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Agardhipeptins A and B, Two New Cyclic Hepta- and Octapeptide, from the Cyanobacterium *Oscillatoria agardhii* (NIES-204)

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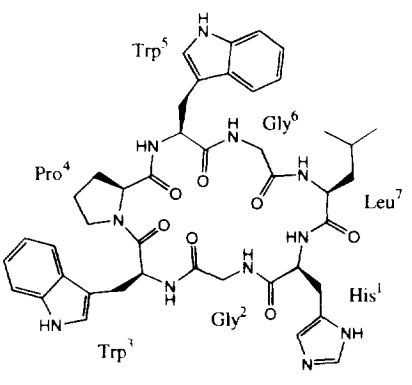
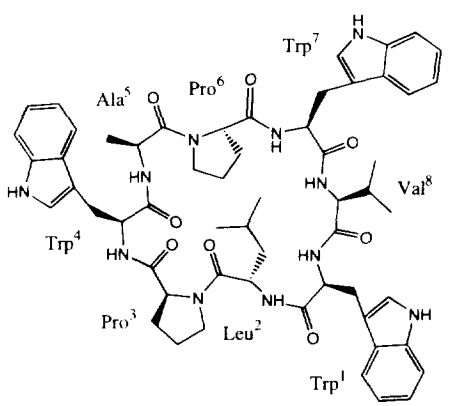
Abstract: New cyclic hepta- and octapeptide, agardhipeptins A (**1**) and B (**2**), were isolated from the cyanobacterium *Oscillatoria agardhii* (NIES-204). The structures of **1** and **2** were elucidated to be *cyclo*(-His[1]-Gly[2]-Trp[3]-Pro[4]-Trp[5]-Gly[6]-Leu[7]-) and *cyclo*(-Trp[1]-Leu[2]-Pro[3]-Trp[4]-Ala[5]-Pro[6]-Trp[7]-Val[8]-), respectively, by extensive 2D NMR data and chemical degradation. Agardhipeptin A (**1**) inhibited plasmin with an IC_{50} of 65 μg/mL but agardhipeptin B (**2**) had no inhibitory activity.

Cyclic peptides and/or depsipeptides are rapidly emerging in importance as sources of potentially important new drugs. Cyclic peptides having interesting biological activities and novel structures have been isolated from various origins. Some of the most exciting natural products discovered in recent years, cyclosporin A[1] and FK-506[2] from microorganisms, cyclotheonamides,[3] didemnins,[4] and dolastatins[5] from marine invertebrates, are amino acid derived metabolites. Because of their attractive biological profile, didemnins have been the focus of considerable attention. Didemnin B was indeed the first marine natural product to enter clinical trials.

As part of our ongoing search for biologically active metabolites from cyanobacteria,[6] it was found that crude methanol extracts of *Oscillatoria agardhii* (NIES-204) exhibited considerable plasmin inhibitory activity. Assay-guided fractionation resulted in the isolation of plasmin inhibitory cyclic peptide agardhipeptin A (**1**) and related compound agardhipeptin B (**2**). Here we describe the isolation and structure elucidation of novel cyclic hepta- (**1**) and octapeptide (**2**).



1



2

Successive extraction of the freeze-dried cyanobacterium with 80% MeOH and MeOH followed by ODS flash chromatography of the BuOH soluble material gave a mixture of agardhipeptins, which were purified by HPLC on ODS L-column to give **1** and **2**.

Agardhipeptin A (**1**) had the molecular formula $C_{43}H_{51}N_{11}O_7$, as determined by HRFAB-MS and NMR data. The ^1H and ^{13}C NMR spectra of **1** (Table 1) contained resonances that were characteristic of peptide. The ^{13}C NMR spectrum contained 43 signals including 2 methyl, 9 methylene, 18 methine, and 14 quaternary carbons (of which 7 are carbonyl carbons). Amino acid analysis indicated the presence of Pro, His, Leu, and each two residues of Trp and Gly. Presence of the amino acids was confirmed by the 2D NMR employing HMQC,[7] HMBC (8.3 Hz),[8] NOESY, and HOHAHA experiments. The relatively high intensity of the molecular ion peak observed in the FAB-MS spectrum suggested that the presumed heptapeptide might be cyclic. The molecular formula of **1** required 24 unsaturations. Since only 23 unsaturations could be accounted for by functionality present in the seven individual amino acids, it was apparent that **1** was a cyclic peptide. The failure of **1** to react with ninhydrin was consistent with a cyclic structure. The amino acid sequence of **1** was determined by the HMBC and NOESY data (Fig. 1). There were HMBC cross peaks between the NH protons (or α-H) and the carbonyl carbons of the adjacent amino acids, viz., Gly^2 NH/His^1 CO, Trp^3 NH/Gly^2 CO, Pro^4 α-H/Trp^3 CO, Trp^5 NH/Pro^4 CO, Gly^6 NH/Trp^5 CO, that established the partial sequences His^1-Gly^2-Trp^3-Pro^4-Trp^5-Gly^6. The remaining Leu^7 residue was connected with His^1 by the NOESY cross peak between His^1 NH and Leu^7 α-H. Although no HMBC or NOESY cross peaks were observed between Leu^7 and Gly^6, the amino acid sequence of **1** was determined as *cyclo*(-His^1-Gly^2-Trp^3-Pro^4-Trp^5-Gly^6-Leu^7-) from the fact that **1** should be cyclic peptide.

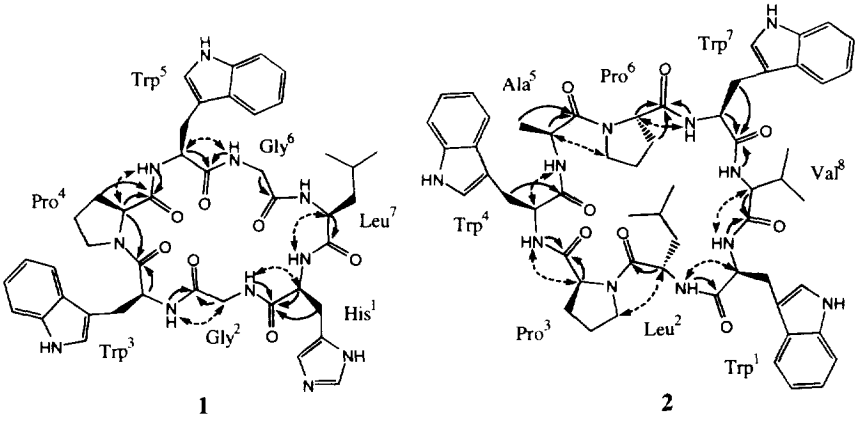


Fig. 1. Selected HMBC (half arrows) and NOESY (dashed arrows) correlations for agardipeptins A (**1**) and B (**2**).

Proline *cis-trans* isomers can be distinguished in solution by the chemical shift difference of the β- and γ-carbons.[9] In a *cis* X-pro, these signals are further separated than in a *trans* X-pro. The chemical shifts of β- and γ-carbons in Pro^4 differ by 4.5 ppm, indicating that the peptide bond

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

Units	^{1}H (mult, *J* Hz)	^{13}C (mult)	HMBC correlations	Units	^{1}H (mult, *J* Hz)	^{13}C (mult)	HMBC correlations
His^{1}				4	1.54 (m)	24.1 (t)	Pro^{4} 3, 5
1		170.1 (s)	His^{1} 2, 3, Gly^{2} NH		1.69 (ddd, 12.1,7.0, 7.0)		
2	4.23 (ddd, 8.8, 6.2, 2.9)	54.6 (d)	His^{1} 3	5	3.47 (m)	45.9 (t)	Pro^{4} 3, 4
3	3.03 (m)	25.6 (t)			3.55 (m)		
	3.17 (m)			Trp^{5}			
4		128.8 (s)	His^{1} 2, 3, 5, 7	1		171.4 (s)	Trp^{5} 2, Gly^{6} NH
5	7.41 (s)	117.1 (d)	His^{1} 3, 7	2	4.53 (m)	53.5 (d)	Trp^{5} 3
7	8.88 (d, 4.9)	134.0 (d)	His^{1} 5, NH[a]	3	2.92 (dd, 14.3, 12.1)	27.2 (t)	
NH[a]	8.74 (d, 4.9)				3.33 (m)		
NH	9.13 (brs)			1'	10.79 (d, 1.9)		
Gly^{2}				2'	7.09 (d, 1.9)	123.2 (d)	Trp^{5} 3, 1'
1		168.4 (s)	Gly^{2} 2, Trp^{3} NH	3'		110.7 (s)	Trp^{5} 2', 4'
2	3.18 (m)	42.5 (t)		4'	7.56 (d, 8.0)	118.2 (d)	Trp^{5} 6', 7'
	3.90 (dd, 16.5, 7.7)			5'	6.98 (t, 7.3)	118.2 (d)	Trp^{5} 6', 7'
NH	8.61 (m)			6'	7.05 (t, 7.3)	120.9 (d)	Trp^{5} 4', 5'
Trp^{3}				7'	7.32 (d, 8.1)	111.4 (d)	Trp^{5} 5', 6'
1		171.0 (s)	Trp^{3} 2, Pro^{4} 2	8'		127.2 (s)	Trp^{5} 1', 2', 4', 5', 7'
2	4.55 (ddd, 10.3, 10.3, 3.9)	54.9 (d)	Trp^{3} 3	9'		136.1 (s)	Trp^{5} 1', 2', 4'
3	2.83 (dd, 14.3, 10.3)	29.3 (t)	Trp^{3} 2	NH	7.82 (d, 9.2)		
	3.19 (dd, 14.3, 3.9)			Gly^{6}			
1'	10.79 (d, 2.2)			1		167.6 (s)	Gly^{6} 2
2'	7.17 (d, 2.2)	123.7 (d)	Trp^{3} 3, 1'	2	3.88 (dd, 17.6, 3.7)	42.8 (t)	
3'		109.7 (s)	Trp^{3} 2, 3, 2', 4'		4.17 (dd, 17.6, 3.7)		
4'	7.51 (d, 8.1)	117.8 (d)	Trp^{3} 6', 7'	NH	8.15 (t, 3.7)		
5'	6.98 (t, 7.3)	118.2 (d)	Trp^{3} 7'	Leu^{7}			
6'	7.05 (t, 7.3)	120.8 (d)	Trp^{3} 7'	1		171.3 (s)	Leu^{7} 2, 3
7'	7.31 (d, 8.1)	111.3 (d)	Trp^{3} 5'	2	4.54 (m)	51.3 (d)	Leu^{7} 4
8'		127.0 (s)	Trp^{3} 1', 2', 4', 5', 7'	3	1.43 (m)	41.6 (t)	Leu^{7} 5, 5'
9'		136.0 (s)	Trp^{3} 1', 2', 4', 6', 7'		1.60 (m)		
NH	8.23 (d, 10.3)			4	1.59 (m)	24.0 (d)	Leu^{7} 3, 5, 5'
Pro^{4}				5	0.67 (d, 6.2)	23.1 (q)	Leu^{7} 5'
1		170.97 (s)	Pro^{4} 2, 3, Trp^{5} NH	5'	0.92 (d, 6.2)	21.8 (q)	Leu^{7} 3, 5
2	3.95 (dd, 7.9, 7.0)	61.2 (d)	Pro^{4} 3, 4	NH	7.38 (d, 7.0)		
3	1.14 (m)	28.6 (t)	Pro^{4} 2, 4, 5				
	1.85 (m)						

[a] Imidazole NH

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

Units	^{1}H (mult, *J* Hz)	^{13}C (mult)	HMBC correlations	Units	^{1}H (mult, *J* Hz)	^{13}C (mult)	HMBC correlations
Trp^{1}				5'	7.00 (m)	118.4 (d)	Trp^{4} 7'
1		169.1 (s)	Leu^{2} NH	6'	7.08 (m)	121.0 (d)	Trp^{4} 4'
2	3.65 (m)	55.2 (d)	Trp^{1} 3, NH	7'	7.34 (d, 8.1)	111.6 (d)	Trp^{4} 4', 5'
3	3.31 (m)	22.4 (t)		8'		127.2 (s)	Trp^{4} 1', 2', 4'
	3.40 (dd, 15.4, 3.3)			9'		136.0 (s)	Trp^{4} 1', 2', 4'
1'	10.8 (brs)			NH	7.28 (d, 8.4)		
2'	7.01 (m)	122.3 (d)	Trp^{1} 3, 1'	Ala^{5}			
3'		111.6 (s)	Trp^{1} 3, 1', 2', 4'	1		171.4 (s)	Ala^{5} 2, 3
4'	7.38 (m)	117.8 (d)	Trp^{1} 6'	2	4.27 (m)	47.1 (d)	Ala^{5} 3
5'	6.92 (t, 7.3)	118.2 (d)	Trp^{1} 7'	3	0.91(d, 6.2)	16.5 (q)	Ala^{5} 2
6'	7.02 (m)	120.8 (d)	Trp^{1} 4', 5'	NH	7.14 (d, 7.2)		
7'	7.28 (d, 8.4)	111.2 (d)	Trp^{1} 5'	Pro^{6}			
8'		127.3 (s)	Trp^{1} 1', 2', 5', 6', 7'	1		174.2 (s)	Pro^{6} 2, 3, Trp^{7} NH
9'		136.0 (s)	Trp^{1} 1', 2', 4',	2	4.14 (dd, 7.6, 7.6)	62.1 (d)	Pro^{6} 3, 4
NH	7.56 (d, 8.1)			3	1.70 (m)	29.0 (t)	Pro^{6} 2, 4
Leu^{2}					2.07 (m)		
1		173.0 (s)	Leu^{2} 2	4	1.82 (2H, m)	24.4 (t)	Pro^{6} 3, 5
2	4.80 (m)	47.9 (d)		5	3.26 (m)	46.9 (t)	
3	0.92 (m)	40.2 (t)	Leu^{2} 5, 5'		3.49 (m)		
	1.08 (m)			Trp^{7}			
4	1.67 (m)	22.9 (d)	Leu^{2} 5, 5'	1		171.5 (s)	Trp^{7} 2, 3, Val^{8} NH
5	0.78 (d, 7.0)	23.2 (q)	Leu^{2} 5'	2	4.24 (m)	56.45 (d)	Trp^{7} 3, NH
5'	0.88 (d, 6.6)	20.7 (q)	Leu^{2} 5	3	3.15 (d, 7.7)	25.5 (t)	Trp^{7} 2, NH
NH	7.03 (d, 8.8)				3.18 (d, 5.1)		
Pro^{3}				1'	11.0 (brs)		
1		171.5 (s)	Pro^{3} 2, Trp^{4} NH	2'	7.09 (m)	123.2 (d)	Trp^{7} 3, 1'
2	3.95 (t, 8.1)	62.3 (d)	Pro^{3} 3	3'		109.5 (s)	Trp^{7} 3, 1', 2', 4'
3	1.71 (m)	28.5 (t)	Pro^{3} 2	4'	7.56 (m)	117.8 (d)	Trp^{7} 6'
	2.05 (m)			5'	7.03 (m)	118.6 (d)	Trp^{7} 7'
4	1.86 (m)	24.9 (t)	Pro^{3} 5	6'	7.12 (m)	121.3 (d)	Trp^{7} 4'
	2.03 (m)			7'	7.37 (m)	111.6 (d)	Trp^{7} 5'
5	3.33 (m)	46.6 (t)	Pro^{3} 3	8'		127.0 (s)	Trp^{7} 1', 2', 5', 7'
	3.53 (m)			9'		136.0 (s)	Trp^{7} 1', 2', 4'
Trp^{4}				NH	8.0 (brd, 5.5)		
1		170.2 (s)	Trp^{4} 3	Val^{8}			
2	4.36 (m)	53.6 (d)	Trp^{4} 3	1		169.6 (s)	Val^{8} 2, Trp^{7} NH
3	3.22 (m)	25.2 (t)		2	4.30 (dd, 9.9, 3.3)	56.54 (d)	Val^{8} 4, 4', NH
	3.28 (m)			3	2.41 (m)	27.6 (d)	Val^{8} 4, 4'
1'	10.9 (brs)			4	0.73 (d, 7.0)	17.2 (q)	Val^{8} 2, 4'
2'	6.99 (brs)	123.0 (d)	Trp^{4} 3, 1'	4'	0.75 (d, 6.2)	19.6 (q)	Val^{8} 2, 4
3'		110.0 (s)	Trp^{4} 3, 1', 2', 4'	NH	7.38 (d, 3.3)		
4'	7.52 (d, 7.7)	118.0 (d)	Trp^{4} 6'				

between Trp^3 and Pro^4 has *trans* conformation.

Agardhipeptin B (**2**) was isolated as a colorless amorphous solid. The molecular formula of **2** was deduced for $C_{57}H_{69}N_{11}O_8$, which was consistent with the carbon and hydrogen numbers counted in the NMR data. The peptidic nature of **2** was suggested by the NMR spectra. Standard amino acid analysis of **2** revealed the presence of Ala, Val, Leu, Pro, and Trp. The ^1H NMR spectrum in DMSO-d_6 contained 6 amide NH signals between δ 7.03 and 8.00, and 3 indole NH signals between δ 10.8 and 11.0. The remaining two nitrogen atoms were accounted for two Pro. The ^{13}C NMR spectrum revealed 8 carbonyls, all of them was involved in amide linkages, that account for all of the oxygen atoms. A detailed analysis of the 2D NMR data, including COSY, HMQC, HMBC, NOESY, and HOHAHA, for **2** showed that it contained each one residue of Ala, Val, Leu, two Pro and three Trp residues (Table 2). The molecular formula suggested the presence of total 29 unsaturation equivalents in the molecule but 28 unsaturations could be accounted for by functionality. Thus the remaining 1 unsaturation equivalent should be due to cyclic form. The amino acid sequence of **2** was determined by the interpretation of inter-residual HMBC and NOESY correlations (Fig. 1). Two partial sequences, -Pro^3-Trp^4- (**a**) and -Pro^6-Trp^7-Val^8-Trp^1-Leu^2- (**b**), were determined by the HMBC correlations (Pro^3 CO/Trp^4 NH, Pro^6 CO/Trp^7 NH, Trp^7 CO/Val^8 NH, Val^8 CO/Trp^1 NH, Trp^1 CO/Leu^2 NH). Partial sequences **a** and **b** were connected by the NOESY correlation between Leu^2 α-H and Pro^3 δ-H. NOESY correlations between Trp^4 α-H and Ala^5 NH, and between Ala^5 α-H and Pro^6 δ-H were also observed. At this stage the amino acid sequence of **2** was determined as *cyclo*(-Trp^1-Leu^2-Pro^3-Trp^4-Ala^5-Pro^6-Trp^7-Val^8-). Carbon chemical shifts of the β- and γ-carbons of the proline rings confirmed the *trans* conformation about Leu^2-Pro^3 and Ala^5-Pro^6 bonds.

Absolute stereochemistry of amino acid residues in **1** and **2** was determined by HPLC analysis of the acid hydrolyzates derivatized with Marfey's reagent.[10] All the amino acids present in agardhipeptins had the L configuration.

Agardhipeptin A (**1**) inhibited plasmin with an IC_{50} of 65 μg/mL but agardhipeptin B (**2**) had no plasmin inhibitory activity. Both **1** and **2** did not inhibited thrombin, trypsin, chymotrypsin, elastase, and papain at 100 μg/mL. Further biological evaluations are in progress.

Agardhipeptins A and B are related to phakellistatins,[11] hymenamides,[12] axinastatins,[13] and hymenistatin 1,[14] which were all isolated from marine sponges. To our best knowledge, however, tryptophan-rich structures of agardhipeptins have not been described in any naturally occurring molecule until now.

Experimental section

Instrumentation

NMR spectra were recorded on a JEOL JNM-A500 NMR spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C. ^1H and ^{13}C NMR chemical shifts were referenced to solvent peaks: δ_H 2.49 and δ_C 39.5 for DMSO-d_6. FAB mass spectra were measured by using polyethyleneglycol sulfate and glycerol as matrixes on a JEOL JMS SX-102 mass spectrometer. Amino acid analyses

were carried out with a Hitachi L-8500A amino acid analyzer. High performance liquid chromatography (HPLC) was performed on Shimadzu LC-6A liquid chromatograph with ODS L-column (10 × 250 mm, Chemicals Inspection and Testing Institute, Japan). Ultraviolet spectrum was measured on a Hitachi 330 spectrometer. Optical rotations were determined with a JASCO DIP-140 digital polarimeter.

Strain and culture condition

Oscillatoria agardhii (NIES-204, Cyanophyceae) was obtained from the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and cultured in 10 L glass bottles containing CB medium[15] with aeration (filtered air, 0.3 L/min) at 25℃ under illumination of 250 μE/m^2·s on a 12L:12D cycle. Cells were harvested after 10-14 days incubation by continuous flow centrifugation at 10,000 rpm. Harvested cells were lyophilized and kept in a freezer at −20℃ until extraction.

Extraction and isolation

Freeze-dried cells (138 g from 400 L of culture) were extracted three times with 80% MeOH and one time with MeOH. The extracts were combined and concentrated to give a crude extract (31.3 g). This extract was partitioned between ether and water. The water soluble fraction was further partitioned between *n*-BuOH and water. The *n*-BuOH layer (18.7 g) was subjected to ODS flash chromatography (AM 120-230/70, YMC CO., LTD, 12×10.5 cm) and eluted with aqueous MeOH, MeOH and CH_2Cl_2. MeOH fraction (4.36 g), which showed plasmin inhibitory activity, was resubjected to ODS flash chromatography and fractionated with 70%, 80% MeOH, and MeOH followed by CH_2Cl_2. Active 70% and 80% MeOH fractions were combined and evaporated to give bright yellow solid (172 mg). Final purification was achieved by reversed-phase HPLC on an ODS L-column (linear gradient of CH_3CN in H_2O containing 0.05% TFA, 30% to 80% in 50 min; flow rate 1.5 mL/min; UV detection at 210 nm) to yield agardhipeptins A (**1**, 8.0 mg, tR: 28.9 min) and B (**2**, 6.7 mg, tR: 52.0 min).

Agardhipeptin A (**1**): $[\alpha]_D^{20}$ -9° (*c* 0.1, MeOH); UV (MeOH) λmax 221 nm (ε 40,800), 282 (6,700); FAB-MS *m/z* 834 $(M+H)^+$, 704; HRFAB-MS *m/z* 834.4062 $(M+H)^+$ calcd. for $C_{43}H_{52}N_{11}O_7$ (△ +1.1 mmu).

Agardhipeptin B (**2**): $[\alpha]_D^{20}$ -80° (*c* 0.1, MeOH); UV (MeOH) λmax 221 nm (ε 55,000), 282 (9,800); FAB-MS *m/z* 1036 $(M+H)^+$, 906; HRFAB-MS *m/z* 1036.5414 $(M+H)^+$ calcd. for $C_{57}H_{70}N_{11}O_8$ (△ +0.5 mmu).

Amino acid analysis

Hydrolysis with 1% phenol in 6 N HCl.[16] Each 100 μg of **1** and **2** was dissolved in 1% phenol in 6 N HCl (500 μL) and sealed in reaction vials. The vials were heated at 100℃ for 10 hours. The solution was evaporated in a stream of dry nitrogen with heating and redissolved in 0.1 N HCl to subject to amino acid analysis.

Derivatization of amino acids and HPLC analysis

Each 100 μg of **1** and **2** was dissolved in 500 μl of 1% phenol in 6 N HCl and heated at 110℃ for 5 hours. After removal of HCl in a stream of dry nitrogen, the residues were treated with 10% acetone solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (L-FDAA, Marfey's reagent) in 1 M $NaHCO_3$ at 80-90℃ for 3 min followed by neutralization with 50 μL of 2 N HCl. The reaction mixtures were dissolved in 50 % MeCN and subjected to reverse-phase HPLC: column; Cosmosil MS (Nacalai Tesque Co., 4.6 × 250 mm), gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (50:50:0.1) in 60 min, flow rate 1 mL/min, UV (340 nm). Retention times (min) of standard amino acids were found as follows: D-His (35.1), L-His (37.4), L-Ala (45.6), D-Ala (48.1), L-Pro (46.5), D-Pro (48.0), L-Val (51.4), D-Val (55.5), L-Leu (54.2), D-Leu (60.1), L-Trp (55.1), D-Trp (57.1).

Protease inhibitory activity assay

Serine and cysteine protease inhibitory activities were determined by the method previously described.[6]

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