Antibacterial Activity of Amphipathic Basic Peptides with a Long Acyl Group and Their Interaction with Phospholipid Bilayers

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The antibacterial activities of $CH_3(CH_2)_nCO$-(Leu-Ala-Arg-Leu)$_3$-$NHCH_3$ (**1**, n=0; **2**, n=3; **3**, n=8; **4**, n=14) and their interaction with phospholipid bilayers were examined. Their order in the antibacterial activity was **1**>**2**>**3**>**4** against Gram-positive bacteria and **2**>**1**>**3**>**4** against Gram-negative bacteria: the activity of **4** was essentially negative. The CD study indicated that the α-helical content of **3** and **4** was higher than that of **1** and **2**. Peptides **1**—**3** effectively showed a dye-leakage ability, while the ability of **4** was weak. The DSC measurement indicated that the membrane-stabilizing effect of the acyl group in **4** fairly compensated for the destabilizing effect of the hydrophobic peptide moiety. These results imply that no antibacterial activity of **4** is correlated to a strong interaction of the acyl group in **4** with the acyl chain in DPPC to stabilize the membrane.

Through many studies of the structure-activity relationship and action mechanism of biologically active and amphipathic peptides, it has been pointed out that their amphipathy is often important in showing their activity.[1] Indeed, an amphipathic α-helical structure, which is induced by the interaction of peptides with membranes, has been found to be a common factor in melittin,[2] mastoparan,[3-5] cecropin,[6,7] magainin,[8,9] and so on. Furthermore, these peptides are also characterized by a high content of basic amino acids.

We previously reported that Ac-(Leu-Ala-Arg-Leu)$_3$-$NHCH_3$[10] (**1**), a basic model peptide designed from a common feature of the extension peptides of mitochondrial protein precursors, showed an inhibition of the import of precursors into mitochondria, a perturbation of the mitochondrial membrane and phospholipid bilayers, a fusogenic activity toward liposomes,[11,12] and an antibacterial activity against Gram-positive bacteria.[13] These abilities were found to be correlated to the α-helical amphipathy of **1**.[13] Furthermore, we found that an increase in the quantity of the Lys residue generally leads to a decrease in the antibacterial activity against Gram-positive bacteria and an increase in that against Gram-negative bacteria using various Lys-containing peptides.[14] In this connection, PM-B, a cyclic basic decapeptide possessing five basic moieties and a long acyl group, i.e., 6-methyloctanoyl or 6-methylheptanoyl, in the molecule (Fig. 1) shows a strongly antibacterial activity against Gram-negative bacteria. The acyl group was found to play an important role in the activity.[15] Since **1** and PM-B are basically the same peptide, the difference in the antimicrobial activity may come from that of the acyl group. We, therefore, speculated that the peptides with a longer acyl group than **1** would be effective against Gram-negative bacteria, because **1** showed little activity against Gram-negative bacteria.[13]

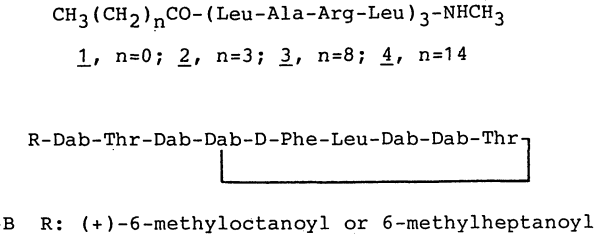

Fig. 1. Structures of acylpeptides **1**—**4** and PM-B.

In the present study, we designed and synthesized three acylpeptides with different alkyl chain-lengths, **2**—**4** (Fig. 1) and examined their antibacterial activity and the effect of the acyl groups on the interaction of the peptides with lipid bilayers.

Experimental

Thin-layer chromatography was carried out on Merck silica gel G with the following solvent systems: R_f^1, $CHCl_3$-MeOH-AcOH (50:10:2), R_f^2 n-BuOH-pyridine-AcOH-H_2O (4:1:1:2). Paper electrophoresis was performed on a Toyo Roshi No. 52 paper with the solvent system of HCOOH-AcOH-MeOH-H_2O (1:3:6:10, pH 1.8) for 4 h at 600 V; the mobility of the peptides is given relative to Arg (R_{Arg}). The optical rotation was measured with a Union high sensitivity polarimeter, PM-71. Amino acid analysis was performed on a Hitachi KLA-5 amino-acid analyzer after hydrolysis in 6 M HCl (1 M=1 mol dm^{-3}) in sealed tubes at 110 °C for 24 h.

Synthesis of Peptides. Valeryl-(Leu-Ala-Arg(Tos)-Leu)$_3$-$NHCH_3$ (5). To a stirred solution of H-(Leu-Ala-Arg-(Tos)-Leu)$_3$-$NHCH_3$·TFA[16] (216 mg, 0.11 mmol) in pyridine (15 ml) were added valeryl chloride (132 mg, 1.1 mmol) and Et_3N (0.015 ml, 0.11 mmol). Stirring was then continued at room temperature overnight. After subsequent evaporation, the residue was solidified with water, collected by filtration, and washed with water and then ether. The

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product was recrystallized from MeOH-ether; yield, 175 mg (82%); mp 147—151 °C; R_f^1, 0.71; $[\alpha]_D^{22}$ −3.4° (*c* 0.5, MeOH). Found: C, 54.77; H, 7.59; N, 15.51%. Calcd for $C_{90}H_{148}O_{19}N_{22}S_3 \cdot 2H_2O$: C, 54.75; H, 7.76; N, 15.61%.

Decanoyl-(Leu-Ala-Arg(Tos)-Leu)$_3$-NHCH$_3$ (**6**) and palmitoyl-(Leu-Ala-Arg(Tos)-Leu)$_3$-NHCH$_3$ (**7**) were similarly prepared. **6**: Yield, 91%; mp 136—139 °C; R_f^1 0.71; $[\alpha]_D^{22}$ −2.7° (*c* 0.5, MeOH). Found: C, 55.67; H, 7.82; N, 14.83%. Calcd for $C_{95}H_{158}O_{19}N_{22}S_3 \cdot 2H_2O$: C, 55.81; H, 7.99; N, 15.07%. **7**: Yield, 77%; mp 127—133 °C; R_f^1 0.76; $[\alpha]_D^{22}$ −3.7° (*c* 0.5, MeOH). Found: C, 56.97; H, 8.20; N, 14.30%. Calcd for $C_{101}H_{170}O_{19}N_{22}S_3 \cdot 2H_2O$: C, 56.99; H, 8.24; N, 14.48%.

Valeryl-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ · 3AcOH (2 · 3AcOH). Compound **5** (150 mg, 0.076 mmol) was treated with HF as has been described previoualy;[16] yield, 44 mg; R_f^2 0.77; R_{Arg} 0.89; amino-acid ratios, Leu 2.14, Ala 1.00, Arg 0.98. Compounds **3** and **4** were prepared in the same way from **6** and **7** respectively. **3**: R_f^2 0.62; R_{Arg} 0.84; amino-acid ratios, Leu 2.27, Ala 1.00, Arg 1.06. **4**: R_f^2 0.64; R_{Arg} 0.80; amino acid ratios, Leu 2.22, Ala 1.00, Arg 0.95.

CD Measurement of Peptides. The CD measurements were made on a JASCO J-40A spectropolarimeter using a cell with a pathlength of 1 mm, as has been previously described.[14] The spectra in a 20 mM Tris-HCl buffer (pH 7.4) were measured at a peptide concentration of 100 μM. DPPC and DPPC-DPPG (3 : 1) vesicles (a mixture of uni- and multilamellar vesicles) were obtained by sonication at 50 °C for 30 min in a 20 mM Tris-HCl buffer (pH 7.4). The peptides were dissolved at a peptide concentration of 10 μM in the 20 mM Tris-HCl buffer containing 0.9 mM DPPC or DPPC-DPPG (3 : 1) vesicles. All the measurements were performed at 25 °C. The CD spectra are expressed as mean residue ellipticities.

Dye-Leakage from Phospholipid Vesicles by the Action of Peptides. The DPPC and DPPC-DPPG (3 : 1) vesicles containing 5(6)-carboxyfluorescein were prepared and the phase-transition release experiments were performed as has been previously described.[14] Fluorescence measurements were made on a Hitachi 650-10S spectrofluorophotometer. Carboxyfluorescein was excited at 470 nm, and emission was recorded at 515 nm.

DSC Measurement. For the preparation of a sample, DPPC (7.34 mg, 10 μmol) was dissolved in $CHCl_3$ (2 ml), and the solvent was evaporated to dryness, first under nitrogen at 50 °C and then in a vacuum at room temperature overnight. Two ml of the Tes buffer (2 mM Tes/2 mM His/0.1 mM EDTA, pH 7.4) containing peptide in a desired concentration was added to the lipid film. The above mixture was subsequently vigorously vortexed for 15 min, during which time the sample was warmed and cooled repeatedly through the phase-transition temperature. The sample was then left to stand overnight around the phase-transition temperature. DSC measurements were made on a Seiko DSC-10 apparatus equipped an SSC thermal controller, using 60 μl of a DPPC multilamellar vesicle solution with or without the peptide and with a scanning rate of 0.5 °C per min from 20 °C up to 50 °C. The peak-mid point was defined as the T_m. The $\Delta T_{1/2}$ was calculated from the phase-transition start (T_s) and end (T_l) temperatures according to this equation: $\Delta T_{1/2}=(T_s+T_l)/2$. The enthalpy of transition was obtained from the area under the peak, which was calculated by weighing the paper cut-outs.

Antibacterial Assay. Antibacterial assays were carried out by the dilution method using a trypticase soy agar medium according to the procedure of Okonogi et al.[17]

Results and Discussion

Figure 2A shows the CD spectra of **1**—**4** in the 20 mM Tris-HCl buffer (pH 7.4). Compounds **1** and **2**

Table 1. Helix Content (%) of Acylpeptides[a]

Petide	Tris-HCl buffer	DPPC	DPPC-DPPG (3 : 1)
1	23	49	68
2	16	30	49
3	41	64	83
4	61	61	84

a) 100% helicity: $[\theta]_{222}^{MRW}=-3.1\times10^4$ deg cm^2 dmol^{-1} (Ref. 18).

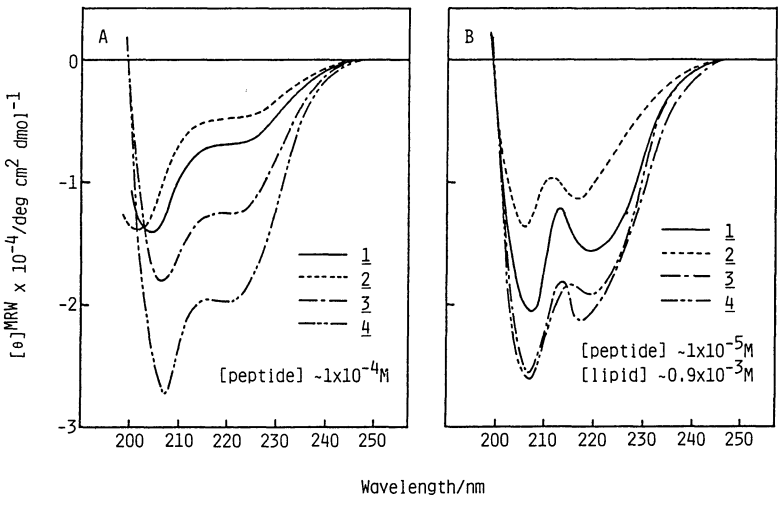

Fig. 2. CD spectra of acylpeptides in Tris-HCl buffer (pH 7.4) (A) and in the buffer in the presence of DPPC liposomes (B).

were found to take no α-helical conformation, or a negligible one, while the CD spectra of **3** and **4**, with a long acyl group, showed, to a considerable extent, the presence of an α-helical conformation (Table 1), indicating that self-aggregation induced by the longer acyl group in the buffer solution seems to result in an increase in the α-helical content.[13] In the presence of DPPC liposomes, all the peptides showed a typical α-helical pattern, with negative peaks around 208 and 222 nm, as is shown in Fig. 2B. Similar CD spectra were obtained in the cases of DPPC-DPPG (3 : 1) liposomes (data not shown). It is likely that the helical conformation was induced by the interaction of the peptides with lipid bilayers. The values of the negative ellipticity of **3** and **4** were greater than that of **1** (Table 1), indicating that the peptides with a longer acyl group tend to have a stronger capacity for α-helix formation. One might assume that no interaction of **4** with lipid bilayers takes place, because the α-helical content of **4** was the same in a buffer and in the presence of liposomes. However, this assumption is obviated by the facts that a homolog of **1** was present in the aggregated state in the buffer and in the monomer state in lipid bilayers, as was shown in a previous paper,[19] and that the nature of the phospholipid bilayers changed in the presence of **4**, as will be described later in the DSC study. It is interesting that **2** shows the smallest α-helical content, although we can not explain this phenomenon at present. The helical contents of **1**—**4** in the DPPC-DPPG (3 : 1) liposomes were higher than those in the DPPC liposomes. These results suggest that an electrostatic interaction of the peptides with the lipid-bilayer interface also plays a significant role in the formation of the α-helical structure.

Profiles of the carboxyfluorescein leakage from the DPPC and DPPC-DPPG (3 : 1) vesicles by the action of **1**—**4** are shown in Fig. 3. Peptides **1**—**3** effectively leaked the dye at the phase-transition temperature of the DPPC vesicles in the order of **1>3>2**, while the degree of the leakage by **4** was about a half of those by **1**—**3**, as may be seen in Fig. 3A. It is noteworthy that **3** and **4** slightly but distinctly induced the dye-leakage at a much lower temperature than the phase-transition temperature, but no such phenomenon was observed in the cases of **1** and **2**. Similar leakage profiles were obtained in acidic DPPC-DPPG (3 : 1) vesicles (Fig. 3B). Although the leakage ability of **3** and **4** at low temperatures increased fairly much, no rapid increase in the dye-leakage at the phase-transition temperature was observed. These observations suggest that the interaction of **3** and **4** with lipid

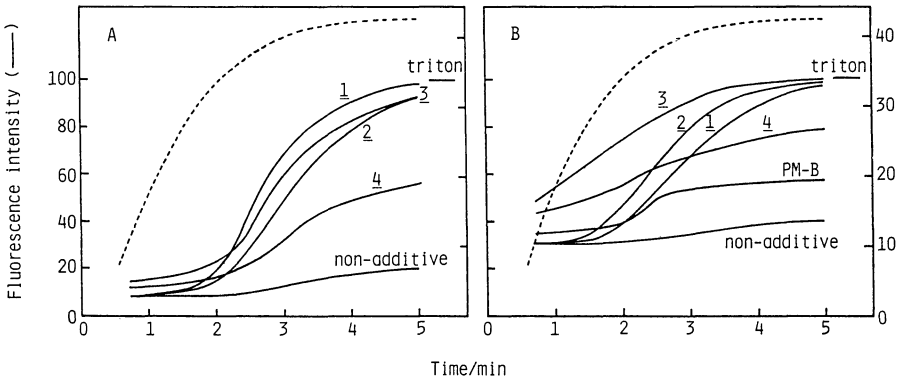

Fig. 3. Profiles of carboxyfluorescein leakage from DPPC vesicles (A) and DPPC-DPPG (3 : 1) vesicles (B) by acylpeptides. (A); Hepes buffer (pH 7.6), 0—42 °C, [peptide]=0.2 μg ml^{-1}, [lipid]=70 μM. (B); Hepes buffer (pH 7.6), 0—42 °C, [peptide]=0.5 μg ml^{-1} or [PM-B]=1 μg ml^{-1}, [lipid]=70 μM.

Table 2. Antibacterial Activity of Acylpeptides, PM-B and GS[a]

Organism	Minimum inhibitory concentration/μg ml^{-1}					
	1	**2**	**3**	**4**	PM-B	GS
S. aureus FDA 209P	6.25	6.25	50	>100	>50	3.13
S. aureus 1840	6.25	12.5	50	>100	>50	3.13
S. subtilis PC 219	3.13	6.25	25	100	6.25	3.13
E. coli O-111	>100	100	>100	>100	1.56	>100
S. flexneri EW-10	50	25	100	>100	0.39	3.13
S. sonnei EW-33	>100	25	>100	>100	0.78	100
P. vulgaris IFO3988	>100	>100	>100	>100	>50	>100

a) Medium: trypticase soy agar. Inoculum: 5 μl of a bacterial suspension containing 10^7 CFU ml^{-1}.

bilayers is different from that of **1** and **2**. Under the conditions tested, PM-B showed a weak dye-leakage ability on the whole, as is shown in Fig. 3B. PM-B, like **3** and **4**, leaked the dye at low temperatures but a clear change in the leakage profile at the phase-transition was observed.

The antibacterial activities of **1**—**4**, PM-B and GS are shown in Table 2. GS, which has a cationic amphipathic structure with an antiparallel β-sheet and a β-turn, is strongly active against Gram-positive bacteria. Peptide **1** showed an activity against Gram-positive bacteria similar to that of GS; the order of the activity of the acylpeptides was **1**>**2**>**3**>**4**. Peptide **4** showed no activity against Gram-positive bacteria, however. With respect to Gram-negative bacteria, only **2** showed something of an activity against *S. flexneri* and *S. sonnei*. The other peptides showed very weak or no activity. It should be noted that the antibacterial activity of PM-B is very strong against Gram-negative bacteria. Chihara et al. reported that the enzymatic removal of the aliphatic side chain from colistin caused it to lose its antibacterial activity,[20] showing that not only the basic domain in the peptide but also the hydrophobic domain is important in the activity. Among the PM-family analogs containing acetyl to stearoyl groups, the greatest activity against Gram-negative bacteria was observed with the octanoyl derivative.[15] In the present study, the valeryl derivative **2** showed the strongest activity among the model peptides against Gram-negative bacteria, indicating that the appropriate chain length of acyl groups is effective for the antibacterial activity, though it is unclear just why the valeryl group is effective. The activity may be related to the slightly unusual behavior of **2** in CD and to the lack of any release of dye at temperatures below the phase-transition of lipid, as has been described above. In the cases of **3** and **4**, which have decanoyl and palmitoyl groups respectively, these long acyl groups may strongly interact with the lipid membrane, in view of the α-helical formation by CD and the release of dye at low temperatures shown by the leakage experiment. In particular, it is likely that the palmitoyl group in **4** penetrates into the lipid bilayers and acts as a constituent of the bilayers to make the bilayers stable, because the leakage and antibacterial abilities of **4** were weak in spite of its strong interaction with the lipid bilayers.

In order to confirm the above view, we compared the effects of **1** and **4** on the thermotropic properties of DPPC by means of DSC measurements. The results are shown in Fig. 4. Two tracings (4A and 4D) were obtained with only DPPC vesicles dispersed in the Tes buffer (pH 7.4). The tracings marked 4B and 4C were obtained in the presence of **1** and **4** respectively in a molar ratio of 70 : 1 of DPPC to the peptide, those in 4E and 4F being gained in the presence of an increasing amount of the peptides (molar ratio of DPPC to the peptide, 10 : 1). In all cases except F, the T_m of the main peak remained essentially unaltered, although $\Delta T_{1/2}$ became broader at high peptide-to-lipid ratios and with an increase in the length of the acyl chain of the peptides, as is shown in Table 3. The calculated ΔH value for the endothermic peak in each case is also shown in Table 3. Neither peptide caused any perturbation of the lipid-molecule packing of the bulk lipid, because there was no decrease or increase in T_m.

A charge interaction between the hydrophilic part in the peptide and the head group in phospholipid stabilizes membranes, while a hydrophobic interaction between the hydrophobic part in peptide and the acyl chain in phospholipid perturbs them.[21] With respect to **1** a slight increase and a slight decrease in ΔH were observed in molar ratios of 70 : 1 and 10 : 1 respectively of DPPC to **1**. This result indicates that the charge interaction between **1** and the DPPC head group acted to stabilize the membrane structure, without any change of T_m in low peptide-to-lipid ratios, while both the charge and hydrophobic interactions caused a slight destabilization in the membrane in high peptide-to-lipid ratios. With respect to **4**, the ΔH was slightly reduced at 70 : 1 of DPPC to **4**, and there was no peak at 10 : 1. This observation implyies that, although the acyl group in **4** strongly interacted with the acyl chain in DPPC to show a stabilizing effect for the membrane, the membrane seems to be fluid at high peptide-to-lipid ratios, resulting in showing no phase-transition temperature.

Papahadjopoulos et al. reported that the embedded

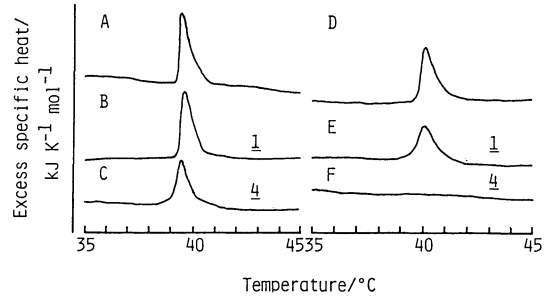

Fig. 4. DSC of DPPC in the absence and presence of acylpeptides. (B) and (C); [peptide] : [lipid]=1 : 70. (E) and (F); [peptide] : [lipid]=1 : 10.

Table 3. Effects of **1** and **4** on Thermotropic Property of DPPC

	T_m/°C	$\Delta T_{1/2}$/°C	ΔH/kJ mol^{-1}
[DPPC] : [peptide]=70 : 1			
Without peptide	39.5	1.0	31.8
1	39.6	1.3	34.7
4	39.5	1.6	29.3
[DPPC] : [peptide]=10 : 1			
Without peptide	40.1	1.2	31.0
1	40.1	1.6	26.8
4	No peak appeared		

or penetrated proteins or polypeptides in lipid bilayers caused an enthalpy change in the transition and fluidization of bilayers, but did not change the transition temperature appreciably.[21] Therefore, it is likely that **1** and **4** also interacted with the hydrophobic part of the membrane by penetration. This is consistent with the previous findings that the hydrophilic side of **1** in the helix interacts with the acidic moiety of phospholipid in the membrane and that the hydrophobic side is immersed into the membrane shallowly and horizontally.[13,21] The lack of antibacterial activity of **4** may be correlated to the stabilizing effect for the membrane induced by the penetration of the acyl group into the membrane to compensate for the destabilizing effect of the hydrophobic part of the peptide in the helix.

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10) Abbreviations according to the IUPAC-IUB Commission, *Eur. J. Biochem.*, **138**, 9 (1984), are used throughout. Other abbreviations: Dab, 2,3-diaminobutyric acid; DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DPPG, dipalmitoyl-DL-α-phosphatidylglycerol; DSC, differential scanning calorimetry; Et_3N, triethylamine; GS, gramicidin S; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PM-B, polymyxin B; Tes, 2-[tris(hydroxymethyl)methylamino]-ethanesulfonic acid. All amino acid symbols except D-Phe denote the L-configuration.
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