Synthesis of [L-α-Aminomyristic Acid$^{3,3'}$]gramicidin S and Its Interaction with Phospholipid Bilayer

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A lipophilized gramicidin S (GS) analog was synthesized by introducing L-α-aminomyristic acid (Amy) residues instead of L-leucine residues by the conventional solution method. Influences of lipophilization of GS on interaction with phospholipid liposomes were examined by the peptide-induced dye-leakage assays using carboxyfluorescein (CF)-entrapped liposomes of dipalmitoyl-DL-α-phosphatidylcholine (DPPC). The [Amy$^{3,3'}$]GS (Amy-GS) had the enhanced ability of CF-leakage below the phase-transition temperature of DPPC liposomes as compared with GS, while it showed weaker leakage ability at higher temperature. Conformation of Amy-GS in solution and in the presence of liposomes was similar to that of GS, which was elucidated by CD and ^{1}H NMR measurements. The Amy residue can be utilized to enhance the affinity of a peptide to membrane environment without changing conformation of an original peptide.

Biologically active peptides which have a possibility to interact with biological membrane, for instance, hormones, antibiotics, toxins, and signal peptides, have been energetically investigated on interaction with model membrane systems such as liposomes.[1] Higher lipophilization of peptides is interesting to investigate the interaction between peptides and membranes. For this purpose, it is necessary to introduce a lipophilic amino acid which does not change conformation of original peptides. We have found, by synthesizing a series of designed cyclic hexapeptide ionophores containing L-α-aminomyristic acid (L-Amy) (Fig. 1),[2,3] that Amy has highly lipophilic property with its dodecyl side chain and the effect of the Amy residue on peptide conformation is the same as that of an Ala residue. In order to examine the influences of introduction of Amy to the biologically active peptides, we attempted to synthesize [L-Amy$^{3,3'}$]gramicidin S (Amy-GS) (Fig. 1) and to examine its interaction with dipalmitoyl-DL-α-phosphatidylcholine (DPPC) liposomes. GS, antimicrobial cyclic decapeptide (Fig. 1), has rigid cyclic structure and amphiphilic feature in its whole conformation.[4] To increase its lipophilicity, two Leu residues of GS were replaced with Amy. This GS analog was subjected to the experiments for peptide–lipid interaction.

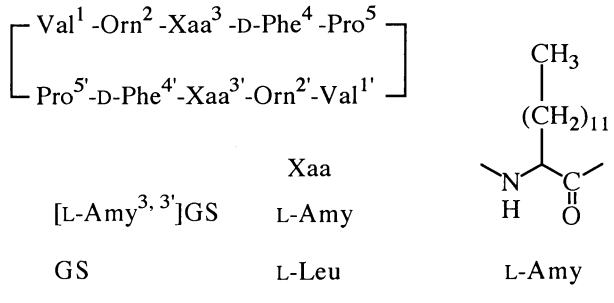

Fig. 1. Structure of [L-Amy$^{3,3'}$]GS.

Results and Discussion

Synthesis. Synthesis of Amy-GS (**1**) was carried out according to the strategy as shown in Fig. 2. The pentapeptide **7** was prepared by the solution method and dimerization of **8** and **9** by fragment coupling to give the decapeptide **10**. The decapeptide was cyclized by the HONSu active ester method in pyridine[5] (1 mM peptide concentration, 1 M=1 mol dm^{-3}) followed by hydrogenation to give Amy-GS (**1**). Amy-GS was fairly soluble in water as well as in MeOH in spite of its high lipophilicity, which can be attributed to the amphiphilic structure of **1**. Lipophilization of GS with Amy was indicated by the retardation of the elution time on reversed-phase HPLC (see Experimental).

Conformation. CD spectra of Amy-GS in MeOH and in the presence of DPPC vesicles are shown in Fig. 3. Amy-GS in MeOH showed a spectrum with double minima at 207 and 217 nm which are typically attributed to the GS backbone conformation (type II′ β-turn and β-sheet structure).[4] Ellipticities of Amy-GS were as almost same as those of GS. In the presence of DPPC vesicles, Amy-GS also showed similar spectra to those of GS at both temperature over (54 °C) and below (25 °C) the phase-transition temperature of DPPC, though the shape of the spectra was slightly different from that in MeOH.[6]

Temperature dependence of NH proton chemical shifts of Amy-GS in DMSO-d_6 were measured and temperature coefficients were shown in Table 1 with the reported values of GS.[6] These values of Amy-GS were

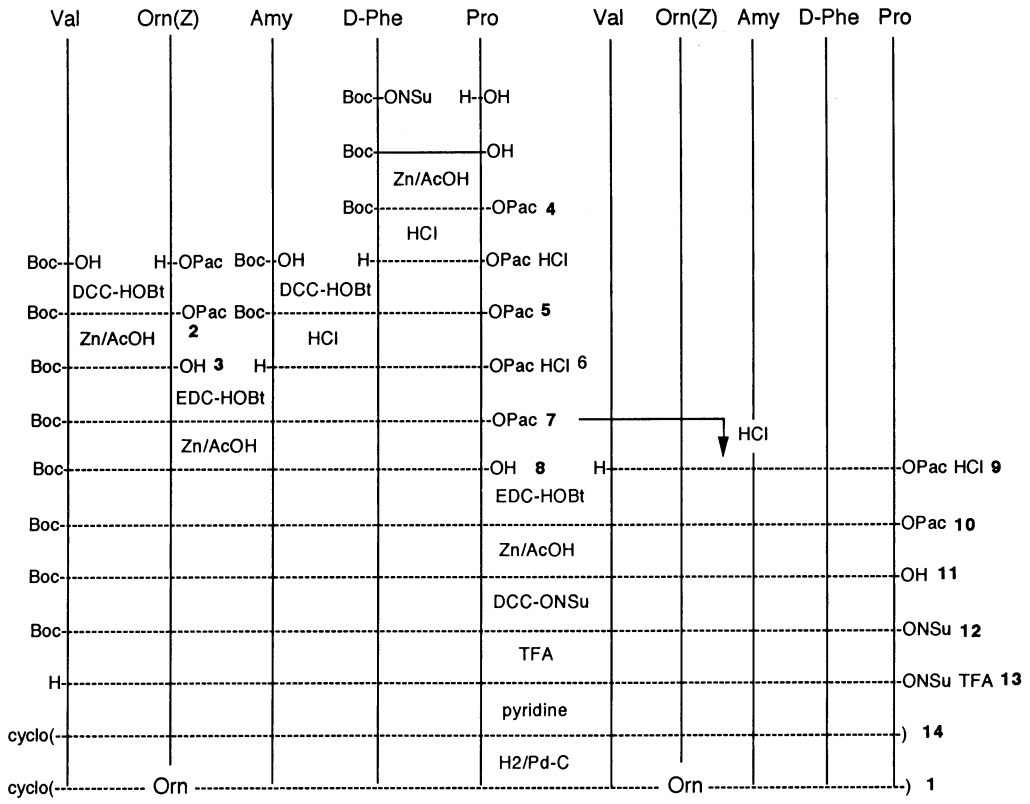

Fig. 2. Synthetic scheme of [L-Amy$^{3,3'}$]GS.

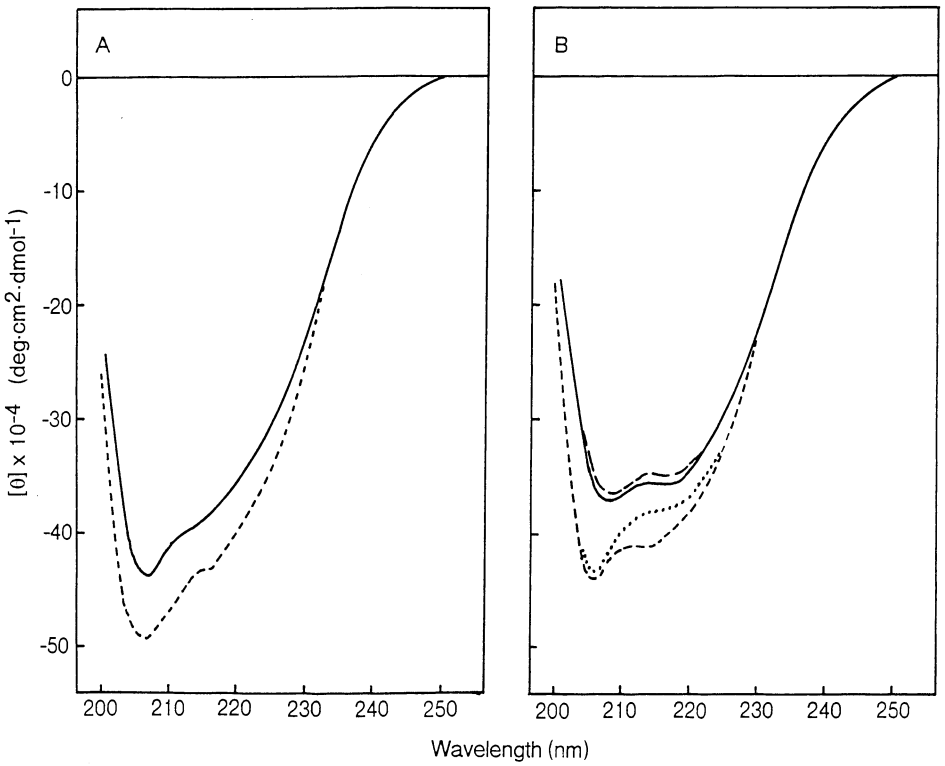

Fig. 3. CD spectra of Amy-GS and GS in MeOH (A) and in 10 mM Hepes buffer with DPPC vesicles at 25 and 54°C (B). (A) (—) Amy-GS; (-----) GS. (B) (—) Amy-GS, 25°C; (— — —) Amy-GS, 54°C; (-----) GS, 25°C; (·····) GS, 54°C.

Table 1. Temperature Coefficients of NH Proton Chemical Shifts of Amy-GS in DMSO-d_6

NH proton	Temperature coefficient (10^{-3} ppm/°C)	
	Amy-GS	GS[a)]
Val	−2.1	−2.2
Orn	−5.4	−5.4
Amy	−3.3	
Leu		−3.3
D-Phe	−7.3	−8.1

a) Data from Ref. 6.

little different from those of GS in spite of the substitutions of L-Amy for L-Leu residues, indicating the existence of the four hydrogen bonds between Val NH and Amy CO, and Amy NH and Val CO. These CD and NMR data demonstrate that the backbone conformation of Amy-GS is quite similar to that of GS even in the membrane environment. This fact indicates that Amy-GS has a highly amphiphilic secondary structure as GS and the hydrophobic face is much more lipophilic than that of GS by two dodecyl side chains.

Interaction with Phospholipid Bilayer. It is recognized that the antimicrobial activity of GS is derived from the perturbation ability of phospholipid bilayer of the peptide.[7-9] The perturbation ability of Amy-GS was estimated by the dye-release experiments from carboxyfluorescein-entrapped DPPC vesicles[10,11] and compared with the ability of GS (Figs. 4 and 5). The CF-leakage from DPPC vesicles by Amy-GS and GS took place mainly through the phase-transition temperature (Fig. 4). Amy-GS caused the leakage from lower temperature than GS and GS showed more rapid leakage

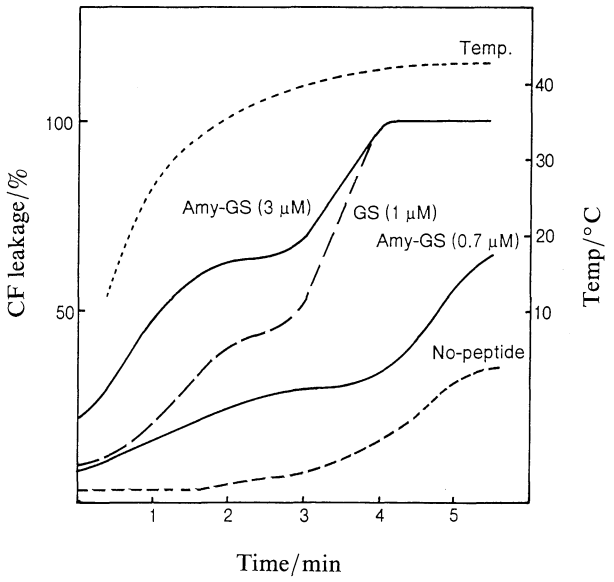

Fig. 4. Time course of CF-leakage from DPPC vesicles by Amy-GS and GS with changing temperature.

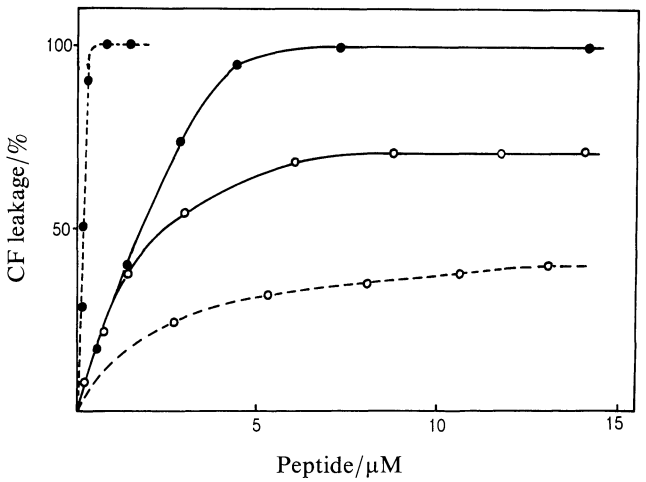

Fig. 5. Concentration dependence of GS and Amy-GS on CF-leakage from DPPC vesicles at 25 and 50°C. (–○–) Amy-GS, 25°C; (–●–) Amy-GS, 50°C; (--○--) GS, 25°C; (--●--) GS, 50°C.

near the phase-transition temperature than Amy-GS. To estimate the membrane disturbing activity of Amy-GS more precisely, concentration dependences of CF-leakage at the temperature over (50°C) and below (25°C) the phase transition were measured (Fig. 5). Both peptides caused the complete leakage at 50°C but not 100% leakage at 25°C. Because the memberane is gel-state below the phase-transition temperature, the peptides can not deeply penetrate into the membrane to disturb the membrane structure completely. This observation of GS was comparable to the results reported by others.[12,13] At 25°C, Amy-GS has twice higher ability of the leakage than GS. Amy-GS could be distributed more preferentially into the membrane with its lipophilic side chains so that the peptide can cause higher leakage than GS. On the other hand, Amy-GS required higher concentration to achieve the complete leakage than GS at 50°C. Amy has a linear hydrocarbon chain which is also the constituents of membrane, though Leu has branched side chain, isobutyl. Therefore, Amy-GS inserts its dodecyl groups into the hydrocarbon regions of membrane (Fig. 6), more

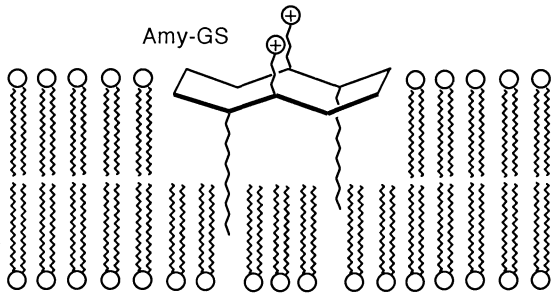

Fig. 6. Proposed interaction of Amy-GS with phospholipid bilayer.

easily than GS does. Thus, the high affinity of Amy-GS to the bilayer results in stabilization of the membrane structure. This would explain the weaker ability in perturbation of the membrane over the phase-transition temperature.

Amy-GS did not show antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus*, and *Escherichia coli* upto 100 μg ml^{-1} in solution media,[14] while GS showed the activity of 3.13, 3.13, and 12.5 μg ml^{-1}, respectively, as minimum inhibition concentrations. Amy-GS was a weaker perturbation agent for liquid crystalline state membrane as discussed above (also see Fig. 5). Thus, the weak ability in perturbing phospholipid bilayer seems to be responsible for the loss of antimicrobial activity.

In conclusion, we demonstrated the interesting roles of lipophilic amino acid, Amy, in GS. Amy-GS showed different effects depending on the temperature (below and over the phase-transition temperture) to the lipid bilayer, which is responsible to the linear hydrocarbon side chain of the Amy residue. Lipophilization of biologically active peptides becomes very interesting to investigate the enhanced interaction of the peptides with biological membranes as well as with receptors. This hydrophobic non-proteinous amino acid can be applied to various biologically active peptides which are known to interact with membrane.

Experimental

Synthesis of Peptides. L-Amy. L-Amy was prepared by the resolution of Ac-DL-Amy with *Aspergillus genus* acylase (Tokyo Kasei),[15] $[\alpha]_D^{20}$ +24.9° (*c* 2.0, $CHCl_2COOH$).

Boc-L-Val-L-Orn(*Z*)-OPac (2). To a solution of a mixed anhydride prepared from Boc-L-Val-OH (4.4 g, 20 mmol), isobutyl chloroformate (2.7 ml, 20 mmol) and Et_3N (2.8 ml, 20 mmol) in THF (40 ml) was added a solution of H-L-Orn(*Z*)-OPac·HCl (8.4 g, 20 mmol) and Et_3N (2.8 ml, 20 mmol) in $CHCl_3$ (60 ml) at −10 °C. The mixture was stirred at room temperature for 2 h and the solvent was evaporated in vacuo. The residue was dissolved in EtOAc, and the solution was washed with 10% citric acid, 4% $NaHCO_3$ and water, and then dried over $MgSO_4$. After evaporation, the residue was crystallized from ether-petroleum ether; 9.5 g (81%); mp 148—150 °C; $[\alpha]_D^{20}$ −26.2° (*c* 1.0, MeOH); R_f 0.53 ($CHCl_3$–MeOH 9:1, v/v). Anal. ($C_{31}H_{41}O_8N_3$) C, H, N.

Boc-L-Val-L-Orn(*Z*)-OH (3). To a solution of **2** (2.0 g, 3.5 mmol) in 90% AcOH (30 ml) was added Zn powder (2.0 g) at room temperature. The mixture was stirred at room temperature for 2 h. After filtration, the filtrate was evaporated. To the residue was added 10% citric acid, and the separated oil was extracted with EtOAc and washed with water, and then dried over $MgSO_4$. After evaporation in vacuo, the oily residue was washed with ether–petroleum ether (1:3, v/v) by decantation; 1.6 g (98%); R_f 0.38 ($CHCl_3$–MeOH 9:1, v/v). This compound was used for the next step without further purification.

Boc-D-Phe-L-Pro-OPac (4). To a chilled EtOAc solution of Boc-D-Phe-L-Pro-OH (4.3 g, 12 mmol) which was prepared from Boc-D-Phe-ONSu and L-Pro was added 2-bromoacetophenone (2.4 g, 12 mmol) and Et_3N (1.7 ml, 12 mmol). The mixture was stirred at room temperature for 3 h and washed with 4% $NaHCO_3$ and water, and then dried over $MgSO_4$. Evaporation of the solvent gave an oily product; 5.7 g (100%); R_f 0.78 ($CHCl_3$–MeOH 9:1, v/v).

Boc-L-Amy-D-Phe-L-Pro-OPac (5). The Boc group of **4** (5.7 g, 12 mmol) was removed by the treatment with 2 M (1 M=1 mol dm^{-3}) HCl in dioxane (60 ml) for 2 h followed by precipitation with ether to give H-D-Phe-L-Pro-OPac·HCl (5.0 g, 100%). To a chilled solution of the HCl salt, Boc-L-Amy-OH (3.7 g, 11 mmol), HOBt (1.5 g, 11 mmol) and Et_3N (1.54 ml, 11 mmol) in $CHCl_3$ (30 ml) was added DCC (2.5 g, 12 mmol). The mixture was stirred at 0 °C for 2 h and at room temperature overnight. After removal of the solvent, the residue was dissolved in EtOAc and washed with 10% citric acid, 4% $NaHCO_3$ and water, and then dried over $MgSO_4$. After evaporation, the product was obtained as an oil; 6.6 g (85%); R_f 0.59 ($CHCl_3$–MeOH 9:1, v/v).

Boc-L-Val-L-Orn(*Z*)-L-Amy-D-Phe-L-Pro-OPac (7). The Boc group of **3** (2.5 g 3.5 mmol) was removed by the treatment with 2 M HCl in dioxane (20 ml) for 2 h to give an oily product, H-L-Amy-D-Phe-L-Pro-OPac·HCl (**6**) (2.2 g, 98%). To a chilled solution of **6**, Boc-L-Val-L-Orn(*Z*)-OH (1.6 g, 3.5 mmol), HOBt (570 mg, 4.2 mmol), and Et_3N (0.5 ml, 3.5 mmol) in DMF (10 ml) was added EDC·HCl (800 mg, 4.2 mmol). The mixture was stirred at 0 °C overnight. After evaporation, the residue was dissolved in EtOAc, and washed with 10% citric acid, 4% $NaHCO_3$ and water, and then dried over $MgSO_4$. After evaporation, the residue was precipitated with ether–petroleum ether; 3.0 g (81%); mp 82—85 °C; $[\alpha]_D^{20}$ −34.8° (*c* 0.4, MeOH); R_f 0.60 ($CHCl_3$–MeOH 9:1, v/v). Anal. ($C_{59}H_{84}O_{11}N_6 \cdot H_2O$) C, H, N.

Boc-L-Val-L-Orn(*Z*)-L-Amy-D-Phe-L-Pro-OH (8). To a solution of **7** (1.4 g, 1.3 mmol) in 90% AcOH (20 ml) was added Zn powder (2.0 g) at room temperature. The mixture was stirred at room temperature for 2 h. After filtration, the filtrate was evaporated, and the residue was precipitated with 10% citric acid and collected. The solid was washed with water and dried in vacuo, and then washed with ether–petroleum ether (1:2, v/v); 1.1 g (82%); mp 100—103 °C; $[\alpha]_D^{20}$ −44.4° (*c* 0.3, MeOH); R_f 0.34 ($CHCl_3$–MeOH 9:1, v/v). Anal. ($C_{51}H_{78}O_{10}N_6 \cdot H_2O$) C, H, N.

H-L-Val-L-Orn(*Z*)-L-Amy-D-Phe-L-Pro-OPac (9). The compound **7** (1.2 g, 1.14 mmol) was treated with 2 M HCl in dioxane (10 ml) at room temperature for 2 h and the solvent was evaporated. The residue was precipitated with ether and washed twice with ether by decantation; 1.0 g (84%); R_f 0.42 ($CHCl_3$–MeOH 9:1, v/v). This compound was used for the next step without further purification.

Boc-(L-Val-L-Orn(*Z*)-L-Amy-D-Phe-L-Pro)$_2$-OPac (10). To a chilled solution of **8** (940 mg, 1.0 mmol), **9** (990 mg, 1.0 mmol), HOBt (200 mg, 1.5 mmol), and Et_3N (0.14 ml, 1.0 mmol) in DMF (10 ml) was added EDC·HCl (290 mg, 1.5 mmol). The mixture was stirred at 0 °C for 2 h and at room temperature overnight. After evaporation, the residue was precipitated by adding 4% $NaHCO_3$, and washed with 4% $NaHCO_3$, 10% citric acid and water, and then dried in vacuo; 1.8 g (96%); mp 102—105 °C; $[\alpha]_D^{20}$ −96.2° (*c* 0.6, MeOH); R_f 0.44 ($CHCl_3$–MeOH 9:1, v/v). Anal. ($C_{105}H_{152}O_{18}N_{12} \cdot H_2O$) C, H, N.

Boc-(L-Val-L-Orn(*Z*)-L-Amy-D-Phe-L-Pro)$_2$-OH (11). To a solution of **10** (1.8 g, 0.9 mmol) in 90% AcOH (30 ml) was added Zn Powder (2.0 g) at room temperature. The mixture

was stirred at room temperature for 2 h. After filtration, the filtrate was evaporated. To the residue was added 10% citric acid, and the separated oil was extracted with EtOAc and washed with water, and then dried over $MgSO_4$. After evaporation in vacuo, the residue was precipitated with ether–petroleum ether; 1.5 g (92%); mp 106—109 °C; $[\alpha]_D^{20}$ −37.5 ° (*c* 0.5, MeOH); R_f 0.31 ($CHCl_3$–MeOH 9:1, v/v). Anal. ($C_{97}H_{146}O_{17}N_{12}\cdot 2H_2O$) C, H, N.

***cyclo*(-L-Val-L-Orn(*Z*)-L-Amy-D-Phe-L-Pro-)$_2$ (14).** To a chilled solution of **11** (1.4 g, 0.8 mmol) and HONSu (180 mg, 1.6 mmol) in DMF (5 ml) was added DCC (330 mg, 1.6 mmol). The mixture was stirred at 0 °C overnight. After removal of *N*,*N*′-dicyclohexylurea, the solvent was evaporated and the residue was washed with ether–petroleum ether by decantation to give an oily product, Boc-(L-Val-L-Orn(*Z*)-L-Amy-D-Phe-L-Pro)$_2$-ONSu (**12**). The compound **12** was treated with TFA (2 ml) at 0 °C for 30 min. After evaporation, the residue was washed with petroleum ether by decantation to give H-(L-Val-L-Orn(*Z*)-L-Amy-D-Phe-L-Pro)$_2$-ONSu·TFA (**13**). The compound **13** was dissolved in DMF (5 ml) and the solution was added dropwise into pyridine (800 ml) at room temperature. After 24 h, the solvent was removed. The residue was dissolved in EtOAc and washed with 2% HCl and water, and then dried over $MgSO_4$. After evaporation, the crude oily product was purified by silica-gel chromatography (Merck 60, 2.0×40 cm, $CHCl_3$–MeOH 40:1, v/v as an eluent) and crystallized from MeOH; 500 mg (38%); mp 195—197 °C; $[\alpha]_D^{20}$ −240 ° (*c* 0.1, $CHCl_3$); R_f 0.54 ($CHCl_3$–MeOH 9:1, v/v). Anal ($C_{92}H_{136}O_{14}N_{12}\cdot H_2O$) C, H, N.

***cyclo*(-L-Val-L-Orn-L-Amy-D-Phe-L-Pro-)$_2$·2HCl (Amy-GS) (1).** The compound **14** (250 mg, 0.15 mmol) was dissolved in AcOH (20 ml), and the solution was hydrogenated in the presence of 5% Pd–C at room temperature overnight. After removal of the catalyst, the solvent was evaporated and the residue was dissolved in MeOH and 4 M HCl in dioxane (0.1 ml) was added. The solvent was evaporated and the residue was powdered with ether–petroleum ether; 180 mg (83%); mp 219—221 °C (decomp); $[\alpha]_D^{20}$ −112 ° (*c* 0.4, MeOH); R_f 0.20 ($CHCl_3$–MeOH–AcOH 50:10:2, v/v). Found: C, 58.21; H, 8.79; N, 10.54%. Calcd for $C_{76}H_{126}O_{10}N_{12}Cl_2\cdot 7H_2O$: C, 58.33; H, 9.02; N, 10.74%.

The same relative mobility as GS was observed on a paper electrophoresis with the solvent system of HCOOH–AcOH–MeOH–H_2O (1:3:6:10, v/v, pH 1.8) for 2 h at 600 V. On a reversed-phase HPLC column (Vydac C4 column (4.6×250 mm)), Amy-GS was eluted at 15.40 min with the linear gradient system of 46—100% *i*-PrOH/0.1% TFA over 30 min (10.90 min for GS). ^{1}H NMR spectrum (DMSO-d_6) indicated the correct structure.

Preparation of DPPC Vesicles. DPPC (Sigma) (22 mg) was dissolved in $CHCl_3$–MeOH (1:1, v/v) (2 ml) and dried by breathing of nitrogen in a conical glass tube. The dried lipid was hydrated in 10 mM Hepes/100 mM NaCl (pH 7.5) (3 ml) with repeated vortex-mixing at 50 °C for 15 min. The suspension was sonicated at 50 °C for 30 min using a Branson Sonifier 250 and stood at room temperature for 1 h. This sample was used for the CD measurements. The carboxyfluorescein-entrapped DPPC vesicles were prepared by the same method as described above except that the dried lipid was hydrated with the same buffer (2 ml) containing 100 mM carboxyfluorescein (purchased from Kodak). After sonication, the mixture was subjected to gel-filtration through a Sephadex G-75 (1.5×20 cm) in the same buffer. The separated vesicles were utilized for leakage experiments.

The CF-Leakage Experiments. The CF-leakage experiments were carried out according to the procedure reported previously.[11] To 2 ml of 10 mM Hepes/100 mM NaCl (pH 7.5) was added 20 μl of DPPC vesicles containing 100 mM CF to give a final concentration of 100 μM phospholipid. Aliquots of the peptide solution were added to the vesicle mixture, and increase of the fluorescence intensity was recorded on a Hitachi 650-10S fluorescence spectrophotometer. The fluorescence intensity derived from 100% dye-release was determined by adding 10 μl of 20% Triton X-100 solution in water to the vesicle mixture. The percentage of CF-leakage caused by the peptides was evaluated by equation, $100(F-F_0)/(F_t-F_0)$, where F is the fluorescence intensity achieved by the peptides, F_0 and F_t are intensities without the peptides and of post-Triton X-100 treatment, respectively.

CD Measurements. CD spectra were recorded on a JASCO J-500A spectropolarimeter equipped with a TAIYO thermo supplier EZ-100 using a quartz cell of 1 mm pathlength. Spectra in MeOH and in the presence of DPPC vesicles (1 mM) were measured at a peptide concentration of 100 and 20 μM, respectively.

NMR Measurements. ^{1}H NMR spectra were measured in DMSO-d_6 solution on a JEOL JNM GX-400 spectrometer at 23.5—60 °C using tetramethylsilane as an internal reference. Sample concentration was 10 mM. Assignments were carried out by the homodecoupling method and the comparison with those of GS.

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2) Abbreviations used are according to IUPAC-IUB Commissions, *Eur. J. Biochem.*, **138**, 9 (1984). Other abbreviations: Amy, L-α-aminomyristic acid; CF, 5(6)-carboxyfluorescein; DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GS, gramicidin S; HOBt, 1-hydroxybenzotriazole; Pac, phenacyl; HONSu, *N*-hydroxysuccinimide; TFA, trifluoroacetic acid.

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