Nostopeptins A and B, Elastase Inhibitors from the Cyanobacterium *Nostoc minutum*

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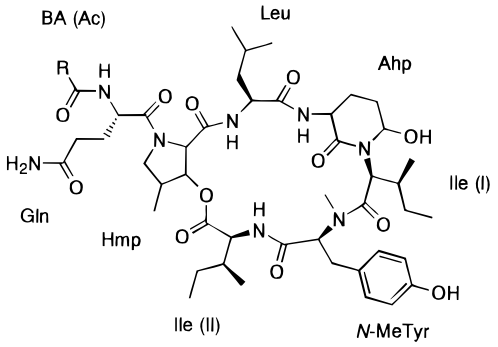
Nostopeptins A and B were isolated from the cultured freshwater cyanobacterium *Nostoc minutum* (NIES-26). Their structures were elucidated on the basis of 2D NMR data and chemical degradation. These cyclic depsipeptides containing Ahp (3-amino-6-hydroxy-2-piperidone) inhibited elastase and chymotrypsin potently.

Cyanobacteria have been known to be a rich source of biologically active peptides, which include toxins, anticancer agents, fungicides, and enzyme inhibitors.[2] In the course of our continuous screening program for protease inhibitors from microalgae, we have reported an angiotensin-converting enzyme inhibitor microginin[3] and a variety of inhibitors of serine proteases[4] such as trypsin, plasmin, elastase, and chymotrypsin. Elastase is suggested to be involved in pulmonary emphysema, rheumatoid arthritis, adult respiratory distress syndrome, and other inflammatory states.[5] Its inhibitors might be useful chemotherapeutic agents for these diseases. In our search, an Ahp (3-amino-6-hydroxy-2-piperidone)-containing depsipeptide, oscillapeptin,[6] and tricyclic peptides, microviridins,[4,7] showed elastase inhibition. We have also found new elastase inhibitors designated as nostopeptins A (**1**) and B (**2**) from the cultured freshwater cyanobacterium *Nostoc minutum* (NIES-26) (Nostocaceae). In this paper, we describe the isolation and structure elucidation of **1** and **2**.



1 R=$CH_3(CH_2)_2$
2 R=CH_3

The first mass culture of *N. minutum* yielded 161 g (dry wt) of algal cells from 380 L of culture. The freeze-dried alga was extracted with MeOH, and the extract was partitioned between Et_2O and H_2O. The Et_2O layer was partitioned between aqueous MeOH and the solvent series of hexane, CCl_4, and $CHCl_3$. The $CHCl_3$-soluble fraction, which inhibited elastase, was subjected to ODS flash chromatography followed by reversed-phase HPLC to yield nostopeptin A (**1**, 20 mg).

In an additional search for elastase inhibitors from this alga, a second mass culture of *N. minutum* was conducted. The extract of the alga (231 g) from 590 L of culture was also partitioned between Et_2O and H_2O, and both layers showed elastase inhibition. From the Et_2O layer, nostopeptin A (**1**, 61 mg) was isolated by the above-mentioned procedure. Then the aqueous fraction was partitioned between BuOH and H_2O. The BuOH-soluble fraction was subjected to ODS flash chromatography and reversed-phase HPLC similarly to the procedure for the first mass culture, to afford **1** (8.5 mg) and nostopeptin B (**2**, 8.5 mg).

The molecular formula of nostopeptin A (**1**) was established as $C_{48}H_{74}N_8O_{12}$ by HRFABMS. Its peptidic nature was suggested by its ^{1}H- and ^{13}C-NMR spectra (Table 1). In addition, amino acid analysis of the acid hydrolysate gave residues of Glu, Leu, and two Ile. Extensive NMR analyses including ^{1}H−^{1}H COSY, HOHAHA, HMQC, and HMBC (J_{CH} = 5, 10 Hz) spectra revealed the spin systems of Gln, Ile, and Leu. The other Ile was suggested to be an *N, N*-disubstituted derivative, because its amide proton was not observed. Furthermore, the presence of butyric acid (BA), *N*-MeTyr, and Ahp (3-amino-6-hydroxy-2-piperidone) was indicated by 2D NMR spectral data. Ahp was assigned by comparison with the same residue found in micropeptins[8] and was deduced to constitute a part of a hemiaminal structure formed from glutamate γ-semialdehyde and another amino acid. The last spin system, (N)−CH_2−CH(CH_3)−CH(O)−CH(N)(CO), was suggested by a ^{1}H−^{1}H COSY spectrum. An HMBC correlation (δ_H 4.30/δ_C 64.0) supported the presence of a substituted pyrrolidine, and the downfield chemical shift of an oxygen-bearing methine (δ_H 5.15) indicated ester formation. These results determined the last unit to be esterified 3-hydroxy-4-methylproline (Hmp). It is noted that the upfield chemical shift (δ_H −0.11) of the methyl protons of Ile (I) is caused by an anisotropic effect due to the benzene ring of *N*-MeTyr.

The sequence of **1** was deduced primarily by HMBC correlations (Table 1). The HMBC cross peaks from Ahp H-3, H-4, H-6, and Ile(I) H-2 to Ahp CO and another peak between Ile(I) H-2 and Ahp C-6 confirmed the Ahp−Ile connection. Two carbonyl carbons were observed at the same chemical shift (δ_C 169.3), but determination of the Ahp−Ile connection enabled us to distinguish the carbonyl carbon of Ahp from that of *N*-MeTyr. All the α-protons except that of Hmp were correlated with the carbonyl carbons of the adjacent

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Table 1. ^{1}H- and ^{13}C-NMR Data of Nostopeptin A (**1**) in DMSO-d_6

position		^{1}H	J (Hz)	^{13}C	HMBC[a]
BA	1			172.1 (s)	BA 2, 3, Gln 2, NH
	2	2.06 (m)		36.6 (t)	BA 3, 4
	3	1.47 (qt)	7.4, 7.0	18.5 (t)	BA 2, 4
	4	0.83 (t)	7.4	13.5 (q)	BA 2, 3
Gln	1			170.9 (s)	Gln 2, 3
	2	4.34 (m)		49.6 (d)	Gln 3, 4, NH
	3	1.75 (m)		27.7 (t)	Gln 2, 4, NH
	4	2.04 (m)		30.3 (t)	Gln 2, 3, NH_2
		2.11 (m)			
	5			173.8 (s)	Gln 3, 4, NH_2
	NH	8.16 (d)	7.0		
	NH_2	7.01 (br)			
		7.24 (br)			
Hmp	1			167.4 (s)	Hmp 2, Leu 2, NH
	2	4.42 (d)	3.2	64.0 (d)	Hmp 3, 5
	3	5.15 (m)		77.5 (d)	Hmp 5, Me
	4	2.42 (m)		36.9 (d)	Hmp 5, Me
	5	3.40 (m)		51.5 (t)	Hmp 3, Me
		4.30 (m)			
	Me	1.04 (d)	6.5	10.9 (q)	Hmp 5
Leu	1			171.0 (s)	Leu 2, Ahp 3, NH
	2	4.30 (m)		50.2 (d)	Leu 3, NH
	3	1.43 (m)		39.5 (t)	Leu 2, 5, 5′
		1.77 (m)			
	4	2.06 (m)		22.6 (d)	Leu 2, 3, 5, 5′
	5	0.75 (d)	6.1	21.0 (q)	Leu 3, 5′
	5′	0.86 (m)		24.1 (q)	Leu 3, 5
	NH	8.42 (d)	9.0		
Ahp	2			169.3 (s)	Ahp 3, 4, 6, Ile(I) 2
	3	4.47 (m)		48.4 (d)	Ahp NH
	4	1.72 (m)		21.3 (t)	Ahp 3, 6
		2.69 (m)			
	5	1.74 (m)		29.7 (t)	
	6	4.90 (br)		73.7 (d)	Ile(I) 2
	NH	7.08 (d)	9.3		
	OH	6.10 (br)			
Ile(I)	1			169.8 (s)	Ile(I) 2, MeTyr 2, Me
	2	4.38 (d)	10.6	54.1 (d)	Ile(I) 4, Me
	3	1.78 (m)		32.8 (d)	Ile(I) 2, 4, Me
	4	0.62 (m)		23.7 (t)	Ile(I) 3, 5, Me
		1.09 (m)			
	5	0.63 (m)		10.3 (q)	Ile(I) 4
	Me	−0.11 (d)	6.4	13.9 (q)	Ile(I) 2, 4
N-MeTyr	1			169.3 (s)	MeTyr 2, Ile(II) 2, NH
	2	5.13 (d)	11.6	60.4 (d)	MeTyr 3
	3	2.64 (dd)	12.9,11.6	33.3 (t)	MeTyr 2, 5, 9
		3.14 (d)	12.9		
	4			127.3 (s)	MeTyr 2, 3, 6, 8
	5,9	6.98 (d)	8.3	130.3 (d)	MeTyr 3
	6,8	6.63 (d)	8.3	115.3 (d)	
	7			156.2 (s)	MeTyr 5, 6, 8, 9
	Me	2.69 (s)		30.4 (q)	MeTyr 2
	OH	9.16 (br)			
Ile(II)	1			173.2 (s)	Ile(II) 2, Hmp 3
	2	4.81 (dd)	4.4, 9.6	55.6 (d)	Ile(II) 3, 4, Me, NH
	3	1.90 (m)		38.0 (d)	Ile(II) 2, 4, 5, Me
	4	0.95 (m)		23.7 (t)	Ile(II) 2, 3, 5, Me
		1.17 (m)			
	5	0.78 (t)	7.4	11.3 (q)	Ile(II) 3, 4
	Me	0.86 (d)	6.6	16.1 (q)	Ile(II) 2, 3, 4
	NH	7.59 (d)	9.5		

[a] Protons correlated to the carbon of the raw.

amino acids. Correlations between the NH-protons and the carbonyl carbons of the adjacent amino acids were also detected. The correlation between Hmp H-3 and Ile(II) C-1 confirmed the ester formation between Hmp OH and Ile(II) CO_2H. These HMBC results assembled two partial structures: BA−Gln− and cyclic [Hmp−Leu−Ahp−Ile−*N*-MeTyr−Ile−O]. A connection between Gln and Hmp satisfied the molecular formula and was supported by a ROESY peak between Hmp H-5 and Gln H-3.

Chiral gas chromatographic analysis of the *N*-trifluoroacetyl isopropyl ester derivatives of the acid hydrolysate clarified that all of the four usual amino acid residues in **1** were in the L-form, in comparison with commercially available standard amino acids. The stereochemistry of *N*-MeTyr was also determined to be L by Marfey's method using the conventional and D-Marfey's reagents.[9,10] The stereochemistries of Ahp and Hmp have not been determined. Therefore, the gross structure of nostopeptin A was determined to be **1**.

The molecular formula of nostopeptin B (**2**) was determined to be $C_{46}H_{70}N_8O_{12}$ by HRFABMS. The presence of four usual amino acid residues, Leu, Gln, and two Ile, and three modified amino acids, Ahp, *N*-MeTyr, and Hmp, was established based on analyses of ^{1}H, ^{13}C, ^{1}H−^{1}H COSY, HMQC, and HMBC spectra (Table 2). The amide proton of Ile(I) was not observed, which again suggested that a nitrogen of Ile(I) and glutamate γ-semialdehyde formed a hemiaminal structure of Ahp. The remaining C_2H_3O portion was deduced to be an acetyl moiety in place of the butyryl moiety in **1**, from methyl and carbonyl NMR signals. The stereochemistries of the four usual amino acids (Gln, Leu, and two Ile) and *N*-MeTyr were determined to be L by chiral gas chromatographic analysis and Marfey's method.

HMBC correlations (Table 2) allowed us to construct two partial sequences: Ac−Gln−Hmp−Leu− and −Ahp−Ile(I)−*N*-MeTyr−. The connections of Ahp to Leu and of *N*-MeTyr to Ile(II) were suggested from NOESY peaks with Ahp NH to Leu H-2 and NH, and between *N*-MeTyr H-2 and Ile(II) NH. Finally, the downfield chemical shift of Hmp H-3 (δ_H 5.15) supported ester formation with Ile(II). Thus, the structure **2** was assigned to nostopeptin B.

The effects of **1** and **2** against elastase and the other proteolytic enzymes were investigated. Depsipeptides **1** and **2** inhibited elastase (IC_{50}; 1.3 and 11.0 μg/mL) and chymotrypsin (IC_{50}; 1.4 and 1.6 μg/mL), respectively, while neither compound inhibited papain, trypsin, thrombin, or plasmin, even at 100 μg/mL.

These compounds are characterized by the unusual Ahp component. The first example of an Ahp-containing peptide is dolastatin 13, isolated from the sea hare *Dolabella auricularia*.[11] Recently, freshwater cyanobacteria have been found to produce Ahp-containing peptides such as micropeptins,[8,12] aeruginopeptins,[13] microcystilide A,[14] cyanopeptolins,[15,16] A90720A,[17] and oscillapeptin.[6] Oscillapeptin inhibits elastase and chymotrypsin similarly to **1** and **2**, whereas micropeptins A, B, and 90, A90720A, and cyanopeptolin S inhibit trypsin−plasmin group serine proteases, and oscillapeptin G[18] inhibits tyrosinase. Nostopeptins are also characteristic of being esterified by Hmp. Hmp is present as a constituent of echinocandin B[19] and pneumocandin A[20] isolated from fermentation broth of fungi, but their Hmp is not esterified.

Experimental Section

General Methods. NMR spectra were recorded on a Bruker AM600 NMR spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C and on a JEOL JNM-A500 NMR spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C. ^{1}H- and ^{13}C-NMR chemical shifts were referenced to solvent peaks: δ_H 2.49 and δ_C 39.5 for DMSO-d_6. Optical rotations were determined by a JASCO DIP-371 and DIP-3000 digital polarimeters. UV

Table 2. ^{1}H- and ^{13}C-NMR Data of Nostopeptin B (**2**) in DMSO-d_6

position		^{1}H	*J* (Hz)	^{13}C	HMBC[a]
Ac	1			169.2 (s)	Ac 2, Gln NH
	2	1.80 (s)		22.3 (q)	
Gln	1			169.8 (s)	Gln 2, Hmp 2
	2	4.35 (m)		49.6 (d)	Gln 3, NH
	3	1.75 (m)		27.9 (t)	Gln 2, 4
	4	2.03 (m)		30.4 (t)	Gln 3, NH_2
		2.11 (m)			
	5			173.6 (s)	Gln 3, 4, NH_2
	NH	8.23 (d)	7.3		
	NH_2	7.00 (br)			
		7.24 (br)			
Hmp	1			167.4 (s)	Hmp 2, Leu NH
	2	4.41 (d)	3.3	64.0 (d)	Hmp 3, 5
	3	5.15 (dd)	5.5, 3.3	77.5 (d)	Hmp 5, Me
	4	2.42 (m)		36.9 (d)	Hmp 5, Me
	5	3.41 (m)		51.5 (t)	Hmp 3, Me
		4.29 (dd)	9.1, 4.0		
	Me	1.04 (d)	6.6	10.9 (q)	Hmp 5
Leu	1			170.9 (s)	Leu 2, 3
	2	4.30 (dd)	9.2, 2.9	50.3 (d)	Leu 3, NH
	3	1.43 (m)		39.5 (t)	Leu 2, 5, 5′
		1.77 (m)			
	4	2.06 (m)		22.6 (d)	Leu 3, 5, 5′
	5	0.77 (d)	7.3	21.0 (q)	Leu 3, 5′
	5′	0.85 (m)		24.1 (q)	Leu 3, 5
	NH	8.42 (d)	9.2		
Ahp	2			169.3 (s)	Ile(I) 2
	3	4.47 (ddd)	12.1, 9.2, 6.6	48.4 (d)	
	4	1.73 (m)		21.3 (t)	
		2.70 (m)			
	5	1.74 (m)		29.7 (t)	
	6	4.90 (m)		73.9 (d)	Ile(I) 2
	NH	7.08 (d)	9.2		
	OH	6.10 (d)	3.3		
Ile(I)	1			169.8 (s)	Ile(I) 2, MeTyr Me
	2	4.38 (d)	10.9	54.1 (d)	Ile(I) Me
	3	1.78 (m)		32.8 (d)	Ile(I) 2, 4, Me
	4	0.62 (m)		23.7 (t)	Ile(I) 5, Me
		1.09 (m)			
	5	0.63 (m)		10.3 (q)	Ile(I) 4
	Me	−0.11 (d)	6.6	13.9 (q)	Ile(I) 4
N-MeTyr	1			169.3 (s)	MeTyr 2, Ile(II) 2, NH
	2	5.13 (m)		60.4 (d)	MeTyr 3
	3	2.64 (m)		33.4 (t)	MeTyr 5, 9
		3.14 (m)			
	4			127.4 (s)	MeTyr 3, 6, 8
	5, 9	6.98 (d)	8.4	130.3 (d)	MeTyr 3
	6, 8	6.63 (d)	8.4	115.3 (d)	MeTyr OH
	7			156.3 (s)	MeTyr 5, 6, 8, 9
	Me	2.70 (s)		30.4 (q)	
	OH	9.16 (s)			
Ile(II)	1			173.2 (s)	Ile(II) 2
	2	4.81 (dd)	9.6, 4.4	55.7 (d)	Ile(II) Me
	3	1.90 (m)		38.0 (d)	Ile(II) 2, 5, Me
	4	0.95 (m)		23.6 (t)	Ile(II) 2
		1.17 (m)			
	5	0.79 (t)	7.3	11.3 (q)	
	Me	0.85 (m)		16.1 (q)	Ile(II) 2
	NH	7.59 (d)	9.6		

[a] Protons correlated to the carbon of the raw.

spectra were measured on a Hitachi 330 spectrophotometer. FABMS were measured by using glycerol as a matrix on a JEOL SX102 mass spectrometer. Amino acid analyses were carried out with Hitachi 835 and L-8500A amino acid analyzers.

Culture of *N. minutum*. *N. minutum* (NIES-26) was obtained from the NIES Collection and cultured in 10-L glass bottles containing CB medium [$Ca(NO_3)_2$·4 H_2O 15 mg, KNO_3 10 mg, β-Na_2glycerophosphate 5 mg, $MgSO_4$·7 H_2O 4 mg, vitamin B_{12} 0.01 μg, biotin 0.01 μg, thiamine HCl 1 μg, PIV metals 0.3 mL, Bicine 50 mg, and distilled H_2O 99.7 mL, pH 9.0] with aeration at 25 °C under illumination of 200 μE/m^2·s on a 12L:12D cycle. After 23−35 days, the algal cells were filtered by 90-μm nylon plankton nets (Swiss Silk Bolting Cloth Mfg. Co., Ltd.), lyophilized, and kept in a freezer at −20 °C until extraction. The yield was 0.4 g/L on an average. From the first culture, 161 g of algal cells were obtained from 380 L of culture, and the second culture yielded 231 g of algal cells from 590 L of culture.

Extraction and Isolation. Freeze-dried alga of the first mass culture was extracted with MeOH, concentrated, and partitioned between Et_2O and H_2O. The Et_2O-soluble fraction was partitioned between *n*-hexane and MeOH−H_2O (9:1), and the 90% MeOH layer was subsequently partitioned between CCl_4 and MeOH−H_2O (8:2). The 80% MeOH layer was further partitioned between $CHCl_3$ and MeOH−H_2O (6:4). The $CHCl_3$ layer was subjected to ODS flash chromatography with increasing amounts of MeOH in H_2O (20−100%). The fractions eluted with 40−80% MeOH were purified by HPLC on Capcell Pak C_{18} (10 × 250 mm, mobile phase MeCN−H_2O−TFA 33:67:0.05, flow rate 3.0 mL/min, UV 210 nm) to yield nostopeptin A (**1**, 20 mg).

The Et_2O fraction of the extract of the second mass culture was purified by the same procedure to afford **1** (61 mg). The aqueous fraction was partitioned between H_2O and BuOH. The BuOH-soluble fraction was subjected to ODS flash chromatography (20−100% MeOH). The 60% MeOH eluate was purified by linear-gradient system of HPLC on Capcell Pak C_{18} (10 × 250 mm, mobile phase MeCN−H_2O−TFA 27:73:0.05−37:63:0.05, flow rate 3.0 mL/min, UV 210 nm) to afford **1** (8.5 mg) and nostopeptin B (**2**, 8.5 mg).

Amino Acid Analyses. Each compound (100 μg) was dissolved in 6 N HCl (1 mL) and sealed in a test tube. The test tubes were heated at 110 °C for 16 h. The solution was evaporated and redissolved in 0.1 N HCl to be subjected on an automatic amino acid analyzer.

The hydrolysate of **1** was heated in 10% HCl in *i*-PrOH (0.5 mL) at 100 °C for 30 min and then treated with trifluoroacetic anhydride in CH_2Cl_2 [1:1 (v/v), 0.6 mL] at 100 °C for 5 min. Chiral gas chromatography was carried out by using a Chirasil Val III capillary column (0.32 mm × 25 m) with a flame ionization detector (FID). Column temperature was kept at 50 °C for 10 min and increased to 200 °C at a rate of 4 °C/min. Helium was used as carrier gas. Retention times (minutes): D-*a*Ile (21.4), D-Ile (22.1), L-Ile (23.0), D-Leu (24.3), L-Leu (26.3), D-Glu (35.2), L-Glu (36.1).

For determination of the stereochemistry of *N*-MeTyr, 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA) or D-FDAA in Me_2CO (10 mg/mL) and 100 μL of 1 M $NaHCO_3$ were added to the acid hydrolysate of a 300-μg portion of each compound, and the mixture was kept at 80 °C for 3 min. To the reaction mixture, 50 μL of 2 N HCl and 300 μL of 50% MeCN were added, and the mixture was analyzed by reversed-phase ODS−HPLC: column Cosmosil MS (Nacalai Tesque) (4.6 × 250 mm); mobile phase MeCN−H_2O−TFA (40:60:0.1); UV (340 nm); flow rate (1 mL/min). Retention times (min): *N*-Me-L-Tyr-L-FDAA (23.6), *N*-Me-L-Tyr-D-FDAA (25.1).

Nostopeptin A (1): colorless amorphous powder; $[\alpha]^{23}$D −114° (*c* 0.08, MeOH); HRFABMS *m*/*z* 937.5324 ([M − OH]$^+$, calcd for $C_{48}H_{73}N_8O_{11}$ 937.5399); UV

(MeOH) λ max 278 (ϵ 1200). For ^{1}H- and ^{13}C-NMR data, see Table 1.

Nostopeptin B (2): colorless amorphous powder; $[\alpha]^{23}_D$ −91° (*c* 0.1, MeOH); HRFABMS *m/z* 927.5252 ($[M + H]^+$, calcd for $C_{46}H_{71}N_8O_{12}$ 927.5191); UV (MeOH) λ max 278 (ϵ 1300). For ^{1}H- and ^{13}C-NMR data, see Table 2.

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