



Synthesis of Novel Unnatural Amino Acid as a Building Block and Its Incorporation into an Antimicrobial Peptide

Jong Eun Oh and Keun Hyeung Lee*

Protein Chemistry Laboratory, Mogam Biotechnology Research Institute, 341 Pojung-Ri, Koosung-Myun, Yongin-City, Kyunggi-Do, 449-910, South Korea

Received 21 June 1999; accepted 14 August 1999

Abstract—Considering the biological mechanism and in vivo stability of antimicrobial peptides, we designed and synthesized novel unnatural amino acids with more positively charged and bulky side chain group than lysine residue. The unusual amino acids, which were synthesized by either solution phase or solid phase, were incorporated into an antimicrobial peptide. Its effect on the stability, activity, and the structure of the peptide was studied to evaluate the potential of these novel unnatural amino acids as a building block for antimicrobial peptides. The incorporation of this unusual amino acid increased the resistance of the peptide against serum protease more than three times without a decrease in the activity. Circular dichroism spectra of the peptides indicated that all novel unnatural amino acids must have lower α helical forming propensities than lysine. Our results indicated that the unnatural amino acids synthesized in this study could be used not only as a novel building block for combinatorial libraries of antimicrobial peptides, but also for structure–activity relationship studies about antimicrobial peptides. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

Recent advances of biological techniques made it possible to identify novel biological active compounds from various natural sources.¹ For developing the novel compounds as therapeutic agents, rational design and synthesis of novel candidate molecules, originated from the natural form, were required. This process, however, should overcome the following obstacles such as a time-consuming optimization process, a nonlinear relationship between structure and activity, and a lack of information on the structure of compounds in target sites. As an alternative method to overcome these limitations, combinatorial libraries have been developed and proved to be an efficient method to screen novel compounds as well as to optimize activity.^{2,3} The recent increase of the use of the combinatorial library technique has demanded the novel building block because increasing the number of building blocks resulted in the exponential increase of combination mixtures and the increase of probability for identifying novel compounds.

The recent emergence of multidrug-resistant bacteria has stimulated the development of novel antibacterial molecules with unexploited mechanisms of action.⁴ A large number of defense peptides produced in eukaryotic systems have been isolated and their functions characterized.^{5–7} Some of them have selectivity between prokaryotic and eukaryotic cells and a broad range of the activity against bacteria and fungi. Although the mode of the action of these peptides is not fully understood, it is suggested that the peptides fulfil their biological function by enhancing the permeability of lipid membranes of pathogenic cells.^{8–10} In the first step, the positively charged peptides bind to the negatively charged lipid membranes of the pathogen mainly by charge interactions and then they adopt mostly α helical structure or β sheet structure. In the next step, the peptides increase the permeability of the lipid membranes either by ion channel formation¹¹ or by the perturbation of the structure of the bilayer,¹² resulting in the death of target cells. As the peptides have a different mode of action from classical antibiotics, many combinatorial libraries screening for peptides active against bacteria and fungi have been used for developing novel therapeutic agents.^{13–15} However, the relatively low activity and rapid enzymatic degradation of antimicrobial peptides, compared with classical antibiotics, are still regarded as the main limitations.

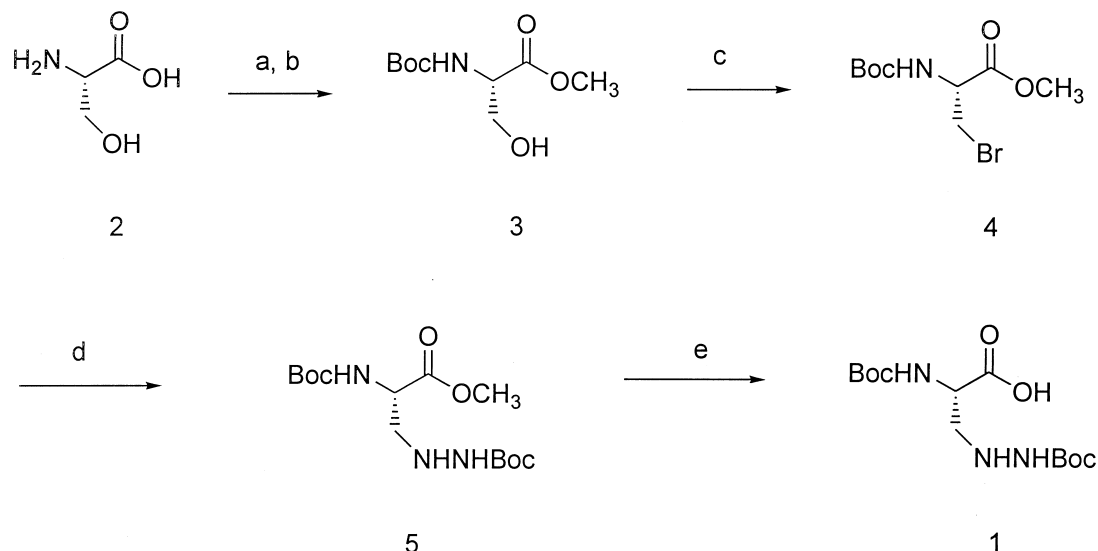
Key words: Amino acids and derivatives; antimicrobial compounds; depsipeptides; solid phase synthesis.

* Corresponding author. Tel.: +82-331-260-9814; fax: +82-0331-262-6622; e-mail: lkh@kgcc.co.kr

In the present study, to improve activity and in vivo stability of antimicrobial peptides, we designed and synthesized novel unnatural amino acids that had more positively charged and bulky side chain group than that of lysine residue on the basis of the following facts. The increase of the net positive charge of an antimicrobial peptide enhanced its binding to lipid membranes, resulting in the improvement of its activity^{16,17} and the peptide containing the unnatural amino acid with the bulky side chain group should have more resistance against enzymatic digestion than the peptide consisting of natural amino acids. We incorporated the novel unnatural amino acids into a well-characterized antimicrobial peptide, MP,¹⁸ and studied the activity, stability in the presence of serum, and the secondary structure of the peptide to evaluate their effectiveness as novel building blocks for the combinatorial libraries to screen for peptides active against microorganisms.

Chemistry

Antimicrobial peptides containing unnatural amino acids were synthesized by two different methods. In the first method, we synthesized unnatural amino acid(s) in solution phase and then incorporated it into the model peptide in solid phase peptide synthesis (SPPS). The unnatural amino acid **1** was prepared from L-serine **2** as described in Scheme 1. The amino group of L-serine **2** was protected with Boc group and then the carboxyl group was converted to methyl ester **3**.¹⁹ *N*-Boc serine methyl ester **3** was converted to brominated compound **4** in 60% yield by treatment with CBr₄ and PPh₃.²⁰ The treatment of activated intermediate **4** with Boc hydrazine and followed by hydrolysis provided protected unnatural amino acid **1**. No racemization was observed in this step by using HPLC with a chiral column. The incorporation of unnatural amino acid into the peptide was performed by SPPS in the presence of PyBOP and TEA in DMF.



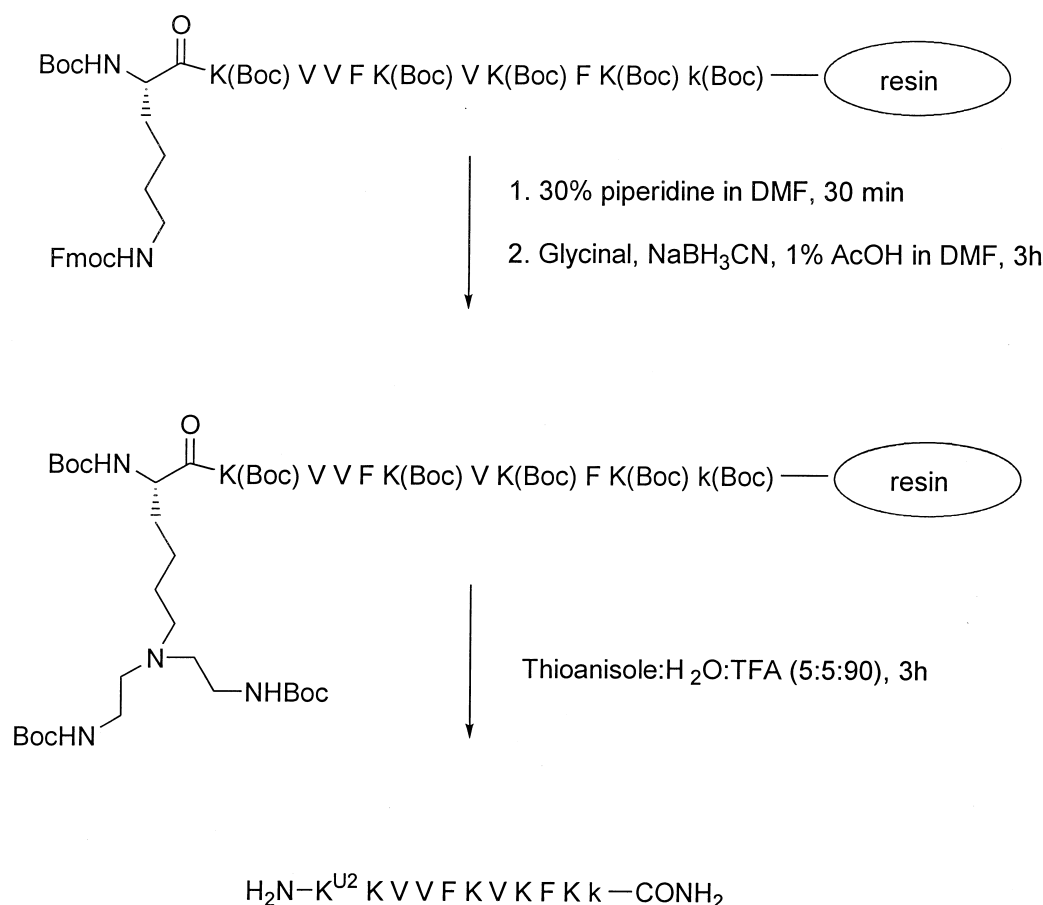
Scheme 1. Synthesis of unusual amino acid in solution phase. (a) (Boc)₂O, NaOH, H₂O, 24 h, 95%; (b) DCC, HOBT, MeOH, 5 h, 72%; (c) CBr₄, PPh₃, CH₂Cl₂, 1 h, 66%; (d) BocNHNH₂, TEA, CH₂Cl₂, 4 h, 57%; (e) LiOH, THF/MeOH/H₂O (3/1/1), 3 h, 37%.

In the second method, the peptide containing unnatural amino acids was synthesized in solid phase peptide synthesis as described in Scheme 2. Classical reductive alkylation of aldehyde with the amino group of the peptide has been used for the modification of the side chain in Lys of the peptide attached to the resin.²¹ The reductive alkylation using Boc-glycinal and NaBH₃CN, yielded a more bulky and positively charged unnatural amino acid than a natural amino acid, Lys. When Fmoc-Lys(Fmoc)-OH instead of Boc-Lys(Fmoc)-OH was introduced at the N-terminal of the peptide attached to the resin, an α -amino group as well as an ϵ -amino group of side chain in lysine was also modified by the same procedure. The completion of the reaction was monitored by a ninhydrin test.²² All synthesized peptides containing unnatural amino acid were purified by preparative RP-HPLC and characterized by analytical HPLC and mass spectroscopy.

The first method can provide a variety of positively charged unnatural amino acids with various side chains by substitution reaction of intermediate **4** with various nucleophiles. Primary or secondary amine was successfully introduced to the side chain of the amino acid by substitution reaction. For example, *tert*-butyl *N*-(2-aminoethyl) carbamate, *tert*-butyl *N*-(3-aminopropyl) carbamate, and 4-piperidonopiperidine²³ were reacted with intermediate **4** yielding the corresponding unnatural positively charged amino acids (data not shown). The second method directly modified the side chain in Lys of the peptide in SPPS and, therefore, it was not necessary to synthesize and purify the unnatural amino acid. In addition, there is no risk of racemization. Also, the second method can be easily applied to other antimicrobial peptides containing Lys residue, in SPPS.

Results and Discussion

The model peptide, MP consisting of eleven amino acid residues, was reported to have potent antimicrobial



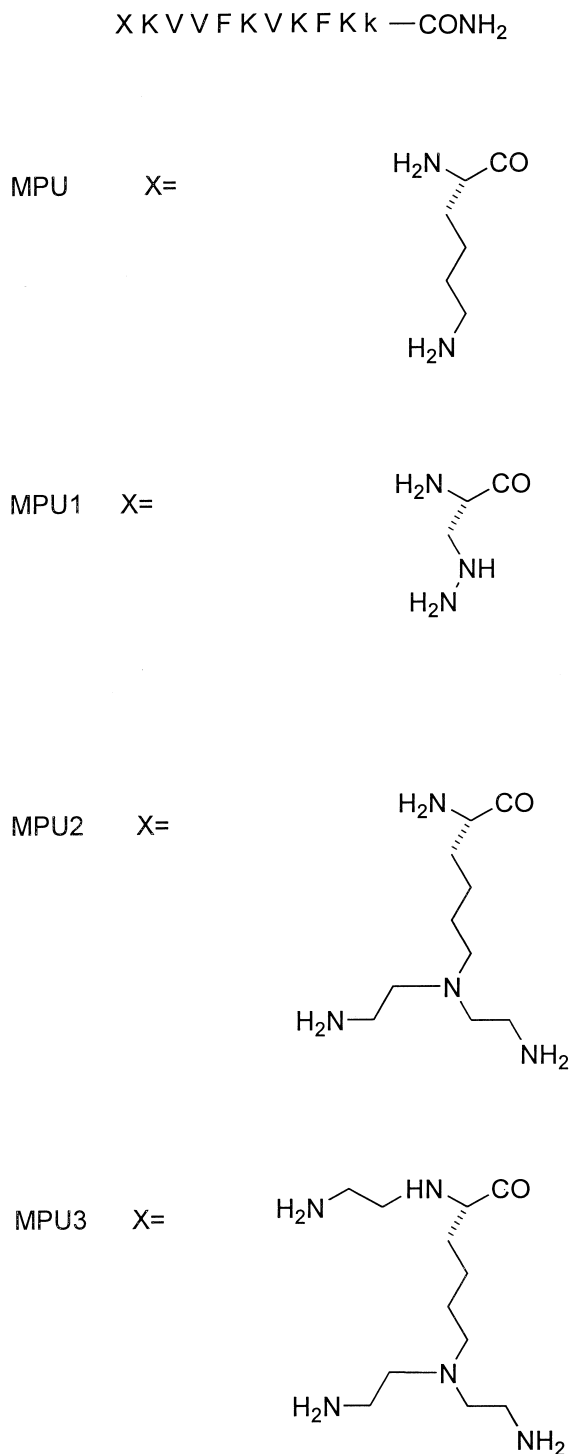
Scheme 2. Synthesis of MPU analogues in solid phase.

activity without hemolytic activity.¹⁸ We synthesized several MP analogues containing the unnatural amino acid (Scheme 3) and investigated the unnatural amino acid effect on the activity, stability, and secondary structure of the peptide. To investigate the effect of the unnatural amino acid on the stability, we measured the half-life of the peptides in the presence of serum. Our previous study indicated that exoprotease in the serum played a major role in the degradation of the antimicrobial peptide with a primary structure similar to that of MP.²⁴ In the present study, we incorporated the unusual amino acid at the N-terminal and simultaneously C-terminal amino acid was replaced by D-amino acid for easy monitoring the half-life in the presence of serum. As shown in Figure 1, MPU had a very short half-life (6 min), whereas MPU2 (21 min) and 3 (24 min) had a much longer half-life than MPU. This result suggests that the bulky side chain of the unnatural amino acid must play a major role in the increase of the resistance against exoprotease in the serum.

As shown in Table 1, the incorporation of unnatural amino acids resulted in the increase of the net positive charge of the peptide from 1 to 3. This incorporation, however, did not significantly affect antimicrobial activity. As many structure–activity relationship (SAR) studies indicated that net positive charge and α helical structure played a crucial role in antimicrobial activity,^{16,17} we investigated the secondary structure of each

peptide containing unnatural amino acid in membrane-mimetic environment to elucidate the real net charge effect on the activity. As shown in Figure 2, CD spectra of MPU 1-3, measured in the presence of 50% TFE, indicated that MPU had 54% α helicity whereas MPU1, MPU2, and MPU3 had 29, 42, and 33% α helicity, respectively. The secondary structure change of the peptide containing the unnatural amino acid indicated that the introduction of novel unnatural amino acid resulted in the decrease of α helicity. As the other minor factors for the activity such as hydrophobicity and amphiphilicity were regarded to be similar, we could find out the relationship between the activity and structural parameters in terms of net positive charge and α helical structure. All MP analogues (MPU1-3) employed in this study retained antimicrobial activities in spite of the decrease of α helicity, which suggested that the increase of net positive charge must compensate for the decrease of α helicity, resulting in the retention of the activity. CD spectra also revealed that the novel unnatural amino acid had a lower α helical forming propensity than lysine. It is due to the fact that the novel unnatural amino acid has a different number and location of amino groups, which can participate in hydrogen bonding, and a different bulkiness in the side chain from those of lysine residue.

A positively charged amino acid, for example Lys residue, had been added to the N- or C-terminal of



Scheme 3. Structure of MPU and its analogues.

antimicrobial peptides and various amino acids in antimicrobial peptides had been replaced by Lys residue to study structural parameters for the activity such as net positive charge, α helicity, and hydrophobicity.^{8–10,16,17} However, the augmentation of Lys residue and the multiple amino acid replacement by Lys residue must result in the change of several structural parameters such as hydrophobicity, size, and angle subtended by the charged helix face. Therefore, it is difficult to evaluate the independent effect of net positive

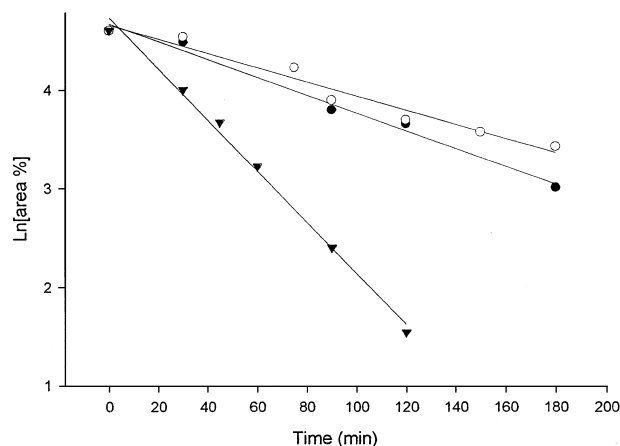


Figure 1. Stabilities of MPU and its analogues in the presence of serum half-life was measured in the presence of 25% mouse serum (v/v). MPU (▼), MPU2 (●), MPU3 (○).

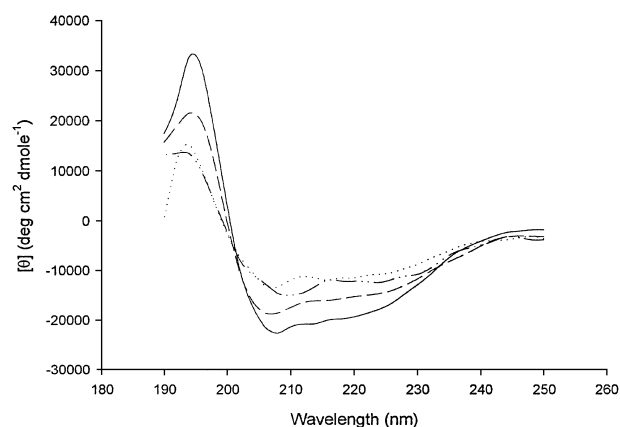


Figure 2. CD spectra of MPU and its analogues in 50% TFE (v/v). CD spectra were measured at the concentration of 100 μ g/mL sample in 10 mM sodium phosphate buffer (pH 7.4) including 50% TFE (v/v).

charge on the antimicrobial activity. In the present study, the single amino acid replacement by the unnatural amino acid resulted in the increase of net positive charge (1–3) of the peptide with little change of size and angle subtended by the charged helix face. This result suggests that the unnatural amino acids described here can be useful to study the relationship between the activity and net positive charge of the novel antimicrobial peptides.

In conclusion, our result indicated that the unnatural amino acid as a novel building block for antimicrobial peptide could improve the activity against bacteria and fungi as well as the stability of the peptide in serum. We also suggest that this unusual amino acid can be used to investigate the structural parameter of the antimicrobial peptides on the activity.

Experimental

Methyl *N*-(*tert*-butyloxycarbonyl)-L-serinate (3). To a stirred solution of *N*-(*tert*-butyloxycarbonyl)-L-serine in MeOH, which was prepared from L-serine **2** using

Table 1. Antimicrobial activity of MPU and its analogues

Name	Net charge	Minimum inhibitory concentration ($\mu\text{g/mL}$) ^a				
		<i>S. aureus</i> ATCC6538	MRSA ^b Sr1550	<i>M. luteus</i> ATCC 9341	<i>P. aeruginosa</i> ATCC 9027	<i>C. albicans</i> ATCC 36232
MPU	+7	4	25	4	6	4
MPU1	+8	8	—	5	8	10
MPU2	+9	5	25	3	13	4
MPU3	+10	5	25	3	9	4
MagaininII		40	>100	50	20	50

^a Average MIC values were calculated from three independent experiments performed in duplicate, which provided a standard deviation below 30%.

^b MRSA is methicillin resistant *S. aureus*.²⁹

(BOC)₂O, were added DCC and HOBt under N₂. After 5 h, the mixture was filtered. The combined filtrate was concentrated and the residue purified by FC. Yield: 72%. oil. *R_f* 0.37 (hexane:EtOAc, 7:3). ¹H NMR (CDCl₃): 1.43 (s, 9H), 2.45 (m, 1H), 3.76 (s, 3H), 3.80–3.94 (m, 2H), 4.36 (m, 1H), 5.42 (m, 1H)

Methyl-3-bromo-*N*-(*tert*-butyloxycarbonyl)-L-alaninate (4). To a stirred solution of **3** (0.98, 4.5 mmol) and CBr₄ (1.84 g, 5.5 mmol) in CH₂Cl₂ (10 mL) was added portionwise PPh₃ (1.75 g, 6.6 mmol) at 0°C. The mixture was stirred for 20–30 min, and then the solvent was evaporated. The residue was triturated with Et₂O (20 mL), the mixture filtered, and the filter cake washed with Et₂O (3×10 mL). The combined filtrate was concentrated and the residue purified by FC. Yield: 66%. White solid. *R_f* 0.2 (hexane:EtOAc, 9:1). Mp 50–52°C ¹H NMR (CDCl₃): 1.42 (s, 9H); 3.65–4.93 (m, 5H); 4.70–4.80 (m, 1H); 5.40 (m, 1H).

Methyl-3-[2'-(*tert*-butyloxycarbonyl)-hydrazino]-L-alanine (5). To a stirred solution of **4** (0.4 g, 1.5 mmol) in CH₂Cl₂ (10 mL) was added *t*-butyl carbazate (0.23 g, 1.8 mmol) and triethylamine (0.31 mL, 2.25 mmol). After 4 h, the mixture was diluted with CH₂Cl₂ (30 mL). The organic layer was washed with 5% HCl and brine, dried (Na₂SO₄), and evaporated. The residue was purified by FC. Yield: 57%. oil. *R_f* 0.29 (hexane:Et₂O, 95:5). [α]₂₅ (–11°, CHCl₃) ¹H NMR (CDCl₃): 0.82–0.88 (m, 2H); 1.25 (s, 9H), 1.49 (m, 10H); 3.83 (s, 3H); 5.729–5.734 (m, 1H); 6.16 (s, 1H); 7.02 (s, 1H).

3-[2'-(*tert*-Butyloxycarbonyl)-hydrazino]-L-alanine (1). To a stirred solution of **5** in mixed solution (8 mL) of THF and MeOH (3:1) was added LiOH in H₂O (2 mL) at 0°C. After 1.5 h, the mixture was stirred at room temperature for 2 h. THF and MeOH were removed by evaporation and the residue in H₂O was acidified with 10% citric acid and extracted with EtOAc. The combined organic layer was washed with H₂O and brine, dried (Na₂SO₄), and concentrated. The compound **1** was dried on vacuum and purified by FC. The extent of racemization of the unusual amino acid (>95%) was confirmed by HPLC with Chirex (D) penicillamine column (10×250 mm, phenomenex, Torrance, CA, USA). The amino acid was eluted using solvent A consisting of water and solvent B consisting of methanol and monitored by absorbance at 214 nm. The amino acid was

analyzed using linear gradient of 0–70% B in 70 min. Yield: 37%. Oil. TLC *R_f* 0.5 (CH₂Cl₂:MeOH, 4:1). [α]₂₅ (–19°, MeOH) ¹H NMR (CDCl₃): 0.85–0.88 (m, 2H); 1.46 (s, 18H), 1.93–2.06 (m, 1H); 5.85 (s, 1H); 6.20 (s, 1H); 7.07 (s, 1H); 8.4 (s, 1H).

Synthesis of peptides

Peptides were prepared by stepwise solid-phase synthesis on an Applied Biosystems model 431A automatic peptide synthesizer. The peptide chain was assembled on PAL resin with a Fmoc/*tert*-butyl strategy.²⁵

MPU1 was synthesized as follows; unusual amino acid (**1**), which was synthesized in solution phase, was incorporated into the peptide, MPU by the coupling of unusual amino acid (**1**) with free amino groups of resin bound peptides, in the presence of benzotriazole-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and triethylamine (TEA) for 5 h at 25°C. The reaction was repeated until no color change was observed in the ninhydrin test. MPU2 and MPU3 were synthesized in solid phase by three steps. First, *N*-Boc-Lys(Fmoc)-OH in MPU2 or *N*-Fmoc-Lys(Fmoc)-OH in MPU3 was incorporated into the peptide. Second, the Fmoc-group was selectively deprotected in the presence of 30% piperidine in *N,N*-dimethylformamide (DMF). Third, the side chain of Lys was modified by reductive alkylation of *tert*-butoxycarbonyl (Boc)-Glycinal with free amino group of resin bound peptides, in the presence of NaBH₃CN (1 M solution in tetrahydrofuran) in 1% acetic acid in DMF.

Deprotection was achieved by treatment with a mixture of trifluoroacetic acid (TFA):water:thioanisole (9:0.5:0.5, v/v/v) at room temperature for 2–4 h. After filtration of the resin and washing with TFA, a gentle stream of nitrogen was used to remove the excess TFA. The crude peptide was triturated with diethyl ether chilled at –20°C and was centrifuged at 3000×g for 10 min. Diethyl ether was decanted and crude peptide was dried under nitrogen. The peptide was purified by high performance liquid chromatography with a Phenomenex C₁₈ column (21.2×250 mm; Phenomenex, Torrance, CA, USA). The homogeneity of the peptides (>95%) was checked by analytical HPLC with a Waters Delta Pak C₁₈ column (3.9×150 mm; Waters, Milford, MA, USA). Mass spectrometry on Platform II (Fisons

instruments, Manchester, United Kingdom) was used to measure the mass of the purified peptide. MPU (ES-MS:1376.43 $[M+H]^+$, calculated mass 1377.92), MPU1 (ES-MS:1349.14 $[M+H]^+$, calculated mass 1350.92), MPU2 (ES-MS:1462.25 $[M+H]^+$, calculated mass 1463.92), MPU3 (ES-MS:1504.92 $[M+H]^+$, calculated mass 1505.92)

Antifungal and antibacterial assay

In vitro antimicrobial assays were performed by the modified checkboard microdilution method by following the recommendation of the National Committee for Clinical Laboratory Standards.²⁶ Antibiotic medium 3 (M3; pH 7 at 25°C, Difco) was used as the antibacterial assay media. Bacteria cells freshly grown on antibiotic medium 3 agar plate were suspended in physiological saline to 10^4 cells per 1 mL and used as the inoculum. The test solution was added (100 μ L per well) and serially diluted by twofold. After inoculation (100 μ L per well, 5×10^3 cells per 1 mL), plates were incubated at 37°C for 24 h and the absorbance was measured at 620 nm using an ELISA reader (Spectra, SLT, Salzburg, Austria) to assess cell growth. Antifungal assay was done in RPMI 1640 media (pH 7 at 25°C) and the plates were incubated at 30°C for 24 h. Minimal inhibition concentration (MIC) was defined as the lowest concentration exhibiting no visible growth of the test organism. All MICs were measured from three independent experiments performed in duplicate.

CD measurement

Circular dichroism (CD) spectra were recorded on a J-715 spectropolarimeter (Jasco, Tokyo, Japan) using a quartz cell of 1 mm path length, at wavelengths ranging from 190 to 245 nm. CD spectra were obtained with a 0.5 nm bandwidth and a scan speed of 10 nm/min at room temperature. Two scans were averaged to improve the signal to noise ratio. CD spectra were expressed as the mean residue ellipticity and the α helicity was calculated from the mean residue ellipticity $[\theta]$ at 222 nm.²⁷

Half-lives measurement in the presence of serum

1 mL of 25% mouse serum/RPMI media (v/v) in 1.5 mL Eppendorf tube was temperature-equilibrated at 37°C for 15 min before adding 10 μ L of peptide stock solution (10 mg/mL) to make the final peptide concentration 100 μ g/mL. The initial time was recorded and 100 μ L of reaction solution was removed at known time intervals and added into 100 μ L of 10% aqueous trichloroacetic acid (TCA) solution. The cloudy reaction sample was cooled at 4°C for 15 min and spun at 13,000 g for 15 min to precipitate serum protein.²⁸ Peptide analysis was carried out by reverse phase HPLC with Waters C₁₈ column. Kinetic analysis was carried out by a linear least square analysis of the logarithm of the peak area versus time. Each half-life was determined from two

independent experiments performed in duplicate and all pseudo-first plots were linear showing correlation coefficient greater than 0.96.

References and Notes

- Pirrung, M. C. *Chemtracts-org. Chem.* **1995**, 8, 5.
- Terrett, N. K.; Gardner, M.; Gordon, D. W.; Kobylecki, R. J.; Steele, J. *Tetrahedron* **1995**, 51, 8135.
- Youngquist, R. S.; Fuentes, G. R.; Lacey, M. P.; Keough, T. *J. Am. Chem. Soc.* **1995**, 117, 3900.
- Maloy, W. L.; Kari, P. U. *Biopolymer* **1995**, 37, 105.
- Steiner, H.; Hulmark, D.; Engstrom, A.; Bennich, H.; Boman, H. G. *Nature* **1981**, 292, 246.
- Kusuhara, T.; Nakajima, Y.; Natsuyama, K.; Natori, S. *J. Biochem.* **1990**, 107, 514.
- Yamada, K.; Natori, S. *J. Biochem.* **1993**, 291, 275.
- Juvvadi, P.; Vummam, S.; Merrifield, R. B. *J. Am. Chem. Soc.* **1996**, 118, 8989.
- Matsuzaki, K.; Sugishita, K.; Fujii, N.; Miyajima, K. *Biochemistry* **1995**, 34, 3423.
- Datthe, M.; Schumann, M.; Wieprecht, T.; Winkler, A.; Beyermann, M.; Krause, E.; Matsuzaki, K.; Murase, O.; Bienert, M. *Biochemistry* **1996**, 35, 12612.
- Sansom, M. S. P. *Prog. Biophys. Mol. Biol.* **1991**, 55, 139.
- Matsuzaki, K.; Murase, O.; Fujii, N.; Miyajima, K. *Biochemistry* **1996**, 35, 11361.
- Blondelle, S. E.; Houghten, R. A. *TIBTECH* **1996**, 14, 60.
- Blondelle, S. E.; Takahashi, E.; Weber, P. A.; Houghten, R. A. *Antimicrob. Agents Chemother.* **1994**, 38, 2280.
- Hong, S. Y.; Oh, J. E.; Kwon, M. Y.; Choi, M. J.; Lee, J. H.; Lee, B. L.; Moon, H. M.; Lee, K. H. *Antimicrob. Agent Chemother.* **1998**, 42, 2534.
- Kiyota, T.; Lee, S.; Sugihara, G. *Biochemistry* **1996**, 35, 13196.
- Datthe, M.; Wieprecht, T.; Nikolenko, H.; Handel, L.; Maloy, L. W.; MacDonald, D. L.; Beyermann, M.; Bienert, M. *FEBS Letter* **1997**, 403, 208.
- Hong, S. Y.; Oh, J. E.; Lee, K. H. *Antimicrob. Agent Chemother.* **1999**, 43, 1704.
- Tarbell, D. S.; Yamamoto, Y.; Pope, B. M. *Proc. Nat. Acad. Sci. USA* **1972**, 69, 730.
- Savithri, D.; Leumann, D.; Scheffold, R. *Hel. Chim. Acta* **1996**, 79, 288.
- Sasaki, Y.; Murphy, W. A.; Heiman, M. L.; Lance, V. A.; Coy, D. H. *J. Med. Chem.* **1987**, 30, 1162.
- Kaiser, E.; Colscott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, 34, 595.
- N-(tert-butoxycarbonyl)-3-[4-piperidopiperidyl] alanine* ES/MS 356.22 $[M+H]^+$.
- Oh, J. E.; Hong, S. Y.; Lee, K. H. *J. Peptide Res.* **1999**, 54, 129.
- Fields, G. G.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, 35, 161.
- Nakajima, R.; Kitamura, A.; Someya, K.; Tanaka, M.; Sato, K. *Antimicrob. Agents Chemother.* **1995**, 39, 1517.
- Chen, Y. H.; Yang, J. T.; Chau, K. H. *Biochemistry* **1974**, 13, 3350.
- Powell, M. F.; Stewart, T.; Otvos, L. Jr.; Urge, L.; Gaeta, F. C.; Sette, A.; Arrhenius, T.; Thomson, D.; Soda, K.; Colon, S. M. *Pharm. Res.* **1993**, 10, 1268.
- Murakami, K.; Nomura, K.; Doi, M.; Yosida, T. *Antimicrob. Agents Chemother.* **1987**, 31, 1307.