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Oscillapeptins A to F, Serine Protease Inhibitors from the Three Strains of *Oscillatoria agardhii*

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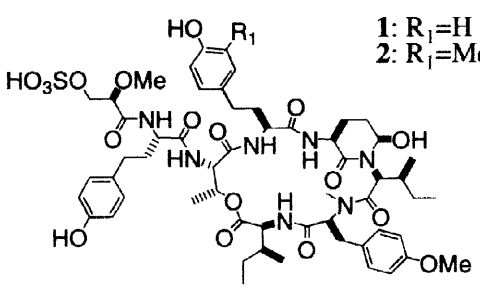
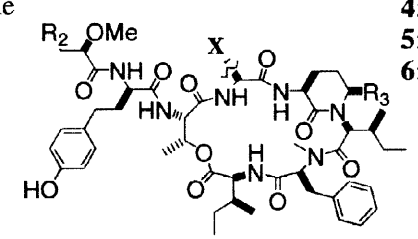
Abstract: Oscillapeptins B (**2**) to F (**6**), which were congeners of oscillapeptin A (**1**), were isolated from the three strains of cultured cyanobacterium *Oscillatoria agardhii*. These structures were established by spectroscopic analysis including the 2D NMR techniques. The absolute configurations of oscillapeptins were determined by the spectral and chemical methods. Oscillapeptins A (**1**) to E (**5**) inhibited chymotrypsin and/or elastase, and oscillapeptin F (**6**) inhibited trypsin and plasmin.

The search for new enzyme inhibitors from natural sources has led to the discovery of structurally diverse and biologically operative compounds for structure-based drug design. In this regard, we have reported a number of serine protease inhibitors from cyanobacteria including radiosumins,[1] microviridins,[2] aeruginosins,[3] and micropeptin-type peptides. Micropeptins A and B,[4] cyclic depsipeptides containing a 3-amino-6-hydroxy-2-piperidone (Ahp) unit, were first isolated from *Microcystis aeruginosa* (NIES-100) as potent trypsin inhibitors. Related protease inhibitors are widely distributed among other several freshwater cyanobacteria, such as nostopeptins from *Nostoc minutum*,[5] micropeptin 103 from *M. viridis*,[6] micropeptins 88-A to 88-F from *M. aeruginosa*,[7] and oscillapeptin A (**1**) from *Oscillatoria agardhii* (NIES-204), which potently inhibited elastase and chymotrypsin.[8]

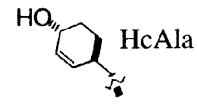
In our continuous survey of freshwater cyanobacteria for protease inhibitors, we isolated oscillapeptin B (**2**) and oscillapeptins C (**3**) to E (**5**) from *O. agardhii* (NIES-204 and 205, respectively) as chymotrypsin and/or elastase inhibitors. Furthermore, oscillapeptin F (**6**) was isolated from *O. agardhii* (NIES-596) as a trypsin and plasmin inhibitor. In this paper we report the isolation and structure elucidation of **2** to **6** and the determination of absolute configurations of **1** to **4** and **6**.



1: R_1=H
2: R_1=Me



3: R_2=OH, R_3=OMe, X=HcAla
4: R_2=OSO_3H, R_3=OH, X=HcAla
5: R_2=OSO_3H, R_3=OH, X=Hty
6: R_2=OSO_3H, R_3=OH, X=Lys



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O. agardhii (NIES-205), from which trypsin and plasmin inhibitors aeruginosins 205-A and –B were isolated,[3b] showed the potent inhibitory activities against elastase and chymotrypsin. Assay-guided fractionation resulted in the isolation of oscillapeptin C (**3**, 3.8 mg) from the 60% MeOH fraction, oscillapeptins D (**4**, 5.0 mg) and E (**5**, 4.1 mg) from the 50% MeOH fraction.

Oscillapeptin D (4). The molecular formula of **4** was deduced as $C_{54}H_{77}N_7O_{17}S$ by HRFABMS and the fragment ion of negative FABMS [*m/z* 1046, $(M\text{-}SO_3\text{-}H)^-$] indicated the presence of sulfate as **1**. Amino acid analysis of acid hydrolyzate of **4** revealed the presence of one residue of Thr and two residues of Ile. The ^1H and ^{13}C NMR spectra in DMSO-d_6 suggested depsipeptidic nature of **4**, showing five amide protons, seven amide carbonyl groups, and one ester carbonyl group. The NMR spectra of **4** were similar to those of **1**, except for a set of disubstituted double bond signals (δ_H 5.41, brd, J=10.3 Hz, and δ_H 5.55, brd, J=10.3 Hz) observed in ^1H NMR spectrum. The interpretation of 2D NMR analyses including ^1H-^1H COSY, HMBC[9] and HMQC[10] assigned three usual amino acids (Ile (1), Ile (2) and Thr), although the amide proton of Ile (1) was not recognized. Other structural units, i.e. a 2-*O*-methylglyceric acid 3-*O*-sulfate (Mgs), a homotyrosine (Hty), a *N*-methylphenylalanine (Nmf), and a 3-(4'-hydroxy-2'-cyclohexenyl)alanine (HcAla),[7] were established by 2D NMR experiments. The presence of Ahp was also deduced on the basis of ^1H-^1H COSY and HMBC spectra. Since Ile (1) was suggested to be *N,N*-disubstituted by the absence of an amide proton, it was supposed that Ahp involved the amino group of the Ile (1) moiety, which was confirmed by HMBC correlations (Ile (1) H-2/Ahp CO, Ile (1) H-2/Ahp C-6). This result and HMBC correlations between adjacent units (Ile (2) NH/Nmf CO, Nmf *N*-Me/Ile (1) CO, Ahp NH/HcAla CO, HcAla NH/Thr CO, Thr NH/Hty CO, Hty NH/Mgs CO) allowed us to establish the sequence of eight segments, (Ile (2)-Nmf-Ile (1)-Ahp-HcAla-Thr-Hty-Mgs). In addition, HMBC correlation (Thr H-3/Ile (2) CO) indicated the lactone structure between the Thr hydroxy group and the Ile (2) carbonyl group. It was consistent with both the absence of the hydroxy proton signal and downfield shift (δ 5.46) of the methine proton of Thr. Thus the gross structure of **4** was determined as Fig. 1.

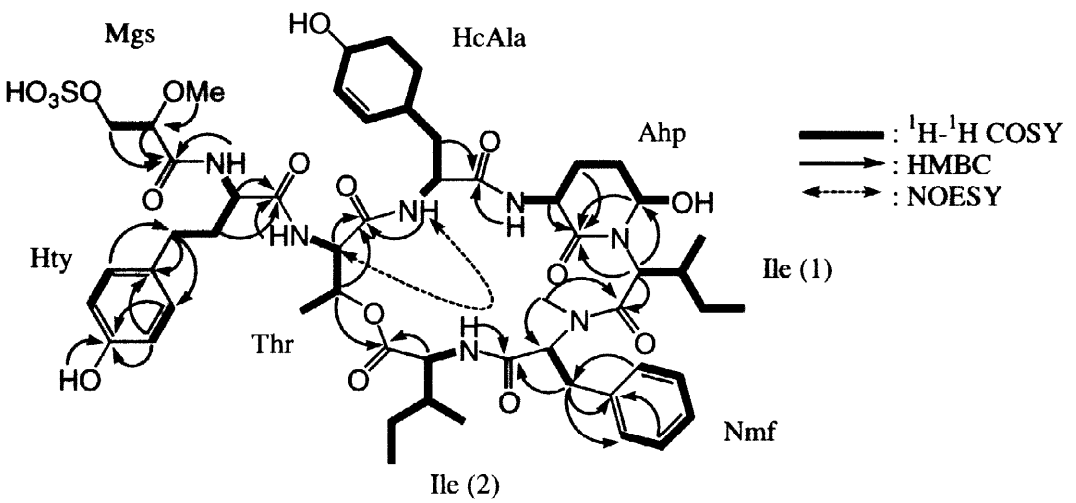

Fig. 1. Selected ^1H-^1H COSY, HMBC, and NOESY correlations for **4**

Oscillapeptin C (3). The ^1H NMR spectrum of **3** was similar to that of **4**. However a hydroxy proton signal of Ahp was absent and a methine (H-6) proton signal was shifted upfield (δ_H 4.43), furthermore a new methoxy signal appeared at 3.01 ppm. These differences could be explained by the presence of *O*-Me at C-6 of Ahp, which was confirmed by HMBC correlations (H-6/*O*-Me, *O*-Me/C-6). Thus a 3-amino-6-methoxy-2-

piperidone (Amp) unit was established. The fragment peak of negative FABMS of **3** did not indicate the presence of sulfate. In the ^{1}H NMR spectrum, the chemical shifts of glyceric acid portion was shifted upfield in comparison with those of **4**, and a new hydroxy proton signal appeared at 4.84 ppm. These facts and COSY data assigned a 2-*O*-methylglyceric acid (Mga) unit instead of Mgs in **4**, and thus the gross structure of **3** was determined as depicted.

Oscillapeptin E (5). The negative FABMS data indicated that the molecular weight of **5** differed from **1** by 30 mass units. The ^{1}H and ^{13}C NMR spectra of **5** and **1** differed only slightly. A methoxy signal of a *N,O*-dimethyltyrosine (Dmy) unit was missing from the ^{1}H and ^{13}C NMR spectra of **5**. This fact suggested that Nmf was present in **5** instead of Dmy in **1**. Amino acid analysis and NMR analysis confirmed the proposed structure.

Oscillapeptin B (2). **2** (5.6 mg) was isolated from the cultured *O. agardhii* (NIES-204), from which oscillapeptin A (**1**) was isolated previously.[8] The negative FABMS data indicated that the molecular weight of **2** differed from **1** by 14 mass units. The ^{1}H and ^{13}C NMR spectra of **2** and **1** were very similar. The ^{1}H NMR spectra of **2** showed one more methyl signal at 1.19 ppm, which was attached to C-7 of Hty by HMBC correlations [7-Me/C-6, 7-Me/C-8, H-6/7-Me]. The interpretation of 2D NMR analyses revealed that a 7-methylhomotyrosine (Mhty) unit was present in **2** instead of Hty between Thr and Ahp in **1**.

Oscillapeptin F (6). *O. agardhii* (NIES-596), isolated from Veluwemeer in Holland, was mass cultured in our laboratory. The crude extracts from this alga showed the potent inhibitory activities against plasmin and trypsin. Freeze-dried alga (135.9 g from 400 L of culture medium) was extracted twice with 80% MeOH and once with 100% MeOH. The extracts were concentrated and partitioned between Et_2O and H_2O. The active H_2O fraction was further partitioned between *n*-BuOH and H_2O. The *n*-BuOH-soluble material was subjected to ODS flash chromatography and eluted with aqueous MeOH. The active 80% MeOH fraction was purified by HPLC to yield oscillapeptin F (**6**, 38.3 mg) as the active principal.

The ^{1}H and ^{13}C NMR spectra indicated that **6** was an analogue of **4**. Amino acid analysis and 2D NMR experiments of **6** showed the presence of Lys instead of HcAla. Amino acid analysis and NMR analysis confirmed the proposed structure.

Absolute stereochemistry. The absolute configurations of oscillapeptins were determined by the spectral and chemical methods. The stereochemistry of usual and *N*-methyl amino acids (Ile, Thr, Lys, Dmy, and Nmf) in **1** to **6** was determined by HPLC analysis of the acid hydrolyzates derivatized with Marfey's reagent,[11] which allowed us to assign the L configurations for all these amino acids. In order to determine the absolute configurations of Hty in **1** to **6** by Marfey's analysis, Hty standard was prepared from the acid hydrolyzate of anabaenopeptin F,[12] a cyclicpeptide isolated from *O. agardhii* (NIES-204). Prepared Hty was ozonized using an oxidative workup, followed by derivatization with Marfey's reagent to give L-Glu, indicating that prepared Hty was the L-form. By using the prepared L-Hty as standard, the stereochemistry of Hty in **1** and **2** was determined as the L configuration, and that in **3**, **4** and **6** was the D configuration. But Marfey's analysis of **5** revealed the presence of both L- and D-Hty. The partial acid hydrolysis (3N HCl/EtOH=1:1, 80°C, 48 h) of **5** (1.0 mg) was carried out, but unfortunately the expected fragments were not obtained. In all other Ahp-containing metabolites, however, an amino acid residue between Ahp and Thr was the L configuration without exception. Therefore, one Hty between Ahp and Thr in **5** was presumed to be the L configuration, and the other between Thr and Mgs to be the D configuration. Ozonolysis of **2** using an oxidative workup, followed by hydrolysis and derivatization gave 1.8 equiv. of L-Glu, which must be derived from Hty and Mhty in **2**. Therefore the absolute stereochemistry of Mhty in **2** is also the L configuration.

The relative stereochemistry of Amp in **3** was deduced as shown in Fig. 2 by NOESY correlations. **3** was oxidized with CrO_3 in AcOH, followed by hydrolysis to give L-Glu. Therefore, the absolute stereochemistry of Amp in **3** was decided to be 3*S*, 6*R* configuration.

Because the chemical shifts of H-4a and H-5 (2H) in Ahp were overlapped in ^{1}H NMR spectrum of **1**, **2**, **4**, **5** and **6**, the configuration of OH at C-6 was not determined by NOESY data. However, in the case of all other related compounds the OH in Ahp was the axial configuration, and the OH in Ahp have an important role for the inhibitory mechanism against protease. In consideration of these points, the configuration of the OH of Ahp in **1**, **2**, **4**, **5** and **6** was also to be axial, so the relative stereochemistry of Ahp was deduced as shown in Fig. 2. The reduction of **1**, **2**, **4**, **5** and **6** with $NaBH_4$ followed by hydrolysis produced both L-pentahomoserine and L-Pro, which was confirmed by Marfey's analysis.[7] Therefore, the absolute chemistry of Ahp was also deduced as 3*S*, 6*R*.

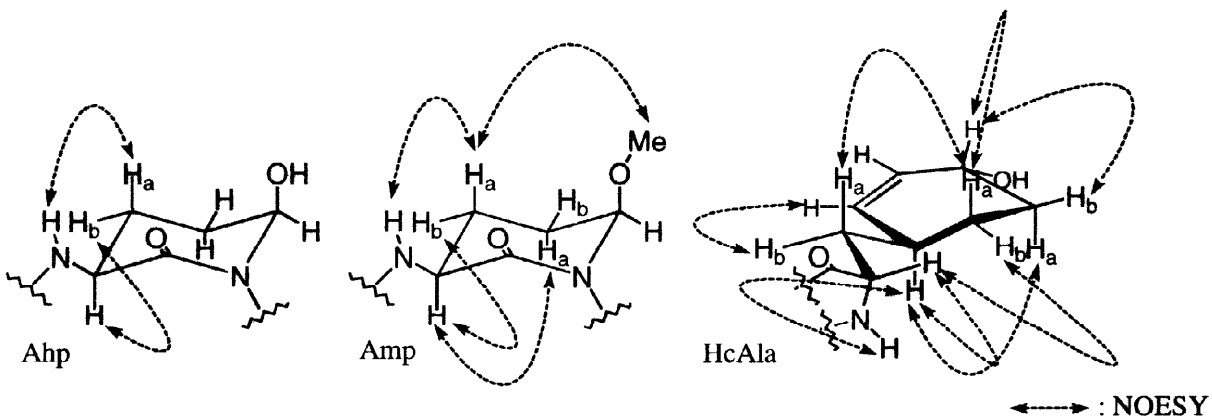

Fig. 2. The relative stereochmistry of Ahp, Amp, and HcAla

The absolute stereochemistry of HcAla in **3** and **4** was determined by same procedures described in the structure elucidation of micropeptins 88-A and -D.[7] The relative stereochemistry of HcAla was deduced as shown in Fig. 2 by NOESY correlations. **4** triacetate was hydrogenated with Pd-black, followed by hydrolysis to produce 2-amino-3-cyclohexylpropionic acid, which was proved to be the *S* configuration at C-2 by Marfey's analysis compared with synthetic enantiomers. Therefore, the absolute configuration of HcAla in **4** was determined to be (2*S*, 1'*S*, 4'*R*)–3-(4'-hydroxy-2'-cyclohexenyl)alanine. The absolute stereochemistry of HcAla in **3** was determined to be identical with that of **4** by the same procedure.

The determination of the absolute stereochemistry of Mgs and Mga in **1** to **6** was achieved by chiral HPLC analysis. The each hydrolyzate of **1** to **6** was esterified with *p*-bromophenacyl bromide to detect at 280 nm on HPLC, which was the effective method to separate a racemic carboxylic acid.[13] Comparison by chiral HPLC of the each derivatized hydrolyzate with standard, which was synthesized from DL-glyceric acid using Me_3OBF_4 in the presence of proton sponge,[14] showed that the absolute stereochemistry of Mgs and Mga was the D configuration.

Discussion

Oscillapeptins A (**1**), B (**2**), C (**3**), D (**4**), and E (**5**) inhibited chymotrypsin with IC_{50}'s of 2.2, 2.1, 3.0, 2.2, and 3.0 μg/mL, respectively. Compounds **1**, **2**, **4**, and **5** also inhibited elastase with IC_{50}'s of 0.3, 0.05, 30, and 3.0 μg/mL, respectively, whereas **3** did not show any inhibitory activities at 100 μg/mL. Although oscillapeptin

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

Position		^{1}H J (Hz)	^{13}C	HMBC
Mgs	1		169.0 (s)	
	2	3.86 (dd, 6.8, 3.0)	81.0 (d)	Mgs C-1, 3, *O*-Me
	3a	3.76 (dd, 10.7, 6.8)	66.2 (t)	Mgs C-1, 2
	3b	3.97 (dd, 10.7, 3.0)		
	O-Me	3.35 (s)	57.7 (q)	Mgs C-2
Hty	1		172.1 (s)	
	2	4.53 (ddd, 8.5, 8.5, 5.6)	52.3 (d)	Hty C-1, 3, 4
	3a	1.82 (m)	35.0 (t)	Hty C-1, 2, 4, 5
	3b	1.93 (m)		
	4	2.42 (2H, m)	30.4 (t)	Hty C-2, 3, 5, 6, 10
	5		131.4 (s)	
	6, 10	6.96 (d, 8.1)	129.2 (d)	Hty C-4, 5, 7, 8, 9
	7, 9	6.62 (d, 8.1)	115.0 (d)	Hty C-5, 6, 8, 10
	8		155.1 (s)	
	NH	8.01 (d, 8.5)		Mgs C-1, Hty C-2
	OH	9.06 (s)		Hty C-7, 8, 9
Thr	1		169.1 (s)	
	2	4.62 (d, 9.4)	55.0 (d)	Hty C-1, Thr C-1, 3
	3	5.46 (q, 6.8)	71.6 (d)	Thr C-1, 2, 4, Ile (2) C-1
	4	1.17 (d, 6.8)	17.6 (q)	Thr C-2, 3
	NH	8.12 (d, 9.4)		Hty C-1, Thr C-1
HcAla	1		170.7 (s)	
	2	4.30 (br)	49.8 (d)	
	3a	1.50 (m)	36.5 (t)	HcAla C-1, 5
	3b	1.80 (m)		
	4	2.00 (m)	(d)	HcAla C-5
	5	5.41 (brd, 10.3)	131.9 (d)	HcAla C-3
	6	5.55 (brd, 10.3)	132.7 (d)	HcAla C-8
	7	3.94 (m)	65.1 (d)	HcAla C-6
	8a	1.20 (m)	31.4 (t)	HcAla C-6
	8b	1.74 (m)		
	9a	0.93 (m)	26.1 (t)	HcAla C-5
	9b	1.70 (m)		
	NH	8.43 (d, 9.0)		Thr C-1
Ahp	2		169.8 (s)	
	3	4.41 (ddd, 11.9, 9.4, 6.4)	49.0 (d)	Ahp C-2, 4
	4a	1.70 (m)	21.7 (t)	Ahp C-2, 3, 5, 6
	4b	2.55 (m)		
	5	1.70 (2H, m)	29.7 (t)	Ahp C-3, 4, 6
	6	4.89 (br)	74.0 (d)	Ahp C-2, 4, 5
	NH	7.34 (d, 9.0)		HcAla C-1, Ahp C-3
	OH	6.06 (d, 3.4)		Ahp C-6
Ile (1)	1		169.7 (s)	
	2	4.38 (d, 10.7)	54.0 (d)	Ahp C-2, 6, Ile (1) C-1, 3
	3	1.70 (m)	23.0 (d)	Ile (1) C-2, 4, 6
	4a	0.58 (m)	23.6 (t)	Ile (1) C-3, 5, 6
	4b	1.04 (m)		
	5	0.60 (d, 4.0)	10.2 (q)	Ile (1) C-3, 4
	6	-0.28 (d, 6.4)	13.8 (q)	Ile (1) C-2, 3, 4
Nmf	1		169.2 (s)	
	2	5.12 (dd, 11.1, 3.4)	60.3 (d)	Nmf C-1, 3
	3a	2.81 (dd, 14.5, 11.1)	34.2 (t)	Nmf C-1, 2, 4, 5, 9
	3b	3.24 (dd, 14.5, 3.4)		
	4		137.2 (s)	
	5, 9	7.20 (d, 7.3)	129.5 (d)	Nmf C-3, 4, 6, 7, 8
	6, 8	7.23 (t, 7.3)	128.6 (d)	Nmf C-4, 5, 7, 9
	7	7.18 (t, 7.3)	126.6 (d)	
	N-Me	2.71 (s)	30.0 (q)	Ile (1) C-1, Nmf C-2
Ile (2)	1		172.4 (s)	
	2	4.74 (dd, 9.4, 5.1)	55.4 (d)	Nmf C-1, Ile (2) C-1, 3, 4, 6
	3	1.70 (m)	37.5 (d)	Ile (2) C-1, 2, 4, 5, 6
	4a	0.98 (m)	24.6 (t)	Ile (2) C-2, 3, 5, 6
	4b	1.22 (m)		
	5	0.78 (t, 7.3)	11.2 (q)	Ile (2) C-3, 4
	6	0.81 (d, 6.9)	16.0 (q)	Ile (2) C-2, 3, 4
	NH	7.63 (d, 9.4)		Nmf C-1, Ile (2) C-2

Table 2. ^{1}H and ^{13}C NMR Data for Oscillapeptins C (**3**) and E (**5**) in DMSO-d_6

		Oscillapeptin C (**3**)				Oscillapeptin E (**5**)	
Position		^{1}H J (Hz)	^{13}C	Position		^{1}H J (Hz)	^{13}C
Mga	1		169.8 (s)	Mgs	1		168.8 (s)
	2	3.70 (m)	84.0 (d)		2	3.88 (dd, 6.4, 2.6)	80.8 (d)
	3a	3.56 (m)	62.5 (t)		3a	3.80 (dd, 10.7, 6.4)	66.1 (t)
	3b	3.65 (m)			3b	4.01 (dd, 10.7, 2.6)	
	OH	4.84 (dd, 9.8, 5.1)			*O*-Me	3.36 (s)	57.5 (q)
	O-Me	3.35 (s)	57.5 (q)	Hty (1)	1		170.4 (s)
Hty	1		172.1 (s)		2	4.57 (m)	52.0 (d)
	2	4.53 (ddd, 8.6, 8.6, 4.7)	52.5 (d)		3a	1.92 (m)	35.0 (t)
	3a	1.85 (m)	34.9 (t)		3b	1.96 (m)	
	3b	1.94 (m)			4	2.45 (2H, m)	30.4 (t)
	4	2.45 (2H, m)	30.4 (t)		5		131.5 (s)
	5		131.0 (s)		6, 10	6.96 (d, 8.1)	129.2 (d)
	6, 10	6.94 (d, 8.5)	129.1 (d)		7, 9	6.62 (d, 8.1)	115.0 (d)
	7, 9	6.65 (d, 8.5)	115.2 (d)		8		155.6 (s)
	8		154.0 (s)		NH	8.04 (d, 8.5)	
	NH	7.94 (d, 8.6)			OH	9.08 (s)	
	OH	9.12 (s)		Thr	1		172.4 (s)
Thr	1		169.2 (s)		2	4.72 (d, 9.4)	55.1 (d)
	2	4.64 (d, 9.4)	55.1 (d)		3	5.50 (q, 6.8)	71.6 (d)
	3	5.52 (q, 6.3)	72.0 (d)		4	1.21 (d, 6.8)	17.8 (q)
	4	1.19 (d, 6.3)	17.8 (q)		NH	8.18 (d, 9.4)	
	NH	8.13 (d, 9.4)		Hty (2)	1		170.1 (s)
HcAla	1		170.7 (s)		2	4.13 (dt, 10.7, 8.5, 2.8)	51.5 (d)
	2	4.33 (br)	50.0 (d)		3a	1.68 (m)	32.3 (t)
	3a	1.51 (m)	36.5 (t)		3b	2.21 (m)	
	3b	1.83 (m)			4a	2.35 (m)	30.3 (t)
	4	2.01 (m)	31.7 (d)		4b	2.52 (m)	
	5	5.42 (brd, 10.3)	132.0 (d)		5		131.5 (s)
	6	5.58 (brd, 10.3)	132.9 (d)		6, 10	6.90 (d, 8.4)	129.2 (d)
	7	3.97 (m)	65.2 (d)		7, 9	6.61 (d, 8.4)	114.9 (d)
	8a	1.22 (m)	31.3 (t)		8		155.0 (s)
	8b	1.79 (m)			NH	8.47 (d, 8.5)	
	9a	0.98 (m)	25.9 (t)		OH	9.08 (s)	
	9b	1.70 (m)		Ahp	2		168.8 (s)
	NH	8.49 (d, 9.0)			3	4.42 (ddd, 11.5, 9.4, 6.4)	48.8 (d)
Amp	2		169.0 (s)		4a	1.73 (m)	21.7 (t)
	3	4.47 (ddd, 11.6, 9.5, 6.5)	48.5 (d)		4b	2.56 (m)	
	4a	1.72 (m)	21.7 (t)		5	1.73 (2H, m)	29.8 (t)
	4b	2.40 (m)			6	4.90 (br)	74.0 (d)
	5a	1.67 (m)	23.6 (t)		NH	7.32 (d, 9.4)	
	5b	2.06 (m)			OH	6.06 (d, 3.0)	
	6	4.43 (br)	83.0 (d)	Ile (1)	1		169.8 (s)
	NH	7.22 (d, 8.0)			2	4.38 (d, 10.7)	54.0 (d)
	O-Me	3.01 (s)	55.2 (q)		3	1.73 (m)	32.5 (d)
Ile (1)	1		169.5 (s)		4a	0.60 (m)	23.6 (t)
	2	4.42 (d, 10.7)	54.2 (d)		4b	1.06 (m)	
	3	1.79 (m)	32.6 (d)		5	0.59 (d, 3.9)	10.2 (q)
	4a	0.60 (m)	23.6 (t)		6	-0.26 (d, 6.4)	13.8 (q)
	4b	1.06 (m)		Nmf	1		168.8 (s)
	5	0.59 (d, 3.9)	10.0 (q)		2	5.13 (dd, 11.5, 3.0)	60.4 (d)
	6	-0.29 (d, 6.8)	13.5 (q)		3a	2.82 (dd, 15.0, 11.5)	34.1 (t)
Nmf	1		169.0 (s)		3b	3.26 (m)	
	2	5.18 (dd, 11.5, 3.4)	60.5 (d)		4		137.6 (s)
	3a	2.81 (dd, 14.1, 11.5)	34.1 (t)		5, 9	7.22 (d, 7.3)	129.4 (d)
	3b	3.27 (dd, 14.1, 3.4)			6, 8	7.24 (dd, 7.5, 7.3)	128.6 (d)
	4		137.0 (s)		7	7.18 (t, 7.5)	126.5 (s)
	5, 9	7.22 (d, 7.3)	129.5 (d)		*N*-Me	2.72 (s)	30.0 (q)
	6, 8	7.27 (t, 7.3)	128.7 (d)	Ile (2)	1		172.4 (s)
	7	7.18 (t, 7.3)	126.8 (d)		2	4.75 (dd, 9.4, 5.6)	55.2 (d)
	N-Me	2.73 (s)	30.1 (q)		3	1.76 (m)	37.2 (d)
Ile (2)	1		172.4 (s)		4a	1.02 (m)	24.6 (t)
	2	4.72 (m)	55.2 (d)		4b	1.24 (m)	
	3	1.73 (m)	37.8 (d)		5	0.80 (t, 7.3)	11.2 (q)
	4a	1.09 (m)	24.9 (t)		6	0.86 (d, 6.8)	15.9 (q)
	4b	1.31 (m)			NH	7.63 (d, 9.4)	
	5	0.82 (t, 7.3)	11.0 (q)				
	6	0.86 (d, 7.0)	16.0 (q)				
	NH	6.92 (d, 8.6)					

Table 3. ^1H and ^{13}C NMR Data for Oscillapeptins B (2) and F (6) in DMSO-d_6

		Oscillapeptin B (2)			Oscillapeptin F (6)	
Position		^1H J (Hz)	^{13}C	Position	^1H J (Hz)	^{13}C
Mgs	1		169.0 (s)	Mgs	1	168.9 (s)
	2	3.94 (m)	80.2 (d)		2	3.93 (dd, 6.4, 2.6)	80.3 (d)
	3a	3.77 (m)	66.2 (t)		3a	3.78 (dd, 10.2, 6.4)	66.4 (t)
	3b	3.93 (m)			3b	3.96 (dd, 10.2, 2.6)	
	O-Me	3.32 (s)	57.4 (q)		*O*-Me	3.33 (s)	57.4 (q)
Hty	1		172.1 (s)	Hty	1		172.1 (s)
	2	4.53 (ddd, 8.1, 8.1, 5.2)	52.0 (d)		2	4.59 (ddd, 8.1, 8.1, 4.7)	52.1 (d)
	3	1.88 (2H, m)	34.4 (t)		3a	1.87 (m)	35.0 (t)
	4a	2.42 (m)	30.5 (t)		3b	1.95 (m)	
	4b	2.52 (m)			4	2.47 (2H, m)	30.5 (t)
	5		131.5 (s)		5		131.5 (s)
	6, 10	6.94 (d, 8.4)	129.0 (d)		6, 10	6.96 (d, 8.1)	129.2 (d)
	7, 9	6.62 (d, 8.4)	115.0 (d)		7, 9	6.63 (d, 8.1)	115.0 (d)
	8		155.3 (s)		8		155.0 (s)
	NH	8.02 (d,7.7)			NH	8.08 (d, 8.1)	
	OH	9.06 (s)			OH	9.12 (s)	
Thr	1		169.2 (s)	Thr	1		172.4 (s)
	2	4.74 (d, 10.3)	55.1 (d)		2	4.62 (d, 9.0)	55.0 (d)
	3	5.51 (q, 6.6)	71.8 (d)		3	5.49 (q, 6.4)	71.7 (d)
	4	1.22 (d, 6.6)	17.7 (q)		4	1.20 (d, 6.4)	17.6 (q)
	NH	8.12 (d, 10.3)			NH	8.30 (d, 9.0)	
Mhty	1		170.4 (s)	Lys	1		170.2 (s)
	2	4.15 (ddd, 10.7, 8.7, 2.8)	51.6 (d)		2	4.28 (m)	51.2 (d)
	3a	1.65 (m)	32.5 (t)		3a	1.43 (m)	29.8 (t)
	3b	2.21 (dddd, 13.4, 8.2, 8.2, 3.3)			3b	2.04 (m)	
	4a	2.27 (ddd, 13.4, 8.2, 8.2)	30.4 (t)		4	1.28 (2H, m)	22.1 (t)
	4b	2.47 (m)			5	1.50 (2H, m)	26.1 (t)
	5		130.9 (s)		6	2.73 (2H, m)	38.6 (t)
	6	6.76 (d, 8.4)	130.5 (d)		NH_2	7.68 (m)	
	7		123.4 (s)		NH	8.46 (d, 8.3)	
	8		153.3 (s)	Ahp	2		169.2 (d)
	9	6.61 (d, 8.4)	115.0 (d)		3	4.44 (ddd, 11.9, 8.9, 6.8)	48.9 (d)
	10	6.94 (d, 8.4)	129.0 (d)		4a	1.72 (m)	21.7 (t)
	7-Me	1.99 (s)	16.0 (q)		4b	2.56 (m)	
	NH	8.44 (d, 8.4)			5	1.72 (2H, m)	29.7 (t)
	OH	8.93 (s)			6	4.92 (br)	73.9 (d)
Ahp	2		169.2 (s)		NH	7.34 (d, 8.9)	
	3	4.42 (ddd, 11.6, 9.5, 6.5)	48.8 (d)		OH	6.08 (d, 2.6)	
	4a	1.72 (m)	21.7 (t)	Ile (1)	1		169.7 (s)
	4b	2.58 (m)			2	4.39 (d, 10.7)	54.0 (d)
	5	1.72 (2H, m)	29.7 (t)		3	1.75 (m)	33.0 (d)
	6	4.91 (br)	73.9 (d)		4a	0.59 (m)	23.6 (t)
	NH	7.38 (d, 8.0)			4b	1.06 (m)	
	OH	6.09 (br)			5	0.59 (d, 3.8)	10.1 (q)
Ile (1)	1		169.7 (s)		6	-0.25 (d, 6.4)	13.7 (q)
	2	4.38 (d, 10.6)	54.1 (d)	Nmf	1		168.8 (s)
	3	1.75 (m)	33.0 (d)		2	5.13 (dd, 11.5, 3.0)	60.4 (d)
	4a	0.62 (m)	23.6 (t)		3a	2.82 (dd, 14.1, 11.4)	34.2 (t)
	4b	1.08 (m)			3b	3.28 (dd, 14.1, 3.0)	
	5	0.62 (d, 5.5)	10.3 (q)		4		137.7 (s)
	6	-0.19 (d, 6.6)	13.8 (q)		5, 9	7.21 (d, 7.3)	129.2 (d)
Dmy	1		169.2 (s)		6, 8	7.24 (dd, 7.7, 7.3)	128.6 (d)
	2	5.04 (dd, 11.4, 2.9)	60.6 (d)		7	7.18 (t, 7.7)	126.3 (d)
	3a	2.75 (dd, 15.0, 11.4)	33.2 (t)		*N*-Me	2.72 (s)	30.0 (q)
	3b	3.21 (dd, 15.0, 2.9)		Ile (2)	1		172.3 (s)
	4		129.4 (s)		2	4.79 (dd, 9.4, 5.1)	55.3 (d)
	5, 9	7.12 (d, 8.8)	130.5 (d)		3	1.78 (m)	37.6 (d)
	6, 8	6.81 (d, 8.8)	114.1 (d)		4a	1.02 (m)	24.5 (t)
	7		158.3 (s)		4b	1.23 (m)	
	N-Me	2.71 (s)	30.1 (q)		5	0.80 (t, 7.3)	11.1 (q)
	O-Me	3.66 (s)	55.1 (q)		6	0.86 (d, 6.9)	16.0 (q)
Ile (2)	1		172.7 (s)		NH	7.66 (d, 9.4)	
	2	4.72 (dd, 9.5, 5.5)	55.4 (d)				
	3	1.81 (m)	37.3 (d)				
	4a	1.01 (m)	24.4 (t)				
	4b	1.24 (m)					
	5	0.81 (t, 7.3)	11.1 (q)				
	6	0.85 (d, 7.0)	16.0 (q)				
	NH	7.65 (d, 9.5)					

F (**6**) did not inhibit both chymotrypsin and elastase, it showed the strong inhibitory activities against trypsin and plasmin with IC_{50}'s of 0.2 and 0.03 μg/mL, respectively. These results suggested that an amino acid residue between Thr and Ahp played an essential role to be recognized in selectivity of the inhibitory activities.

The structure of oscillapeptins is closely related to that of A90720A, an Ahp-containing depsipeptide isolated from the terrestrial cyanobacterium *Microchaete loktakensis*. A90720A was reported to have the inhibitory activities against trypsin, thrombin, and plasmin.[15] Clardy *et* al. reported the atomic structure of the trypsin-A90720A complex by X-ray crystallography and discussed putative inhibitory mechanisms.[16] In that the 19-membered ring of A90720A was determined to be in elliptical shape by two hydrogen bonds, one between the lactone carbonyl and the Ahp NH, and the other between the OH of Ahp and the Val NH which corresponds to the Ile (2) NH in oscillapeptins. In consideration of these results, a lack of the inhibitory activity against elastase in **3** may be explained by a change in the shape of the 19-membered ring due to the methylation of the OH of Ahp. The fact that the ^{1}H NMR signal of Ile (2) NH was unusually shifted upfield by 0.7 ppm suggested a change of the hydrogen bond between these atoms. It should be noted that in the case of A90720A the glyceric sulfate of the side chain added two hydrogen bonds to interact with trypsin, while that in **3** was missing which might also attenuated the inhibitory activity against elastase.

Experimental Section

Instrumentation. Optical rotation was determined on a JASCO DIP-1000 digital polarimeter. UV spectra were measured on a Hitachi 330 spectrometer. NMR spectra were recorded on a JEOL JNM-A600 or -A500 NMR spectrometer using DMSO-d_6 as solvent at 27 °C. ^{1}H- and ^{13}C-NMR chemical shifts were referenced to residual solvent peaks of DMSO-d_6 at δ_H 2.49 and δ_C 39.5. FABMS spectra were measured by using glycerol as matrix on a JEOL JMS SX-102 mass spectrometer. Amino acid analysis was carried out with a Hitachi L-8500A amino acid analyzer.

Culture Conditions. Culture conditions of *O. agardhii* (NIES-204 and -205) were the same as previously described.[8, 3b] *O. agardhii* (NIES-596) was obtained from the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and cultured in 10 L glass bottles containing CT medium[17] with aeration (filtered, 0.3 mL/min, without CO_2). Cultures were illuminated with fluorescent lights on a 12L:12D cycle at an intensity of 250 μ/m^2·s at 25 °C for 10-14 days. After incubation, cells were harvested by continuous flow centrifugation at 10 000 rpm. Harvested cells were lyophilized and kept in a freezer at −20 °C until extraction.

Extraction and Isolation. Extraction of *O. agardhii* (NIES-204) and isolation of oscillapeptin A (**1**) were previously described.[8] Oscillapeptin B (**2**, 5.6 mg) was isolated from the 60% MeOH fraction by rpHPLC (ODS L-column, 10 × 250 mm; 35% MeCN containing 0.05% TFA; flow rate 2.0 mL/min; UV detection at 210 nm). Extraction of *O. agardhii* (NIES-205) was previously described.[3b] The 60% MeOH fraction (617 mg) was purified by rpHPLC (Cosmosil C_{18} MS column, 10 × 250 mm; 20-60% MeCN containing 0.05% TFA in 50 min; flow rate 2.0 mL/min; UV detection at 210 nm) to yield oscillapeptin C (**3**, 3.8 mg). The 50% MeOH fraction (327 mg) was purified by reversed-phase HPLC (Cosmosil C_{18} MS column, 10 × 250 mm; 20-60%MeCN containing 0.05% TFA in 50 min; flow rate 2.0 mL/min; UV detection at 210 nm) to yield oscillapeptin D (**4**, 5.0 mg) and oscillapeptin E (**5**, 4.1 mg).

Freeze-dried cyanobacterium *O. agardhii* (NIES-596) (135.9 g dry weight from 400 L of culture medium) was

extracted twice with 80% MeOH and once with 100% MeOH. The extracts were combined and concentrated to give a crude extract (27.2 g), which was partitioned between Et_2O and H_2O. The H_2O fraction was further partitioned between *n*-BuOH and H_2O. The *n*-BuOH-soluble material (4.41 g) was subjected to ODS flash chromatography and eluted with aqueous MeOH, 100% MeOH, and CH_2Cl_2. The 80% MeOH fraction (160 mg) was purified by rpHPLC (CAPCELL PAK C_{18} UG column, 10 × 250 mm; 20-80% MeCN containing 0.05% TFA in 60 min; flow rate 2.0 mL/min; UV detection at 210 nm) to yield oscillapeptin F (**6**, 38.3 mg).

Oscillapeptin B (2): white powder; $[\alpha]_D$ –30.2° (*c* 0.2, MeOH); UV (MeOH) λ_{max} 278 nm (ε 2600); For ^1H and ^{13}C data, see Table 3; HRFABMS *m/z* 1180.4956 (M - H)$^-$ calculated for $C_{57}H_{78}N_7O_{18}S$ (Δ -1.2 mmu).

Oscillapeptin C (3): white powder; $[\alpha]_D$ –26.6° (*c* 0.05, MeOH); UV (MeOH) λ_{max} 278 nm (ε 1100); For ^1H and ^{13}C data, see Table 2; HRFABMS *m/z* 1060.5531 (M - H)$^-$ calculated for $C_{55}H_{78}N_7O_{14}$ (Δ -7.6 mmu).

Oscillapeptin D (4): white powder; $[\alpha]_D$ –23.4° (*c* 0.05, MeOH); UV (MeOH) λ_{max} 278 nm (ε 1800); For ^1H and ^{13}C data, see Table 1; HRFABMS *m/z* 1126.5013 (M - H)$^-$ calculated for $C_{54}H_{76}N_7O_{17}S$ (Δ -0.5 mmu).

Oscillapeptin E (5): white powder; $[\alpha]_D$ –25.1° (*c* 0.05, MeOH); UV (MeOH) λ_{max} 278 nm (ε 2400); For ^1H and ^{13}C data, see Table 2; HRFABMS *m/z* 1136.4882 (M - H)$^-$ calculated for $C_{55}H_{74}N_7O_{17}S$ (Δ +2.0 mmu).

Oscillapeptin F (6): white powder; $[\alpha]_D$ –56.1° (*c* 1.0, MeOH); UV (MeOH) λ_{max} 278 nm (ε 1300); For ^1H and ^{13}C data, see Table 3; HRFABMS *m/z* 1187.5024 (M - H)$^-$ calculated for $C_{51}H_{75}N_8O_{16}S$ (Δ +0.3 mmu).

Amino Acid Analysis of Oscillapeptin B (2) to F (6). A solution of **2** to **6** (100 μg) in 6 N HCl (500 μL) was heated to 110 °C in a each sealed tube for 16 h and then cooled. The solvent was removed in a stream of dry N_2 with heating, and the residue was dissolved in 0.02 N HCl (500 μL) and subjected to amino acid analysis. Retention times (min) in amino acids analysis: Thr (15.06), Ile (45.76), Lys (85.49). Under this condition, Ile and allo-Ile were separated clearly. Retention times (min): allo-Ile (43.68) and Ile (45.76).

L-Hty Standard. Anabaenopeptin F (30 mg)[12] was hydrolyzed and subjected to rpHPLC (Cosmosil MS, 10 × 250 mm; 0-60% MeOH containing 0.05% TFA; flow rate 2.0 mL/min; UV detection at 210 nm) to yield Hty (3.4 mg); $[\alpha]_D$ +48.6° (*c* 0.1, MeOH); FABMS (matrix: glycerol, positive) *m/z* 196 $[M + H]^+$, ^1H NMR (DMSO-d_6), δ_H 9.20 (s), 6.96 (d, 8.1), 6.67 (d, 8.1), 3.34 (m), 2.53 (2H, m), 1.96 (m), 1.83 (m), ^{13}C NMR (DMSO-d_6) δ_C 170.0, 155.3, 130.8, 129.2, 115.5, 52.6, 32.7, 29.8. Hty (1 mg) was treated with O_3 in MeOH at 0 °C for 30 min. After removal of O_3 by N_2, 2 mL of H_2O_2 and 1 drop of concd HCl were added to the reaction mixture. After incubation at room temperature for 16 h, excess solvents were removed under high vacuum to yield L-Glu, which was confirmed by amino acid analysis and Marfey's analysis as described bellow. Retention times (min): L-Glu (41.2), D-Glu (42.4).

HPLC Analysis of the Marfey's Derivatives. To each acid hydrolyzate of a 100 μg portion of **1** to **6**, 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (L-FDAA) in acetone (50 μL) and 1M $NaHCO_3$ (100 μL) were added. The mixture was kept at 80 °C for 3 min followed by neutralization with 2 N HCl (50 μL). The reaction mixture were dissolved in 50% MeCN and subjected to rpHPLC (Cosmosil C_{18} MS column, 4.6 × 250 mm; 0-60% MeCN containing 0.1% TFA in 60 min; flow rate 1 mL/min, UV detection at 340 nm). The identity of each peak was confirmed by coinjection with a solution of a standard that had been derivatized in the same manner, but L-Hty-D-FDAA derivative was substituted for D-Hty-L-FDAA derivative. Retention times (min): D-Lys (34.6), L-Lys (36.0), L-Thr (36.5), L-allo-Thr (36.8), D-allo-Thr (38.2), D-Thr (39.2), L-Ile (50.2), D-Ile (54.4), L-Dmy (51.4), D-Dmy (52.4), L-Hty (62.8), D-Hty (65.2). In order to separate DL-Nmf clearly on HPLC, an isocratic elution (35% MeCN containing 0.1% TFA) was used. Retention times (min): L-Nmf (23.0), D-Nmf (24.4).

Ozonolysis and Oxidation of Oscillapeptin B (2). Compound **2** (500 μg) was treated with O_3 in MeOH at 0 °C for 30 min. After removal of O_3 by a stream of N_2, 2 mL of H_2O_2 and 1 drop of conc. HCl were added to the reaction mixture. After incubation at room temperature for 16h, excess solvents were removed under high vacuum, and the residue was hydrolyzed to yield 1.8 equiv. of L-Glu derived from both Hty and Mhty in **2**, which was confirmed by amino acid analysis and Marfey's analysis as described above. Retention times (min): L-Glu (41.0), D-Glu (42.2).

2-*O*-Methylglyceric acid *p*-bromophenacyl ester. DL-glyceric acid (530 mg, 5 mmol) was added to a solution of KF (581 mg, 10 mmol) and *p*-bromophenacyl bromide (1668 mg, 6 mmol) in DMF (5 mL).[18] After being stirred for 30 min, the mixture was diluted with EtOAc and washed with H_2O. The organic layer was concentrated *in vacuo*, and chromatographed (SiO_2, EtOAc) to afford DL-glyceric acid *p*-bromophenacyl ester (914 mg) as a white powder. To this ester (360 mg, 1.19 mmol) in CH_2Cl_2 (3 mL), *t*-butyldimetylsilyl (TBDMS) (1.3 mL, 1.3 mmol), Et_3N (250 μL, 1.5 mmol)) and DMAP (8 mg) were added under Ar. After being stirred for overnight, the reaction mixture was diluted with Et_2O and washed with saturated $NaHCO_3$ and saturated NH_4Cl. The organic layer was concentrated *in vacuo*, and chromatographed (SiO_2, $CHCl_3$) to afford DL-3-*O*-TBDMS-glyceric acid *p*-bromophenacyl ester (266 mg, 0.64 mmol) as yellow oil. To a solution of this ester in CH_2Cl_2 (7 mL), proton sponge (800 mg) and Me_3OBF_4 (200 mg) were added,[14] and the reaction mixture was stirred for 12 h and then chromatographed (SiO_2, $CHCl_3$) to afford DL-3-*O*-TBDMS-2-*O*-methylglyceric acid *p*-bromophenacyl ester (241.4 mg, 0.56 mmol) as yellow oil. This was dissolved in H_2O/THF/AcOH (1:1:1, 3.0 mL) and stirred for 24 h at room temperature. The reaction mixture was extracted with EtOAc and washed with saturated $NaHCO_3$. The organic layer was concentrated *in vacuo*, and chromatographed (SiO_2, EtOAc) to yield DL-2-*O*-methylglyceric acid *p*-bromophenacyl ester (165.7 mg, 0.52 mmol) as a white powder. L-2-*O*-methylglyceric acid *p*-bromophenacyl ester was also synthesized by same procedures. L-2-*O*-methylglyceric acid *p*-bromophenacyl ester: $[\alpha]_D$ –28.1° (*c* 1.0, MeOH); FABMS (matrix: glycerol, positive) *m/z* 317 $[M + H]^+$; 319 $[M(^{81}Br) + H]^+$, ^1H NMR (CD_3OD), δ_H 7.89 (d, 8.6, 2H), 7.71 (d, 8.6, 2H), 5.51 (d, 3.9, 2H), 4.07 (dd, 6.0, 3.4), 3.90 (dd, 11.5, 3.4), 3.81 (dd, 11.5, 6.0), 3.49 (s); ^{13}C NMR (CD_3OD), δ_C 192.8, 171.9, 134.3, 133.3, 130.6, 129.9, 83.1, 67,8, 63.9, 58.8.

Chiral HPLC Analysis of *p*-bromophenacyl ester Derivatives. Each acid hydrolyzate of **1** to **6** (100 μg) was added to a stirring solution of KF and *p*-bromophenacyl bromide in DMF (300 μL). After 30 min, the solution was diluted with EtOAc and washed with H_2O. The organic layer was evaporated and chromatographed (SiO_2, EtOAc). The EtOAc fraction was concentrated *in vacuo* and the residue was subjected to Chiral HPLC (CHIRALCEL OJ column, 4.6 × 250 mm; 100% EtOH; flow rate 0.8 mL/min, UV detection at 280 nm). Retention times (min): D-2-*O*-methylglyceric acid *p*-bromophenacyl ester (7.0), L-2-*O*-methylglyceric acid *p*-bromophenacyl ester (8.4).

Reduction of Oscillapeptins A (1), B (2), D (4), E (5), and F (6). Each of **1**, **2**, **4**, **5**, and **6** (500 μg) was dissolved in MeOH (1.0 mL), and an excess amount of $NaBH_4$ was added to the solution with stirring at room temperature. After 3 h, H_2O was added in the reaction mixture followed by concentration *in vacuo*. The residue was subjected to a disposable ODS column (YMC Dispo SPE C 18; H_2O-50% MeCN) and the 50% MeCN fraction was concentrated *in vacuo*. The residue was hydrolyzed, derivatized and analyzed by the Marfey's method as described above.[7] L-Pentahomoserine-D-FDAA derivative was substituted for D-pentahomoserine-L-FDAA derivative. Retention times (min): L-pentahomoserine (39.3), D-pentahomoserine (39.8), L-Pro (42.4), D-Pro (43.4).

Oxidation by CrO_3 of Oscillapeptin C (3). **3** (500 μg) was dissolved in AcOH (500 μL) and combined with 2 mg of CrO_3. After being stirred at room temperature for 2 h, the reaction mixture was subjected to a disposable ODS column (YMC Dispo SPE C 18; H_2O-MeOH). The MeOH fraction was hydrolyzed to yield L-Glu which was confirmed by Marfey's analysis as described above. Retention times (min): L-Glu (41.0), D-Glu (42.2).

Acetylation and Hydrogenation of Oscillapeptin D (4). To **4** (2.5 mg) dissolved in pyridine (0.5 mL), Ac_2O (0.5 mL) was added at room temperature. After being stirred for 5 h, the reaction mixture was concentrated *in vacuo* and subjected to rpHPLC (Cosmosil C_{18} MS column, 10 × 250 mm; gradient elution 50-100% MeCN containing TFA 0.05% in 50 min; flow rate 2 mL/min, UV detection at 210 nm) to yield triacetate **4** (1.5 mg). ^{1}H NMR (CD_3OD), Mgs [δ 4.05 (H-2, dd, 5.1, 3.0), 4.20 (H-3a, dd, 10.7, 5.1), 4.39 (H-3b, dd, 10.7, 3.0), 3.53 (*O*-Me, s)], Hty (OAc) [δ 4.60 (H-2, dd, 8.6, 5.1), 2.13 (H-3a, m), 2.20 (H-3b, m), 2.75 (H-4, m), 7.28 (H-6,10, d, 8.6), 6.96 (H-7,9, d, 8.6), 2.20 (Ac, s)], Thr [δ 4.66 (H-2, br), 5.60 (H-3, m), 1.39 (H-4, m)], HcAla (OAc) [δ 4.55 (H-2, m), 1.72 (H-3a, m), 2.02 (H-3b, m), 2.28 (H-4, m), 5.65 (H-5, brd, 10.3), 5.72 (H-6, brd, 10.3), 5.22 (H-7, m), 1.58 (H-8a, m), 2.02 (H-8b, m), 1.26 (H-9a, m), 1.87 (H-9b, m), 2.00 (Ac, s)], Ahp (OAc) [δ 4.57 (H-3, m), 1.85 (H-4a, m), 2.75 (H-4b, m), 1.89 (H-5, m), 5.04 (H-6, br), 2.02 (Ac, s)], Ile (1) [δ 4.53 (H-2, d, 10.6), 1.89 (H-3, m), 0.74 (H-4a, m), 1.15 (H-4b, m), 0.68 (H-5, t, 6.8), -0.09 (H-6, d, 6.4)], Nmf [δ 5.28 (H-2, dd, 11.5, 2.6), 2.86 (H-3a, m), 3.54 (H-3b, m), 7.31 (H-5,9, d, 7.5), 7.26 (H-6,8, dd, 7.5, 6.8), 7.20 (H-7, t, 6.8), 2.87 (*N*-Me, s)], Ile (2) [δ 4.43 (H-2, d, 8.1), 1.89 (H-3, m), 1.22 (H-4a, m), 1.46 (H-4b, m), 0.88 (H-5, t, 7.2), 0.93 (H-6, d, 6.8)].

To a solution of triacetate **4** in EtOH (1.0 mL), palladium black (5.0 mg) was added at room temperature under H_2. After 5 h with stirring, the reaction mixture was filtered and subjected to rpHPLC (Cosmosil C_{18} MS column, 10 × 250 mm; gradient elution 50-100% MeCN containing TFA 0.05% in 50 min; flow rate 2 mL/min, UV detection at 210 nm) to yield **7** (300 μg). ^{1}H NMR spectrum of **7** showed the absence of both an olefin and OAc signals in HcAla. **7** was hydrolyzed and analyzed by the Marfey's method. (2*S*)-2-amino-3-cyclohexylpropionic acid-D-FDAA was substituted for (2*R*)-2-amino-3-cyclohexylpropionic acid-L-FDAA. Retention times (min): (2*S*)-2-amino-3-cyclohexylpropionic acid (60.8), (2*R*)-2-amino-3-cyclohexylpropionic acid (65.2).

Protease Inhibitory Assay. Serine protease inhibitory activities were determined by the method previously described.[2b]

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