Notes

Design and Synthesis of Antibacterial Pseudopeptides with a Potent Antibacterial Activity and More Improved Stability from a Short Cationic Antibacterial Peptide

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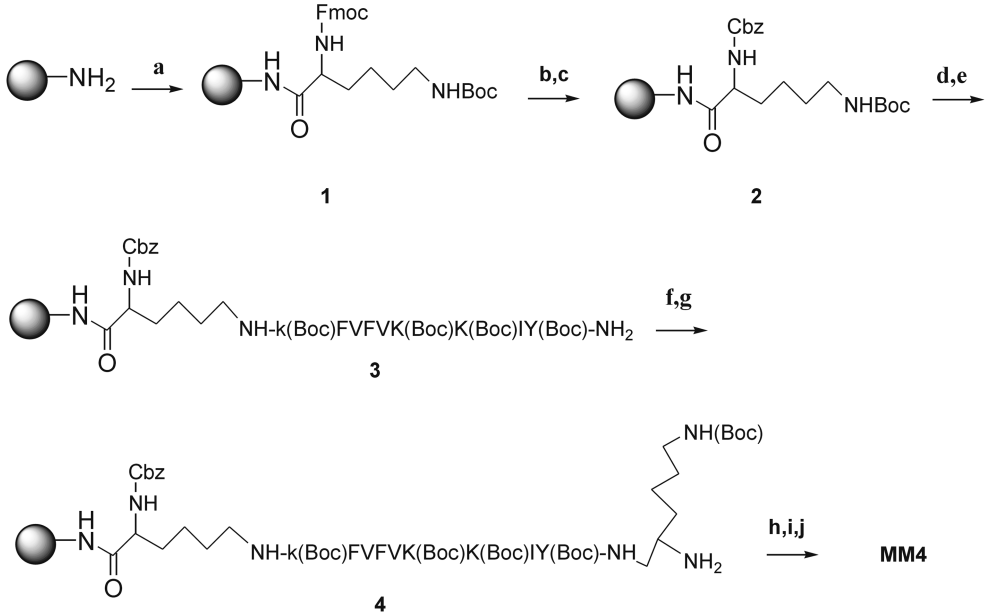
The problem of increasing microorganisms resistant to current antibiotics urgently demanded the development of novel antimicrobial agents.[1] A large number of cationic antibacterial peptides and proteins have been identified from various natural sources as a key component of immune system in addition to antibody, phagocyte, and cell immunity.[2] A number of studies indicated that most of antibacterial peptides exerted their activities by enhancing the permeability of pathogenic cell membranes and this kind of antibacterial peptides may be difficult to induce resistant strains of pathogens compared to classic antibacterial agents.[3,4] As cationic antibacterial peptides and proteins can be novel candidates to fight against microbes, they have been extensively studied in the past two decades.[3] In the development process, the minimum active fragment was identified from parent antibacterial peptides or proteins and then several analogs of the active fragment were developed by replacement, appending, and/or deletion of amino acids to enhance its spectrum, potency, and therapeutic index.[3] However, these antibacterial peptides may not be used as therapeutic agents because of their poor *in vivo* stability and a risk of degradation by enzymes released from pathogens. Previously, we first reported the incorporation of amide bond isosters including reduced amide bond, carbamate bond, and *N*-substituted glycine into antimicrobial peptides.[5,6] According to the previous results,[5,6] the incorporation of amide bond isosters into antimicrobial peptides was an efficient method to improve bioavailability as well as to change the spectrum of antimicrobial activity and the selectivity between bacteria and mammalian cell.

In the present study, we chose a short antibacterial peptide as a model peptide and simultaneously incorporated reduced amide bond as well as unnatural amino acid into the peptide and investigate the stability in the presence of serum and antibacterial activity. The model peptide (MM1) and its diastereomer (MM6) were synthesized by the Fmoc-chemistry in solid phase peptide synthesis according to the literature procedure.[7] The pseudopeptides containing reduced amide bonds (MM2 and MM3) were also synthesized in solid phase synthesis as follows. The peptide chain was assembled on Rink Amide MBHA resin with a Fmoc/Boc strategy. The reduced amide bond ψ(CH_2NH) was introduced by the reductive alkylation of the preformed Fmoc-protected amino aldehyde with the free amino terminal of the resin bound peptide, in the presence of an excess $NaBH_3CN$ (1.0 M solution in tetrahydrofuran) in 1% acetic acid in *N*,*N*-dimethylformamide (DMF).[8] Cleavage and deprotection were achieved by treatment with a mixture of trifluoroacetic acid (TFA)/H_2O/thioanisole (9/0.5/0.5, v/v/v) at room temperature for 8-9 h. The pseudopeptides (MM4 and MM5) containing unnatural amino acid were synthesized in solid phase synthesis described in Scheme 1. Fmoc-Lys(Boc)-OH was assembled on MBHA resin and then the Fmoc protection group of the amino acid bound on resin **1** was replaced by Cbz protection group. Deprotection of Boc group of the resin bound amino acid and then Fmoc amino acid coupling and deprotection in solid phase synthesis provided **3**. The reduced amide bond ψ(CH_2NH) was introduced by the reductive alkylation of the preformed Fmoc-protected amino aldehyde with the free amino terminal of the resin bound peptide **3**, in the presence of an excess $NaBH_3CN$ (1.0 M solution in tetrahydrofuran) in 1% acetic acid in *N*,*N*-dimethylformamide (DMF).[8] Cleavage and deprotection achieved by treatment with a mixture of TMSBr/EDT/m-cresol/thioanisole/TFA (1.32/0.5/0.1/1.17/7.5, v/v/v/v/v) at 4 °C for 12 h provided the crude pseudopeptide. The crude peptide and pseudopeptide were purified by preparative HPLC. The yields of each product were over 40% and the products were obtained in high purity (generally > 95% by RP analytical HPLC, $UV_{214\,nm}$). The success of synthesis of peptides and pseudopeptides was confirmed by ESI mass.

The prediction of *in vivo* peptide pharmacokinetics is quite difficult because there are many factors that determine peptide fate, including absorption rate, first-pass metabolism, hepatic and renal clearance, cellular binding and uptake, and circulating peptidase.[9] *In vivo* peptide stability in blood can be gauged by determining the *in vitro* stability in serum.[9] Thus, the half-life of the peptides and its pseudopeptides in the presence of serum was investigated. As shown in Table 1, the half-life of MM1 was about 6 minutes, which meant that this peptide must require more stability for



Scheme 1. (a) 3 equiv Fmoc-Lys(Boc)-OH, DCC, HOBt, DMF; (b) 25% Piperidine, DMF; (c) 3 equiv Cbz-Cl, DIEA, DMF; (d) 50% TFA, DMF; (e) 3 equiv Fmoc-AA-OH, DCC, HOBt, DMF and then 25% Piperidine, DMF; (f) 3 equiv Fmoc-Lys(Boc)-CHO, $NaBH_3CN$, 1% CH_3COOH, DMF; (g) 25% Piperidine, DMF (h) 3 equiv Fmoc-(D)-Lys(Boc)-OH, DCC, HOBt, DMF; (i) 25% Piperidine, DMF (j) TMSBr/EDT/m-cresol/thioanisole/TFA (1.32/0.5/0.1/1.17/7.5, v/v/v/v/v). MBHA resin was used as solid support.

in vivo efficacy test and therapeutic agents. Several researches revealed that N and/or C-terminal region of peptides must be the most susceptible site to the enzyme in the serum.[10] Thus, amino acids in the susceptible sites of the model peptide were replaced by D-amino acids and the stability of diastereomers (MM6) was investigated. As shown in Table 1, MM6 where both lysines at the N-terminal and C-terminal were replaced by D-lysines showed 9 times increased half-life in the presence of serum and exhibited the same antibacterial activity as that of MM1. On the basis of this result, we synthesized pseudopeptide containing one or two reduced amide bond(s) at the N or C-terminal region of the peptide. We successfully prepared the pseudopeptide (MM2 and MM3) containing one or two reduced amide bond(s) at the N-terminal by using solid phase synthesis. However, the pseudopeptide containing one or two reduced amide bond at the C-terminal was not obtained by using the same solid phase synthesis. This may be due to interference of amino acid coupling reaction by reduced amide bond and/or leading to branched peptide from reduced amide bond in the amino acid coupling reaction. Both pseudopeptides (MM2 and MM3) containing reduced amide bond at the N-terminal showed an increased-stability in the presence of serum without a loss of antibacterial activity. To synthesize more stable analog of the peptide, we introduced unnatural amino acid at the C-terminal and incorporate reduced amide bond and D-amino acid at the N-terminal of the peptide (MM4). The pseudopeptide showed more than 24 times longer half-life in the presence of serum without a great loss of the activity. This result revealed that the stability of the peptide could be synergistically increased by simultaneous introducing reduced amide bond and unnatural amino acid at the

Table 1. Sequence, net positive charges, antibacterial and hemolytic activities of the antibacterial peptide and its pseudopeptides

Name	Sequence[a]	Net charge	MIC (μg/mL) for *S. aureus* ATCC 6538[b]	HD_{50} (μg/mL)[c]	Stability in the presence of serum Half-life (min)[d]
MM1	KKYIKKVFVFK-$CONH_2$	+6	3.13	>100	6
MM2	KKψ[CH_2NH]YIKKVFVFK-$CONH_2$	+7	3.13	>100	31
MM3	Kψ[CH_2NH]Kψ[CH_2NH]YIKKVFVFK-$CONH_2$	+8	3.13	>100	64
MM4	**k**Kψ[CH_2NH]YIKKVFVF**k**-UK-$CONH_2$	+8	6.25	>100	146
MM5	Ab-**k**Kψ[CH_2NH]YIKKVFVF**k**-UK-$CONH_2$	+8	12.6	>100	>150
MM6	**k**KYIKKVFVF**k**-$CONH_2$	+6	3.13	>100	55
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$	+6	3.13	3.13	ND

[a]Small and bold letter indicated D-amino acid. Ab; 4-aminobenzoic acid, UK; NH_2-$(CH_2)_4CHNH_2$-$CONH_2$. [b,c]Average MIC and HD_{50} values were calculated from three independent experiments performed in duplicate, which provided a standard deviation below 20%. [d]Half-life of each compound in the presence of serum was calculated by a linear least square analysis of the logarithm of the peak area versus time, which provided a standard deviation below 30%.

N and C-terminal of the peptide. To improve the stability of MM4, we introduced 4-aminobenzoic acid into the N-terminal of the pseudopeptide. Generally, acetic acid and benzoic acid were frequently used for capping of N-terminal amino acid of peptide to increase stability against enzyme. However, as the net positive charge of α amino acid of the antibacterial peptide was regarded as an important factor for the activity, we used 4-aminobenzoic acid for capping of N-terminal amino acid of the pseudopeptide. The half-life of MM5 was increased more than 25 times while antibacterial activity for *S. aureus* was decreased by 4 fold. To investigate mammalian cell toxicity, we measured hemolytic activity of the peptides and pseudopeptides as described in the experimental section. However, all compounds including the model peptide did not show hemolytic activity in this condition so we could not characterize the effect of reduced amide bond on the mammalian cell toxicity.

In summary, we successfully synthesized novel antibacterial pseudopeptides that exhibited a longer half-life than the model peptide in the presence of serum as well as a considerable activity against test bacteria. Our result indicated that simultaneous incorporation of reduced amide bond and unnatural amino acid into the N and C-terminal region of peptides may be a useful tool to improve *in vivo* stability of the short antibacterial peptide without a great loss of the activity.

Experimental Section

Preparation of *N*-Methoxy-*N*-methyl-N^{α}-Fmoc Hydroxamates. Nα-Fmoc-Lys(Nε-Boc)-OH (0.854 mmol, 400 mg), PyBop (1.1 equiv, 488 mg), DMAP (0.1 equiv, 10.4 mg), TEA (1.5 equiv, 180 μL) were dissolved in 5 mL of DCM. After 5 min of stirring at room temperature, a solution of N,O-dimethylhydroxylamine hydrochloride (1.2 equiv, 100 mg), TEA (1.5 equiv, 180 μL) in 5 mL DCM was added. After 3 h, 20 mL of DCM was added and the organic phase was washed once with water, once with a saturated aqueous solution of $NaHCO_3$, and once with 1 N HCl, before being dried with brine and anhydrous $MgSO_4$. The solvent was removed under vacuum. The crude product was purified by flash column chromatography (SiO_2, 40%-60% EtOAc: Hexane). TLC (90% $CHCl_3$/8% CH_3OH/2% CH_3CO_2H, Rf 0.53). ^{1}H-NMR (DMSO-d_6, 400 MHz): 1.19-1.15 (m, 1H), 1.41-1.31 (m, 12H), 1.53 (br, 2H), 1.99 (s, 1H), 2.89 (br, 2H), 3.10 (s, 3H), 3.72 (s, 2H), 4.26-4.22 (m, 3H), 4.42 (br, 1H), 6.77 (br, 1H), 7.35-7.31 (m, 2H), 7.44-7.40 (m, 2H), 7.62-7.60 (m, 1H), 7.73-7.72 (m, 2H), 7.90-7.88 (m, 2H). ESI-MS: calcd 534.26, obsd 534.30 $[M+Na]^+$.

Preparation of N^{α}-Fmoc Amino Aldehyde. The weinreb amide (0.5 mmol, 256 mg) was dissolved in 10 mL of dry THF. $LiAlH_4$ (1 M solution in THF, 1 mL) was added to the solution for 10 min at 4 °C. The solution was stirred for 10 min at room temperature and 10% citric acid was added slowly. Solvent was removed and then 20 mL of DCM was added and the organic phase was washed and dried as described above. The amino aldehyde was used without further characterization.

Incorporation of the Reduced Amide Bond into the Peptide. The amino aldehyde (0.3 mmol. 3 equiv) was dissolved in 4 mL of 1% AcOH in DMF and added to the peptide-resin (0.1 mequiv). And then, 1.0 M solution of $NaCNBH_3$ in 0.6 mL THF was added and stirred for 4 h. If necessary, the reaction was repeated until no color change of the resin was monitored in ninhydrin test. The resin was washed and dried and then the peptide synthesis was continued.

Synthesis and Purification of Peptide and Pseudopeptide. Peptide and pseudopeptide syntheses were performed on a 0.1 mmol scale by the solid phase method as reported previously.[7] Briefly, amino acid (3 equiv), HOBt (3 equiv), and dicyclohexylcarbodiimide (3 equiv) were added into DMF solution. After activation, the solution was added to the resin DMF solution and agitated for 5h. Each coupling reaction in solid phase was repeated until no color change of the resin was monitored in ninhydrin test. After cleavage and deprotection of the compound from resin, the resin was filtered and the excess TFA was removed by a gentle sream of N_2. 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 the crude peptide was dried under nitrogen. The product was purified prep HPLC with a Vydac C_{18} column (22 mm × 250 mm) using a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient (5-45% acetonitrile during 40 min). The yields of each product were over 40% and the products were obtained in high purity (generally >95% by RP analytical HPLC, UV_{214nm}). The success of synthesis of peptides and pseudopeptides was confirmed by ESI mass (Platform II, micromass, Manchester, UK). MM1 (ESI-MS: calcd 1425.91, obsd 1426.77 $[M+H]^+$), MM2 (ESI-MS: calcd 1411.90, obsd 1412.78 $[M+H]^+$), MM3 (ESI-MS: calcd 1397.89, obsd 1399.04 $[M+H]^+$). MM4 (ESI-MS: calcd 1540.00, obsd 1540.66 $[M+H]^+$), MM5 (ESI-MS: calcd 1659.02, obsd 1660.02 $[M+H]^+$) MM6 (ESI-MS: calcd 1425.91, obsd 1426.42 $[M+H]^+$).

Antibacterial Activity and Hemolytic Activity. Antibiotic medium 3 (M3; pH 7.0 at 25 °C, Difco) was used as antibacterial assay media. Freshly grown cells on antibiotic medium 3 agar plate were suspended in physiological saline to 10^4 cells per 1 mL of 2 x-concentrated medium and used as the inoculum. Compound was added to the 96 wells (100 μL/well) in microplate and serially diluted by twofold. After inoculation, plates were incubated at 37 °C for 24 hrs and the absorbance at 620 nm was measured by ELISA reader (Spectra, SLT, Salzburg, Austria) to assess cell growth. Antibacterial activity was expressed as the minimal inhibitory concentration (MIC), which was defined as the concentration at which 100% inhibition was observed. All MICs were determined from two independent experiments performed in duplicate. Mouse blood was centrifuged and packed mouse erythrocytes were washed three times with buffer. Various concentration of compounds were incubated with the erythrocyte suspension [final erythrocyte concentration of 1%] for 1 hour 37 °C. And then after centrifugation

at 4000 × g for 5 minutes, the absorbance of the supernatant was measured at 540 nm and lysed erythrocytes by 0.1% Triton X-100 were considered as the standard for 100% hemolysis. Melittin, well-know antibacterial peptide, was used as a reference compound in antibacterial and hemolytic assay.

Compound Stability in the Presence of Serum. After adding 10 μL of peptide stock solution (10 mg/mL) into1 mL of 25% mouse serum at 37 ^{o}C, the initial time was recorded and 100 μL of each reaction solution was removed at known time intervals and added into 100 μL of 10% aqueous trichloroacetic acid solution. The sample solution was cooled at 4 ^{o}C and spun to precipitate serum protein. Peptide analysis was carried out by reverse phase HPLC with Waters C_{18} column. Kinetic analysis for half-life of the sample was carried out by a linear least square analysis of the logarithm of the peak area versus time.

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