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Note

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Stigonemapeptin, an Ahp-Containing Depsipeptide with Elastase Inhibitory Activity from the Bloom-Forming Freshwater Cyanobacterium *Stigonema* sp.

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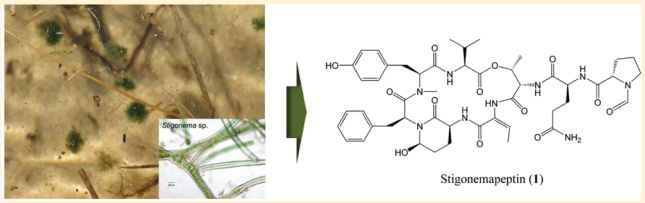
Supporting Information

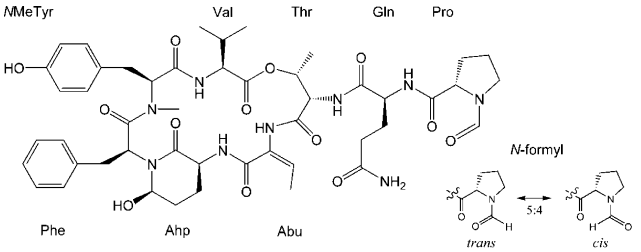
ABSTRACT: Stigonemapeptin (**1**), a depsipeptide containing an Ahp (3-amino-6-hydroxy-2-piperidone) residue, was isolated from a bloom sample of the freshwater cyanobacterium *Stigonema* sp. collected from North Nokomis Lake in the Highland Lake District of northern Wisconsin. The planar structure was determined by 1D and 2D NMR experiments as well as HRESIMS analysis. The absolute configurations of the amino acids were determined using the advanced Marfey's method after acid hydrolysis. Stigonemapeptin (**1**), characterized by the presence of the Ahp residue, also contained the modified amino acids Abu (2-amino-2-butenoic acid) and *N*-formylated Pro. Stigonemapeptin (**1**) showed *in vitro* elastase and chymotrypsin inhibitory activity, with IC_{50} values of 0.26 and 2.93 μM, respectively.



Cyanobacteria have been shown to be a prolific source of secondary metabolites.[1−3] One of the major classes of cyanobacterial secondary metabolites is cyclic depsipeptides. This class of compounds commonly contains modified and unusual amino acid residues. One such example is Ahp (3-amino-6-hydroxy-2-piperidone)-containing depsipeptides, which have been isolated from taxonomically diverse cyanobacteria.[4−8] Production of Ahp-containing depsipeptides has frequently been observed in bloom samples from both marine and freshwater cyanobacteria.[9−11] Due to the ability of Ahp-containing depsipeptides to inhibit serine proteases, their ecological role is believed to be as inhibitors of digestive enzymes and a chemical defense against crustacean predators.[12] However, studies done by Sedmak et al. showed that the Ahp-containing depsipeptide planktopeptin BL1125 induced cyanobacterial cell lysis, leading to the hypothesis that these protease inhibitors could play an important ecological role by controlling cyanobacterial population density in the natural environment.[13,14]

Most of the Ahp-containing depsipeptides have shown inhibitory activity against serine proteases such as trypsin, chymotrypsin, and elastase with different selectivity depending on variation in their amino acid composition. Ahp-containing depsipeptides with an Abu (2-amino-2-butenoic acid) residue neighboring the Ahp residue have been found to selectively inhibit elastase as compared to other serine proteases.[15−17] Herein, we report the isolation, structural elucidation, and serine protease inhibitory activity of a new Ahp-containing depsipeptide, named stigonemapeptin (**1**).

The sample of the freshwater cyanobacterium was collected from North Nokomis Lake in the Highland Lake District of northern Wisconsin in August 2010. Taxonomic identification was carried out on the basis of morphological observation and phylogenetic analysis using 16S rRNA gene sequence and indicated this strain to be *Stigonema* sp. (see Supporting Information for detail). The cyanobacterial cells were manually cleaned from debris and freeze-dried. The freeze-dried cells (5 g) were extracted with a mixture of CH_2Cl_2 and MeOH (1:1). LC-MS analysis of the extract indicated the presence of a potentially new nitrogen-containing compound with a molecular weight of 973.5 Da. The cell extract was subsequently fractionated using Diaion HP-20 resin with an increasing amount of iPrOH in H_2O. The fraction eluting at 40% iPrOH was found to contain the nitrogen-containing compound by LC-MS analysis and was further purified by reversed-phase HPLC to yield stigonemapeptin (**1**, 4.1 mg, 0.08%).



Stigonemapeptin (**1**)

Stigonemapeptin (**1**) was obtained as a colorless, amorphous powder. The molecular formula of **1** was determined as $C_{48}H_{63}N_9O_{13}$ by HRESIMS analysis. The peptidic nature of **1** was suggested by the signal distribution pattern observed in the

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Table 1. NMR Spectroscopic Data of Stigonemapeptin (1) in DMSO-d_6

		trans conformer			*cis* conformer				
		δ_C[b]	δ_H[a]	mult. (J in Hz)	δ_C[b]	δ_H[a]	mult. (J in Hz)	HMBC[a]	ROESY[a]
Val	1	172.4			172.4				
	2	56.0	4.73,	overlapped	56.1	4.69,	dd (9.0, 4.2)	1, 1_{NMeTyr}, 3, 4, 5	H-3, H-5, NH
	3	30.8	2.10,	m	30.5	2.09,	m	2, 4, 5	H-2, H-4, H-5
	4	17.2	0.72,	d (6.6)	17.2	0.72,	d (6.6)	2, 3, 5	H-2
	5	19.3	0.86,	d (6.6)	19.3	0.86,	d (6.6)	2, 3, 4	H-2
	NH		7.50,	d (9.6)		7.51,	d (9.0)	1_{NMeTyr}, 2	H-2, H-2_{NMeTyr}
*N*MeTyr	1	169.4			169.4				
	2	60.9	4.86,	dd (11.4, 2.7)	60.9	4.86,	dd (11.4, 2.7)	3, *N*-Me	H-3b, H-5/9, NH_{Val}
	3a	32.8	2.70,	dd (13.2, 11.4)	32.8	2.70,	dd (13.2, 11.4)	2, 4, 5/9	H-2, H-5/9
	3b		3.09,	dd (13.2, 2.7)		3.09,	dd (13.2, 2.7)	2, 4, 5/9	H-2, H-5/9
	4	127.5			127.5				
	5/9	130.4	6.99,	dd (8.4, 1.8)	130.4	6.99,	dd (8.4, 1.8)	3, 5/9, 7	H-2, H-3, H-2_{Phe}
	6/8	115.3	6.77,	d (8.4)	115.3	6.77,	d (8.4)	4, 6/8, 7	
	7	156.3			156.3				
	N-Me	30.5	2.76,	s	30.5	2.76,	s	1_{Phe}, 2	H-2, H-5/9, NH_{Val}
	OH		nd[c]			nd[c]			
Phe	1	170.5			170.5				
	2	50.3	4.74,	overlapped	50.3	4.74,	overlapped	2_{Ahp}, 3, 6_{Ahp}	H-3a, H-3b, H-5/9
	3a	35.3	1.82,	overlapped	35.3	1.82,	overlapped	4, 5/9	H-3b
	3b		2.88,	t (13.8)		2.88,	t (13.8)	2, 4, 5/9	H-2, H-3a, H-5/9, H-6_{Ahp}
	4	136.8			136.8				
	5/9	129.5	6.85,	d (7.8)	129.5	6.85,	d (7.8)	3, 5/9, 7	H-2, H-3, H-$5a_{Ahp}$, H-6_{Ahp}
	6/8	127.8	7.20,	t (7.8)	127.8	7.20,	t (7.8)	4, 6/8	
	7	126.3	7.16,	t (7.8)	126.3	7.16,	t (7.8)	5/9	
Ahp	2	168.9			168.9				
	3	48.0	3.76,	m	48.0	3.76,	m	2, 4	H-4a, NH
	4a	21.8	1.56,	overlapped	21.9	1.56,	overlapped	2, 6	H-3, H-4b
	4b		2.38,	m		2.38,	m		H-4a, NH
	5a	29.2	1.58,	brd (13.2)	29.2	1.58,	brd (13.2)		H-6, H-5/9_{Phe}
	5b		1.71,	brd (13.2)		1.71,	brd (13.2)	3, 6	H-3, H-5a, H-6
	6	73.8	5.08,	brs	73.8	5.08,	brs	2, 4	H-5a, H-5b, H-$3b_{Phe}$
	NH		7.20,	overlapped		7.20,	overlapped	1_{Abu}	H-3, H-4b
	OH		nd[c]			nd[c]			
Abu	1	163.0			163.1				
	2	128.0			128.0				
	3	133.4	5.75,	q (7.5)	133.7	5.77,	q (7.5)	1, 2	H-4, NH
	4	13.9	1.94,	d (4.5)	13.9	1.95,	d (7.5)	2, 3	H-3
	NH		9.15,	s		9.29,	s		H-3, H-2_{Thr}, H-3_{Thr}
Thr	1	168.6			168.6				
	2	55.4	4.48,	d (9.0)	55.4	4.50,	d (9.0)	1, 1_{Gln}, 3, 4	H-3, H-4, NH, NH_{Abu}
	3	72.0	5.38,	q (6.5)	72.0	5.41,	q (6.5)	1_{Val}, 4	H-2, H-4
	4	18.3	1.19,	d (6.5)	18.3	1.18,	d (6.5)	2, 3	H-2, H-3
	NH		7.74,	d (9.0)		7.87,	d (9.0)	1_{Gln}	H-2, H-2_{Gln}
Gln	1	172.0			172.1				
	2	52.6	4.29,	m	52.5	4.37,	m	1, 3, 4	H-3b, H-4, NH_{Thr}
	3a	27.0	1.80,	m	27.2	1.80,	m	1, 5	
	3b		1.94,	m		1.96,	m	1, 5	
	4	31.5	2.17,	m	31.6	2.15,	m	2, 3, 5	H-2, H-3b, NH_2
	5	173.9			174.0				
	NH		8.32,	d (7.3)		8.40,	d (7.2)	1_{Pro}	H-2_{Pro}
	NH_2		6.80,	brs		6.79,	brs	5	H-4
			7.27,	brs		7.32,	brs		H-4
Pro	1	171.6			172.1				
	2	57.2	4.25,	dd (8.5, 4.5)	58.9	4.44,	dd (8.2, 3.9)	3, 4	NH_{Gln}, $(H_{N\text{-}formyl})^{cis}$
	3a	29.6	1.85,	m	30.3	1.94,	m		
	3b		2.16,	m		2.14,	m		
	4a	23.7	1.78,	m	22.6	1.81,	m		
	4b		1.89,	m		1.82,	m		
	5a	46.4	3.55,	m	43.8	3.33,	m	2, 3, 4	$(H_{N\text{-}formyl})^{trans}$

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Table 1. continued

		trans conformer			*cis* conformer				
		δ_C [b]	δ_H [a]	mult. (J in Hz)	δ_C [b]	δ_H [a]	mult. (J in Hz)	HMBC[a]	ROESY[a]
	5b		3.61,	m		3.36,	m	2, 3, 4	$(H_{N\text{-formyl}})^{trans}$
N-Formyl	CHO	161.2	8.21,	s	161.5	8.08,	s	2_{Pro}, 5_{Pro}	$(H_2\text{-}5_{Pro})^{trans}$, $(H\text{-}2_{Pro})^{cis}$

[a]Recorded at 600 MHz. [b]Recorded at 226 MHz. [c]nd: not detected probably due to the high content of water in the sample.

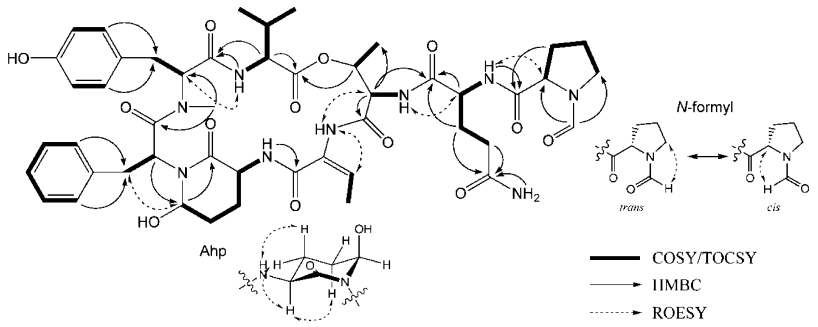

Figure 1. Key 2D NMR correlations used for the determination of the planar structure of **1**.

^{1}H NMR spectrum (DMSO-d_6), which included exchangeable amide NH signals (6−10 ppm), amino acid α-proton signals (4−6 ppm), and aliphatic methylene and methyl signals (0−3 ppm). Signal doubling was observed for some of the signals in the ^{1}H NMR spectrum (integration ratio of 5:4) and implied the presence of two conformers likely arising from one part of the molecule. The structure determination of **1** was carried out using the major conformer. Combined analysis of the COSY and TOCSY spectra identified the structures of six standard amino acids: Val, Tyr, Phe, Thr, Gln, and Pro (Table 1 and Figure 1). An HMBC correlation observed from *N*-Me (δ_H 2.76) to Tyr C-2 (δ_C 60.9) indicated that the Tyr residue was *N*-methylated. The presence of an Ahp residue was evident by the appearance of a broad singlet of H-6 (δ_H 5.08) along with sequential COSY correlations from H-6 to NH (δ_H 7.20) and an HMBC correlation from H-6 to C-2 (δ_C 168.9). An amide NH singlet proton signal (δ_H 9.15) and an upfield amide carbonyl carbon signal (δ_C 163.0), as well as a COSY correlation between H-3 (δ_H 5.75) and H-4 (δ_H 1.94), and HMBC correlations from H-3 to C-1 and C-2 (δ_C 128.0), indicated the presence of an Abu residue. An NOE correlation observed between NH and H-3 assigned the *E* geometry to the Abu residue. Lastly, a singlet proton signal (δ_H 8.21), which showed an HSQC correlation with a carbonyl carbon (δ_C 161.2), and HMBC correlations with Pro C-2 (δ_C 57.2) and C-5 (δ_C 46.4) suggested that a formyl group was attached to the amino group of Pro. The complete sequence of eight amino acid residues in **1** was established by combined analysis of the HMBC and ROESY spectra. HMBC correlations from Val NH (δ_H 7.50) and H-2 (δ_H 4.73) to *N*MeTyr C-1 (δ_C 169.4), from *N*-Me (δ_H 2.76) to Phe C-1 (δ_C 170.5), from Phe H-2 (δ_H 4.74) to Ahp C-6 (δ_C 73.8), from Ahp NH (δ_H 7.20) to Abu C-1 (δ_C 163.0), and from Thr H-3 (δ_H 5.38) to Val C-1 (δ_C 172.4), along with a ROESY correlation between Abu NH (δ_H 9.15) and Thr H-2 (δ_H 4.48), established the structure of a cyclic depsipeptide core with an ester linkage formed between the Thr hydroxy and the Val carboxylic acid. This cyclic depsipeptide core was further branched from Thr NH with the sequence Gln-Pro-*N*-formyl by HMBC correlations from Thr H-2 (δ_H 4.48) to Gln C-1 (δ_C 172.0) and from Gln NH (δ_H 8.32) to Pro C-1 (δ_C 171.6) along with NOE correlations observed between Thr NH (δ_H 7.74) and Gln H-2 (δ_H 4.29) and between Gln NH and Pro H-2 (δ_H 4.25), completing the planar structure of **1**. The noticeable doubling of NMR signals was observed for the *N*-formylated Pro and Gln residues. These two *N*-formyl proton signals (δ_H 8.21 and 8.08) showed different NOE correlations, one with Pro H_2-5 and the other with Pro H-2, thus indicating that this signal doubling was due to the restricted rotation of the *N*-formyl group between *trans* and *cis* conformations (Figure 1). The same phenomenon has been reported for structurally related compounds anabaenopeptilide 202-B and kempopeptin A, possessing *N*-formylated Pro and *N*-acetylated Pro, respectively.[6,18]

The absolute configurations of the amino acids were assigned using the advanced Marfey's method after acid hydrolysis. Two equal portions of the acid hydrolysate of **1** were derivatized with L- and DL-FDLA, respectively, and LC-MS comparison between two derivatives assigned an L configuration for all of the amino acid residues except for the Ahp residue. The relative configuration of Ahp C-3 and C-6 was determined by analysis of NOE correlations. A broad singlet proton signal observed for H-6 suggested small coupling constants between H-6 and two diastereotopic protons H-5a_{eq} and H-5b_{ax}, indicating H-6 to be in an equatorial position. NOE correlations observed between H-3 and H-5b_{ax}, between NH and H-4b_{ax}, and between H-3 and H-4a_{eq} supported the *chair* conformation of the piperidone ring with an axial hydroxy at C-6 and the equatorial amide NH at C-3, as depicted in Figure 1. To determine the absolute configuration of the Ahp residue, CrO_3 oxidation was carried out prior to acid hydrolysis, and subsequent analysis of the acid hydrolysate by the advanced Marfey's method exclusively identified the presence of L-Glu, thereby assigning the 3*S*,6*R* configuration for the Ahp residue. This configuration was further supported by nearly identical ^{1}H and ^{13}C NMR chemical shifts of the Ahp residue in **1** to those reported for the lyngbyastatins and somamides, which shared the same depsipeptide core structure except for the geometry of the Abu residue.[15,16,19]

Many of the Ahp-containing depsipeptides isolated from cyanobacteria have been known to inhibit serine proteases with different selectivity profiles against trypsin, chymotrypsin, and elastase. Accordingly, we tested stigonemapeptin (**1**) for its

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inhibitory activity against these three enzymes. Stigonemapeptin (**1**) showed inhibition of elastase and chymotrypsin with IC_{50} values of 0.26 and 2.93 μM, respectively, whereas no inhibition was found against trypsin at the highest concentration tested (10 μM). This result is in good agreement with activity profiles of known Ahp-containing depsipeptides. Co-crystallographic data of elastase with scyptolin A suggested that the selectivity of this class of compounds for the inhibition of serine proteases varies depending on the type of amino acids located between Ahp and Thr due to different binding preferences to the enzyme's specificity pocket.[20] Preferences for the inhibition of chymotrypsin and elastase are conferred by a bulky hydrophobic amino acid (Phe, Tyr, or Trp) and a small neutral amino acid (Ala, Gly, or Val), respectively, whereas a positively charged amino acid (Arg or Lys) is preferred for the inhibition of trypsin. Therefore, the selective inhibitory activity of **1** against elastase can be attributed to the presence of the Abu residue between Ahp and Thr, as an Abu residue is considered to be relatively small and neutral. Stigonemapeptin (**1**) was also tested for its inhibitory activity against the 20S proteasome and cytotoxicity against HT-29 cancer cells, but showed no activities at the highest concentration tested (25 μM).

In summary, we have isolated a new Ahp-containing depsipeptide, which possesses nonstandard amino acids Abu and *N*-formylated Pro, from a bloom sample of the freshwater cyanobacterium *stigonema* sp. (collection ID WI53). The structure of stigonemapeptin (**1**) differed from other known Ahp- and Abu-containing depsipeptides by the geometry of the Abu residue and the amino acid composition of the branching residues. Stigonemapeptin (**1**) showed selective inhibition of elastase and chymotrypsin with 10-fold higher selectivity for elastase.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation was measured on a Perkin-Elmer 241 polarimeter. UV and IR spectra were recorded on a Shimadzu UV spectrometer UV2401 and a Thermo Nicolet 6700 FT-IR spectrometer, respectively. 1D and 2D NMR spectra including ^{1}H NMR, COSY, TOCSY, HSQC, HMBC, and T-ROESY spectra were obtained on a Bruker Avance DRX 600 MHz NMR spectrometer with a 5 mm CPTXI Z-gradient, whereas a Bruker Avance II 900 MHz NMR spectrometer with a 5 mm ATM CPTCI Z-gradient probe was used to acquire the DEPT-Q spectrum. ^{1}H 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 experiment and 200 ms for the T-ROESY experiment. The HMBC spectrum was recorded with the average $^3J_{CH}$ of 8 Hz, and the HSQC spectrum was measured with the average $^1J_{CH}$ of 145 Hz. HRESIMS and LC-MS data were obtained on a Shimadzu IT-TOF LC-MS spectrometer.

Sample Collection and Morphological Study. A bloom sample of *Stigonema* sp. (collection ID WI53) was collected from North Nokomis Lake in the Highland Lake District of northern Wisconsin in August 2010 (N 45°50.4812′, W 89°26.4920′). A voucher specimen has been retained at UIC under the collection ID WI53. Morphological analysis was performed using a Zeiss Axiostar Plus light microscope equipped with a Canon PowerShot A620 camera.

DNA Extraction and Phylogenetic Analysis of 16S rRNA Gene Sequence. For genomic DNA isolation, a sample was re-collected at the same site in August 2011, and the production of stigonemapeptin (**1**) was confirmed by LC-MS analysis. The kit-based DNA extraction method routinely used in our laboratory[21] failed to yield genomic DNA in a sufficient quantity and quality for PCR amplification possibly due to the presence of a firm mucilaginous sheath. Therefore, the CTAB-based method was employed as previously described for DNA extraction from cyanobacteria of the genus *Arthrospira* with a slight modification; freeze−thaw cycles were replaced by grinding in liquid nitrogen.[22] This extraction method yielded a sufficient amount of the PCR-amplifiable template DNA. The partial 16S rRNA gene was amplified from the isolated genomic DNA using the cyanobacteria-specific primers 106F and 1509R.[23] The reaction volume was 26 μL containing 10.5 μL of nuclease-free H_2O, 12.5 μL of PCR Master Mix (Promega M7502), 1 μL of each primer (10 μM), and 1 μL of the template DNA. The PCR was performed in a Bio-Rad C1000 thermal cycler as follows: initial denaturation for 30 s at 98 °C, 35 amplification cycles of 10 s at 98 °C, 30 s at 53 °C, and 30 s at 72 °C, and a final extension for 10 min at 72 °C. PCR products were purified using a MinElute PCR purification kit (Qiagen) and sequenced using the cyanobacteria-specific primers 106F and 1509R as well as the internal primer 359F. The resulting sequence was deposited in the NCBI GenBank under the accession number JQ435860.

Extraction and Isolation. The cyanobacterial sample was manually cleaned from debris and freeze-dried. The freeze-dried sample (5 g) was extracted with the solvent mixture of CH_2Cl_2 and MeOH (1:1) and concentrated *in vacuo* to yield the organic extract (0.5 g). The resulting extract was fractionated using Diaion HP-20 resin and an increasing amount of iPrOH in H_2O. The fraction eluting at 40% iPrOH was found to contain a new nitrogen-containing peptide with a molecular weight of 973.5 Da by LC-MS analysis and subjected to reversed-phase HPLC (Varian C_8 semipreparative column, 10 mm × 250 mm, 3 mL/min) to afford stigonemapeptin (**1**, 4.1 mg, 0.08%).

Stigonemapeptin (1): colorless, amorphous powder; $[\alpha]^{25}_D$ −61 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.37), 223 (4.08), 272 (3.23) nm; IR (neat) ν_{max} 3276, 2962, 2936, 1734, 1653, 1533, 1517 cm^{-1}; ^{1}H and ^{13}C NMR, HMBC, and ROESY data, see Table 1; HRESIMS *m*/*z* 996.4439 [M + Na]$^+$ (calcd for $C_{48}H_{63}N_9NaO_{13}$, 996.4443).

Absolute Configuration of Amino Acids and Ahp by the Advanced Marfey's Method. Approximately 0.3 mg of **1** was hydrolyzed with 6 N HCl (500 μL) for 16 h at 110 °C. The resulting acid hydrolysate 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 mixed with 20 μL of 1 N $NaHCO_3$ and 20 μL of L-FDLA or DL-FDLA (10 mg/mL in acetone). Then, acetone was added to the final volume of 200 μL, and the reaction mixtures were heated to 40 °C and stirred for 1 h. After cooling to room temperature (rt), 20 μL of 1 N HCl was added, and the resulting reaction mixtures were air-dried and redissolved in CH_3CN. LC-MS analysis was performed on a reversed-phase column (Alltima C_{18}, 250 × 4.6 mm, 5 μm, 1.0 mL/min) with a linear gradient from 20% to 65% aqueous CH_3CN containing 0.1% formic acid for 50 min. The selective ion chromatograms of L-FDLA and DL-FDLA for each amino acid derivative were compared for the assignment of amino acid configurations. Two peaks corresponding to the L- and D-FDLA derivatives of each amino acid were observed as follows: Val 35.9 min (L) and 44.2 min (D); Glu 27.4 min (L) and 28.9 min (D); *N*MeTyr 24.1 min (L) and 25.2 min (D); Phe 40.7 min (L) and 46.9 min (D); Pro 30.4 min (L) and 34.3 min (D); Thr 24.6 min (L) and 30.7 min (D). The L-FDLA derivative gave one peak for each amino acid at 35.9, 27.4, 24.1, 40.6, 30.5, and 24.6 min, confirming the L configuration for Val, Gln, *N*MeTyr, Phe, Pro, and Thr. The presence of L-Thr was further confirmed by chromatographic comparison of the L-FDLA derivative of the acid hydrolysate with those of the amino acid standards L-Thr, D-Thr, L-*allo*-Thr, and D-*allo*-Thr.

For the absolute configuration of the Ahp residue, CrO_3 oxidation was carried out prior to acid hydrolysis as previously described.[15,24] Briefly, approximately 0.4 mg of **1** was dissolved in 0.4 mL of glacial AcOH, and CrO_3 (2 mg) was added. The mixture was stirred at rt for 5 h and purified on a C_{18} SPE cartridge eluting with H_2O and MeOH. The resulting oxidized product was hydrolyzed and subjected to advanced Marfey's analysis in the same way as described above. LC-MS analysis exclusively identified the presence of L-Glu, assigning the *S* configuration to Ahp C-3.

Protease Inhibition Assays. The inhibitory activity of **1** against three serine proteases including elastase, chymotrypsin, and trypsin

was evaluated according to the previously described method.[11] Elastase inhibitory activity was tested using elastase from porcine pancreas (Sigma E0258) and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma S4760) as a substrate. The assay mixture composed of 79 μL of the assay buffer (1 M Tris-HCl, pH 8.0), 5 μL of elastase solution (75 μg/mL in assay buffer), and 1 μL of **1** (various concentrations in DMSO) was preincubated in a microtiter plate at rt for 15 min, and then 15 μL of substrate solution (2 mM in assay buffer) was added to each well. The increase in absorbance was measured every 30 s for 30 min at 405 nm using a microplate reader.

For chymotrypsin- and trypsin-inhibition assays, α-chymotrypsin from bovine pancreas (Sigma C4129) and trypsin from porcine pancreas (Sigma T0303) were used with substrates *N*-succinyl-Gly-Gly-Phe-*p*-nitroanilide (Sigma S1899) for chymotrypsin and *N*α-benzoyl-DL-arginine-4-nitroanilide (Sigma B4875) for trypsin. The assay buffer including 50 mM Tris-HCl, 100 mM NaCl, and 1 mM $CaCl_2$ (pH 7.8) was used for both assays. To the preincubated mixture of 39 μL of assay buffer, 10 μL of enzyme solution (100 μg/mL in assay buffer), and 1 μL of **1** (various concentrations in DMSO) at 37 °C for 15 min, 50 μM substrate solution (1.5 mM in assay buffer) was added. The absorbance increase was recorded every 30 s for 30 min at 405 nm. Activity was calculated as a percentage inhibition by comparing the slope of an initial linear progress curve of enzyme reactions with **1** at various concentrations to that of the control reaction without compounds.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Taxonomic identification, photomicrograph and phylogenetic tree of the sample WI53; ^{1}H NMR, DEPT-Q, COSY, TOCSY, HSQC, HMBC, and T-ROESY spectra of **1**. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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