson, and Jimmy Orjala*

Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 833 S. Wood Street, Chicago, Illinois 60612, United States

Supporting Information

ABSTRACT:



Four cyclic decapeptides, minutissamides A−D (**1**−**4**), were isolated from the cultured cyanobacterium *Anabaena minutissima* (UTEX 1613). The planar structures were determined using various spectroscopic techniques including HRESIMS and 1D and 2D NMR experiments. The absolute configurations of the α-amino acid residues were assigned using Marfey's method after acid hydrolysis. The absolute configuration of a β-amino acid residue was assigned by a combination of the advanced Marfey's method, *J*-based configurational analysis, and ROE spectroscopic analysis. The structures of minutissamides A−D (**1**−**4**) were characterized by the presence of three nonstandard α-amino acid residues (two α,β-dehydro-α-aminobutyric acids and one *N*-methylated Asn) and one β-amino acid residue (2-hydroxy-3-amino-4-methyldodecanoic acid or 2-hydroxy-3-amino-4-methylhexadecanoic acid). Minutissamides A−D (**1**−**4**) exhibited antiproliferative activity against the HT-29 human colon cancer cell line with IC_{50} values of 2.0, 20.0, 11.8, and 22.7 μM, respectively.

Cyanobacteria (blue-green algae) have been shown to be prolific producers of bioactive secondary metabolites.[1−3] A major class of secondary metabolites from cyanobacteria is polyketide-conjugated nonribosomal peptides, commonly known as lipopeptides.[4,5] Numerous cyclic lipopeptides have been isolated from both freshwater and marine cyanobacteria with ring sizes of up to 12 amino acid residues. Cyclic deca- and undecapeptides, containing 10 and 11 residues, have been reported to exhibit a wide spectrum of biological activities, including antifungal,[6,7] cardiotonic,[8,9] and cytotoxic.[10,11] A common structural feature of cyclic deca- and undecapeptides is the occurrence of one lipophilic β-amino acid residue with a structurally unique side chain.[12] Amination of fatty acids at C-3 forming β-amino acids provides the branching point via an amide bond with other α-amino acids, whereas C-2 is usually a methylene, methylated or hydroxylated. Further fatty acid modifications include methylation, oxidation, chlorination, and/or hydroxylation of the side chain, resulting in the high diversity of modified β-amino fatty acids. Most of the cyclic lipopeptides in this class also possess nonstandard amino acids, such as *N*-methylated α-amino acids, α-hydroxy or β-hydroxy amino acids, and Dhb (α,β-dehydro-α-aminobutyric acid).

In our continuing search for secondary metabolites from laboratory-cultured cyanobacteria, we evaluated the cell extract of cultured *Anabaena minutissima* (UTEX 1613). Herein we report the isolation, structure elucidation, and biological activity of four new cyclic decapeptides, named minutissamides A−D, each possessing two Dhbs, one *N*-methylated Asn, and one β-amino acid, Hamd (2-hydroxy-3-amino-4-methyldodecanoic acid) or modified Hamh (2-hydroxy-3-amino-4-methylhexadecanoic acid). The planar structures were determined using a combination of spectroscopic techniques including HRESIMS, tandem MS, and 1D and 2D NMR (COSY, TOCSY, HSQC, HMBC, and ROESY) experiments. The absolute configurations of the α-amino acid residues were determined by Marfey's method, whereas the stereoconfiguration of the Hamd residue

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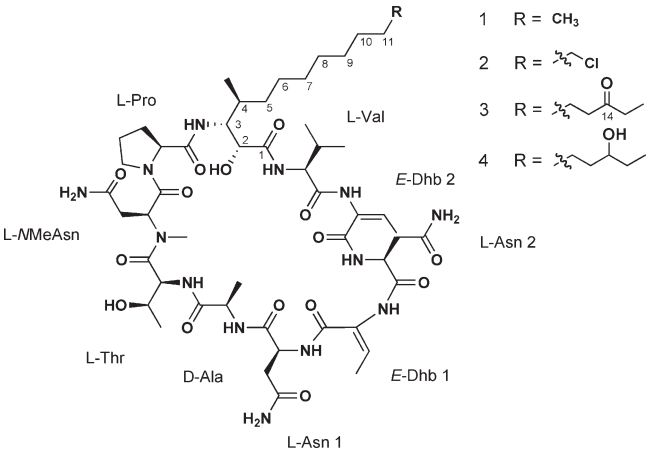
in **1** was solved by a combination of the advanced Marfey's method, *J*-based configurational analysis, and ROE correlations.



RESULTS AND DISCUSSION

A. minutissima (UTEX 1613) was grown in Z media.[13] The freeze-dried cells were extracted with a mixture of CH_2Cl_2 and MeOH (1:1) and dried in vacuo. The cell extract was subsequently fractionated by column chromatography using Diaion HP-20 resin and an increasing amount of isopropyl alcohol (IPA) in H_2O. LC-MS analysis of the third fraction (40% IPA) indicated the presence of peptides with molecular weights of 1118, 1152, 1188, and 1190 Da. Subsequent Sephadex LH-20 column chromatography followed by reversed-phased HPLC yielded minutissamides A−D (**1**−**4**).

Minutissamide A (**1**) was obtained as a colorless, amorphous powder. The molecular formula of **1** was determined as $C_{51}H_{83}N_{13}O_{15}$ by HRESIMS analysis (*m*/*z* 1140.6049 $[M + Na]^+$). The ^1H NMR spectrum in DMSO-d_6 showed a signal distribution typical for lipopeptides, including several exchangeable amide NH signals (6.5−10.5 ppm), signals in the α-proton region (4−6 ppm), methylene signals (1.2−1.3 ppm), and numerous aliphatic doublet and triplet methyl signals (0.5−2.0 ppm). The structures of the amino acid residues were elucidated by interpretation of the COSY and TOCSY spectra (Figure 1 and Table 1) and indicated the presence of 10 residues, including nine α-amino acid residues and one *β*-amino acid residue. Six of the α-amino acid residues were identified as the standard amino acids proline (Pro), threonine (Thr), alanine (Ala), valine (Val), and two asparagines (Asn). In addition, the structures of three nonstandard amino acid residues were determined by analysis of COSY, HSQC, and HMBC data (Figure 1). An HMBC correlation from the *N*-methyl singlet (δ_H 2.96) to the C-2_{NMeAsn} (δ_C 49.4) in combination with a COSY correlation between *N*MeAsn H-2 (δ_H 5.53) and *N*MeAsn H_2-3 (δ_H 2.00 and 3.02) identified the structure of *N*-methylasparagine (*N*MeAsn). The presence of two Dhb residues was deduced by COSY correlations observed between the methyl protons (H_3-4_{Dhb1}, δ_H 1.90 and H_3-4_{Dhb2}, δ_H 1.66) and the corresponding olefinic proton (H-3_{Dhb1}, δ_H 5.71 and H-3_{Dhb2}, δ_H 5.30), and HMBC correlations from the methyl protons (H_3-4_{Dhb1} and H_3-4_{Dhb2}) to the corresponding olefinic carbon (C-2_{Dhb1}, δ_C 130.1 and C-2_{Dhb2}, δ_C 133.0). The 2-hydroxy-3-amino-4-methyl portion in Hamd was identified by sequential COSY correlations between H-2 (δ_H 4.18)/H-3 (δ_H 3.93)/H-4 (δ_H 1.65), as well as COSY correlations between H-3

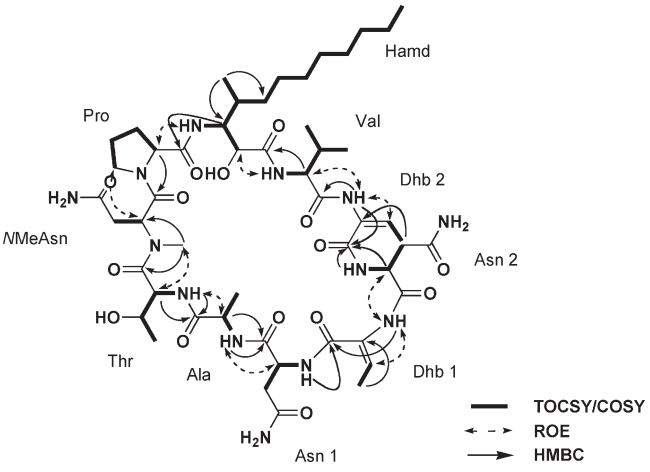


Figure 1. Key 2D correlations of minutissamide A (**1**) that were used for the determination of the planar structure of **1**.

(δ_H 3.93) and 3-NH (δ_H 6.85) and between H-4 (δ_H 1.65) and 4-Me (δ_H 0.58). Further COSY correlations from H-4 (δ_H 1.65) to H_2-5 (δ_H 1.17 and 1.61), H_2-5 to H_2-6 (δ_H 1.18 and 1.24), and H_2-6 to the region of highly overlapped methylene signals (H_2-7−11, δ_H 1.26−1.27), corresponding to five carbons in the HSQC spectrum (δ_C 30.1, 29.2, 29.7, 31.8, and 22.6), and finally to the methyl triplet protons H_3-12 (δ_H 0.87), completed the structure of Hamd.

The sequence of the 10 residues was established by analysis of the ROESY and selective HMBC spectra (Figure 1 and Table 1). A selective HMBC spectrum for the carbonyl region was used to resolve the overlapped amide carbonyl signals.[14] HMBC correlations from Dhb_2-NH (δ_H 9.17) to Val C-1 (δ_C 169.2), from Val H-2 (δ_H 4.29) to Hamd C-1 (δ_C 170.1), and from Hamd H-3 (δ_H 3.93) to Pro C-1 (δ_C 171.9) suggested a partial sequence of Dhb_2-Val-Hamd-Pro. ROE correlations between Dhb_2-NH (δ_H 9.17) and Val H-2 (δ_H 4.29), between Val-NH (δ_H 6.85) and Hamd H-2 (δ_H 4.18), and between Hamd-NH (δ_H 6.85) and Pro H-2 (δ_H 4.19) confirmed this partial sequence. HMBC correlations from Pro H-2 (δ_H 4.19) to *N*MeAsn C-1 (δ_C 168.3) and from *N*MeAsn N-Me (δ_H 2.96) to Thr C-1 (δ_C 170.4), together with ROE correlations between Pro H_2-5 (δ_H 4.10) and *N*MeAsn H-2 (δ_H 5.53) and between *N*MeAsn N-Me (δ_H 2.96) and Thr H-2 (δ_H 4.63), further expanded the sequence to Dhb_2-Val-Hamd-Pro-*N*MeAsn-Thr. HMBC correlations from Thr NH (δ_H 7.30) to Ala C-1 (δ_C 172.5) and from Ala NH (δ_H 8.27) to Asn1 C-1 (δ_C 171.2), together with ROE correlations between Thr-NH (δ_H 7.30) and Ala H-2 (δ_H 4.29) and between Ala-NH (δ_H 8.27) and Asn_1 H-2 (δ_H 4.40), established the sequence of Dhb_2-Val-Hamd-Pro-*N*MeAsn-Thr-Ala-Asn_1, leaving two residues (Dhb_1 and Asn_2) unassigned. The complete sequence of Dhb_2-Val-Hamd-Pro-*N*MeAsn-Thr-Ala-Asn_1-Dhb_1-Asn_2 was deduced by an HMBC correlation from Asn_1-NH (δ_H 8.45) to Dhb_1 C-1 (δ_C 163.4) and a ROE correlation between Dhb_1-NH (δ_H 10.18) and Asn_2 H-2 (δ_H 4.92). Lastly, HMBC correlations from Asn_2 H-2 (δ_H 4.92) and NH (δ_H 8.73) to Dhb_2 C-1 (δ_C 163.6) closed the ring, completing the planar structure of **1**.

Mass fragmentation analysis was performed to further verify the structure of **1**.[15] The ESIMS/MS analysis, using a quadrupole-time-of-flight (Q-TOF) tandem mass spectrometer, supported the proposed planar structure of **1**. The parent peak was observed at *m*/*z* $[M + H]^+$ 1118.6. The ring-opening occurred

Table 1. NMR Spectroscopic Data for Minutissamides A and B (1 and 2) in DMSO-d_6

		minutissamide A (1)						minutissamide B (2)[a]		
		δ_C[b]	δ_H[c]	mult. (J in Hz)	COSY	HMBC	ROESY	δ_C[f]	δ_H[c]	mult.(J in Hz)
Hamd or Hamcd	1	170.1						170.0		
	2	70.0	4.18	d (4.8)	3	3		70.0	4.18	overlapped
	3	56.6	3.93	m	2, 4	1,[e] 2, 1_{Pro}[e]		56.6	3.92	M
	4	32.6	1.65	m	3, 5, 4-Me			32.6	1.65	m
	5	33.8	1.17	m	4, 6			33.7	1.17	m
			1.61	m					1.61	m
	6	25.9	1.18	m	5, 6			25.9	1.16	m
			1.24	m					1.23	m
	7	30.1	1.26	m	6, 8			30.1	1.23	m
	8	29.2	1.26	m	7, 9			29.7	1.23	m
	9	29.7	1.26	m	8, 10			29.1	1.26	m
	10	31.8	1.26	m	10, 11			26.6	1.37	m
	11	22.6	1.27	m	11, 12			32.4	1.70	m
	12	14.5	0.87	t (7.2)	11	10, 11		45.8	3.62	t (6.6)
	2-OH		5.51	br s	2					nd[d]
	3-NH		6.85	partly overlapped	3	1_{Pro}[e]	3, 2_{Pro}		6.88	partly overlapped
	4-Me	16.3	0.58	d (6.6)	4	3, 4, 5	3	16.4	0.57	d (6.6)
Pro	1	171.9						171.9		
	2	60.5	4.19	m	3	3, 4, 1_{NMeAsn}		60.4	4.18	m
	3	30.7	1.91	m	2, 4	1		30.7	1.92	m
	4	24.2	1.85	m	3, 5			24.2	1.83	m
			1.92	m					1.92	m
	5	47.3	3.26	m	4	2	2_{NMeAsn}	47.3	3.25	m
			4.10	m					4.09	m
*N*MeAsn	1	168.3						168.3		
	2	49.4	5.53	dd (12.0, 3.0)	3	1,[e] 3, 4[e]	5_{Pro}, 3, N-Me	49.3	5.53	br d (12.0)
	3	34.4	2.00	m	2, 4	1[e], 2[e], 4[e]		34.4	2.00	m
			3.02	dd (15.6, 12.0)					3.01	m
	4	172.1						172.1		
	N-Me	30.7	2.96	s		2, 1_{Thr}[e]	2_{Thr}, 3_{Thr}	30.7	2.95	s
	NH_2		5.92	s		3			6.03	m
			7.52	s					7.52	m
Thr	1	170.4						170.4		
	2	55.3	4.63	dd (8.4, 3.6)	2-NH, 3	1,[e] 3, 1_{Ala}[e]	N-Me, 3, 4	55.3	4.62	m
	3	66.8	3.92	m	2, 4			66.8	3.92	m
	4	20.0	1.00	d (6.0)	3	2, 3		19.9	0.99	d (6.6)
	3-OH		5.13	br s						nd[d]
	NH		7.30	d (7.8)	2	1_{Ala}	2, 3		7.30	d (8.4)
Ala	1	172.5						172.5		
	2	48.9	4.29	q (7.2)	2-NH, 3	1,[e] 1_{Asn1}[e]		48.7	4.28	m
	3	17.3	1.23	d (7.2)	2	1,[e] 2		17.3	1.22	d (7.2)
	NH		8.27	d (8.4)	2	2, 1_{Asn1}	2, 2_{Asn1}, NH_{Thr}, NH_{Asn1}			
Asn1	1	171.2						171.2		
	2	50.5	4.40	m	2-NH, 3	3	3	50.3	4.39	m
	3	36.2	2.56	m	2	1,[e] 2, 4[e]		36.2	2.53	m
			2.87	dd (18.6, 7.2)					2.88	dd (16.8, 7.2)
	4	173.2						173.2		
	NH		8.45	d (7.2)	2	1_{Dhb1}	2		8.45	d (7.2)
	NH_2		7.04	s		3			7.04	s
			7.58	s					7.59	s
Dhb1	1	163.4						163.4		
	2	130.1						130.1		

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Table 1. Continued

		minutissamide A (**1**)						minutissamide B (**2**)[a]		
		δ_C[b]	δ_H[c]	mult. (*J* in Hz)	COSY	HMBC	ROESY	δ_C[f]	δ_H[c]	mult.(*J* in Hz)
	3	125.6	5.71	q (7.2)	4	1, 2, 4	NH	125.6	5.71	q (7.2)
	4	13.7	1.90	d (7.2)	3	2, 3		13.6	1.89	d (7.2)
	NH		10.18	s		1, 1_{Asn2}[e]	3, 2_{Asn2}		10.20	s
Asn2	1	172.4						172.4		
	2	49.4	4.92	q (7.2)	2-NH, 3	1,[e] 3, 1_{Dhb2}[e]	3	49.4	4.91	q (7.8)
	3	39.1	2.67	dd (15.0, 7.2)	2	1,[e] 2, 4[e]		39.2	2.67	dd (15.0, 7.2)
			2.77	dd (15.0, 7.8)					2.77	dd (15.0, 7.8)
	4	172.6						172.7		
	NH		8.73	d (7.8)	2	1_{Dhb2}			8.74	d (7.8)
	NH_2		7.11	s		3			7.12	s
			7.58	s					7.59	s
Dhb2	1	163.6						163.6		
	2	133.0						133.0		
	3	116.5	5.30	q (7.2)	4	1, 2, 4	NH	116.4	5.29	q (7.2)
	4	13.5	1.66	d (7.2)	3	2, 3		13.3	1.65	d (7.2)
	NH		9.17	s		1,[e] 3, 1_{Val}[e]	3, 2_{Val}		9.21	s
Val	1	169.2						169.2		
	2	56.2	4.29	d (10.8)	2-NH, 3	1,[e] 1_{Hamd}[e]		56.2	4.28	m
	3	33.1	1.75	m	2, 4	1[e]		33.1	1.74	m
	4	19.4	0.83	d (6.6)	3	2, 3, 4′		19.3	0.82	d (6.6)
	4′	19.1	0.90	d (6.6)	3	2, 3, 4		19.0	0.89	d (6.6)
	NH		6.85	partly overlapped	2		2_{Hamd}		6.88	partly overlapped

[a] Complete NMR table for minutissamide B (**2**) including 2D NMR data can be found in the Supporting Information. [b] Carbon chemical shifts were assigned from DEPT-Q spectrum measured at 226 MHz. [c] Measured at 600 MHz. [d] nd: not detected. [e] Assigned from the selective HMBC spectrum. [f] Assigned from the HSQC and HMBC spectra.

between Pro and *N*MeAsn, forming an acylium ion. The continuous fragmentation yielded the fragment ions at *m/z* 990.6 $[M + H - NMeAsn]^+$, 889.5 $[M + H - NMeAsn - Thr]^+$, 704.4 $[M + H - NMeAsn - Thr - Ala - Asn_1]^+$, 621.4 $[M + H - NMeAsn - Thr - Ala - Asn_1 - Dhb_1]^+$, 507.4 $[M + H - NMeAsn - Thr - Ala - Asn_1 - Dhb_1 - Asn_2]^+$, and 396.3 $[M + H - NMeAsn - Thr - Ala - Asn_1 - Dhb_1 - Asn_2 - Dhb_2]^+$ (Figure 2). This fragmentation pattern was in complete agreement with the structure determined by 2D NMR analysis.

Minutissamide A (**1**) contained three different types of stereogenic elements, the geometric configuration of Dhbs, the configurations of the α-amino acid residues, and the configurations of the three consecutive asymmetric centers in the Hamd residue. A number of techniques were used for the assignment of stereoconfiguration of **1**, including Marfey's method and the advanced Marfey's method, *J*-based configurational analysis, and ROE correlations. A strong ROE correlation between the respective NH (δ_H 10.18 or δ_H 9.17) and the corresponding olefinic proton (H-3, δ_H 5.71 or δ_H 5.30) was observed for both Dhb_1 and Dhb_2, assigning the geometric configuration of both double bonds as *E* (Figure 1). The absolute configurations of the common amino acids and *N*MeAsn were assigned as L-Pro, L-Thr, L-Asn_1, L-Asn_2, L-Val, D-Ala, and L-*N*MeAsn by chromatographic comparison of Marfey's derivatives of the acid hydrolysate of **1** and appropriate amino acid standards.[16] The relative configurations of the three asymmetric centers (C-2, C-3, and C-4) in the Hamd residue were established by a combination of *J*-based configurational analysis and ROE correlations (Figure 3). Homonuclear ($^3J_{H-H}$) coupling constants were acquired from the DQF-COSY spectrum, and the phase-sensitive HMBC spectrum was used to obtain the heteronuclear ($^2J_{CH}$ and $^3J_{CH}$) coupling constants.[17] The small $^3J_{HH}$ (5.6 Hz) between H-2 and H-3, the large $^3J_{CH}$ (5.4 Hz) between H-3 and C-1, and the large $^2J_{CH}$ (−5.1 Hz) between H-3 and C-2 allowed for two possible conformations (A2 and A6) out of the six conformations shown in Figure 3a. The conformation A6 was found to be a correct conformation based on a ROE correlation observed between H-2 and H-4, assigning the relative configuration between C-2 and C-3 as "*erythro*". The same method was applied for the assignment of the relative configuration between C-3 and C-4. On the basis of the large $^3J_{HH}$ (11.0 Hz) measured between H-3 and H-4, only two conformations (B3 and B4) were possible, as shown in Figure 3b. A ROE correlation observed between H-2 and H_2-5 confirmed B3 as the correct confirmation, assigning the relative configuration between C-3 and C-4 as "*threo*". The nearly identical carbon chemical shifts of C-1, C-2, C-3, and C-4 of Hamd (δ_C 170.1, 70.0, 56.6, and 32.6) to those of Hamh (δ_C 169.8, 69.9, 56.2, and 32.4) in puwainaphycin E provided further evidence for this relative configuration.[10] Because the relative configurations of C2−C3 and C3−C4 were *erythro* and *threo*, respectively, the absolute configurations of the three stereogenic centers (C-2, C-3, and C-4) in the Hamd residue could be either "*RRS*" or "*SSR*". The advanced Marfey's method was applied to assign the absolute configuration of the Hamd residue.[18,19] The acid hydrolysate of **1** was derivatized with L- and DL-FDLA, respectively. Subsequent LC-MS analysis of DL-FDLA derivatives identified two peaks of the FDLA derivatives of Hamd (*m/z* 538 $[M - H]^-$) at 43.3 and 48.8 min, while the L-FDLA

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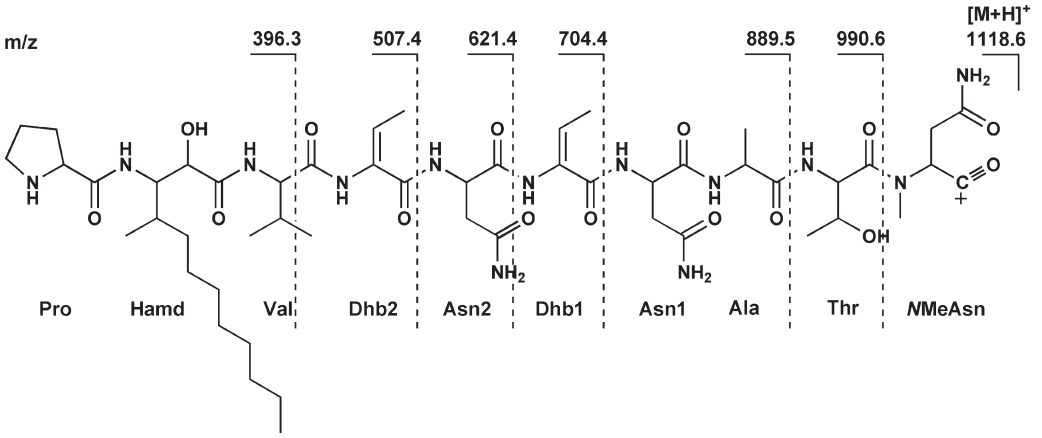

Figure 2. ESIMS/MS fragmentation of minutissamide A (**1**).

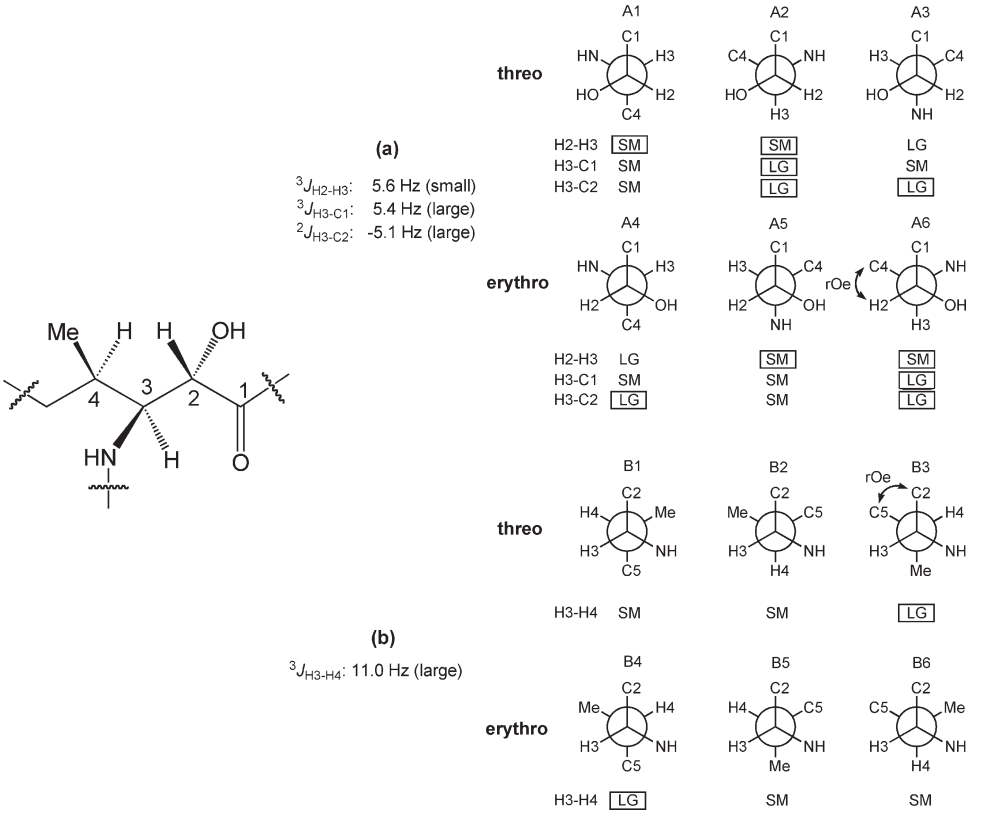

Figure 3. Newman projections for (a) C-2/C-3 and (b) C-3/C-4. All possible relative conformations are shown. The DQF-COSY and phase-sensitive HMBC spectra were used for the calculation of homo- and heteronuclear coupling constants. Labels below projections denote the predicted size of coupling constants. The predicted values that are consistent with observed values are highlighted by a box. Observed ROE correlations are presented as double-headed arrows: (a) H-2/H-4 and (b) H-2/H_2-5.

derivative of Hamd resulted in one peak at 48.8 min. Previous application of this method for the determination of the absolute configuration of Ahda (amino-2-hydroxydecanoic acid) identified that the *R*-L and *S*-D derivatives are more hydrophobic than the *R*-D and *S*-L derivatives.[20] In our experiment, the L-FDLA derivative of **1** corresponded to the later peak of DL-FDLA derivatives, suggesting the presence of *R*-L. As a result, the absolute configuration of C-3 in Hamd was assigned as *R*, which allowed for the assignment of the absolute configurations at C-2, C-3, and C-4 in Hamd as "*RRS*".

Minutissamide B (**2**) was obtained as a colorless, amorphous powder. The molecular formula of **2** was deduced as $C_{51}H_{82}ClN_{13}O_{15}$ on the basis of HRESIMS analysis (*m*/*z* 1174.5680 $[M + Na]^+$). A M + 2 isotope peak verified the presence of chlorine in **2**. The ^1H and ^{13}C NMR spectra of **2** were almost identical to those of **1** except for the Hamd residue, where the terminal methyl proton signal had been replaced by a new triplet methylene resonance at 3.62 ppm. This indicated that chlorine was positioned on the terminal carbon of Hamd, thus renaming it Hamcd (2-hydroxy-3-amino-4-methyl-12-chlorododecanoic acid). The sequential COSY correlation network of

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Table 2. ^{1}H and ^{13}C NMR Data for Minutissamides C and D (3 and 4) in DMSO-d_6

		minutissamide C (**3**)[a]			minutissamide D (**4**)[a]		
		δ_C[b]	δ_H[c]	mult. (*J* in Hz)	δ_C[b]	δ_H[c]	mult. (*J* in Hz)
Hamoh or Hamhh	1	170.0			170.1		
	2	70.0	4.17	d (4.8)	70.0	4.18	d (4.2)
	3	56.5	3.92	m	56.6	3.92	m
	4	32.6	1.65	m	32.6	1.65	m
	5	33.8	1.17	m	33.9	1.17	m
			1.62	m		1.61	m
	6	25.9	1.16	m	25.9	1.16	m
			1.23	m		1.23	m
	7	30.1	1.23	m	30.1	1.25	m
	8	29.1	1.23	m	29.1	1.25	m
	9	29.7	1.23	m	29.7	1.25	m
	10	29.4	1.23	m	29.4	1.25	m
	11	29.4	1.23	m	29.4	1.25	m
	12	23.6	1.44	q (7.2)	25.8	1.16	m
						1.35	m
	13	41.9	2.38	t (7.2)	35.4	1.26	m
						1.32	m
	14	211.5			71.5	3.28	m
	15	35.4	2.40	q (7.8)	30.1	1.16	m
						1.35	m
	16	8.2	0.90	t (7.8)	10.6	0.83	t (7.2)
	2-OH			nd[d]			nd[d]
	3-NH		6.84	partly overlapped		6.87	partly overlapped
	4-CH_3	16.4	0.57	d (6.6)	16.5	0.57	d (6.6)
Pro	1	171.9			172.0		
	2	60.6	4.18	m	60.6	4.18	m
	3	30.8	1.92	m	30.8	1.92	m
	4	24.2	1.84	m	24.3	1.83	m
			1.91	m		1.91	m
	5	47.3	3.26	m	47.3	3.25	m
			4.10	m		4.09	m
*N*MeAsn	1	168.2			168.3		
	2	49.4	5.52	dd (12.0, 3.0)	49.1	5.53	dd (12.0, 3.0)
	3	34.4	2.00	m	34.4	1.99	m
			3.01	dd (16.2, 12.6)		3.01	dd (15.6, 12.6)
	4	172.1			172.1		
	N-Me	30.8	2.95	s	30.8	2.95	s
	NH_2		5.95	s		6.01	s
			7.52	s		7.52	s
Thr	1	170.4			170.4		
	2	55.3	4.62	dd (9.0, 3.6)	55.3	4.62	dd (8.4, 3.6)
	3	66.8	3.93	m	66.8	3.91	m
	4	20.0	0.99	d (6.0)	20.0	0.98	d (7.8)
	3-OH			nd[d]			nd[d]
	NH		7.29	d (9.0)		7.30	d (7.8)
Ala	1	172.6			172.6		
	2	48.9	4.28	m	49.0	4.28	m
	3	17.3	1.22	d (7.2)	17.4	1.22	d (7.2)
	NH		8.26	d (8.4)		8.26	d (8.4)
Asn1	1	171.2			171.2		
	2	50.5	4.39	td (7.2, 3.0)	50.5	4.39	td (7.2, 3.0)
	3	36.2	2.53	m	36.2	2.52	m

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Table 2. Continued

		minutissamide C (**3**)[a]			minutissamide D (**4**)[a]		
		δ_C[b]	δ_H[c]	mult. (*J* in Hz)	δ_C[b]	δ_H[c]	mult. (*J* in Hz)
			2.87	dd (16.8, 7.2)		2.87	dd (16.8, 7.2)
	4	173.2			173.2		
	NH		8.45	d (7.2)		8.44	d (7.2)
	NH_2		7.04	s		7.04	s
			7.58	s		7.59	s
Dhb1	1	163.4			163.4		
	2	130.1			130.1		
	3	125.6	5.70	q (7.2)	125.8	5.70	q (7.8)
	4	13.7	1.89	d (7.2)	13.7	1.89	d (7.8)
	NH		10.18	s		10.19	s
Asn2	1	172.4			172.4		
	2	49.4	4.90	q (7.2)	49.5	4.91	q (7.2)
	3	39.0	2.66	dd (15.0, 7.2)	39.2	2.67	dd (15.0, 7.2)
			2.76	dd (15.0, 7.8)		2.76	dd (15.0, 7.8)
	4	172.6			172.7		
	NH		8.73	d (7.8)		8.73	d (7.8)
	NH_2		7.12	s		7.12	s
			7.59	s		7.59	s
Dhb2	1	163.6			163.7		
	2	133.0			133.0		
	3	116.4	5.29	q (7.2)	116.5	5.29	q (7.2)
	4	13.5	1.65	d (7.2)	13.5	1.64	d (7.2)
	NH		9.18	s		9.19	s
Val	1	169.2			169.3		
	2	56.2	4.28	m	56.3	4.28	m
	3	33.1	1.74	m	33.1	1.74	m
	4	19.4	0.82	d (6.6)	19.4	0.82	d (6.6)
	4′	19.1	0.89	d (6.6)	19.1	0.89	d (6.6)
	NH		6.84	partly overlapped		6.86	partly overlapped

[a] Complete NMR tables for minutissamides C (**3**) and D (**4**) including 2D NMR data can be found in the Supporting Information. [b] Carbon chemical shifts were assigned from the DEPT-Q spectrum measured at 226 MHz. [c] Measured at 600 MHz. [d] nd: not detected.

H_2-12/H_2-11/H_2-10 and the slightly downfield chemical shifts of H_2-11 (δ_H 1.70) and H_2-10 (δ_H 1.37) further supported the structure of **2**. The stereoconfiguration of **2** was determined by comparison of proton and carbon chemical shifts as well as specific rotation to those of **1**. The carbon chemical shifts of all of the stereogenic centers in **2** were similar to those observed for **1** (deviation <0.2 ppm), suggesting the same relative configurations. The specific rotations of both **1** and **2** displayed positive values (**1**, $[\alpha]_D$ +4; **2**, $[\alpha]_D$ +5). On the basis of these data and a shared biosynthesis, we propose that the absolute configurations of all of the stereogenic centers in **2** are the same as those found in **1**.

Minutissamide C (**3**) was obtained as a colorless, amorphous powder. The HRESIMS analysis (*m/z* 1210.6440 $[M + Na]^+$) suggested the molecular formula of **3** to be $C_{55}H_{89}N_{13}O_{16}$. Detailed comparison of the NMR spectra indicated that compounds **1** and **3** share the same cyclic decapeptide core structure. The difference was the replacement of the dodecanoic acid residue found in **1** with an oxidized hexadecanoic acid residue in **3** (Table 2). The ^{1}H NMR spectrum of **3** displayed three additional methylene protons, H_2-12 (δ_H 1.44), H_2-13 (δ_H 2.38), and H_2-15 (δ_H 2.40), as compared to **1**. In addition to three methylene carbon signals (δ_C 23.6, 41.9, and 35.4), a signal characteristic of a ketone (δ_C 211.5) was also apparent in the ^{13}C NMR spectrum. The molecular weight difference of 70 between **3** and **1** corresponded to three methylenes and one ketone, indicating the presence of a Hamoh (2-hydroxy-3-amino-4-methyl-14-oxohexadecanoic acid) moiety in **3**. The location of the ketone group at C-14 in Hamoh was determined by the proton chemical shifts of H_2-13 (δ_H 2.38) and H_2-15 (δ_H 2.40) and a TOCSY correlation observed between H_2-15 (δ_H 2.40) and H_3-16 (δ_H 0.90). An HMBC correlation from the terminal methyl protons (H_3-16, δ_H 0.90) to the ketone carbon (C-14, δ_C 211.5) further confirmed this location. The stereoconfiguration of **3** was assigned by comparison of spectroscopic data to those of **1**. No significant difference (deviation <0.1 ppm) was found for the ^{13}C chemical shifts of all of the stereogenic centers in **3** when compared to **1**. Also, compound **3** displayed a similarly small positive specific rotation ($[\alpha]_D$ +3) to **1**. Together, these data suggested the absolute configurations of the stereogenic centers in **3** to be identical to those of **1**.

Minutissamide D (**4**) was also obtained as a colorless, amorphous powder. The HRESIMS spectrum of **4** displayed a major ion peak at 1212.6632 $[M + Na]^+$, suggesting a molecular formula of $C_{55}H_{91}N_{13}O_{16}$. The ^{1}H and ^{13}C NMR spectra of **4** were almost identical to those of **3**, except that the ketone in the

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Hamoh moiety had been replaced by resonances for a carbinol moiety (δ_H 3.28 and δ_C 71.5). This, together with the molecular weight difference of 2 between **4** and **3**, suggested that the ketone in Hamoh of **3** had been reduced to a hydroxy group in **4**, forming a Hamhh residue (2-hydroxy-3-amino-4-methyl-14-hydroxyhexadecanoic acid). The structure of Hamhh was further supported by analysis of the COSY and HMBC spectra. Sequential COSY correlations between H_3-16 (δ_H 0.83)/H_2-15 (δ_H 1.16 and 1.35)/H-14 (δ_H 3.28) and an HMBC correlation from H_3-16 (δ_H 0.83) to C-14 (δ_C 71.5) placed the hydroxy group at C-14. The absolute configurations of the amino acid residues and the three asymmetric centers in the Hamhh residue appeared to be the same as those found for **1** by comparison of ^{13}C chemical shifts and specific rotation ($[\alpha]_D$ +2). Compound **4** possessed one more stereogenic hydroxy-bearing carbinol carbon (C-14) in the Hamhh residue. Mosher's ester analysis was attempted in an effort to assign the absolute configuration of this carbinol center. However, the presence of three hydroxy groups (one in Thr and two in Hamhh) resulted in a mixture of products and a very complex ^1H NMR spectrum of the Mosher's ester products of **4**. Hydrolysis of **4** followed by isolation of the Hamhh residue would be necessary for Mosher's ester analysis, but was not attempted due to the limited amount of sample available. Thus, the absolute configuration of the C-13 carbinol carbon in Hamhh could not be assigned conclusively.

The structures of minutissamides A−D (**1**−**4**) were characterized by the presence of a β-amino acid residue, a 2-hydroxy-3-amino-4-methyldodecanoic or -hexadecanoic acid, and three nonstandard amino acid residues (*N*MeAsn and two Dhbs). The β-amino acid residues were further modified by chlorination in **2**, by oxidation to ketone in **3**, or by hydroxylation in **4**. Interestingly, the structures of minutissamides A−D (**1**−**4**) revealed some similarities to the cyclic decapeptides puwainaphycins A−E, isolated from a Hawaiian terrestrial *Anabaena* sp.[9] The sequence of the five residues in the puwainaphycins, including Dhb, Val, β-amino acid unit, Pro, and *N*MeAsn, was conserved in the minutissamides. The other five amino acid residues, OMT (*O*-methyl-Thr), Gly, Gln, Thr-2 (or Val-2), and Thr-1, found in the puwainaphycins were replaced by Thr, Ala, Asn1, Dhb1, and Asn2 in the minutissamides. The core structure of the lipophilic β-amino acid residues, characterized by 2-hydroxy-3-amino-4-methyl substitution, and the chlorination and oxidation patterns were also similar to those observed in the puwainaphycins.

Minutissamides A−D (**1**−**4**) were evaluated for their antiproliferative activity against the HT-29 human colon cancer cell line. Minutissamide A (**1**) displayed antiproliferative activity with an IC_{50} value of 2.0 μM, whereas lower activity was observed for minutissamides B−D (**2**−**4**) with IC_{50} values of 20.0, 11.8, and 22.7 μM, respectively. All four minutissamides have identical cyclic peptide cores and only differ in the lipophilic β-amino acid residue. For example, the only structural difference between minutissamides A (**1**) and B (**2**) was the presence of a chlorine atom at C-12 of this residue, but minutissamide A (**1**) was found to be 10-fold more active in the HT-29 assay. This suggests that the β-amino acid residue plays an important role in the antiproliferative activity of these compounds.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Shimadzu UV spectrometer UV2401 and scanned from 190 to 360 nm. 1D and 2D NMR spectra were obtained on a Bruker Avance DRX 600 MHz NMR spectrometer with a 5 mm CPTXI Z-gradient probe and a Bruker Avance II 900 MHz NMR spectrometer with a 5 mm ATM CPTCI Z-gradient probe. ^1H and ^{13}C NMR chemical shifts were referenced to the DMSO-d_6 solvent signals (δ_H 2.50 and δ_C 39.51, respectively). A mixing time of 60 ms was set for the TOCSY experiments and 200 ms for the T-ROESY experiment. The HMBC spectra were recorded with the $^3J_{C-H}$ set to 8 Hz, and the HSQC spectra were collected with the $^1J_{C-H}$ set to 140 Hz. High-resolution ESI mass spectra were obtained using a Shimadzu IT-TOF LC mass spectrometer. Tandem mass analysis was performed using a Micromass Q-TOF LC mass spectrometer.

Biological Material. *Anabaena minutissima* was acquired from the Culture Collection of Algae at the University of Texas at Austin (UTEX 1613). The cyanobacterium was grown in 20 L flasks containing 18 L of inorganic media (Z media).[13] Cultures were illuminated with fluorescent lamps at 1.03 klx. The temperature of the culture room was maintained at 22 °C. After 6−8 weeks, the biomass of cyanobacteria was harvested by centrifugation and then freeze-dried.

Extraction and Isolation. The freeze-dried biomass (5.2 g) from the total 54 L (3 × 18 L) culture was harvested and extracted with CH_2Cl_2−MeOH (1:1) and concentrated in vacuo to yield 1.1 g of the extract. The extract was fractionated using Diaion HP-20 with increasing amounts of IPA in H_2O to generate eight subfractions (0, 20, 40, 60, 70, 80, 90, 100% aqueous IPA). Fraction 3 (40% IPA) indicated the presence of four peptides by LC-MS. This fraction was further purified by Sephadex LH-20 column chromatography in MeOH. Fractions 7−9 containing the peptides were subjected to reversed-phased HPLC (Varian C_8 semipreparative column, 10 mm × 250 mm, 3 mL/min) eluting with a linear gradient from 60 to 80% aqueous MeOH for 45 min. Minutissamaides A−D (**1**−**4**) were eluted at 37.9 min (**1**, 1.4 mg), 31.6 min (**2**, 0.4 mg), 30.0 min (**3**, 1.0 mg), and 33.5 min (**4**, 0.7 mg), respectively.

*Minutissamide A (**1**):* colorless, amorphous powder; $[\alpha]^{25}_D$ +4 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 243 (3.45) nm; IR (neat) 3317 (br), 2925, 2861, 1681, 1635 (br), 1561, 1541 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); HRESIMS *m*/*z* 1140.6049 $[M + Na]^+$ (calcd for $C_{51}H_{83}N_{13}O_{15}Na$, 1140.6029).

*Minutissamide B (**2**):* colorless, amorphous powder; $[\alpha]^{25}_D$ +5 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 230 (4.24) nm; IR (neat) 3315 (br), 2926, 2852, 1670, 1647 (br), 1545, 1516, 1459, 1402 cm^{-1}; ^1H and ^{13}C NMR (see Table 1); HRESIMS *m*/*z* 1174.5680 $[M + Na]^+$ (calcd for $C_{51}H_{83}ClN_{13}O_{15}Na$, 1174.5640).

*Minutissamide C (**3**):* colorless, amorphous powder; $[\alpha]^{25}_D$ +3 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 242 (2.62) nm; IR (neat) 3315 (br), 2926, 2857, 1664, 1630 (br), 1533, 1447, 1402 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); HRESIMS *m*/*z* 1210.6440 $[M + Na]^+$ (calcd for $C_{55}H_{89}N_{13}O_{16}Na$, 1210.6448).

*Minutissamide D (**4**):* colorless, amorphous powder; $[\alpha]^{25}_D$ +2 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 240 (3.94) nm; IR (neat) 3320 (br), 2926, 2852, 1664, 1636 (br), 1539, 1453, 1379 cm^{-1}; ^1H and ^{13}C NMR (see Table 2); HRESIMS *m*/*z* 1212.6632 $[M + Na]^+$ (calcd for $C_{55}H_{91}N_{13}O_{16}Na$, 1212.6604).

Determination of Amino Acid Configurations. Approximately 0.3 mg of minutissamide A was hydrolyzed using 6 N HCl (500 μL) in a high-pressure tube for 16 h at 110 °C. The hydrolysate was dried under vacuum and redissolved in H_2O. This procedure was repeated three times to completely remove the remaining HCl. For the derivatization with Marfey's reagent (FDAA), the hydrolysate (0.1 mg) or amino acid standard was dissolved in 50 μL of H_2O, and 20 μL of 1 N $NaHCO_3$ and 110 μL of acetone were added. The reaction was initiated by adding 20 μL of FDAA solution (10 mg/mL w/v in acetone), proceeded for 1 h at 40 °C, and was quenched by adding 20 μL of 1 N HCl. The reactant was dried under vacuum and redissolved in CH_3CN for HPLC analysis. Chromatographic analysis of the FDAA

derivatives was performed on an Alltech C_{18} reversed-phased column (5 μm, 250 × 4.6 mm) with the flow rate of 1.0 mL/min. Aqueous CH_3CN containing 0.1% formic acid (FA) was used as a mobile phase eluting with a linear gradient from 10 to 100% (A: H_2O containing 0.1% FA; B: CH_3CN containing 0.1% FA; gradient conditions: 0 min 10% B, 5 min 10% B, 35 min 20% B, 65 min 30% B, 70 min 60% B, 72 min 100% B, and 80 min 100% B). The absolute configurations of the amino acids were assigned by comparing the retention times with those of the corresponding amino acid standards. The retention times of the FDAA derivatives of the authentic amino acid standards were identified at 30.70 (L-Thr and *N*-Me-D-Asp), 32.30 (L-Asp), 36.17 (*N*-Me-L-Asp), 36.94 (D-Asp), 41.06 (L-Ala), 42.54 (D-Thr), 44.57 (L-Pro), 49.84 (D-Pro), 51.76 (D-Ala), 59.09 (L-Val), and 71.45 (D-Val) min. The FDAA derivatives of the amino acids from the hydrolysate of **1** showed the peaks at 32.30, 36.17, 44.57, 51.76, and 59.09 min, corresponding to L-Asp, N-Me-L-Asp, L-Pro, and L-Val, respectively. The peak at 30.70 min was identified as L-Thr by LC-MS analysis (m/z 372, $[M + H]^+$).

Advanced Marfey's Analysis. L- and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide) was synthesized using the protocol published for Marfey's analysis.[21] The acid hydrolysate (0.2 mg) was separated into two equal portions for derivatization with either L-FDLA or DL-FDLA. Each portion was dissolved in 50 μL of H_2O and then mixed with 20 μL of 1 N $NaHCO_3$ and 110 μL of acetone. Finally, 20 μL of L-FDLA or DL-FDLA (10 mg/mL w/v in acetone) was added, and the mixtures were heated to 40 °C for 1 h. The reaction mixtures were cooled to room temperature, and 20 μL of 1 N HCl was added to quench the reaction. After drying, the FDLA derivatives were dissolved in CH_3CN for LC-MS analysis. The chromatograms of L-FDLA and DL-FDLA derivatives were compared for the assignment of the absolute configuration of Hamd in **1**. LC-MS analysis was performed using a Varian reverse-phased C_{18} column (250 × 2.0 mm) at the flow rate of 0.4 mL/min. The linear gradient eluting from 20 to 75% aqueous CH_3CN (0.1% FA) for 50 min was used as a mobile phase, and ESI was used as the ionization method. Two peaks corresponding to the L- and D-FDLA derivatives of Hamd were observed at 43.33 and 48.84 min (m/z 538, $[M - H]^-$), respectively. The L-FDLA derivative of Hamd gave one peak at 48.84 min.

HT-29 Antiproliferative Assay. Antiproliferative activity against the HT-29 cancer cell line was performed according to established protocols.[22]

■ ASSOCIATED CONTENT

Supporting Information. Complete NMR data tables of **2**−**4**; HPLC chromatograms of Marfey's and the advanced Marfey's derivatives of **1**; ^{1}H, COSY, TOCSY, HSQC, and HMBC spectra of **1**−**4**; DEPTQ spectra of **1**, **3**, and **4**; ROESY spectrum of **1**; Q-TOF Tandem MS spectrum of **1**. This information is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +1-312-996-5583. Fax: +1-312-996-7107. E-mail: orjala@uic.edu.

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