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SYNTHESES OF ANTIBACTERIAL PEPTIDES, GRAMICIDIN S ANALOGS AND DESIGNED AMPHIPHILIC OLIGOPEPTIDES

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Abstract. In order to clarify the relationship between antimicrobial activity and peptide-structure, gramicidin S analogs and cationic α-helical model peptides were designed and synthesized. Introduction of cationic side chains in hydrophilic side of gramicidin S increased antimicrobial activity against Gram-negative bacteria. Amphiphilic structures of the α-helical peptides were found to be effective to show antimicrobial activities against Gram-positive bacteria. Increase in number of cationic amino acid residues in the α-helical peptides caused appreciable antimicrobial activities against Gram-negative bacteria, however, induced lower activities against Gram-positive ones.

INTRODUCTION

Gramicidin S, an antibiotic produced by a strain of *Bacillus brevis*, is known to be strongly active against Gram-positive bacteria but little active against Gram-negative ones. As gramicidin S has a unique structure of cyclic decapeptide as shown in Figs. 1 and 2,[1,2] the peptide has been often taken up as a suitable material to study the structure-activity relationship and to evaluate new methods in peptide synthesis.[3] Conformational studies of gramicidin S have shown that the antiparallel β-sheet structure of Val-Orn-Leu sequence is connected by two β-turn (type II') structures of D-Phe-Pro sequence and that gramicidin S has hydrophobic amino acid side chains on one side and hydrophilic ones on the opposite side to form the amphiphilic structure. The amphiphilicity is strongly kept by the rigid ring structure of the molecule and is known to be necessary to exhibit the antimicrobial activity against Gram-positive bacteria.

Abbreviations used are according to IUPAC-IUB Commissions, Eur. J. Biochem. (1984), 138, 9-37. Other abbreviations: DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DPPG, dipalmitoyl-DL-α-phosphatidylglycerol; Dpr, α,β-diaminopropionic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Fmoc-, 9-fluorenylmethyloxycarbonyl; HCO-, formyl; HOBt, 1-hydroxybenzotraizole; -ONSu, succimidooxy; -Pac, phenacyl; TFA, trifluoroacetic acid. All amino acids are of L-configuration unless otherwise noted.

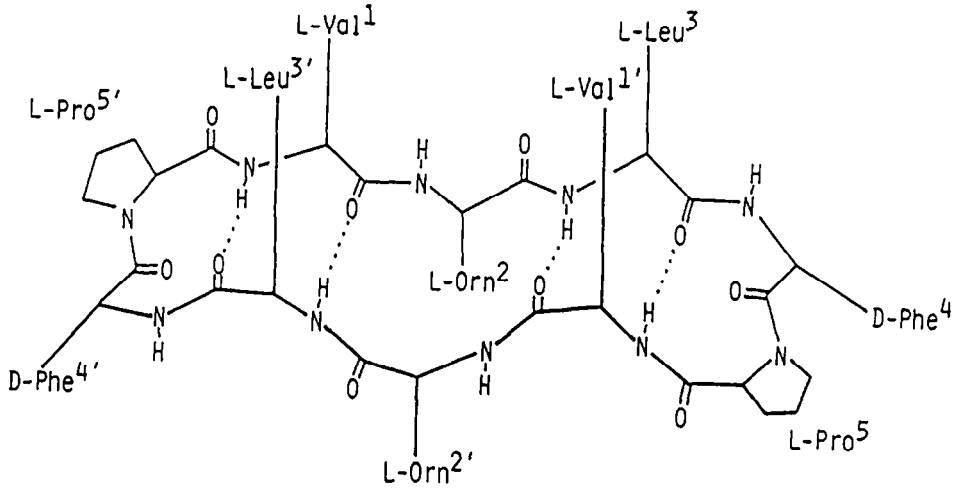

Fig. 1. β-Structure of gramicidin S.

On the other hand, some antibacterial peptides such as cecropins and melittin have been found to have a common factor of amphiphilic α-helical structure. Cecropins are active against a variety of Gram-positive and -negative bacteria and contain 37 amino acid residues with a basic N-terminal region and a hydrophobic C-terminal region.[4] In this connection, DeGrado reported that Fmoc-(Leu-Lys-Lys-Leu-Leu-Lys-Leu)$_2$-OH, a simple α-helical amphiphilic model peptide of cecropins, showed the same antimicrobial activity as cecropins against Gram-positive and -negative bacteria.[5] Recently, we found that Ac-(Leu-Ala-Lys-Leu)$_3$-NHCH$_3$[6] which holds also an amphiphilic α-helical structure showed a strong antimicrobial activity against Gram-positive bacteria but was almost inactive against Gram-negative ones. These results suggest that cecropins and melittin possess unknown factor(s) necessary for exhibiting the activity against Gram-negative bacteria in addition to that against Gram-positive ones, because gramicidin S (1) and Ac-(Leu-Ala-Lys-Leu)$_3$-NHCH$_3$ (4) are little active against Gram-negative bacteria in spite of their amphiphilicity.

We, therefore, intended to obtain gramicidin S analogs having strong activities against Gram-negative bacteria by synthetic modification, and also to find the factor(s) necessary for exhibiting the activity against Gram-negative bacteria using α-helical model peptides. This paper deals with the design, syntheses and properties of more basic analogs of gramicidin S and several cationic α-helical peptides. Additionally, relationships between activities and secondary structures of the peptides are discussed.

RESULTS

Design and syntheses of gramicidin S analogs

As gramicidin S has an antiparallel β-sheet structure connected by two type II' β-turns, there are two possible approaches to design and synthesize the more basic analogs of gramicidin S without losing the amphiphilicity. One approach is described as follows. Gramicidin S consists of the hydrophobic side of Val and Leu, and of the hydrophilic side of Orn as shown in Fig. 1. The side chains of D-Phe are exceptionally directed to the hydrophilic side. Therefore, replacement of the D-Phe residues with basic D-amino acids would increase the basicity in the hydrophilic side and might affect the antimicrobial activity. From this point, a

gramicidin S analog ([D-Dpr$^{4,4'}$]GS, 2 in Fig. 2) containing D-α,β-diaminopropionic acids was designed and synthesized previously, by the deprotection of Z and HCO groups in [Orn(δ-HCO)$^{2,2'}$,D-Dpr(β-Z)$^{4,4'}$]GS.[8] At the present study, this analog (2) was synthesized again in quantity for more detailed studies.

Another approach to increase the basicity in the hydrophilic side is to extend the antiparallel β-sheet structure. From this point, we desinged and synthesized a macro-ring analog (cyclo(-Leu-Orn-Leu-Orn-Leu-D-Phe-Pro-)$_2$, 3) containing four ornithine residues in the hydrophilic side.[9] At the present study, we prepared this analog (3) in quantity by the deprotection of Z groups in cyclo(-Leu-L-Orn(δ-Z)-Leu-Orn(δ-Z)-Leu-D-Phe-Pro-)$_2$.[9]

		Number of basic amino acid residue
cyclo(-Val^1-Orn^2-Leu^3-D-Phe^4-Pro^5-Val$^{1'}$-Orn$^{2'}$-Leu$^{3'}$-D-Phe$^{4'}$-Pro$^{5'}$-)	1 (GS)	2
cyclo(-Val^1-Orn^2-Leu^3-D-Dpr^4-Orn^5-Val$^{1'}$-Orn$^{2'}$-Leu$^{3'}$-D-Dpr$^{4'}$-Pro$^{5'}$-)	2 ([D-Dpr$^{4,4'}$]GS)	4
cyclo(-Leu-Orn-Leu-Orn-Leu-D-Phe-Pro-Leu-Orn-Leu-Orn-Leu-D-Phe-Pro-)	3 (macro-ring analog of GS)	4

Fig. 2. Gramicidin S and its analogs.

		Number of Lys residue
Ac-Leu-Ala-Lys-Leu-Leu-Ala-Lys-Leu-Leu-Ala-Lys-Leu-NHCH$_3$	(4)	3
Ac-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Leu-Lys-Lys-Leu-NHCH$_3$	(5)	5
Ac-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Leu-Ala-Lys-Leu-NHCH$_3$	(6)	4
Ac-Leu-Ala-Lys-Ala-Leu-Lys-Lys-Leu-Leu-Ala-Lys-Leu-NHCH$_3$	(7)	4
Ac-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Ala-Leu-NHCH$_3$	(8)	6
Ac-Leu-Ala-Lys-Leu-Lys-Ala-Lys-Leu-Lys-Ala-Lys-Leu-Leu-Ala-Lys-Leu-NHCH$_3$	(9)	6

Fig. 3. Synthetic model peptides. Amino acid residue with underline means the alternation of a residue in -Leu-Ala-Lys-Leu- sequence.

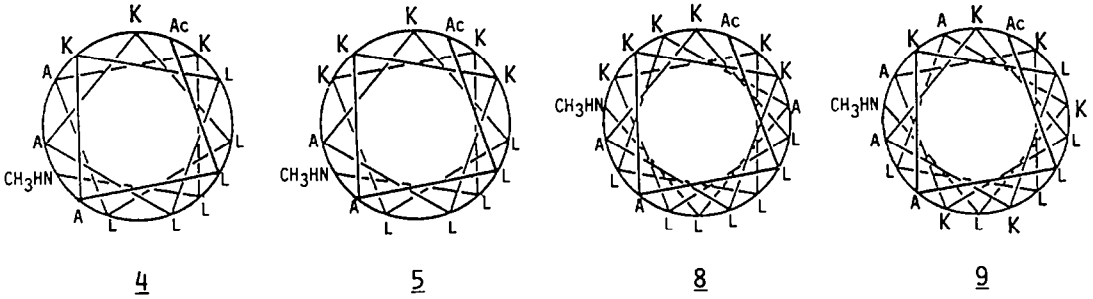

Fig. 4. Amphiphilic α-helical structures of synthetic peptides. A, alanine; K, lysine; L, leucine.

Design and syntheses of amphiphilic linear peptides

On previous planning in syntheses of amphiphilic α-helical peptides, Leu and Ala were used as hydrophobic amino acids, and Lys as a basic amino acid for simple combination. The sequence of -Leu-Ala-Lys-Leu- was selected as a standard unit because peptides containing the unit repeatedly and properly might take amphiphilic α-helical structures. To avoid unexpected influence, N- and C-terminals were protected with acetyl and methylamide groups, respectively. Therefore, peptide 4 (Figs. 3 and 4) was a polymer consisting of the standard unit.[6] Other designed peptides in the present study are shown in Figs. 3 and 4. In peptides 5-7, one or two Lys residues are added to 4. Peptide 8 is amphiphilic and slightly resemble the Fmoc-peptide prepared by DeGrado, while 9 is not amphiphilic when it takes an α-helical structure (see Fig. 4).

Figure 5 shows the synthetic route for 6 as an example. α-Amino and ε-amino group in Lys were protected with Boc and Z group, respectively. The stepwise elongation by DCC-HOBt,[10] mixed anhydride or active ester method[11] was carried out throughout for the synthesis of each unit. The Boc group was removed by use of HCl in dioxane or TFA and the phenacyl ester was cleaved with Zn dust in 90% AcOH.[12,13] Fragment coupling was carried out by EDC-HOBt method. After removal of Boc group in 15, the resulting TFA salt was treated with Ac-ONSu to give Ac-derivative. The Z group in 16 was removed with 25% HBr in AcOH to give the corresponding HBr salt, which was converted to the desired acetate (6) by treatment with Amberlite IR-45 (AcO^- form) followed by lyophilization. The product showed a single spot in paper electrophoresis, and the acid hydrolysate gave satisfactory result in amino acid analysis. The content of peptide in the product and

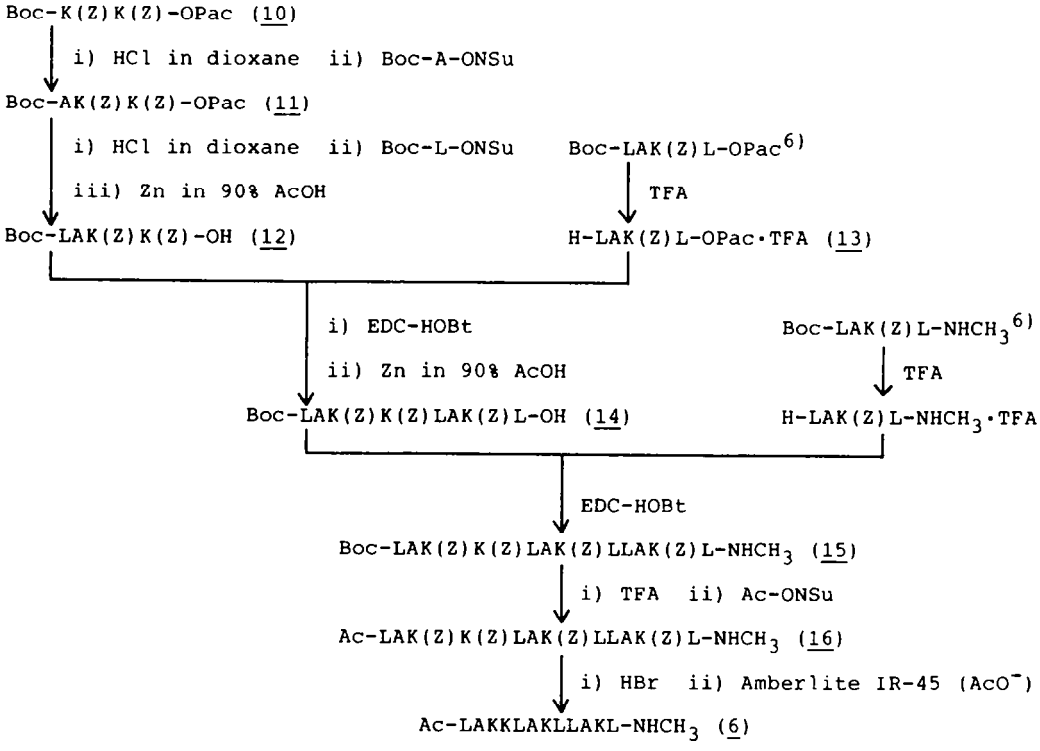

Fig. 5. Synthetic route for Ac-dodecapeptide-$NHCH_3$ (6).
A, alanine; K, lysine; L, leucine.

Table 1. Physical constants of the key intermediates.

Compound[a]	Mp(°C)	$[\alpha]_D^{20}$	R_f^1
Boc-LK(Z)K(Z)L-$NHCH_3$ (17)	207-210	-20.2[b]	0.64
Boc-LAK(Z)ALK(Z)K(Z)L-OH·3/2H_2O (18)	229-234(d)	-22.9[b]	0.52
Boc-LAK(Z)LK(Z)AK(Z)L-OH·3/2H_2O (19)	240-245(d)	-24.4[d]	0.51
Boc-LK(Z)K(Z)LLK(Z)AL-$NHCH_3$·H_2O (20)	255-260(d)	-17.2[b]	0.55
Boc-K(Z)AK(Z)LLAK(Z)L-$NHCH_3$·1/2H_2O (21)	267-270(d)	-25.6[c]	0.64
Ac-LAK(Z)K(Z)LAK(Z)LLK(Z)K(Z)L-$NHCH_3$·2H_2O (22)	293-297(d)	-20.4[c]	0.55
Ac-LAK(Z)ALK(Z)K(Z)LLAK(Z)L-$NHCH_3$·H_2O (23)	251-253(d)	-28.9[c]	0.58
Ac-LAK(Z)K(Z)LAK(Z)LLK(Z)K(Z)LLK(Z)AL-$NHCH_3$·2H_2O (24)	285-290(d)	-12.0[c]	0.60
Ac-LAK(Z)LK(Z)AK(Z)LK(Z)AK(Z)LLAK(Z)L-$NHCH_3$·2H_2O (25)	285-290(d)	-11.6[c]	0.65

[a] Reasonable compositions were estimated from the results of elemental analysis. [b] c 1, DMF. [c] c 0.5, DMSO.

Table 2. Antimicrobial activities of the synthetic peptides, gramicidin S (1) and polymyxin B (PM).

Organism	Minimum inhibitory concentraion (mg/ml)[a]									
	GS(1)	2	3	4	5	6	7	8	9	PM
S. aureus FDA 209P	3.13	100	12.5	6.25	100	100	50	>100	>100	>50
B. subtilis PCI 219	3.13 (0.5)[b]	12.5	6.25	3.13 (0.5)[b]	25 (0.5)[b]	12.5	25	50 (1.25)[b]	50	6.25
E. coli NIHJ JC-2	>100 (5.0)[b]	25	12.5	>100 (6.0)[b]	100 (1.25)[b]	>100	>100	>100 (2.5)[b]	>100	0.78
S. flexneri EW-10	6.25	25	6.25	6.25	6.25	12.5	12.5	50	12.5	0.39
P. vulgaris IFO 3988	>100	-	-	>100	>100	>100	>100	>100	>100	-
S. marcescesn IFO 12648	>100	-	-	>100	>100	>100	>100	>100	>100	-

[a] In the agar dilution method except b). [b] Values are obtained by the serial dilution method in solution media.

reasonable composition were estimated from the result of elemental analysis. Other peptides also were obtained similarly. Physical constants of the key intermediates are shown in Table 1.

Antimicrobial activity

Antimicrobial assays were carried out by agar dilution method[14] and serial dilution method in solution media.[15] The results are shown in Table 2. [D-$Dpr^{4,4'}$]gramicidin S (2) showed a considerable antimicrobial activity against Gram-negative bacteria such as *E. coli*. However, this analog almost lost the activity against Gram-positive *S. aureus* which is sensitive to native gramicidin S.[8] The macro-ring gramicidin S analog (3) also showed an antimicrobial spectrum similar to

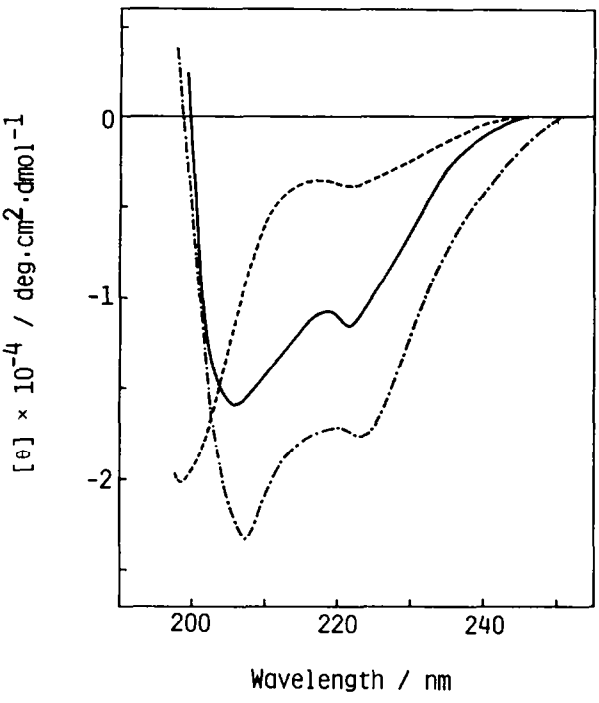

Fig. 6. CD spectra of peptide 5 in water (----) and in the presence of DPPC (——) and DPPC-DPPG (3:1) (—·—) liposomes.

that of 2, although the analog (3) is still effective to *S. aureus*.[9]

In agar dilution method,[14] peptides 5-7 showed weak activities against Gram-positive bacteria such as *S. aureus* and *B. subtilis*, while peptides 8 and 9 showed no or weak activities against them. Only peptide 5 containing five Lys residues showed a very weak but substantial activities against *E. coli*. Other α-helical peptides including 4 showed no activity against Gram-negative bacteria except *S. flexneri*, which was suscestible to all of the peptides tested. Peptides 5-7 containing 12 amino acid residues were clearly more active than peptides 8 and 9 containing 16 residues. On the other hand, in serial dilution method,[15] all the peptides tested here exhibited the activities against Gram-positive and -negative bacteria. Peptide 5 showed again a stronger activity against *E. coli* than the other peptides and gramicidin S.

CD measurement

In order to investigate the structures of the peptides, CD measurements were carried out under several conditions. Gramicidin S analogs (2 and 3) showed similar CD curves to that of gramicidin S, although the ellipticities of two troughs around 205 and 215 nm decreased.[8,9] These analogs are supposed to take similar β-sheet and β-turn structures to those of native gramicidin S. The reduced ellipticities of the CD curves may suggest small structural distortions in the used solvent due to the proximity of four cationic charges in the hydrophilic side.

A typical result of linear peptides is shown in Fig. 6. All the linear peptides in water showed a negative band at 200 nm attributable to disordered structures. The addition of DPPC or DPPC-DPPG (3:1) liposomes into water resulted in marked increase in ellipticities of negative bands at 208 and 222 nm, indicating the formation of α-helical structures in the peptides. α-Helical content of the peptides calculated according to the literature[16] are 30-45% in the presence of neutral DPPC liposomes and 37-60% in the presence of acidic DPPC-DPPG liposomes.

DISCUSSION

From the observations that both [D-Dpr$^{4,4'}$]gramicidin S (2) and macro-ring analog (3) showed CD curves with two minima characteristic to native gramicidin S, these analogs are supposed to maintain secondary structures similar to that of gramicidin S, although the ellipticities of their CD curves are smaller than that of gramicidin S. These results mean that the side chains of the Dpr residues in analog 2 and also those of the Orn residues in analog 3 are located in the hydrophilic sides formed by similar antiparallel β-sheet structures. As shown in Table 2, the increase in the number of cationic charges in the amphiphilic structure of gramicidin S induced considerable antimicrobial activities against Gram-negative bacteria. However, the activities against Gram-positive bacteria such as *B. subtilis* and *S. aureus* decreased, especially against *S. aureus*.

On the other hand, the linear peptides synthesized in this study were found to take the α-helical structures in liposomes as designed, whereas they take random structures in water. These peptides are likely taking α-helical structures and obtaining the amphiphilicity through the interaction with bacterial membranes. The peptides containing increased number of Lys residues also showed α-helical CD curves in liposomes and were found to show lower activities against Gram-positive bacteria (in the agar dilution method), however, simultaneously, appreciable activities against Gram-negative ones (in the serial dilution method) when compared to standard peptide 4. Similarly the analogs (8 and 9) containing 16 amino acid residues showed very weak activities against Gram-positive bacteria. These results are compatible with the antimicrobial activities of the more basic GS analogs mentioned above. Thus, generally speaking, the increase in number of basic amino acid residues in the hydrophilic sides of the amphiphilic structures induced lower antimicrobial activities against Gram-positive bacteria, especially against *S. aureus*, but caused appreciable activities against Gram-negative ones such as *E. coli*. This phenomenon was seen clearly for gramicidin S analogs.

Gramicidin S analogs can take β-sheet structures stabilized by type II' β-turns even in water. On the contrally, α-helical structures of the amphiphilic peptides synthesized in this study are induced through the interaction with membranes. The low activities of these amphiphilic peptides against Gram-negative bacteria might be due to the flexibility of the structures. In this connection, secropins, α-helical amphiphilic peptides, show high antimicrobial activities against Gram-negative bacteria. The α-helical structure of secropins might be more stable because of their long peptide chain length. As another antibiotic peptide, polymyxins are known as amphiphilic, but not α-helical. Polymyxins show high antimicrobial activities agaist not only Gram-negative bacteria but also some Gram-positive ones, although they have no activities against *S. aureus* as shown in Table 2. Similarly, polylysine also takes an α-helical structure and is active against both kinds of bacteria.[17] Peptide 8 becoming α-helical in liposomes showed a weak activity against Gram-positive bacteria but was more active against *E. coli* when compared with 4. Therefore, we at this stage, can not exclude a possibility that the increased numbers of basic amino acids are simply effective to show antimicrobial activities against Gram-negative bacteria.

EXPERIMENTAL

Synthesis

General procedure TLC was carried out on Merck silica gel G with the following

solvent systems: R_f^1, $CHCl_3$-MeOH (5:1); R_f^2, $CHCl_3$-MeOH-AcOH (50:10:2). Optical rotations were measured with a Union high sensitivity polarimeter PM 71. Amino acid analyses were performed on a Hitachi KLA-5 amino acid analyzer after hydrolysis in 6 M HCl in sealed tubes at 110°C for 24 h.

cyclo(-Val-Orn-Leu-D-Dpr-Pro-)$_2$ (2) Compound 2 was prepared as previously described.[8] A part of Boc-Leu-D-Dpr(Z)-Pro-Val-Orn(HCO)-OH prepared by stepwise elongation was converted into Boc-pentapeptide-ONSu and other part into H-pentapeptide-OH. Coupling of the Boc-pentapeptide-ONSu and H-pentapeptide-OH gave Boc-(Leu-D-Dpr(Z)-Pro-Val-Orn(HCO))$_2$-OH. This compound was converted to H-decapeptide-ONSu·TFA. Cyclization of this active ester in pyridine gave the protected cyclic decapeptide. By removal of HCO gruop with 0.5 M HCl in MeOH and Z group by hydrogenation, 2·4HCl was obtained; mp 253-255°C (dec); $[\alpha]_D^{20}$ -112° (c 0.1, MeOH).

cyclo(-Leu-Orn-Leu-Orn-Leu-D-Phe-Pro-)$_2$ (3) Compound 3 was prepared as previously described[9] by cyclization of H-(Leu-Orn(Z)-Leu-Orn(Z)-Leu-D-Phe-Pro)$_2$-N_3 and by subsequent removal of Z groups in the protected cyclic tatradecapeptide by hydrogenation; mp 232-236°C; $[\alpha]_D^{25}$ -90° (c 1, MeOH).

Boc-Lys(Z)-Lys(Z)-OPac (10) To a mixed anhydride prepared from Boc-Lys(Z)-OH (4.94 g, 13 mmol), isobutyl chloroformate (1.70 ml, 13 mmol) and Et_3N (1.82 ml, 13 mmol) in THF (20 ml) was added a solution of H-Lys(Z)-OPac·HCl (5.66 g, 13 mmol) and Et_3N (1.82 ml) in $CHCl_3$ (40 ml) at -10°C. The mixture was stirred at room temperature for 4 h and evaporated in vacuo. The residue was dissolved in EtOAc, and the solution was washed with 10% citric acid, 4% $NaHCO_3$, water, and dried over Na_2SO_4. After removal of the solvent, the residue was recrystallized from EtOAc-petroleum ether; yield, 8.46 g (85%); mp 63-64°C; $[\alpha]_D^{20}$ -36.4° (c 1, DMF); R_f^1 0.44. Anal. calc. for $C_{41}H_{52}O_{10}N_4 \cdot 1/2H_2O$ (769.9): C 63.96; H 6.94; N, 7.28%. Found: C 63.99; H 6.88; N 7.18%

Boc-Ala-Lys(Z)-Lys(Z)-OPac (11) Compound 10 (7.70 g, 10 mmol) was dissolved in 5.6 M HCl in dioxane (20 ml) and the solution was kept at room temperature for 1 h. After evaporation in vacuo, the residue was triturated with a mixture of ether and petroleum ether to give a powder of H-Lys(Z)-Lys(Z)-OPac·HCl (6.98 g, 100%). A solution of Boc-Ala-ONSu (2.86 g, 10 mmol), H-Lys(Z)-Lys(Z)-OPac·HCl (6.98 g) and Et_3N (1.40 ml, 10 mmol) in DMF (30 ml) was stirred at room temperature overnight. After evaporation in vacuo, the residue was solidified by the addition of water. The precipitate was collected by filtration, washed with 10% citric acid, 4% $NaHCO_3$, water, and dried; yield 7.90 g (95%); mp 103-105°C; $[\alpha]_D^{20}$ -17.2° (c 1, DMF); R_f^1 0.75. Anal. calc. for $C_{44}H_{57}O_{11}N_5$ (832.0): C 63.52; H 6.91; N 8.41%. Found: C 63.32, H 6.99, N 8.50%.

Boc-Leu-Ala-Lys(Z)-Lys(Z)-OH (12) Boc-Leu-ONSu (1.80 g, 5.5 mmol) and H-Ala-Lys(Z)-Lys(Z)-OPac·HCl (4.22 g, 5.5 mmol) prepared from 11 were coupled and then the reaction mixture was treated as described above to give Boc-Leu-Ala-Lys(Z)-Lys(Z)-OPac·$1/2H_2O$ (5.04 g, 96%). To a solution of the product (3.59 g, 3.8 mmol) in 90% AcOH (40 ml) was added Zn dust (1.8 g) and the mixture was stirred at room temperature overnight. After removal of insoluble material, the filtrate was evaporated in vacuo. The residue was dissolved in EtOAc, washed with 10% citric acid, water, and dried over Na_2SO_4. The product was recrystallized from MeOH-ether; yield 2.57 g (81%); mp 124-128°C; $[\alpha]_D^{20}$ -13.6° (c 1, DMF); R_f^2 0.79. Anal.

calc. for $C_{42}H_{62}O_{11}N_6 \cdot 1/2H_2O$ (836.0): C 60.34, H 7.60, N 10.05%. Found: C 60.55, H 7.59, N 10.28%.

Boc-Leu-Ala-Lys(Z)-Lys(Z)-Leu-Ala-Lys(Z)-Leu-OH (14) Boc-Leu-Ala-Lys(Z)-Leu-OPac[6] (2.42 g, 3 mmol) was dissolved in TFA (7 ml) at 0°C and the solution was kept at 0°C for 1 h. After evaporation in vacuo, the residue was washed several times with ether by decantation to give H-Leu-Ala-Lys(Z)-Leu-OPac·TFA (13) (2.38 g, 98%). To a chilled solution of 13 (2.26 g, 2.7 mmol), 12 (2.19 g, 2.7 mmol), Et_3N (0.38 ml, 2.7 mmol) and HOBt (0.73 g, 5.4 mmol) in DMF (25 ml) was added EDC·HCl (0.78 g, 4.1 mmol) under stirring. The mixture was stirred at 0°C for 5 h and then at room temperature overnight. After evaporation in vacuo, the residue was solidified by the addition of water. The precipitate was collected by filtration, washed with 10% citric acid, 4% $NaHCO_3$ and water, and dried to afford Boc-Leu-Ala-Lys(Z)-Lys(Z)-Leu-Ala-Lys(Z)-Leu-OPac·H_2O (3.95 g, 96%). The product (3.65 g, 2.4 mmol) was treated with Zn in 90% AcOH as described above. In this case, the product which was insoluble in EtOAc was washed with 10% citric acid and water in solid state, and dried; yield, 3.26 g, (96%); mp 265-268°C (dec); $[\alpha]_D^{20}$ -21.9° (c 1, DMF); R_f^1 0.44. Anal. calc. for $C_{79}H_{113}O_{18}N_{11} \cdot 3/2H_2O$ (1413.7): C 62.30, H 7.61, N 10.11%. Found: C 62.12, H, 7.62, N 10.32%.

Ac-Leu-Ala-Lys(Z)-Lys(Z)-(Leu-Ala-Lys(Z)-Leu)$_2$-$NHCH_3$ (16) Compound 14 (353 mg, 0.25 mmol) and H-Leu-Ala-Lys(Z)-Leu-$NHCH_3$·TFA (176 mg, 0.25 mmol) prepared from Boc-Leu-Ala-Lys(Z)-Leu-$NHCH_3$[6] were coupled as described above to give Boc-Leu-Ala-Lys(Z)-Lys(Z)-(Leu-Ala-Lys(Z)-Leu)$_2$-$NHCH_3$·$3/2H_2O$ (15) (471 mg, 95%). The product 15 (417 mg, 0.21 mmol) was treated with TFA as described above. The TFA salt (395 mg, 0.2 mmol), Ac-ONSu (63 mg, 0.4 mmol) and Et_3N (0.028 ml, 0.2 mmol) were dissolved in a mixture of DMF (4 ml) and DMSO (2 ml), and the solution was stirred at room temperature overnight. After evaporation in vacuo, the residue was solidified by the addition of water, collected by filtration and dried. The product was recrystallized from DMSO-EtOAc; yield 366 mg (94%); mp 280-284°C (dec); $[\alpha]_D^{20}$ -17.2° (c 0.5, DMSO); R_f^1 0.53. Anal. calc. for $C_{98}H_{149}O_{21}N_{17} \cdot 3H_2O$: (1955.4): C 60.19, H 7.99, N 12.17%. Found: C 60.21, H 7.96, N 12.42%.

Ac-Leu-Ala-Lys-Lys-(Leu-Ala-Lys-Leu)$_2$-$NHCH_3$ (6) A solution of 16 (300 mg, 0.153 mmol) in 25% HBr in AcOH (8 ml) was allowed to stand at room temperature for 4 h and evaporated in vacuo. The residue was washed several times with ether. The HBr salt was dissolved in water (5 ml), passed through a column (0.9 × 10 cm) of Amberlite IR-45 (AcO^- form), and eluted with water. Fractions containing the product were lyophilized to give a white powder; yield 204 mg. Amino acid ratios in the hydrolysate: Ala 3.00 (3), Leu 5.19 (5), Lys 3.92 (4). the composition of $C_{66}H_{125}O_{13}N_{17} \cdot 4CH_3COOH \cdot 6H_2O$ was estimated from the result of elemental analysis.

Ac-peptide-$NHCH_3$ These peptides were synthesized essentially by the similar route for 6. The estimated compositions of the peptides from the results of elemental analysis are as follows: Ac-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Leu-Lys-Lys-Leu-$NHCH_3 \cdot 6CH_3COOH \cdot 4H_2O$ (5), Ac-Leu-Ala-Lys-Ala-Leu-Lys-Lys-Leu-Leu-Ala-Lys-Leu-$NHCH_3 \cdot 4CH_3COOH \cdot 5H_2O$ (7), Ac-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Ala-Leu-$NHCH_3 \cdot 6CH_3COOH \cdot 6H_2O$ (8), Ac-Leu-Ala-Lys-Leu-(Lys-Ala-Lys-Leu)$_2$-Leu-Ala-Lys-Leu-$NHCH_3 \cdot 6CH_3COOH \cdot 5H_2O$ (9). Amino acid analysis of the peptides gave satisfacotry results.

Antibacterial assay

Agar dilution method.[14] The minimum inhibitory concentration (MIC) was determined by the standard agar dilution method using Trypticase soy agar. Bacterial suspension (5 ml) containing ca. 10^7 colony forming units/ml was inoculated with a multiple inoculator onto agar plates containing twofold serial dilution of each antibiotic. The plates were incubated at 37°C for 18 h, and the MIC was defined as the lowest concentration of the peptide on which there was either no visible growth or less than four colonies per sopt.
Serial dilution method in solution media.[15] The cell suspension was diluted with the medium to 2×10^6 cells/ml. Various concentrations of the peptide solution were placed in the test tubes, made up to 1 ml with medium, and the cell suspension (1 ml) was added. After incubation at 30°C for 17 h, distilled water (1 ml) was added, and the absorbance at 620 nm was measured.

Phospholipid liposomes
DPPC and DPPG were obtained from Sigma. Preparation of liposomes was carried out as described before.[7]

CD measurements
CD spectra were recorded on a JASCO J-40A spectropolarimeter with a JASCO data processor Model J-DPY using quartz cell of 1 mm pathlength.

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