Micropeptins 478-A and -B, Plasmin Inhibitors from the Cyanobacterium *Microcystis aeruginosa*

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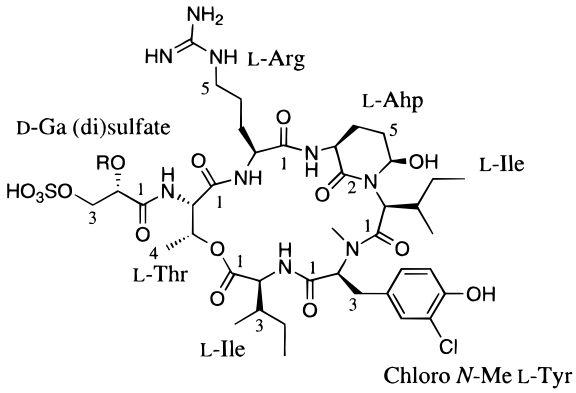
Micropeptins 478-A and -B, plasmin inhibitors, were isolated from the cultured cyanobacterium *Microcystis aeruginosa* (NIES-478). Their structures were elucidated to be **1** and **2** on the basis of 2D NMR data and chemical degradation. These cyclic depsipeptides inhibited plasmin potently with IC_{50} of 0.1 and 0.4 μg/mL, respectively.

Plasmin is a serine protease that regulates blood coagulation and relates closely to cardiovascular diseases such as stroke and coronary artery occlusion. Its inhibitors may be good targets as useful chemotherapeutic agents for these diseases. We have already reported potent plasmin inhibitors, micropeptins, from the freshwater cyanobacterium *Microcystis aeruginosa* (Chroococcaceae) (NIES-90 and 100).[1] Recently, the related compound A90720A was reported obtained from the terrestrial cyanobacterium *Microchate loktakensis* by a Lilly research group.[2] In the course of our screening program for protease inhibitors from microalgae, we isolated potent plasmin inhibitors, micropeptins 478-A (**1**) and -B (**2**), from another strain *M. aeruginosa* (NIES-478). These peptides also were related to such cyanobacteria-derived cyclic depsipeptides as aeruginopeptins,[3] microcystilide A,[4] cyanopeptolins,[5] oscillapeptin,[6] and nostocyclin,[7] as well as dolastatin 13[8] from the cyanobacteria-grazing sea hare *Dolabella auricularia*. Here, we describe the isolation and structure elucidation of **1** and **2**.



1 R=H, 2 R=SO_3H

The 80% methanol extract of freeze-dried alga (27.6 g) was partitioned between water and diethyl ether. The aqueous layer, which inhibited plasmin potently, was further extracted with *n*-butanol and subjected to ODS flash column chromatography followed by reversed-phase HPLC with aqueous MeCN containing 0.05% TFA to yield **1** and **2**.

Micropeptin 478-A (**1**) was isolated as a colorless amorphous powder. The pseudomolecular ions at *m/z* 976/978 $[M + H]^+$ in the FABMS revealed the presence of a chlorine, and desulfated ion at *m/z* 896/898 $[M - SO_3 + H]^+$ was also observed. The molecular formula of **1** was established as $C_{40}H_{61}N_9O_{15}SCl$ by HRFABMS and NMR spectral data (Table 1). Its peptidic nature was suggested by its ^1H and ^{13}C NMR spectra, and amino acid analysis of the hydrolysate indicated the presence of 1 mol each of Thr and Arg and 2 mol of Ile. Extensive NMR analyses including $^1H-^1H$ COSY, HMQC,[9] HOHAHA, and HMBC[10] spectra revealed the spin systems of three of the four amino acids, omitting that of Ile-1. Ile-1 was suggested to be present as a *N*,*N*-disubstituted derivative, since its amide proton was not recognized, and the spin system was deduced by 2D NMR spectra. The structure of 3-amino-6-hydroxy-2-piperidone (Ahp) was deduced as follows. In the COSY and HOHAHA spectra, the connectivity from NH (δ 7.27) to OH (δ 6.04) was determined. C-2 (δ 169.2) was correlated with H-3 (δ 4.45), H-6 (δ 4.92), and α-H of the Ile-1 derivative (δ 4.40) and C-6 (δ 73.9) with α-H of the Ile-1 derivative in the HMBC spectrum. Consequently, Ahp was deduced to be part of a hemiaminal structure formed from glutamate γ-semialdehyde and Ile-1. Units containing chlorine and a sulfate group were identified as 3-chloro *N*-MeTyr and glyceric acid 3-*O*-sulfate (Ga sulfate) by COSY and HMBC spectra, respectively.

The sequence of **1** was deduced primarily by HMBC correlations from α-H to C=O (Figure 1), but the correlations from Arg NH or α-H to Thr C=O could not be observed. The NOESY correlations (Arg α-H/Ahp NH, Thr α-H/Arg NH, Arg NH/Ahp NH) connected Arg to Thr and Ahp (Figure 1). The HMBC correlation from the downfield Thr β-H to Ile-2 C=O confirmed the ester formation between Thr and Ile-2 (Figure 1).

The stereochemistries of all the standard amino acids were determined as L by HPLC analysis of the derivatives of the acid hydrolysate with L-Marfey's reagent.[11] The stereochemistry of chloro *N*-MeTyr, which was synthesized from *N*-Me L-Tyr using sulfuryl chloride,[12] was also determined as L by HPLC analysis of its derivatives with both L- and D-Marfey's reagent.[13] The stereochemistry of the Ga sulfate was determined to be D by chiral GC analysis of *O*-heptafluorobutyryl isopropyl ester derivatives of glyceric acid. The stereochemistry of Ahp was deduced as follows. PCC oxidation of **1** followed by hydrolysis with HCl afforded Glu, which was proved to be the L-form from HPLC analysis of its derivative with L-Marfey's reagent. The relative stereochemistry of Ahp of **1** was decided as in Figure 2 by

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Table 1. ^{1}H and ^{13}C NMR Data of Micropeptins 478-A and -B in DMSO-d_6

	micropeptin 478-A		micropeptin 478-B	
	^{1}H *J*(Hz)	^{13}C	^{1}H *J*(Hz)	^{13}C
Ga sulfate			Ga disulfate	
1		171.7 (s)	1	171.7 (s)
2	4.25 (dd, 6.0, 2.6)	70.4 (d)	2 4.72 (dd, 4.5, 4.3)	74.5 (d)
3	3.83 (dd, 10.2, 6.0)	68.2 (t)	3 3.92 (dd, 9.9, 4.3)	66.3 (t)
	3.97 (dd, 10.2, 2.6)		3.98 (dd, 9.9, 4.5)	
Thr			Thr	
1		168.8 (s)	1	168.8 (s)
2	4.63 (d, 9.0)	54.4 (d)	2 4.62 (d, 9.5)	54.3 (d)
3	5.48 (q, 6.5)	72.1 (d)	3 5.44 (q, 6.4)	71.7 (d)
4	1.22 (d, 6.5)	17.6 (q)	4 1.22 (d, 6.4)	17.6 (q)
NH	7.63 (d, 9.0)		NH 7.72 (d, 9.5)	
Arg			Arg	
1		170.3 (s)	1	170.3 (s)
2	4.31 (m)	51.2 (d)	2 4.30 (m)	51.2 (d)
3	1.46 (m)	26.7 (t)	3 1.46 (m)	26.7 (t)
	2.02 (m)		2.02 (m)	
4	1.42 (m)	24.6 (t)	4 1.42 (m)	24.7 (t)
5	3.05 (m)	40.3 (t)	5 3.05 (m)	40.3 (t)
2-NH	8.57 (d, 8.6)		2-NH 8.50 (br)	
5-NH	7.43 (t, 5.6)		5-NH 7.43 (t, 5.8)	
C=N		156.5 (s)	C=N	156.5 (s)
Ahp			Ahp	
2		169.2 (s)	2	169.2 (s)
3	4.45 (ddd, 12.6, 9.4, 6.0)	48.8 (d)	3 4.45 (ddd, 12.8, 8.5, 6.8)	48.8 (d)
4	1.74 (m)	21.8 (t)	4 1.74 (m)	21.8 (t)
	2.62 (m)		2.62 (m)	
5	1.73 (m)	29.7 (t)	5 1.73 (m)	29.7 (t)
	1.76 (m)		1.76 (m)	
6	4.92 (br)	73.9 (d)	6 4.92 (br)	73.9 (d)
NH	7.27 (d, 9.4)		NH 7.27 (d, 9.4)	
OH	6.04 (d, 2.6)		OH 6.04 (d, 2.6)	
Ile-1			Ile-1	
1		169.7 (s)	1	169.7 (s)
2	4.40 (d, 10.7)	54.2 (d)	2 4.40 (d, 10.7)	54.2 (d)
3	1.82 (m)	32.9 (d)	3 1.82 (m)	32.9 (d)
4	0.64 (m)	23.7 (t)	4 0.64 (m)	23.7 (t)
	1.10 (m)		1.10 (m)	
5	0.64 (m)	10.2 (q)	5 0.64 (t, 7.3)	10.2 (q)
6	−0.12 (d, 6.4)	13.8 (q)	6 −0.12 (d, 6.4)	13.8 (q)
Cl Me Tyr			Cl Me Tyr	
1		168.9 (s)	1	168.9 (s)
2	5.04 (dd, 12.0, 2.6)	60.4 (d)	2 5.04 (dd, 12.0, 3.0)	60.4 (d)
3	2.71 (m)	34.8 (t)	3 2.71 (m)	34.8 (t)
	3.18 (m)		3.18 (m)	
4		129.1 (s)	4	129.1 (s)
5	7.12 (s)	130.6 (d)	5 7.12 (s)	130.6 (d)
6		119.8 (s)	6	119.8 (s)
7		152.0 (s)	7	152.0 (s)
8	6.83 (d, 8.1)	116.7 (d)	8 6.83 (d, 8.1)	116.7 (d)
9	6.97 (d, 8.1)	129.0 (d)	9 6.97 (d, 8.1)	129.0 (d)
N-Me	2.76 (s)	30.1 (q)	*N*-Me 2.76 (s)	30.1 (q)
Ile-2			Ile-2	
1		172.5 (s)	1	172.5 (s)
2	4.62 (dd, 9.4, 6.0)	55.3 (d)	2 4.72 (dd, 9.4, 4.7)	55.2 (d)
3	1.77 (m)	37.7 (d)	3 1.77 (m)	37.7 (d)
4	1.01 (m)	24.5 (t)	4 1.01 (m)	24.5 (t)
	1.22 (m)		1.22 (m)	
5	0.81 (dd, 7.7, 6.8)	11.1 (q)	5 0.81 (t, 7.5)	11.1 (q)
6	0.83 (d, 6.8)	16.0 (q)	6 0.83 (d, 7.3)	16.0 (q)
NH	7.68 (d, 9.4)		NH 7.56 (d, 9.4)	

the NOESY correlations between Ahp NH and H-4 (δ 2.62), H-4 (δ 2.62) and OH, H-4 (δ 1.74) and H-3, and H-3 and H-5 (δ 1.76). Therefore, the stereochemistry of Ahp could be assigned as (3*S*,6*R*)-3-amino-6-hydroxy-2-piperidone (L-Ahp).

The HRFABMS spectrum and NMR data established that micropeptin 478-B (**2**) had a molecular formula of $C_{40}H_{61}N_9O_{18}S_2Cl$. The ^{1}H and ^{13}C NMR spectra of **2** resembled those of **1**, but a dedisulfated ion at *m*/*z* 896 $[M - 2SO_3 + H]^+$ in its FABMS was observed and the ^{1}H and ^{13}C chemical shifts shifted from Ga sulfate H-2 (δ 4.25) and C-2 (δ 70.4) to Ga disulfate H-2 (δ 4.72) and C-2 (δ 74.5). Therefore, the Ga moiety of **2** was determined to be Ga 2,3-*O*-disulfate. The sequence and absolute stereochemistry of **2** were also determined by the above-mentioned procedures.

Micropeptins 478-A and B inhibited plasmin with IC_{50} of 0.1 and 0.4 μg/mL, respectively. These peptides did not inhibit trypsin, thrombin, papain, chymotrypsin, and elastase at 10.0 μg/mL.

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Hitachi 330 spectrophotometer. ^{1}H

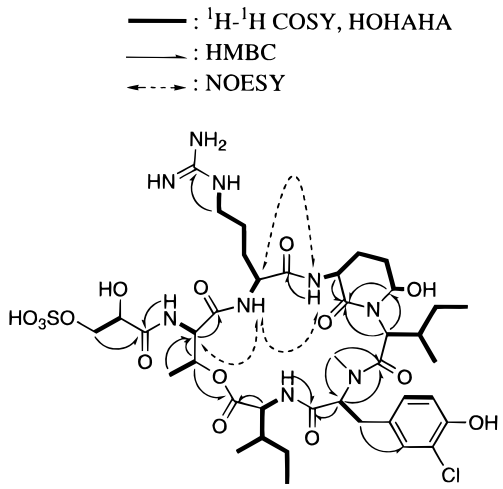

Figure 1. ^{1}H−^{1}H COSY, HOHAHA, HMBC, and NOESY correlations of micropeptin 478-A.

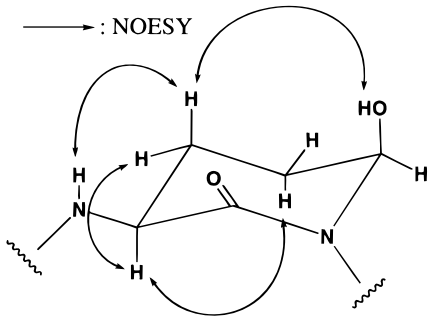

Figure 2. Relative stereochemistry of Ahp in micropeptin 478-A.

and ^{13}C NMR spectra were obtained with JEOL JNM-A500 or 600 in DMSO-d_6 at 27.0 °C. The resonances of residual DMSO-d_6 at δ_H 2.49 and δ_C 39.5 were used as internal references for ^{1}H and ^{13}C NMR spectra, respectively. FAB mass spectra were recorded by employing a JEOL JMS SX-102 mass spectrometer. Absorbances of the protease inhibitory assays were measured at 405 nm on a TOYOSODA MPR4 micro plate reader. Amino acid analyses were carried out with a Hitachi L 8500-A amino acid analyzer.

Culture Conditions. *M. aeruginosa* (NIES-478) was obtained from the NIES-collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan)[14] and cultured in 10 L glass bottles containing MA medium [$Ca(NO_3)_2 \cdot 4H_2O$ 5 mg, KNO_3 10 mg, $NaNO_3$ 5 mg, Na_2SO_4 4 mg, $MgCl_2 \cdot 6H_2O$ 5 mg, β-Na_2glycerophosphate 10 mg, $Na_2EDTA \cdot 2H_2O$ 0.5 mg, $FeCl_3 \cdot 6H_2O$ 0.05 mg, $MnCl_2 \cdot 4H_2O$ 0.5 mg, $ZnCl_2$ 0.05 mg, $CoCl_2 \cdot 6H_2O$ 0.5 mg, $Na_2MoO_4 \cdot 2H_2O$ 0.08 mg, H_3BO_3 2 mg, BICINE 50 mg, distilled water 100 mL, pH 8.6][14] with aeration (filtered air, 0.3 L/min) at 25 °C under illumination of 250 μE/m^2·s on a 12L:12D cycle. Cells were harvested by continuous centrifugation at 10 000 rpm after incubation for 14−21 days. Harvested alga was lyophilized and kept in a freezer at −20 °C until extraction.

Extraction and Isolation. Freeze-dried alga (27.6 g from 100 L of culture) was extracted with 80% MeOH (1 L × 3) and MeOH (1 L × 1). The extracts were evaporated and partitioned between Et_2O and H_2O. The aqueous layer was partitioned between *n*-BuOH and H_2O. The *n*-BuOH layer (1.06 g) was subjected to ODS flash chromatography (YMC-GEL, YMC, 5 × 6 cm) with aqueous MeOH followed by CH_2Cl_2. The 50% and 70% MeOH fractions were subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm, Nacarai tesque; 35−60% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm) and reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 35% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm), respectively. Each active fraction was subjected to reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 28−40% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm) followed by reversed-phase HPLC (Cosmosil MS, 10 × 250 mm; 28% MeCN containing 0.05% TFA; flow rate, 2.0 mL/min; UV detection at 210 nm) to yield micropeptins 478-A (**1**, 1.7 mg) and B (**2**, 1.5 mg).

Micropeptin 478-A (1): colorless amorphous solid; UV (MeOH) λ_{max} 281 nm (ϵ 950); ^{1}H and ^{13}C NMR see Table 1; FABMS (matrix: glycerol, positive) *m*/*z* 976 $[M + H]^+$, *m*/*z* 896 $[M - SO_3 + H]^+$, *m*/*z* 878 $[M - SO_3 - H_2O + H]^+$, *m*/*z* 862 $[M - SO_3 - H_2O - Cl + H]^+$, (matrix: glycerol, negative) *m*/*z* 974 $[M - H]^-$, *m*/*z* 956 $[M - H_2O - H]^-$, *m*/*z* 940 $[M - Cl - H]^-$; HRFABMS (negative) *m*/*z* 974.3641 ($C_{40}H_{62}N_9O_{15}SCl$, Δ −5.6 mmu).

Micropeptin 478-B (2): colorless amorphous solid; UV (MeOH) λ_{max} 282 nm (ϵ 1140); ^{1}H and ^{13}C NMR see Table 1; FABMS (matrix: glycerol, positive) *m*/*z* 976 $[M - SO_3 + H]^+$, *m*/*z* 896 $[M - 2SO_3 + H]^+$, *m*/*z* 878 $[M - 2SO_3 - H_2O + H]^+$, *m*/*z* 862 $[M - 2SO_3 - Cl + H]^+$, (matrix: glycerol, negative) *m*/*z* 1054 $[M - H]^-$, *m*/*z* 1036 $[M - H_2O - H]^-$, *m*/*z* 1020 $[M - Cl - H]^-$, *m*/*z* 974 $[M - SO_3 - H]^-$, *m*/*z* 940 $[M - SO_3 - Cl - H]^-$; HRFABMS (negative) *m*/*z* 1054.3245 ($C_{40}H_{62}N_9O_{18}S_2Cl$, Δ −2.0 mmu).

Acid Hydrolysis of Micropeptins 478-A and -B. For amino acid analysis, 100 μg each of micropeptins 478-A and B were dissolved in 0.5 mL of 6 N HCl and heated at 110 °C for 16 h. The reaction mixture was dried, dissolved in 0.6 mL of 0.02 N HCl, and subjected to amino acid analysis. Retention times in the amino acid analysis of micropeptins 478-A and B (min): Thr (15.12), Ile (46.10), Arg (108.48).

HPLC Analysis of the Marfey Derivatives. To the acid hydrolysate of a 100 μg portion of the peptides were added 50 μL of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in acetone (L-FDAA) (10 mg/mL) and 100 μL of 1 M $NaHCO_3$, and the mixture was kept at 80 °C for 3 min. To the reaction mixture were added 50 μL of 2 N HCl and 300 μL of 50% MeCN, and the resulting mixture was analyzed by reversed-phase ODS-HPLC: column Cosmosil MS (4.6 × 250 mm); gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (60:40:0.1) in 60 min; UV (340 nm). Retention times of the amino acid residues (min): D-Arg (34.8), L-Arg (36.4), L-Thr (37.8), D-Thr (40.6), L-Ile (51.2), D-Ile (55.4). Retention times of the amino acids of micropeptins 478-A and B (min): Arg (36.2), Thr (37.6), Ile (51.0).

Chiral GC Analysis of Glyceric Acid. Each of micropeptins 478-A and -B (100 μg) was dissolved in 0.5 mL of 6 N HCl and heated at 110 °C for 16 h. The reaction mixtures were evaporated in a stream of nitrogen, dissolved in 10% HCl in *i*-PrOH (200 μL), and heated at 100 °C for 30 min. The products were evaporated, dissolved in heptafluoro-*n*-butyric anhydride (100 μL) and CH_2Cl_2 (100 μL), reacted at 100 °C for 10 min, and evaporated. The residues were dis-

solved in CH_2Cl_2 and analyzed by GC with Chirasil-L-Val (Chrompak) column. The oven temperature was maintained at 40 °C for 5 min and raised to 70 °C at 4 °C/min, which was maintained for 25 min. Retention times for glyceric acids (min): D-glyceric acid (15.21), L-glyceric acid (15.40). Retention time of derivatives of the acid hydrolysate of micropeptins 478-A and -B (min): 15.21.

Oxidation of Micropeptins 478-A and -B. Each of the micropeptins 478-A and -B (300 μg) was dissolved in CH_2Cl_2 (2 mL), and then 1.0 mg (1.0 μmol) of PCC/Al_2O_3 in CH_2Cl_2 (1 mL) was added to the solution with stirring at room temperature. After the mixture was stirred for further 6 h, diethyl ether (2 mL) and an excess amount of anhydrous $MgSO_4$ were added, and the resulting mixture was stirred at room temperature for 20 min. After filtration, the solution was evaporated, dissolved in 6 N HCl (0.2 mL), and heated at 110 °C for 6 h to yield Glu. Formation of Glu was confirmed by amino acid analysis.

To the acid hydrolysate of PCC oxyproducts of micropeptins 478-A and -B were added 50 μL of L-FDAA in acetone (10 mg/mL) and 10 μL of 1 M $NaHCO_3$, and the mixture was kept at 80 °C for 3 min. To the reaction mixture were added 50 μL of 2 N HCl and 200 μL of 50% MeCN, and the resulting mixture was analyzed by ODS-HPLC: column Cosmosil MS (4.6 × 250 mm); gradient elution from H_2O/TFA (100:0.1) to MeCN/H_2O/TFA (60:40:0.1) in 60 min; UV (340 nm). Retention times of the standard amino acids (min): L-Glu (39.0), D-Glu (40.1). Retention times of HPLC peaks in the acid hydrolysate of micropeptins 478-A and -B (min): 39.4.

Synthesis and HPLC Analysis of Chloro *N*-Me Tyr. The mixture of 100 mg of *N*-Me-L-Tyr and 2 mL of sulfuryl chloride was warmed at 80 °C (3 min) until gas no longer evolved. Then 2 mL of sulfuryl chloride was added to the reaction flask and warmed at 80 °C (1 h) until gas no longer evolved. The excess sulfuryl chloride was removed by evaporation and freeze-dried. The reaction mixture was subjected to reversed-phase HPLC of Cosmosil MS (10 × 250 mm) with MeCN (5−35%) containing 0.05%TFA to yield chloro *N*-Me-L-Tyr (10%). Chloro-*N*-Me-L-Tyr: $[\alpha]_D$ +12.4° (*c* 0.4, 0.5 N HCl); HRFABMS *m*/*z* 230.0562 ($C_{10}H_{13}NO_3Cl$, Δ −2.2 mmu); ^{1}H NMR (DMSO-d_6), H-2 (δ 4.15), H-3a (δ 3.05, dd, 14.5, 7.0), H-3b (δ 3.15, dd 14.5, 5.1), H-5 (δ 7.24, d, 2.2), H-8 (δ 6.93, d, 8.4), H-9 (δ 7.02, dd, 8.4, 2.2), *N*-Me (δ 2.55, t 4.6).

Chloro-*N*-Me-L-Tyr was derivatized with D- and L-FDAA as described above.[13] The derivatives were analyzed by reversed-phase ODS-HPLC: column Cosmosil MS (4.6 × 250 mm); mobile phase MeCN/H_2O/TFA (40:60:0.1); UV (340 nm). Retention times of standards (min): chloro-*N*-Me-L-Tyr-L-FDAA (29.4), chloro-*N*-Me-L-Tyr-D-FDAA (33.4). Retention time of Chloro-*N*-MeTyr-L-FDAA in the acid hydrolysate of micropeptins 478-A and -B (min): 29.4.

Protease Inhibitory Assay. Plasmin (Sigma Chemical Co.) assay mixture containing 100 μL of 50 mM Tris−HCl buffer (pH 8.3), 30 μL of plasmin solution (1.5 mU/mL in 0.9% NaCl/glycerol 1:1), and 20 μL of test solution was added to each microtiter plate well. Then 50 μL of substrate solution (D-Val-L-Leu-L-Lys-*p*-nitroanilide (Sigma Chemical Co.) 1.4 mg/mL) was added to start the reaction. The absorbance of the well was immediately read at 405 nm. The developed color was measured after 30 min incubation at 37 °C.

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