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Design and Synthesis of Novel Antimicrobial Pseudopeptides with Selective Membrane-Perturbation Activity

Keun-Hyeung Lee* and Jong-Eun Oh

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

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Abstract—By incorporating carbamate bond(s) into a cytolytic peptide, novel pseudopeptides with potent antibacterial activity and low hemolytic activity were synthesized. Circular dichroism spectra suggested that the incorporation of carbamate bond(s) decrease the α helical conformation of the peptide in lipid membrane circumstances, which must be regarded as a major factor for the separation of antibacterial activity from cytotoxic activity for mammalian cell. Experiments in which dye was released from vesicles indicated that the potent antibacterial activity and low hemolytic activity of the pseudopeptides must be due to their great lipid membrane selectivity. The present result suggest that backbone modifications can be a great tool for developing pseudopeptides with improved biological activity and bioavailability from cytolytic peptides.

Introduction

The recent emergence of multi-drug resistant bacteria has stimulated the development of the novel antibacterial molecules with unexploited mechanism of action.[1] A large number of defense peptides produced in eukaryotic systems have been isolated and their functions characterized.[2–5] Some of them had a great selectivity between bacteria and mammalian cell and potent activity against bacteria only. Although the mode of the action of this class of the 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.[6–8] First, the peptides that are positively charged in neutral pH, bind to the negatively charged lipid membrane surface of the cells mainly by charge interactions and then they adopt mostly α helical structure or β sheet structure and interact with lipid membrane by hydrophobic interactions. Sequentially, the peptides enhance the permeability of the lipid membranes either by ion channel formation[9] or by perturbation of the structure of the bilayer.[10] It is understood that the great membrane selectivity of defense peptides is due to the major difference between bacteria lipid membranes and mammalian lipid membranes, i.e. surface charge of lipid membrane, the magnitude of the transmembrane potential, and the content of cholesterol. As the peptides have a different mode of action from classic antibiotic, the synthetic or natural linear α helical antibacterial peptides have been extensively studied for the development of potential therapeutic agents.[11–13] Numerous studies about linear antibacterial peptides[11–13] elucidated that, in spite of diversity of primary structures, a net positive charge, hydrophobicity, and amphipathic α helical structure induced in the lipid membrane played an important role in the activity and selectivity. Even though it is difficult to elucidate each contribution of structural parameters for the activity and selectivity, the high α helicity and/or hydrophobicity were regarded as the major factors critically related to the mammalian cell toxicity rather than antibacterial activity. Several analogues of membrane-active peptides, designed to have perfect amphiphilicity to enhance α helicity, had more increased antibacterial activity. However, the increase of antimicrobial activity also resulted in the increase of cytotoxicity for mammalian cell.[14] Recent several researches by using diastereomers (peptides containing D amino acid residues) devoid of α helical structure or β sheet structure also indicated that amphipathic α helical and β sheet structure was less critical for the antibacterial activity but significant for mammalian cell toxicity.[15] The aforementioned results suggested that if the peptides had high net positive charge and hydrophobicity, modulating hydrophobicity and the net charge was sufficient to control cell selectivity and activity of the membrane-active peptides.

*Corresponding author. Tel.: +82-331-262-3851; fax: +82-331-262-6622; e-mail: lkh@kgcc.co.kr

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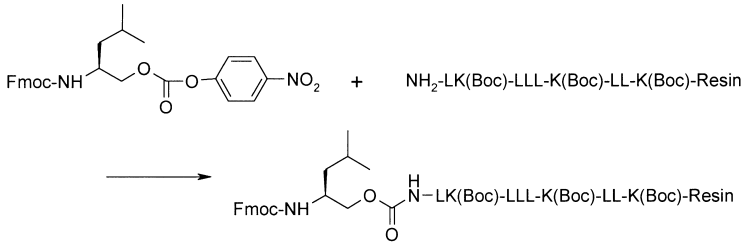
However, to develop antibacterial peptides as therapeutic agents, poor bioavailability mainly due to enzymatic degradation should be improved. Many useful amide bond surrogates have been developed for generating peptide analogues which are resistant to enzymatic degradation.[16] In the present study, by using the characteristic of amide bond surrogate, we tried to design and synthesize novel pseudopeptides which had a more improved bioavailability as well as biological activity than cytolytic peptides. We expect that the insertion of carbamate bond $\psi(CH_2OCONH)$ in the middle of the sequence of the peptide, which resulted in the increase of length corresponding to $-CH_2$–O- bond between the side group of adjacent amino acids in the peptide, can decrease amphipathicity, which will result in the decrease of α helical structure. This change of structural parameter should affect the activity and specificity of the model peptide. To investigate this suggestion, we have synthesized pseudopeptides containing carbamate bond(s) corresponding to the potent cytolytic peptide S1 (Fig. 1). Cytolytic peptide S1 was selected as a model peptide because this peptide, which had an amphipathic α helical structure in lipid membrane circumstances, had cytotoxicity against bacteria and mammalian cell by action on the lipid membranes.[15] We measured biological activities of the peptide and its pseudopeptides and studied their structures by using circular dichroism (CD). The mechanical action of the compounds was also investigated by measuring the leakage potency to induce dye release from vesicles with different surface charge. The results lead to the following conclusion. As we expect, the incorporation of carbamate bond into the peptide resulted in the decrease of α helicity structure and hydrophobic interactions with C_{18} reverse phase HPLC column. This structural change retained antibacterial activity but decreased hemolytic activity. The synthesized novel pseudopeptides had selective lipid membrane-perturbation activity. To the best of our knowledge, it was the first example of developing the pseudopeptide with more improved bioavailability as well as biological activity from cytolytic peptide, which acts on the lipid membrane of cells. This result showed the potential application of backbone modification for cytolytic peptides that acted on the lipid membrane of cells.

Results and Discussion

We synthesized pseudopeptides containing carbamate bond(s) $\psi(CH_2OCONH)$ corresponding to the cytolytic peptide S1[15] consisting of 12 amino acid residues, investigated the secondary structure by CD spectroscopy, and compared the activity against bacteria (*S. aureus, M. luteus, E. coli,* and *P. aeruginosa*), fungi (*C. albicans*), and erythrocyte. As shown in Figure 1, considering the fact that one turn of α helix contain 3.6 amino acid residues, carbamate bond(s) $\psi(CH_2O$-CONH) were introduced after three amino acid residues and/or four amino acid residues in the terminal of the peptide for decreasing amphiphilicity of the target peptide. The pseudopeptides were synthesized by a solid phase approach on the PAL resin using a Fmoc-based strategy. When carbamate bond was introduced, Fmoc-carbonate monomer (10 equiv each) instead of Fmoc amino acid was used in solid peptide synthesis, in the presence of DIEA and HOBt (Fig. 1).[16] This coupling reaction was repeated until no color change was observed in ninhydrin test. Cleavage from the resin and HPLC purification provided the desired pseudopeptide in greater than 20% yield, which is comparable yield of the synthesis of the peptide. The purity of the synthetic peptide and pseudopeptides was above 96% as measured by analytical HPLC and CE, respectively, and the mass of all compounds were characterized by ESI mass spectrometry.

As shown in Table 1, the introduction of carbamate bond did not affect the net positive charge of the peptide. However, incorporation of carbamate bond decreased the retention time of the peptide on the C_{18} reverse HPLC column. It was reported that retention time, which reflected hydrophobic interactions between peptide and C_{18} stationary phase, was parallel to the



S1 KLLLKLLLKLLK-NH_2

SC1 KLLψ [CH_2OCONH] LKLLLKLLK-NH_2

SC2 KLLψ [CH_2OCONH] LKLLLψ [CH_2OCONH] KLLK-NH_2

Figure 1. General method for the synthesis of carbamate bond in SPPS and primary structure of S1 and its pseudopeptides.

hydrophobicity of the peptides or to the α helicity of the peptides with the same amino acid composition.[17] The hydrophobic domain, generated by amphipathic α helical structure of the peptide in hydrophobic environment, affects hydrophobic interactions between the peptides and reverse-phase matrices, which affects the retention time on reverse-phase HPLC. The hydrophobicity of the pseudopeptides should be greater than that of the peptide because carbamate bond(s) itself is more hydrophobic than amide bond. The retention time of the pseudopeptide with carbamate bond, however, was shorter than that of the peptide, which suggested that the incorporation of carbamate bond(s) must decrease α helicity of the peptide.

Many structure–activity studies about linear antimicrobial peptides indicated that the secondary structure in membrane–mimetic environments rather than that in phosphate buffer was correlated well with the activity. The CD spectra of the compounds were measured in phosphate buffer with or without sodium dodecylsulfate (SDS) or trifluoroethanol (TFE), respectively. TFE, structure-inducing solvent, is commonly used for membrane–mimic environment.[18] SDS micelles have been frequently used as a membrane–mimic system because SDS consisting of an aliphatic tail and a negatively charged head group forms micelles that mimic the negatively charged lipid membrane environment.[19,20] As shown in Figure 2a, CD spectra measured in phosphate buffer indicated that the peptide (S1) and the pseudopeptide (SC1) with one carbamate bond had considerable α helical conformations whereas pseudopeptide (SC2) with two carbamate bonds had random conformations. However, as shown in Figure 2b, CD spectra with negative ellipticity at 208 nm and 222 nm revealed that S1 formed a well-defined α helical structure in the presence of sodium dodecylsulfate (SDS) micelles whereas SC1 still formed α helical structure but the content of α helicity (19%) was much lower than that of S1 (51%). As we expected, SC2 did not have α helical conformations and adopted random conformations as a major structure in the presence of SDS micelles. The fractional helicity of the compound measured in the presence of SDS micelles is summarized in Table 1. As shown in Figure 2c, CD spectra, measured in the presence of 50% TFE (v/v), indicated that the α helicity of each molecule was more increased than that obtained in the presence of SDS micelles however, the structural difference trend of each molecule was similar to that obtained in the presence of SDS micelles. CD spectra indicated that the incorporation of carbamate bond seemed to have a medium dependent perturbation effect on the α helical structure and incorporation of two-carbamate bonds into the peptide totally disturbed amphipathic α helical structure. As we expected, the decrease of α helicity was mainly due to the fact that the incorporation of carbamate bonds decreased amphipathicity, resulting in the induction of low α helical conformation in lipid membrane. In addition, there is some possibility that carbamate bond itself should disturb the hydrogen bonding for maintaining α helical structure and even this effect additionally help to disturb α helical structure.

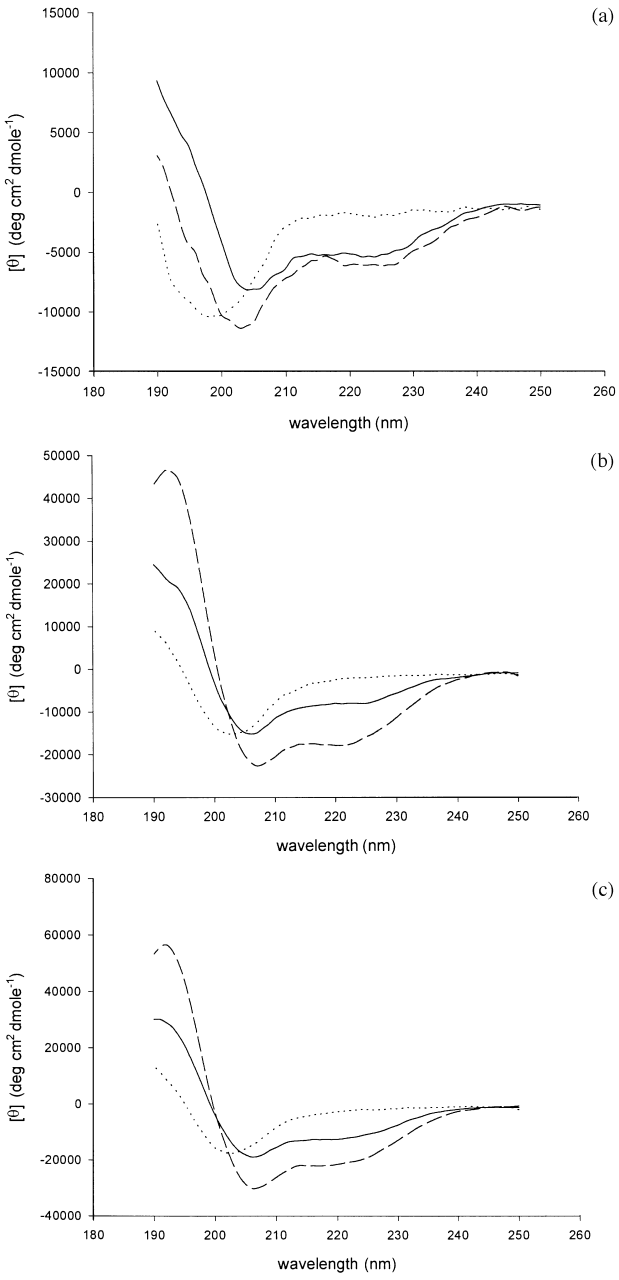


Figure 2. CD spectra of S1 and its pseudopeptides: (a) in 10 mM sodium phosphate buffer (pH, 7.4). (— — —), S1; (——), SC1; (------), SC2; (b) in 10 mM sodium phosphate buffer (pH, 7.4) containing 25 mM SDS. (— — —), S1; (——), SC1; (------), SC2; (c) in 10 mM sodium phosphate buffer (pH, 7.4) containing 50% TFE (v/v). (— — —), S1; (——), SC1; (------), SC2.

As shown in Table 1, the activities of S1 and its pseudopeptides were measured against microorganisms including bacteria and fungi. The model peptide S1 and its pseudopeptides completely inhibited the growth of bacteria and fungi in the assay concentration range. However, the introduction of carbamate bond(s) had a little different effect on the activity against bacteria and fungi, respectively. The single carbamate bond replacement retained antibacterial activity but seemed to decrease antifungal activity; the minimum inhibition concentration (MIC) of S1 for *C. albicans* was 6.3 μg/mL whereas the MIC of SC1 for *C. albicans* was 10.4

Table 1. Antimicrobial activities of S1 and its pseudopeptides[a,b]

Name	net charge	α helicity (%)	Retention Time (min)	Minimum Inhibition Concentration (MIC) (μg/ml)				
				Staphylococcus aureus (ATCC6538)	*Micrococcus luteus* (ATCC9341)	*Escherichia coli* (ATCC2592)	*Pseudomonas aeruginosa* (ATCC9027)	*Candida albicans* (ATCC3623)
S1	5	51	47.05	16.7	6.3	16.7	12.5	6.3
SC1	5	19	45.65	12.5	5.2	12.5	12.5	10.4
SC2	5	0	42.58	12.5	6.3	12.5	10.4	25.0

[a]CD spectra was measured in the presence of SDS micelles. The α helicity of the peptide was determined from the mean reidue ellipticity [θ] at 222 nm according to the relation $[\theta]_{222} = -30300\,[\alpha] - 2340$ (where [α] is the amount of helix).
[b]The MIC values were calculated from three independent experiments performed in duplicate, which provided a standard deviation below 25.

μg/mL. Introduction of two carbamate bonds into the peptide retained the activity against bacteria but decreased the activity against fungi by 4 times; the MIC of S1 for *C. albicans* was 6.3 μg/mL whereas the MIC of SC2 was 25 μg/mL. This activity difference was clearly beyond our normal experimental error. We also tested the cytotoxicity of the peptide and its pseudopeptides against mouse erythrocytes to find out the cytotoxicity for mammalian cell. As shown in Figure 3, the peptide S1 caused 100% lysis of erythrocytes at a concentration greater than 40 μg/mL and SC1 did 100% lysis at a concentration greater than 100 μg/mL. However, SC2 did not cause 100% lysis of erythrocytes even at a concentration up to 200 μg/mL. Well-known cytolytic peptide, melittin[21] caused 100% lysis at concentration greater than 10 μg/mL in the same assay condition. The biological activity indicated that the introduction of carbamate bond into the cytolytic peptide separated bacteria cell toxicity from mammalian cell toxicity.

The membrane-permeable activities of S1 and its pseudopeptides were investigated by measuring dye release from vesicles with different surface charge density. We prepared the neutral charged large unilamellar vesicle (LUV) consisting of egg phosphatidylcholine (PC) and highly negatively charged LUV consisting of egg phosphatidylglycerol (PG) to mimic the surface charge of lipid membrane of mammalian cell and bacterial cell, respectively. After the addition of S1 or its pseudopeptides, the release of fluorescence dye, calcein encapsulated in vesicle was determined by measuring the decrease of self-quenching on spectrofluorometer (Ex: 490 nm, Em: 520 nm). As shown in Figure 4a, all compounds employed in this study showed the similar membrane-perturbation activity on egg PG vesicles in the same assay concentration range, which was consistent with the antibacterial activity measured in this study. As shown in Figure 4b, 2 μg/mL of the peptide (S1) induced 100% leakage of calcein from PC LUV whereas even 5 μg/mL of the pseudopeptide with one carbamate bond (SC1) did not induce 100% of leakage in the PC LUV. The decreased leakage activity for PC LUV was more clearly observed in the pseudopeptide (SC2); SC2 just induced 20% of leakage of the dye at a concentration up to 5 μg/mL. This leakage potency of the peptide and its pseudopeptides for the vesicles with neutral charge density was consistent with hemolytic activity.

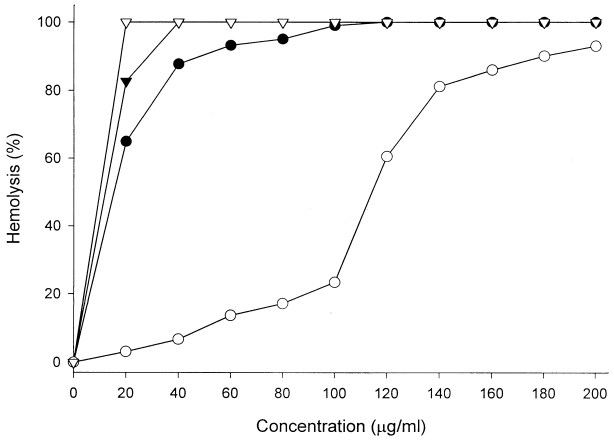


Figure 3. Hemolytic activity of S1 and its pseudopeptides. Erythrocytes were incubated in the Tris buffer (150 mM KCl, 5 mM Tris–HCl, pH 7.4) with various concentration of sample for 1 h at 37 °C: ▼, S1; ●, SC1; ○, SC2; ▽, melittin.

The leakage potency of the molecules was correlated with their biological activity, suggesting that the biological action of the molecules must be carried out through the lipid membrane as a primary target. And the separation of bacterial cell toxicity from mammalian cell toxicity must be due to the great membrane selectivity of the pseudopeptide.

As we mentioned before, the net positive charge, hydrophobicity, and amphipathic α helical structure of the peptide played an important role in the membrane-perturbation activity and selectivity. In this study, we incorporated carbamate bond(s) into the peptide to disturb the amphiphilicity, which resulted in the decrease of α helicity. Amphiphilicity was frequently used as a parameter to modulate α helicity because the amphiphilicity of the peptide played an important role in inducing α helical structure and β sheet structure in lipid membrane.[22] However, under the circumstance without the detail structural information of the pseudopeptides in lipid membrane, we can not remove the possibility that carbamate bond itself disturbed the hydrogen bonding for α helical structure, resulting in the decrease of α helicity.

The synthesized pseudopeptides had a greater selectivity between bacteria and mammalian cell than the peptide without the decrease of antibacterial activity. It was suggested that a great selectivity of some antibacterial peptides is due to the difference between bacteria lipid

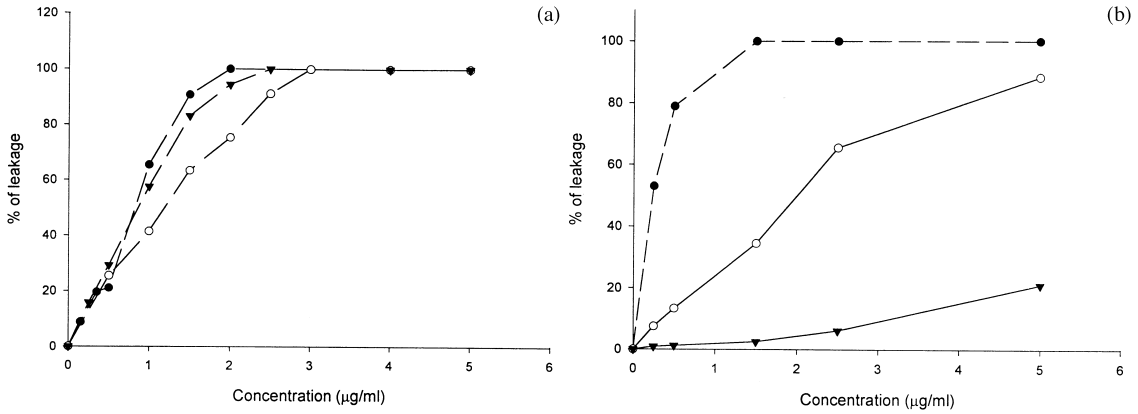


Figure 4. Leakage of the dye from liposomes by S1 and its pseudopeptides. (a) The phospholipid of liposomes (6 μM) consisted of phosphatidylglycerol. Each compound was incubated with liposomes for 0.5 h at 37 °C: ●, S1; ○, SC1; ▼, SC2. (b) The phospholipid of liposomes (12 μM) consisted of phosphatidylcholine. Each compound was incubated with liposomes for 0.5 h at 37 °C: ●, S1; ○, SC1; ▼, SC2.

membranes and mammalian lipid membranes. The surface charge was regarded as a most distinct difference; the outer membranes of erythrocytes consisting of phosphatidylcholine and sphingomyelin were almost electrically neutral whereas the bacterial lipid membranes consisting of mainly phosphatidylglycerol were negatively charged. Our result also indicated that the surface charge difference in the lipid membranes of target cells and the loss of α helical structure of the pseudopeptides accompanying with decrease of hydrophobic interactions to the surface of lipid membranes are main factors for lipid membrane selectivity.

In a previous study, we have for the first time incorporated amide bond surrogate (carbamate bond and reduce amide bond) into the N-terminal of the membrane-active decapeptide[23] and developed potent antimicrobial pseudopeptide with increased stability in the presence of serum.[24] However, in the previous study, as the membrane-active decapeptide itself did not have hemolytic activity and their structures were not greatly changed by an incorporation of amide bond surrogates, we could not observe the real effect of amide bond surrogate on the hemolytic activity. In the present study, we designed and synthesized novel pseudopeptides with selective membrane-perturbation activity by using the characteristics of amide bond surrogates. We did not compare in vivo stability between the peptide and its pseudopeptides described here. However, on the basis of our previous result and the fact that introduction of amide bond surrogate(s) of peptides, located away from a protease labile bond, also increased the resistance against enzymatic degradation,[25] we expect that the bioavailability of the pseudopeptide should be improved. In the present study, the introduction of carbamate bond(s) into the peptide for improving bioavailability simultaneously improved the lipid membrane selectivity resulting in the retention of the antibacterial activity and decrease of mammalian cell toxicity. The present result also suggests that backbone modifications, easily applied in solid phase synthesis, can be a great tool for developing non-peptide or pseudopeptide drug with improved activity as well as improved bioavailability from membrane-active peptides.

Conclusion

By using the characteristic of amide bond surrogate, novel pseudopeptides which have more improved bioavailability as well as improved biological activity were synthesized from a cytolytic peptide that acted on the lipid membrane. The incorporation of the carbamate bonds decreased α helicity structure and hydrophobic interactions to lipid membrane, which must be a major factor for the separation of antibacterial activity from hemolytic activity of the cytolytic peptide. The present study suggested that backbone modifications, easily applied in solid phase synthesis, can be a great tool for developing antibacterial agents from membrane-active peptides.

Experimental

Materials

N-α-9-fluorenylmethoxycarbonyl (Fmoc)-amino acid derivatives for solid phase peptide synthesis were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). 5-(4-Aminomethyl-3,5-dihydroxyphenoxy)valeric acid (PAL) resin was purchased from PerSeptive Biosystems GmbH (Hamburg, Germany). Fmoc-leucinol was purchased from Advanced Chem tech. (Louisville, KY, USA) Piperidine, acetic anhydride, methyl alcohol, dicyclohexylcarbodiimide (DCC), *N*-methylpyrrolidone (NMP), and *N*-hydroxybenzotriazole (HOBt) were obtained from Applied Biosystems, Inc. (Foster city, CA, USA). Phospholipid, NaCl, KCl, ethylenediaminetetraacetic acid (EDTA), calcein, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 was purchased from Gibco BRL (Gaithersburg, MD, USA). Other chemicals were purchased from Aldrich (Milwaukee, WI, USA). All chemicals were reagent grade and used without further purification.

Synthesis of peptide and pseudopeptides. 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 by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry.[26] Side chain protection groups were as follows: Lys, *tert*-butoxylcarbonyl. Carbamate bond was incorporated by the coupling of Fmoc-carbonate monomer with the free amino terminal of the resin bound peptide, in the presence of *N*,*N*-diisopropylethylamine (DIEA) and HOBt in NMP for 4–8 h at 25 °C.[19] The completion of a reaction was monitored by ninhydrin test. 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 3–4 h. After filtration of resin and washing with TFA, a gentle stream of nitrogen was used to remove the excess TFA.

The crude peptide and pseudopeptide were triturated with diethyl ether chilled at −20 °C and were centrifuged at 3000×*g* for 10 min. Diethyl ether was decanted and crude peptide was dried under nitrogen. The peptide and pseudopeptide were purified by high performance liquid chromatography with a Phenomenex C_{18} column (21.2×250 mm; Phenomenex, Torrance, CA, USA). The homogeneity of the peptide and pseudopeptides (>96%) was checked by capillary electrophoresis (CE) from Hewlett Packard using unfused silica capillary column (50 μm×60 mm) and analytical HPLC with a Waters Delta Pak C_{18} column (3.9 mm×150 mm). The peptide and pseudopeptide were eluted using solvent A consisting of 0.1% TFA in water and solvent B consisting of 0.1% TFA in acetonitrile and monitored by absorbance at 214 nm. The peptide was analyzed using linear gradient of 0–70% B in 70 min. Mass spectrometry on Platform II (Micromass, Manchester, UK) was used to measure the mass of the purified peptide and pseudopeptides. S1 (ESI–MS: calcd 1434.06, obsd 1434.94 $[M+H]^+$), SC1 (ESI–MS: calcd 1464.05, obsd 1365.22 $[M+H]^+$), SC2 (ESI–MS: calcd 1494.05, obsd 1494.91 $[M+H]^+$).

Antimicrobial assay. In vitro antifungal assays were performed by the broth microdilution method according to the recommendation of the National Committee for Clinical Laboratory Standards.[27] RPMI 1640 (Gibco BRL, Gaithersburg, MD) was used as the assay medium. *Candida* cells freshly grown on slopes of Sabouraud dextrose agar (logarithmic phase) were suspended in physiological saline, and the cell concentration was adjusted to 10^4 cells per 1 mL of 2×-concentrated medium for use as the inoculum. Peptide solution was added to the wells of a 96-well plate (100 μL per well) and serially diluted 2-fold. The final concentration of peptide mixture ranged from 0.2 to 500 μg/mL. After inoculation (100 μL per well, 5×10^3 cells per mL), the 96-well plate was incubated at 30 °C for 48 h and the absorbance was measured at 620 nm by using an enzyme-linked immunosorbent assay reader (SLT, Salzburg, Austria) to assess cell growth. The MIC was defined as the lowest concentration exhibiting no visible growth compared with the control cell. Each MIC was determined from three independent experiments performed in duplicate. An in vitro antibacterial assay was performed by the aforementioned method for the antifungal assay, with the exception of that the assay media and the incubation temperature were different. In the antibacterial assay, antibiotic medium 3 (pH 7.0 at 25 °C; Difco, Detroit, MI, USA) was used and cells were incubated at 37 °C for 24 h.

Hemolytic assay. Packed mouse erythrocytes were washed three times with buffer (150 mM KCl, 5 mM Tris–HCl, pH 7.4) and then packed erythrocytes were suspended in 10 volumes of the same buffer (Stock cell suspension). For antibiotic treatment, the cell stock suspension was diluted 25-fold with the same buffer and was preincubated in the waterbath at 37 °C for 15 min, and then the test sample was added. After incubation for 1 h, the sample was centrifuged at 4000×*g* for 5 min and the absorbance of the supernatant was determined at 540 nm. The hemolysis effected by 0.1% Triton X-100 was considered 100% hemolysis.

CD measurement. Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) using a quartz cell of 1 mm path length, at wavelengths ranging from 190 to 250 nm. The CD spectrum was recorded at room temperature and was obtained with a 0.5 nm bandwidth and a scan speed of 10 nm/min. Two scans were averaged to improve the signal to noise ratio. CD spectra were expressed as the mean residue ellipticity and the percentage of α helicity was calculated from the mean residue ellipticity [θ] at 222 nm by the method of Chen et al.[28]

Preparation of liposomes. Phospholipid (Sigma, St. Louis, MO) (10 mg) dissolved in chloroform and were dried with a stream of nitrogen gase to form a thin lipid film on the wall of a glass tube. The resulting film was dried overnight under vacuum. The dried film was hydrated with 2 mL of test buffer (10 mM Tris, pH7.4, 154 mM NaCl, 0.1 mM EDTA) containing 70 mM calcein for leakage measurement. The suspension was vortexed for 10 min. This turbid liposome solution was sonicated (under nitrogen, in ice–water) for 10 min (×5) by using a titanium tip sonicator. The solution was freeze–thawed for six cycles. Untrapped calcein was removed from the vesicles by gel filtration on a Sephadex G-50 (Pharmacia, Upsala, Sweden) column that was equilibrated with 10 mM Tris buffer (pH 7.4) containing 154 mM NaCl and 0.1 mM EDTA. The concentration of liposomes was determined on the basis of the method described by Vaskovsky et al.[29]

Leakage measurement. A liposome solution (10 μL) was added to 2 mL of 10 mM Tris buffer (154 mM NaCl, 0.1 mM EDTA, pH 7.4) in the cuvette. To the mixture was added an appropriate concentration of peptides. When the leakage occurred, the calcein was released from the liposomes and emitted the fluorescence. Therefore, the leakage was directly measured by determining the relative change in fluorescence. Fluorescence, excited at 490 nm and emitted at 520 nm, was measured with a Jasco J-777 spectrofluorometer (Jasco, Tokyo, Japan). For determination of 100% dye release, 20 μL of 10% Triton X-100 solution was added to liposome solution. The percent of dye-release caused by sample was evaluated by the equation, $\text{dye-release}\ (\%) = 100 \times (F - F_0)/$

$(F_t - F_0)$, where F was the fluorescence intensity achieved by the peptides, F_0 and F_t are intensities of the fluorescence without the peptides and with Triton X-100, respectively.

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