Antibacterial Activity of Arg/Pro-Rich Bactenecin 5 Model Peptides and Their Interaction with Phospholipid Membranes

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H–Arg–Phe–Arg–Pro–Pro–Ile–Arg–(Arg–Pro–Pro–Phe)$_n$–NH$_2$ (n = 2—5 and 7), models of antibacterial bactenecin 5, were synthesized. The CD measurement showed that they took polyproline II-like conformations in neutral lipid vesicles at 25 and 50 °C, and in acidic ones at 25 °C. However, their conformations changed in acidic lipid vesicles at 50 °C. The membrane perturbation activity of the peptides was also found only for acidic lipid vesicles at 50 °C. The perturbation activity increased with increasing in the peptide chain length. All peptides exhibited negligible hemolytic activity for rabbit erythocytes, whereas they, except for the shortest peptide, had moderate or strong antibacterial activity against Gram-positive and Gram-negative bacteria. Together with the previous results, the N-terminal cationic portion and a certain peptide chain length were found to be important for antibacterial activity.

The peptide-biomembrane interaction is an essential step for various kinds of bioactive peptides, such as hormones, antibiotics, and toxins, to show their functions. Thus, many studies have been carried out to clarify the conformations of such peptides, their functional mechanisms, their behaviors toward and in lipid membranes, and the structure-function relationship. Peptides named Pro/Arg-rich family with unique primary sequences have been found. They are bactenecins from the large granules of the bovine neutrophils,[1] PR-39 from the upper part of bovine small intestine,[2,3] abacin,[4] and apidaecins[5] from the honeybee. Among them, bactenecins have been preferentially investigated. Bactenecin 5 (Bac 5) and Bac 7, cationic antibacterial peptide, contain N-terminal unrepeating portion and successive repeating region of the Arg–Pro–Pro–X and Pro–Arg–Pro–X units, respectively. X means a hydrophobic amino acid residue. Bac 5 and 7 show antibacterial activity mainly against Gram-negative bacteria, and the activity is considered to be attributed to an impairment of the respiratory function.[1]

Gennaro et al. synthesized a variety of fragments covering the entire sequences of Bac 5 and 7, and examined their activity to identify the domain necessary for antibiosis. As a result, the presence of an N-terminal highly cationic portion of the peptides and a minimum length of 18—20 amino acid residues were found to be required.[6] On the other hand, Raj et al. reported that the first six residues of Bac 5 might not be essential for antifungal activity against *Candida albicans*, but the 7—22 residues were a functional domain.[7,8] In this connection, some model peptides of the repeating region of Bac 5, Ac–(Arg–Pro–Pro–Phe)$_n$–NHCH$_3$ (n = 2, 4, 6, 8, and 10) (PR$_2$, PR$_4$, PR$_6$, PR$_8$, and PR$_{10}$) were tested for Gram-negative and Gram-positive bacteria, but showed no activity.[9] These results suggested that a further study on the bioactivity is necessary to obtain a clear explanation. The secondary structures of Bac 5 and the related peptides have been examined by CD[10] and NMR measurements.[7,8] The CD spectra of the peptides showed a negative band at 205 nm with a slight shoulder at around 230 nm in water and in the presence of phospholipids, suggesting that Bac 5 has a polyproline II helix structure. The NMR study also supported this result. A CD measurement of the PR series peptides, Ac–(Arg–Pro–Pro–Phe)$_n$–NHCH$_3$, indicated that more than four repeats of the Arg–Pro–Pro–Phe unit might be required to hold a certain framework.[9]

In this study, we examined whether the N-terminal portion of Bac 5 is essentially important or not for antibacterial activity and tried to clarify the role of the repeating sequence using the model peptides of Bac 5, H–Arg–Phe–Arg–Pro–Pro–Ile–Arg–(Arg–Pro–Pro–Phe)$_n$–NH$_2$ (n = 2, 3, 4, 5, and 7) (BM$_2$—BM$_5$ and BM$_7$). These peptides consist of the N-terminal unrepeating cationic portion of Bac 5 and the model sequence of the repeating region. The property and biological activity of the model peptides were investigated by CD and membrane perturbation measurements, and antibacterial and hemolytic activity assays. The N-terminal portion and a certain peptide chain length of the repeating region were found to be important for antibacterial activity.

Results and Discussion

Peptide Synthesis. We previously synthesized some Bac 5 models, Ac–(Arg–Pro–Pro–Phe)$_n$–NHCH$_3$ (n = 2, 4, 6, 8, and 10). In that case, peptide synthesis was achieved using a combination of the solid-phase synthesis and the solution method; the final products were obtained in moderate yields.[9] Therefore, the same strategy was applied to prepare peptides in the present study. The N-terminal was free, like Bac 5, and the C-terminal was blocked

with an amide group. A tetrapeptide derivative, Boc–Arg-(Tos)–Pro–Pro–Phe–NH_2 (Boc-**1**); two octapeptide derivatives, Boc–(Arg(Tos)–Pro–Pro–Phe)$_2$–OH (**2**) and Boc–(Arg-(Tos)–Pro–Pro–Phe)$_2$–NH_2 (Boc-**3**); and a undecapeptide derivative, Boc–Arg(Tos)–Phe–Arg(Tos)–Pro–Pro–Ile–Arg-(Tos)–Arg(Tos)–Pro–Pro–Phe–OH (**4**), were prepared by solid-phase synthesis using oxime resin.[11,12] Fragment condensation by the solution method with HBTU–HOBt or HATU–HOAt[13,14] was applied for the synthesis of the protected final products (Fig. 1). The intermediates were purified by RP–HPLC and the key compounds were identified by MALDI-TOF-MS. After removing the protecting groups with HF, the final products were purified first by gel chromatography with Sephadex G-25 using aqueous acetic acid, and then RP-HPLC. The purity of the products was confirmed by analytical RP-HPLC to be > 95%. Identification of the products by MALDI-TOF-MS and amino acid analysis gave satisfactory results (Table 1).

CD Study. CD measurements of peptides were performed in a 20 mM Tris-HCl buffer (pH 7.4) (1 M = 1 mol dm^{-3}), TFE and in the presence of DPPC or DPPC/DPPG (3:1) SUVs to examine secondary structures of the peptides. The results are shown in Fig. 2. In the buffer solution at 25 °C, all of the peptides exhibited a negative band at 204 nm with a shoulder at 227 nm (Fig. 2a). Those CD spectra were very similar to those of the previous

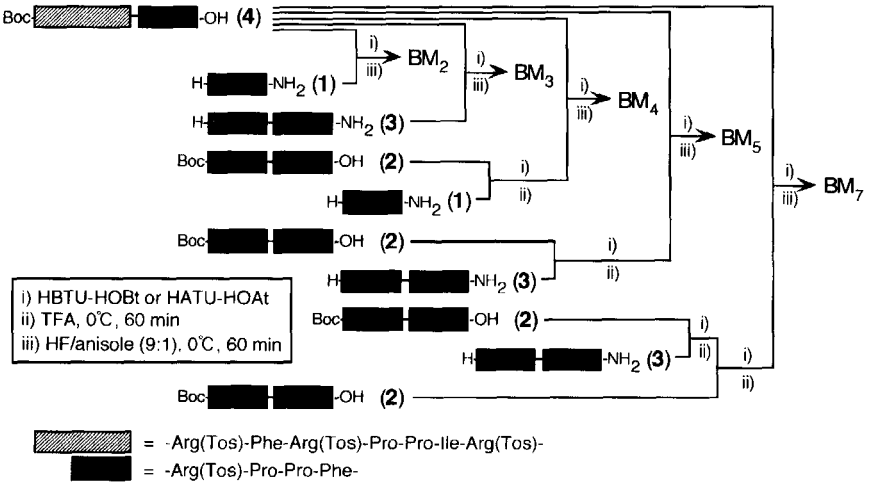

Fig. 1. Synthetic routes for peptides by the fragment condensation method.

Table 1. Analytical Data of Synthetic Peptides

Peptide	Sequence	MALDI-TOF-MS		Amino acid analysis			
		Found	Calcd ([M+H]$^+$)	Ile	Phe	Pro	Arg
BM_2	H–RFRPPIR–(RPPF)$_2$–NH_2	1935.8	1936.4	1.09(1)	3.00(3)	6.03(6)	4.86(5)
BM_3	H–RFRPPIR–(RPPF)$_3$–NH_2	2434.8	2434.0	0.89(1)	4.00(4)	8.23(8)	5.82(6)
BM_4	H–RFRPPIR–(RPPF)$_4$–NH_2	2931.0	2931.6	0.97(1)	5.00(5)	10.23(10)	6.65(7)
BM_5	H–RFRPPIR–(RPPF)$_5$–NH_2	3431.0	3429.2	0.99(1)	6.00(6)	12.20(12)	7.65(8)
BM_7	H–RFRPPIR–(RPPF)$_7$–NH_2	4427.1	4424.4	0.88(1)	8.00(8)	16.50(16)	9.81(10)

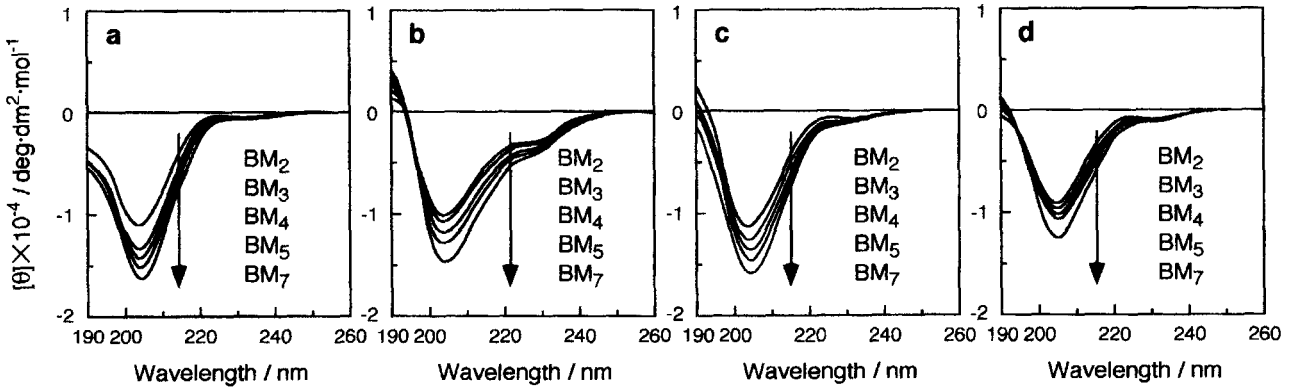

Fig. 2. CD spectra of peptides in 20 mM Tris HCl buffer (pH 7.4) at 25 °C (a), TFE at 25 °C (b), DPPC vesicles at 50 °C (c), and DPPC/DPPG (3:1) vesicles at 50 °C (d). [Peptide] = 20μM, [DPPC] = 1 mM and [DPPC/DPPG (3 : 1)] = 0.25 mM.

PR series model peptides[9] and natural Bac 5.[7,8] Proline-rich polypeptides have often been found to take a polyproline II conformation which has a strong negative band at 204 nm and a shoulder 227 nm.[3,15] These observations indicate that the present model peptides, BM_2—BM_5 and BM_7, also took a polyproline II-like conformation in an aqueous environment. Although the depth at 204 nm gradually increased with increasing peptide chain length, the mean-residue ellipticities at 204 nm ($[\theta]_{204}$) were -11000 deg cm^2 dmol^{-1} for BM_2 and -16000 deg cm^2 dmol^{-1} for BM_7. The values of $[\theta]_{204}$ of proline-rich polypeptides have been reported to have a low of -17,000 to a high of -57,000 (average, more than -30000),[15] indicating that BM_2—BM_5 and BM_7 do not contain a high polyproline II conformation. This may be reasonable, because the proline content in peptides is about half of the whole sequence. The CD spectra of peptides in TFE (Fig. 2b) were different from those in the a buffer. Although the negative band at 204 nm was still observed, a broad shoulder appeared at around 220 nm, instead of a disappearance of the shoulder at 227 nm. TFE is known to induce the formation of an α-helix for which two negative bands at 208 and 222 nm are characteristic. Consequently, all of the peptides seem to have a certain conformation different from polyproline II and α-helix. The CD patterns in the presence of DPPC (neutral) or DPPC/DPPG (3:1, acidic) SUVs at 25 °C were almost the same to those in the buffer at 25 °C (data not shown). Experiments were carried out in the ratios of [peptide]/[lipid] = 10 μM/1 mM and 10 μM/0.25 mM for neutral and acidic SUVs, respectively. Above the DPPC/DPPG (3:1) concentration of 0.25 mM, a slight turbidity appeared at 25 °C. Furthermore, fusogenic activity of the peptides was found for the acidic SUVs at 25 °C, as described later. These facts indicate that peptides interact more strongly with acidic SUVs, at least compared to neutral ones, although the CD curves in the presence of DPPC and DPPC/DPPG (3:1) SUVs are indistinguishable.

At 50 °C, the CD spectra of the peptides in the buffer and in the presence of DPPC SUVs (Fig. 2c) were slightly different from the spectra at 25 °C. The shoulder at 227 nm degenerated slightly at 50 °C, suggesting that a loss of the peptide structure might be caused by an increase in the temperature.[15] In the presence of DPPC/DPPG (3:1) SUVs at 50 °C, the negative bands at 204 nm were fairly shallower (Fig. 2d). This phenomenon indicates that the peptides interacted with acidic lipid membranes to change their structures. However, the structural change of peptides seems not to be so drastic because the fundamental CD spectra are maintained.

CF Leakage. To evaluate the interaction of peptides with lipid vesicles in the buffer (pH 7.4), the leakage of carboxyfluorescein (CF) from DPPC and DPPC/DPPG (3:1) of 70 μM SUVs was examined by changing the [peptide]/[lipid] ratio.[16] The peptides showed a negligible CF leakage activity for both SUVs at 25 °C (data not shown), meaning that they have very weak membrane-purturbing activity under these conditions. These phenomena were consistent with the results in the CD study, where the peptides were found to scarcely interact with the neutral and acidic SUVs at 25 °C. In the presence of DPPC at 50 °C, a weak activity was observed and the leakage percent was below 10% at peptide and lipid concentrations of 50 and 70 μM, respectively (Fig. 3a). On the other hand, the peptides showed strong leakage activities for DPPC/DPPG (3:1) SUVs at 50 °C, as shown in Fig. 3b. The leakage percentage for DPPC/DPPG (3:1) SUVs without peptides was negligible at 50 °C. The order in activity of the peptides was $BM_7 > BM_5 > BM_4 > BM_3 > BM_2$, i.e., the membrane-perturbing activity increased with increasing the peptide chain. BM_7 showed 100% activity at the peptide concentration of 0.8 μM; in other words, complete perturbation was caused at a peptide-to-lipid ratio of 1:9, whereas BM_2 showed 66% activity at 2 μM. In a previous study, we found that Ac–(Arg–Pro–Pro–Phe)$_6$–$NHCH_3$ (PR_6) had moderate leakage activity, but the leakage activity of Ac–(Arg–Pro–Pro–Phe)$_4$–$NHCH_3$ (PR_4) was very weak; we hence concluded that the lack of membrane-perturbing activity of a short peptide may be due to a less-ordered structure and/or an insufficient number of Arg and Phe residues.[9] H–Arg–Phe–Arg–Pro–Pro–Ile–Arg–(Arg–Pro–Pro–Phe)$_{2,3}$–NH_2 (BM_2 and BM_3) could exhibit leakage activity comparable to that of PR_6. These results suggest that the charge interaction of the cationic Arg residues with anionic head groups in DPPG is more effective than the repeating structural motif for the membrane-perturbing activity. In this connection, Skerlavaj et al. previously reported that Bac 5 caused a rapid increase in the permeability of both the outer and inner membranes of *E. coli*.[17]

Membrane Fusion. Peptides and proteins which contain a hydrophobic amino acid cluster and a hydrophilic cationic one often show membrane-fusion activity.[18—20] It is likely that the hydrophobic cluster penetrates into the inside of the membrane and causes membrane fusion with some membrane perturbation. To clarify whether the BM series peptides have the membrane-fusion activity or not, the peptide-mediating membrane fusion was evaluated by electron microscopy[21] and measuring the intermixing phospholipids between vesicles containing either NBD–PE or Rh–PE.[22] The structure of vesicles composed of DPPC/DPPG (3:1) was observed as a relatively homogeneous mass, and their diameters were in the range of about 20—50 nm. After 5 min of incubation with BM_7, a large mass of vesicles was found in the range of about 150—250 nm in diameter. BM_2 also fused acidic

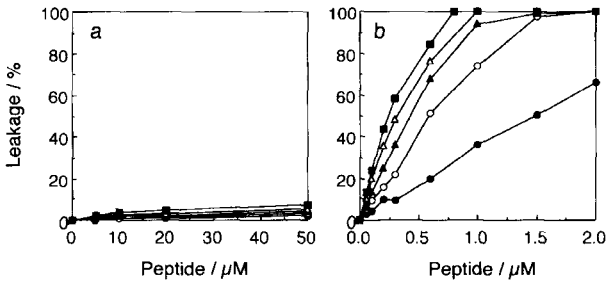

Fig. 3. Carboxyfluorescein leakages from DPPC (a) and DPPC/DPPG (3:1) vesicles (b) as a function of the peptide concentration at 50 °C. [Lipid] = 70 μM, BM_2 (●), BM_3 (○), BM_4 (▲), BM_5 (△), and BM_7 (■).

Table 2. Antibacterial Activities of Peptides

Organisms	MIC/mg ml^{-1} a)									
	BM_2	BM_3	BM_4	BM_5	BM_7	PR_2	PR_4	PR_6	PR_8	PR_{10}
S. aureus IFO 12732	128	16	8	4	4	> 128	> 128	64	8	4
B. subtilis IFO 3134	> 128	64	16	8	4	> 128	> 128	128	16	8
E. coli IFO 12734	128	16	4	4	8	> 128	> 128	64	8	8
P. aeruginosa IFO 12582	> 128	128	64	64	16	> 128	> 128	> 128	64	64

a) Method, serial solution dilution method; medium, TSB medium (pH 7.4); inoculum, 10^4 cells/ml.

vesicles, although the vesicles were smaller (about 100—150 nm) than those induced by BM_7.

Intermixing of the chromophoric phospholipids quenches NBD fluorescence at 530 mm by an energy transfer to rhodamine. Measures were performed with DPPC and DPPC/DPPG (3:1) SUVs of 70 μM at 25 °C by changing the [peptide]/[lipid] ratio. The possibility of quenching of the NBD fluorescence by peptides was tested, as a control experiment, by combining NBD–PE and peptides without Rh–PE. A fusion percentage of 3—10% was observed for the acidic vesicles after 5 min at 25 °C, depending on the concentration and the chain length of the peptides, whereas a negligible fusion percentage (< 1%) was observed for neutral vesicles. The results are shown in Fig. 4. In the presence of DPPC SUVs, BM_7 and BM_5 showed fusion activities of 25—30% at peptide concentrations of 2—5 μM, and weaker activities were observed for BM_4—BM_2 (Fig. 4a). On the other hand, strong and chain length-dependent fusion activity was found in the presence of DPPC/DPPG (3:1) SUVs. At a peptide concentration of 5 μM, all the peptides showed nearly 90% activity (Fig. 4b). As already mentioned, the leakage activity of the peptides was negligible for DPPC and DPPC/DPPG (3:1) SUVs at 25 °C, and CD spectra also remained unchanged in the presence of phospholipids. These results mean that membrane fusion occurred under very mild conditions, which induced negligible structural changes of the lipid vesicles and peptides. Peptides may draw lipid vesicles closer to each other by mainly the peptide's charge interaction with the surface of the vesicles to cause successive membrane fusion. In a previous study, we found that short peptides, PR_2 and PR_4, exhibited no fusion activity for DPPC and DPPC/DPPG (3:1) SUVs at 25 °C,[9] showing that the charge interaction may have a greater effect on the fusion process, because BM_2, which has a chain length similar to that of PR_4 but is more cationic, has stronger fusion activity than PR_4.

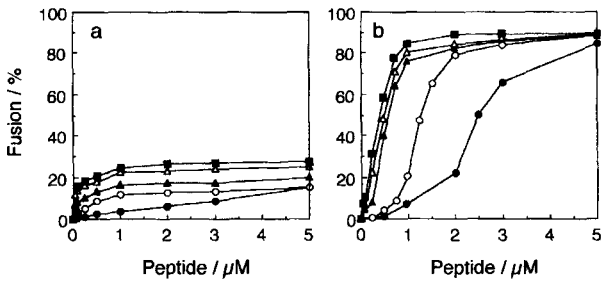

Fig. 4. Fusion activities of peptides for DPPC (a) and DPPC/DPPG (3:1) vesicles (b) at 25 °C. [Lipid] = 70 μM, BM_2 (●), BM_3 (○), BM_4 (▲), BM_5 (△), and BM_7 (■).

Hemolytic and Antibacterial Activities. The hemolytic activity of the peptides was examined using rabbit *erythro*-cytes at 37 °C. Negligible or weak (< 5%) activity was observed for all of the peptides (data not shown). Such weak hemolytic activity is consistent with the result on the membrane-perturbation activity to neutral DPPC SUVs. The absence of acidic phospholipid and the presence of much cholesterol on the outer monolayer in *erythro*-cytes probably protect against a peptide attack.[23]

Bac 5 has a MIC of 12—25 μg ml^{-1} against some Gram-negative bacteria, but is almost inactive against Gram-positive bacteria.[24] In a previous paper, we reported that model peptides (PR_2, PR_4, PR_6, PR_8, and PR_{10}) had no antibacterial activity up to 50 μg ml^{-1} against Gram-negative (9 organisms) and Gram-positive bacteria (11 organisms)[9] by the agar dilution method.[25,26] The same results were obtained for BM_2—BM_5 and BM_7. However, we recently found that not only the BM series peptides, but also the PR series, showed various degrees of antibacterial activity by the serial solution dilution method (Table 2).[26,27] It is interesting that some peptides have antibacterial activity against both of Gram-negative and Gram-positive bacteria, although Bac 5 has been reported to show activity against Gram-negative bacteria exclusively.[16] This difference may be due to the different conditions on the assay. The antibacterial activity generally increased with increasing the peptide chain, and BM_7 had the strongest activity. BM_4 and BM_5, which are more cationic than PR_6 inspite of having similar chain length, exhibited stronger antibacterial activity than PR_6. These findings indicate that the repeating region with a certain chain length has essentially the antibacterial activity, and the presence of the N-terminal cationic portion highly enhances the activity. We conclude that the polyproline II-like structure found for Bac 5 may be important for antibacterial activity, and that the N-terminal portion strengthens the interaction of peptides with lipid vesicles to perturb the lipid structure.

Experimental

Materials. Amino acid derivatives and the reagents for peptide synthesis were puchased from Peptide Institute Inc. and Watanabe Chemical Inductries, Ltd. DPPC and DPPG were from Sigma Chemical Co., and other reagents and solvents were from Wako Pure Chemical Industries, Ltd.

Peptide Synthesis. Peptide synthesis was performed in combination with the solid-phase method and the solution method,

as described by Niidome et al.[9] Peptide fragments were synthesized by the stepwise elongation of Boc-amino acids on *p*-nitrobenzophenone oxime resin, and were liberated from the resin in the form of Boc–peptide–NH_2 or Boc–peptide–Opip, which was converted to Boc–peptide–OH.[11,12] Fragment condensation was carried out by the HBTU–HOBt or HATU–HOAt method.[13,14] The final protected peptides were treated with HF in the presence of anisole at 0 °C for 60 min. After evaporation, the residues were solidified with ether and then chromatographed with a column (1.4×25 cm) of Sephadex G-25 using 10% acetic acid. Further purification was performed by RP–HPLC on a YMC–Pack ODS-A (10×250 mm) with a linear gradient of 100% H_2O/0.1% TFA and 100% acetonitorile/0.08% TFA. The fractions containing pure peptides were lyophilized. The purity of final products was evaluated by analytical RP–HPLC, MALDI–TOF–MS, and amino acid analysis. The results were satisfactory, as shown in Table 1.

Preparation of Phospholipid Vesicles. Small unilamellar vesicles (SUVs) of DPPC and DPPC/DPPG (3:1) were prepared for CD, leakage, and fusion experiments.[28] Phospholipid (7.4 mg) was dissolved in $CHCl_3$/MeOH (2:1 v/v, 2 ml) and then dried by a stream of nitrogen gas. The dried lipid was hydrated in 20 mM Tris HCl buffer (pH 7.4, 10 ml) using a Branson bath-type sonicator. The suspension was sonicated at 50 °C for 20 min using a Titec Ultrasonic Processor VP-5T at 10 W intensity. The vesicles were left for 30 min at 25 °C before the measurements. The lipid concentration was 1.0 mM. The vesicles trapping carboxyfluorescein (CF) were prepared by a procedure described by Lee et al.[28] The dried lipid (20 mg) was hydrated in 20 mM Tris HCl buffer (pH 7.4, 2.0 ml) containing 0.10 M CF. The CF-entrapped vesicles were separated from the free CF by gel filtration using Sephadex G-75 (1.1×24 cm) with the same buffer (pH 7.4).

CD Measurement. CD spectra were recorded on a JASCO J-720 W spectropolarimeter with a thermostatted cell holder using a qualtz cell of 1.0 mm path length. The peptide concentration was 20 μM in 20 mM Tris-HCl buffer (pH 7.4) or in the presence of phospholipid vesicles. The lipid concentrations were 1 mM DPPC and 0.25 mM DPPC/DPPG (3:1). Measurements were done at 25 or 50 °C. The CD data were evaluated by method of Scholtz et al.[29]

CF Leakage. A dye-leakage experiment was performed by a procedure of Lee et al.[30] To 20 mM Tris HCl buffer (pH 7.4, 1.0 ml) in a cuvette was added 20 μl of a vesicle containing 0.1 M CF to give a vesicle solution with a final lipid concentration of 70 μM. To a cuvette placed in a holder (25 or 50 °C) was added 20 μl of an appropriate dilution of the peptides in the buffer. The fluorescence intensities of CF were monitored at 515 nm (excited at 470 nm) and measured 2 min after adding the peptides. To measure the fluorescence intensity for 100% dye release, 10 μl of Triton® X-100 (10% in Tris buffer) was added to dissolve the vesicles. The percentage of the dye release caused by the peptides was evaluated by the equation $100\times(F-F_0)/(F_t-F_0)$, where F is the fluorescence intensity achieved by the peptides, F_0 and F_t are the intensities observed without the peptides and after Triton® X-100 treatment, respectively.

Fusion Assay. Two kinds of vesicles, one containing NBD–PE and the other containing Rh–PE (2.0 mol% each), were prepared by sonication, as desceibed by Lee et al.[30] Equimolar amounts of the vesicles were mixed at 25 °C, and the final lipid concentration was adjusted to 70 μM. The peptides were added to the mixed vesicles, and the decrease in NBD fluorescence was recorded continuously at an excitation wavelength of 450 nm and an emission wavelength of 530 nm. The fluorescence intensity (F) was measured 5 min after adding the peptides. The fluorescence intensity (F_t) for 100% fusion was obtrained using vesicles in which NBD–PE and Rh–PE were pre-mixed. To check quenching of the NBD fluorescence by peptides, a control experiment was performed by combining NBD–PE and peptides without Rh–PE. The fusion percentage was evaluated by the equation $100\times(F_0-F)/(F_0-F_t)-100\times(F'_0-F')/(F'_0-F'_t)$, were F is the fluorescence intensity achieved by the peptides to NBD–PE and Rh–PE, F_0 the intensity observed with NBD–PE and Rh–PE, F' the intensity achieved by the peptides to NBD–PE, and F'_0 the intensity observed only with NBD–PE.

Hemolytic Activity. To fresh rabbit blood (1 ml) was added PBS (pH 7.4, 1 ml); the resulting mixture was centrifuged at 2600 rpm for 5 min, and the precipitates were collected. Washings with PBS were repeated three times. The obtained precipitates were suspended with four-fold volumes of PBS. A hemolytic activity assay was carried out by a procedure of Argiolas and Pisano.[31] To rabbit erythrocyte solution (5 μl) was added PBS solution (pH 7.4, 1 ml) followed by the peptides. The resulting suspension was incubated for 20 min at 37 °C and then centrifuged. The supernatant was monitored at 413 nm.

Antibacterial Activity. The agar dilution method was performed as follows.[25,26] The minimum inhibitory concentration (MIC) was determined by the standard agar dilution method using Trypticase soy agar. About 5 μl of bacterial suspension containing about 10^7 colony forming units/ml was inoculated with a multiple inoculator onto agar plates containing two-fold serial dilution of each antibiotic. The plates were incubated for 18 h at 37 °C, 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 spot. A serial solution dilution method was performed as follows.[26,27] The cell suspension was diluted with TSB medium to 10^4 cells ml^{-1}. Various concentrations of the peptide solution were placed in test tubes, made up to 20 μl with the medium, and the cell suspension (180 μl) was added. After incubation for 20 h at 37 °C, the absorbance at 620 nm was measured. The results of both assays are shown in terms of MIC in Table 2.

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10 Abbreviations used are as follows: Boc, *t*-butyloxycarbonyl; CD, circular dichroism; CF, carboxyfluorescein; DPPC, dipalmitoyl-DL-3-phosphatidylcholine; DPPG, dipalmitoyl-DL-3-phosphatidylglycerol; HATU, 2-(7-azabenbotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, 2-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; MALDI-TOF-MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MIC, minimum inhibitory concentration; NBD-PE, *N*-(7-nitrobenzo[2,1,3]oxadiazol-4-yl)-dipalmitoyl-3-phosphatidylethanolamine; PBS, phosphate-buffered saline; Rh-PE, *N*-[(lissamine rhodamine B)-4-sulfonyl]dipalmitoyl-3-phosphatidylethamolamine; RP-HPLC, reversed phase high-performance liquid chromatography; SUV, small unilamellar vesicle; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Tos, *p*-toluenesulfonyl; TSB, tryptic soy broth.

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