The Effect of D-Amino Acid-Containing Basic Peptides with Different Hydrophobicity on the Antimicrobial and Cytotoxic Activity

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In order to investigate the structure-activity relationships between the hydrophobicity, differences in the number of positive charged amino acids and D-amino acids as well as their action mechanism with different bio-membranes, we designed and synthesized two sets of 12-mer model peptides originating from an amphiphilic antimicrobial model peptide and a D-amino acid-containing non-amphiphilic peptide. The introduction of D-amino acid in peptides led not only to decreases in the hydrophobicity, helical content, and hemolytic activity, but to an increase in the antimicrobial activity against Gram-positive and -negative bacteria. The relative peptide hydrophobicity was estimated in terms of the peptide elution time, expressed by linear trifluoroacetic acid (TFA)–water to TFA–acetonitrile gradients on C18 reverse-phase high-performance liquid chromatography (RP-HPLC). The retention behavior of model peptides on RP-HPLC is correlated with the activity and selectivity for mammalian cells, Gram-positive and -negative bacteria: The α-helical amphiphilic structure is required for cytolytic ability against mammalian cells, but is not necessary for bacterial activity. Additionally, the peptides have different hydrophobicity regions in which they show their optimum activity against both bacteria and erythrocytes. In the highest hydrophobisity region the peptides display high levels of hemolytic activity. At medium hydrophobicity levels, the peptides are able to display to high levels of activity against Gram-positive bacteria. In the lowest hydrophobisity regions the peptides are able to display high levels of activity against Gram-negative bacteria. This may be useful in designing clinically effective antimicrobial peptides with highly potent activity and high bacterial selectivity.

Cytotoxic amphiphilic α-helical peptides are widely found in natural sources, some of which function as cell-lytic agents and some of which function as antibacterial ones. Infectious diseases are bringing about serious climical phenomena in present-day therapy, mainly as the result of an increase in antibiotic-resistant pathogens.[1—3] To combat with those, various antimicrobial peptides have been expected to be used as new antibiotic drugs.[4,5] Because the antimicrobial activity induced by the peptides is not correlated to the lysis of the bacterial cell wall by enzyme inhibitions, the bacteria might not easily acquire resistance to such drugs.[1] Recent studies have also shown that some peptides can penetrate the cell membrane of bacteria and then inhibit cellular functions, resulting in rapid cell death.[6,7] However, there are some proposals for the creation of peptide antibiotics, for instance, with strong antimicrobial activity, but low cytotoxity and stability, in body fluids. Antimicrobial peptides differ markedly in length, sequence and structure, but share two common elements: They are generally polycationic and their active structure is normally amphiphilic.

Studies on the peptide–lipid interaction of cytolytic polypeptides have revealed that an amphiphilic α-helical structure, hydrophobicity, and positive charge are indispensable for their cytolytic activity. Dathe et al.[8] have reported that changes in the hydrophobicity, hydrophobic moment, and angle subtended by charged residues would modulate the antibacterial and hemolytic activities of amphiphilic helical peptides, except for the low hydrophobicity at which the peptides become inactive. However, recent investigations using D-amino acid-substituted analogs of highly potent cytolytic helical peptides, such as pardaxin and melittin, and cationic amphiphilic model peptides containing D-amino acids have revealed that the α-helical amphiphilic structure is required for cytolytic ability against mammalian cells, but is not necessary for bacterial cell lysis; the antimicrobial activity of peptides may be determined only by the balance between the hydrophobicity and the positive charge of amino acids.[9—11]

We have previously shown that a 12 mer-cationic amphiphilic α-helical model peptide 4_3, possesses the potential for cytolytic activities, such as hemolysis and antimicrobial activity against Gram-positive bacteria.[13] The antimicrobial activity of 4_3 was correlated with its helix-forming ability from the antimicrobial activities of its proline-containing analogs in the presence of lipid bilayers.[14] These results conflict with those of recent investigations using the D-amino acid-containing peptides described above.[9,10] Thus, in order to reinvestigate the structure-activity relationship among hydrophobicity, different numbers of positive charged ami-

no acids, and D-amino acids and their action mechanisms with different bio-membranes, we designed and synthesized two sets of 12-mer model peptides originated from an amphiphilic antimicrobial model peptide and a D-amino acid-containing non-amphiphilic peptide. The relative peptide hydrophobicity was estimated in terms of the peptide elution time, expressed by linear trifluoroacetic acid (TFA)–water to TFA–acetonitrile gradients on C18 reverse-phase high-performance liquid chromatography (RP-HPLC). Their interaction mode with membranes was examined through circular dichroism spectroscopy and measurements of the hemolytic and antimicrobial activities.

Experimental

Materials. 9-Fluorenylmethoxycarbonyl (Fmoc)-amino acid–polyethylene glycol–polystyrene (PEG-PS) resin was purchased from Millipore (Tokyo, Japan) and Fmoc amino acids were obtained from Watanabe Chem. Ind., Ltd (Hiroshima, Japan). Egg-yolk phosphatidylcholine (egg PC) and egg-yolk phosphatidylglycerol (egg PG) were purchased from Sigma Chemical Co. (St. Louis, USA). Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemicals (Osaka, Japan). These materials were used as received.

Peptide Synthesis. Peptides were synthesized by the Fmoc strategy based on a solid-phase technique starting from Fmoc–PAL–PEG resin (0.75 g) using a Perseptive 9050 automatic peptide synthesizer, as previously reported.[15] The resin-bound peptides were cleaved from the resin by trifluoroacetic acid (TFA)-containing thioanisole and *m*-cresol (10 : 1 : 0.15) for 1.5 h at room temperature. These crude peptides (approximately 200 mg) were purified by gel filtration (Sephadex G 25, Pharmacia, Sweden) and preparative RP-HPLC (20×250 mm, YMC-Pack ODS-AP, Kyoto, Japan) to obtain approximately 50—170 mg (yield, 25—65%). The purity of the peptides was checked by means of analytical RP-HPLC (4.6×250 mm, Cosmosil 5C18 -AR-300; Nacalai Tesque, Kyoto, Japan), amino acid analysis (JASCO 851-AS, Tokyo, Japan), and FAB mass spectroscopy (JEOL JMX-HX 100, Tokyo, Japan). The peptide concentrations of sample solutions prepared for CD experiments were determined by amino acid analysis.

Peptide Elution Time on RP-HPLC. The peptide elution time was determined by a linear 0.1% TFA–water (solvent A) to 0.1% TFA–acetonitrile (solvent B) gradient on analytical RP-HPLC (4.6×250 mm, Cosmosil 5C18 -AR-300; Nakalai Tesque, Kyoto, Japan). The peptides were dissolved in methanol (1 mg ml^{-1}) and 50 μl of the solutions was injected in a loop of 200 μl in a Hitachi -6200 intelligent pump (Hitachi, Tokyo, Japan). Solvent A was injected for over 5 min, and then 0—20% B in 5 min and 20—70% B in 50 min with a linear gradient at a flow rate of 0.85 ml min^{-1}. Elution peaks were detected at 230 nm on a Hitachi-4200 UV-vis detector. The relative retention times of peptides were determined by applying a mixed solution of all peptides on the analytical RP-HPLC described above.

Preparation of Liposome. Aliquots of a lipid (egg PC or egg PC/eggPG = 3 : 1 mixture, 4.4 mg) solution in chloroform/methanol (9 : 1) were placed in a round-bottom flask. After evaporating the solvent, a residual film was dried under vacuum over night. The lipid film was hydrated with a TES buffer (5 mM (M = mol dm^{-3})) TES/100 mM NaCl, pH 7.4) solution. The suspension was vortexed for 10 min, followed by sonication in ice water with nitrogen flowing for 10 min (×3) using a titanium tip sonicater.

CD Spectroscopy. The CD spectra of the peptides were measured by a JASCO J-600 spectrometer (JASCO, Tokyo, Japan) using a quartz cell with 1 mm path length. The sample solutions of peptides were prepared at concentrations of 40—50 μM in 50% 2, 2,2-trifluoroethanol (TFE) solution, 25 mM sodium dodecyl sulfate (SDS), and in the presence and absence of neutral (egg-PC) or acidic (egg PC/PG = 3/1) liposomes. Lipid concentrations were kept at 1 mM. The α-helical contents were calculated from the following equation:

$$\mathrm{fh} = ([\theta]_{222} - [\theta]^{0}_{222})/[\theta]^{100}_{222},$$

where $[\theta]_{222}$ is the mean residue ellipticity experimentally observed, and $[\theta]^{0}_{222}$ and $[\theta]^{100}_{222}$ are those of 0% and 100% helical contents at 222 nm theoretically estimated to be −2000 and −30000 deg cm^2 mol^{-1}, respectively.[16]

Hemolytic Assay. The hemolytic activities of the peptides were determined using human red blood cells (RBCs). The RBCs were collected from citric acid-treated blood by centrifugation (at 2500 rpm for 5 min) and washed four times with phosphate buffer saline (PBS: 10 mM phosphate buffer/150 mM NaCl at pH 7.4), to remove plasma and buffy coat. Each suspension of 0.3% hematocrit in PBS with or without peptides was incubated for 90 min at 25 °C. Hemolysis was expressed as the hemoglobin content (absorbance at 542 nm) of the supernatant after centrifugation at 2500 rpm for 5 min. Zero hemolysis (blank) was assumed to be the concentration of hemoglobin released in the absence of the peptides, and 100% hemolysis was assumed to be the concentration of hemoglobin released in the presence of the added Triton® X-100 to the each sample. The final concentration of Triton® X-100 was about 0.3%.

Antimicrobial Assay. An antimicrobial assay was performed using an agar-plate dilution method according to the Standard Method of The Japan Society of Chemotherapy. Peptide solutions at a proper concentration in water (1 ml), prepared by 2-fold dilution, were solidified with Mueller Hinton agar (MHA, 9 ml). A bacterial solution of 10^6 colony-forming units (CFU)/ml in Mueller Hinton broth (MHB, 5 μl) was inoculated on the agar plate described above and cultivated for 18—20 h. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of peptide that completely inhibits growth of the test organisms.

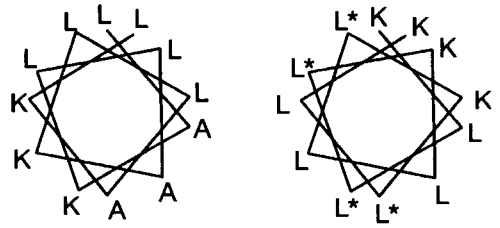
The agar-plate assay method is considered to give MIC values lower by a factor of 2—5, due to the adsorption of peptides to the agar.[17] In order to ascertain it in the present experiment, a broth microdilution method using lactose broth (LB) or Mueller Hinton broth (MHB) was examined for [D]L^{3,4,8,10}-K$_4$L$_8$ against *B. subtilis* PCI 219, *S. aureus* FDA 209P, *E Coli.* NIHJ JC-2, *A. calcoaceticus* ATCC 13006, and *P. aeruginosa* U-31 as described in the literature.[10] Sterile 96-well plates (MICROPLATE, Iwaki Glass, Chiba, Japan) were used in a final volume of 100 μl as follows. Aliquots (50 μl) of a suspension containing bacteria at a concentration of 10^6 CFU ml^{-1} LB or MHB were added to 50 μl of sterilized water, containing the peptide in 2-fold serial dilutions. The MIC was determined by measuring the absorbance at 490 nm with a Microplate reader (Molecular Devices), following incubation for 18—20 h at 37 °C.

Results

Peptide Design. In order to investigate the influence of D-amino acid on biological activity, two sets of 12-mer model peptides, derived from $4^{\prime}_{3}$ (**1**) and [D]L^{3,4,8,10}-K$_4$L$_8$ (**7**) were designed and synthesized.[10,13] The two mother peptides consist of amino acids (Leu, Ala, and Lys) with a high potential for forming an α-helical structure. The sequences, numbers of D-amino acids, and net positive charges of the mother and

designed model peptides are listed in Table 1. Helical wheels of typical model peptides are shown in Fig. 1. The $4'_3$ series model peptides containing different D-amino acids consist of 12 residues with repeats of a tetrapeptide (LAKL) with their N- and C-termini blocked with acetyl- and amide-groups, respectively. Peptides [D] A^6-$4'_3$ (**2**) and [D] $A^{2,6,10}$-$4'_3$ (**3**) were replaced at the positions of L-Ala in $4'_3$ with one and three D-Ala residues, respectively. The peptides K^4, [D] $A^{2,6}K^{10}$-$4'_3$ (**5**) and $K^{4,5}$, [D] $A^{2,6}K^{10}$-$4'_3$ (**6**) were also replaced with one D-Ala and one or two L-Leu residues in the sequence of **3** with D-Lys and one or two L-Lys, respectively. Except for **6**, all peptides can have amphiphilic properties when they take an α-helical structure. In order to investigate the charge effect on the biophysical properties, we also synthesized [DesAC]-$4'_3$ (**4**), which has no N-terminal acetyl group, resulting in a +4 net charge.

[D]$L^{3,4,8,10}$-K_4L_8 (**7**) containing four D-amino acids has been designed and synthesized by Oren et al.[10] It also consists of 12 residues, and its C-terminal is amidated, but it's N-terminal is free. This peptide exhibits high antimicrobial activity against both Gram-positive and Gram-negative bacteria but its hemolytic activity is very low. If the peptide can take an α-helical structure in specific surroundings, like bio-membrane surfaces, it would have an amphiphilic property (see Fig. 1). Thus, in order to further confirm whether or not the amphiphilic helical structure needs to exhibit antimicrobial activity, for the present study, [D]$L^{3,8,10}K^4$-K_4L_8 (**8**) was newly designed. It is composed of similar D-amino acids with the same ratio of lysine-to-leucine residues as **7**, but the sequence is different. The new peptide makes no amphiphilic structure, even though it could take an α-helical structure in a specific surrounding.



$4'_3$ [D]$L^{3,4,8,10}K_4L_8$

Fig. 1. Representation of helical wheels of typical model peptides, $4'_3$ and [D]$L^{3,4,8,10}$-K_4L_8. That of [D]$L^{3,4,8,10}$-K_4L_8 is based on the assumption that it would take α-helical structure. The asterisk (*) represents the substituted D-amino acid residue.

Hydrophobic Character of $4'_3$ and [D]$L^{3,4,8,10}$-K_4L_8 Series Model Peptides. In order to determine the relative hydrophobicity of the peptides used in this study containing D-amino acids, RP-HPLC experiments on a C18 column were performed, using a linear gradient of 20—70% acetonitrile in water containing 0.1% TFA. The retention times of the peptides are listed in Table 1. The retention times of the D-amino acid substituted analogues of $4'_3$ decrease with an increase is D-amino acids, and are shorter than that of the parent peptide, reflecting the more hydrophilic character of the analogs. A decrease in the retention time by introducing D-amino acid may be attributable to a conformational difference reflecting the decrease in the α-helical structure, as shown in the next CD study, because the mean hydrophobicity values of the peptides estimated from the hydrophobicity scale for each amino acid residue is equivalent.[18] In relation to this, a study on the retention times of two sets of amphiphilic and non-amphiphilic peptides, which contain seven to twenty amino acids consisting of Lys, Ala, and Leu, has shown that the secondary structure induction increases the retention time. The minimum length with a secondary structural effect exists between 9 and 13 residues.[19] Krause and co-workers[20] have also reported on a similar observation using 18 mer D-amino acid-containing cationic α-helical peptides.

The introduction of charged amino acids also led to a decrease in the retention time. However, the retention times did not simply depend on the charged residue numbers. When comparing peptides **3** (3 net charges) and **5** (5 net charges) with a difference of two charges, the difference in the retention times was about 13 min. On the other hand, when comparing peptides **6** (6 net charges) and **7** (5 net charges) with a difference of one charge, the former was eluted in a relatively shorter retention time than the latter, and the difference in the retention times was about a 10 min. The retention time of peptide **4** (4 net charges) is slower than that of paptide **1** (3 net charges), however, a 15 min difference in retention time. These differences in the retention times may

Table 1. Sequences, Numbers of Substituted D-Amino Acid,[a] Net Charges and Retention Times (r.t)[b] on HPLC of Model Peptides

Peptide	Sequence			D-AA	Net charge	r.t.[b]	(Δr.t.)
4_3[c]	Ac-LARL	LARL	LARL-$NHCH_3$	0	+3		
$4'_3$	Ac-LAKL	LAKL	LAKL-NH_2 (**1**)	0	+3	52.2	(0)
[D] A^6-$4'_3$	Ac-LAKL	*L*AKL	LAKL-NH_2 (**2**)	1	+3	50.0	(−2.2)
[D] $A^{2,6,10}$-$4'_3$	Ac-*L*AKL	*L*AKL	*L*AKL-NH_2 (**3**)	3	+3	45.8	(−6.4)
[desAc] $4'_3$	LAKL	LAKL	LAKL-NH_2 (**4**)	0	+4	37.0	(−15.0)
K,[4] [D] $A^{2,6}K^{10}$-$4'_3$	Ac-*L*AKK	*L*AKL	L*K*KL-NH_2 (**5**)	3	+5	32.8	(−19.4)
K,[4,5] [D] $A^{2,6}K^{10}$-$4'_3$	Ac-*L*AKK	*K*AKL	L*K*KL-NH_2 (**6**)	3	+6	22.5	(−29.7)
[D]$L^{3,4,8,10}$-K_4L_8[d]	KL*LL*	KLL*L*	K*L*LK-NH_2 (**7**)	4	+5	37.1	(−15.1)
[D]$L^{3,8,10}K^4$-K_4L_8	KL*LK*	LKL*L*	K*L*LL-NH_2 (**8**)	4	+5	39.0	(−13.2)

a) Italic residues represent D-amino acid. b) These were obtained using C18 RP-HPLC at linear gradient of acetonitrile–H_2O containing 0.1% TFA. c) Lee et al.[13] d) Oren et al.[10]

also reflect the conformational affinity of the peptides upon binding to the hydrophobic stationary phase of the HPLC column, as described above.[19,20]

CD Spectroscopy. To investigate the conformational properties of these peptides, we measured the CD spectra in various media (Fig. 2 and Table 2). In a buffer solution, only **1** showed slightly an α-helical structure, but other peptides adopted random structures, as anticipated. In a helix-inducing 50% trifluoroethanol (TFE) solution (Fig. 2a), **1** showed a typical α-helical spectral pattern; negative ellipticity at 206 and 221 nm and a positive CD band below 200 nm. The introduction of D-amino acid(s) or D-(and/or L) Lys residues leads to a decrease in the helical contents in proportion to the increase in the numbers of D-amino acids (Table 2). The extent of the decreasing helical contents depends on their substitution position: the introduction of D-Ala(s) into the hydrophobic region or Lys into the hydrophilic region of an amphiphilic α-helical structure is gradual, but the introduction of D-amino acids into the central parts is more effective. Almost similar results as for TFE were obtained for peptides in small unilamellar egg-PC/egg PG (3 : 1) liposomes and sodium dodecyl sulfate (SDS) micelles (Table 2). However, in the presence of neutral liposome (egg-PC), the increase in charged residue numbers in $4'_3$ series peptides results in a drastic decrease of their secondary structure. It should be mentioned that the helical contents of peptides in the presence of acidic liposomes (egg-PC : egg-PG = 3 : 1) are higher than those in TFE or the neutral liposome, this being indicative of the importance of the charge interaction between peptides and lipids. Interestingly, in the presence of acidic liposomes and 25 mM SDS micelles, compounds **2** and **3** adopted almost the same and relatively high helical contents (65—75%), but in neutral liposomes and TFE they attained considerably different ones. The reduction of the helical structure by the substitution of D-amino acids near to the N- and C-termini might be recovered with their charge interaction between cationic Lys residues and the anionic lipid head group.

Peptide **4**, identical to peptide **1**, except that it is not acetylated at the N-terminus, was less helical than peptide **1**

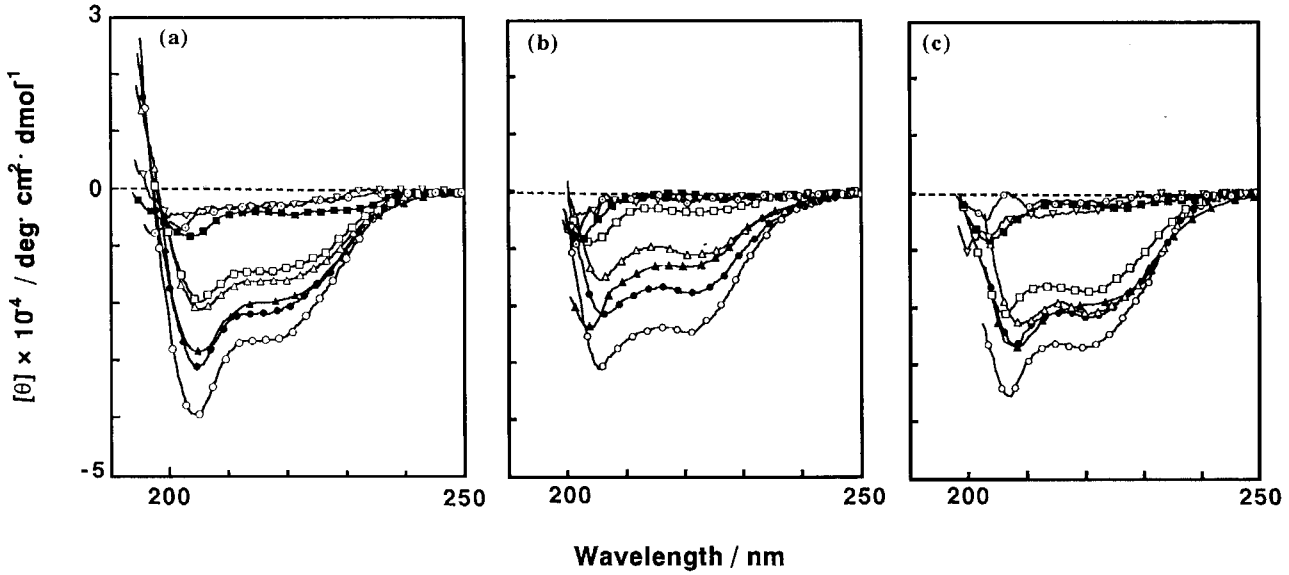


Fig. 2. CD spectra of model peptides in 50% TFE (a), in the presence of egg PC liposomes (b), and egg PC–egg PG (3 : 1) liposome (c). Designations are as follows: ○, **1**; ●, **2**; △, **3**; ▲, **4**; □, **5**; ■, **6**; ▽, **7**; ⊙, **8**.

Table 2. Helical Contents of Model Peptides in Various Media

Peptide	Helical content (%)[a]				
	50% TFE	Buffer	PC liposome	PC/PG liposome[b]	SDS micelle
$4'_3$	71.8	22.7	73.7	83.5	89.7
[D] A^6-$4'_3$	56.1	5.1	51.2	66.8	72.9
[D] $A^{2,6,10}$-$4'_3$	42.6	0.0	30.6	66.0	68.4
[desAc] $4'_3$	21.0	6.3	37.0	57.6	—
K^4[D] $A^{2,6}K^{10}$-$4'_3$	35.9	0.0	4.5	49.8	60.6
$K^{4,5}$[D] $A^{2,6}K^{10}$-$4'_3$	6.8	0.0	0.0	0.0	0.0
[D]$L^{3,4,8,10}$-K_4L_8	0.0	0.0	0.0	0.0	15.0
[D]$L^{3,8,10}K^4$-K_4L_8	0.0	0.0	0.0	0.0	0.0

Peptide concentrations were 50 μM. Concentrations of lipid and SDS were 1 mM and 25 mM, respectively. a) Helical contents were caluculated based on Chen et al.[16] (see Experimental Procedure). b) Molar ratio of egg PC : egg PG was 3 : 1.

in various media. This might be due to a capping effect of the N-terminus; in fact, in a number of cases the helicity of model peptides increases.[21]

However, both **7** and **8** adopted random structures in all media, except for the former in an SDS micellar solution, in which it seemed to take an α-helical structure, with a slight content. The former peptide can have an amphiphilic property, if it takes the α-helical structure. The present results indicate that the **7** peptide probably makes no amphiphilic structure, even in a lipid surrounding.

Antimicrobial and Hemolytic Activities. The peptides were assayed for their antimicrobial activity against Gram-positive and Gram-negative bacteria by an agar-plate dilution method. The results are listed in terms of the minimum inhibitory concentration (MIC) in Table 3. An antibiotic (ampicillin) and a cationic cyclic peptide (gramicidin S) served as controls. There was no marked difference between $4'_3$ and mono- or tri-D-amino acid substituted -$4'_3$ against Gram-positive bacteria, or rather the activity slightly increased with an increase in the D-amino acid. It is noted that $4'_3$ shows no activity against Gram-negative bacteria, but the introduction of D-amino acid(s) into $4'_3$ led to an increase in activity against Gram-negative bacteria. These results were completely compatible with those of **7**.[10] Interestingly, the introduction of Lys residue(s) into **3** strongly reduced the activity. Peptides **7** and **8** had nearly the same activity for Gram-positive, but the latter peptide was more active for Gram-negative bacteria than the former one. These facts indicate that the location of basic residues in the sequence results in a significant effect on the activity and selectivity.

The level of antimicrobial activity has been found to be highly dependent on the type of assay method used; agar-plate assays severely underestimate the activity against Gram-negative bacteria in comparison with a liquid-broth assay.[17,22] In order to confirm this, three different assay methods were performed for **8**: An agar dilution plate method and two liquid broth microdilution methods; one using Mueller Hinton broth (MHB) and one using lactose broth (LB). The results listed in Table 4 indicate that the agar plate was much higher in values of MIC for Gram-negative bacteria than the two liquid-broth methods. Interestingly, different liquid broths also exhibited different MIC values; the medium containing lactose had the lowest MIC. The assay method which we used, utilising a lactose medium, was also 2—10 times as severe as that discussed in the literature.[10] The differences in antimicrobial activity may come from the different species of organisms used. These results indicate that 4_3 and its related peptides are also active against Gram-negative bacteria,[13] and that **3** shows especially potent activity levels.

The dose-response curves of hemolytic activity for the model peptides are shown in Fig. 3. The order of hemolytic activity of $4'_3$ series peptides was found to decrease in proportion to the increase of D-amino acid or Lys residues. The abilities of the $4'_3$ series peptides were parallel to the magnitude of hydrophobicity estimated by RP-HPLC retention time as well as the α-helicity of peptides. Peptide **7** exhibited very low hemolytic activity, as reported by Oren and coworkers,[10] and **8** possessed less activity for hemolysis, but higher activity against Gram-negative bacteria than **7**.

Discussion

In order to experimentally determine the hydrophobicity of peptide antibiotics, RP-HPLC has often been employed.[11,15,23—25] The relative amphiphilicity determined by RP-HPLC is correlated with the hydrophobic interaction occurring during biological processes. In some circumstances, peptides show a good correlation between the retention time and the hemolytic activity,[15,23] and in some circumstances an inverse correlation between the retention time and the antimicrobial activity for a Gram-negative bacterium,

Table 3. Minimum Inhibitory Concentration (MIC) of Model Peptides on Gram-Positive and Gram-Negative Bacteria

Organism	MIC ($\mu g\ ml^{-1}$)									
	1[a)]	**2**	**3**	**4**	**5**	**6**	**7**	**8**	GS	Ampicillin
Staphylococcus aureus FDA 209P	6.25	3.13	3.13	6.25	>100	>100	25	50	3.13	<0.20
S. aureus 1840	6.25	6.25	6.25	12.5	>100	>100	50	100	3.13	1.56
S. epidermidis IFO 12228	12.5	3.13	3.13	6.25	50	>100	12.5	12.5	3.13	0.78
Streptococcus pyogenes C-203	25	12.5	3.13	50	100	>100	25	50	6.25	<0.20
S. pneumoniae TypeIII	>100	>100	100	>100	>100	>100	>100	>100	12.5	<0.20
Enterococcus faecalis LS-101	50	12.5	6.25	12.5	50	>100	12.5	25	6.25	0.78
E. faecium EFMY-28	25	12.5	12.5	50	100	>100	12.5	50	12.5	<0.20
E. avium EAY-30	>100	50	25	>100	100	>100	50	100	12.5	1.56
Bacillus subtilis PCI 219	6.25	3.13	3.13	3.13	6.25	>100	6.25	3.13	1.56	<0.20
Escherichia coli NIHJ JC-2	>100	>100	100	100	100	>100	50	25	>100	6.25
Shigella flexneri EW-10	>100	>100	50	50	100	>100	50	25	>100	1.56
Citrobacter freundii IFO 12681	>100	>100	100	100	>100	>100	50	50	>100	50
Klebsiella pneumoniae DT-S	>100	>100	25	100	100	>100	50	25	50	0.78
Enterobacter cloacae IFO 12937	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Proteus vulgaris IFO 3988	>100	>100	>100	>100	>100	>100	>100	>100	>100	3.13
Serratia marcescens IFO 12648	>100	>100	>100	>100	>100	>100	>100	>100	>100	100
Pseudomonas aeruginosa U-31	>100	>100	100	>100	>100	>100	>100	100	>100	>100

a) The numbers represent model peptides as described in Table 1.

Table 4. The Antimicrobial Activity of [D]$L^{3,4,8,10}$-K_4L_8 (**7**) on Different Assay Methods

Organism	MIC ($\mu g\,ml^{-1}$)			
	Literature value[a)]	Mueller Hinton agar	Mueller Hinton broth	Lactose media
B. subtilis PCI219	0.72	6.25	12.5	6.25
E. coli NIHJ JC-2	5.0	50	50	12.5
A. calcoaceticus ATCC 13006	5.7	100	25	12.5
P. aeruginosa U-31	14.4	>100	100	25

a) The data (μM) of literature[10] were converted into $\mu g\,ml^{-1}$ on the basis of the molecular weight of [D]$L^{3,4,8,10}$-K_4L_8 (1435.0). The species of micro organism were as follows: *B. subtilis* ATCC 6051; *E. coli* D21; *A. calcoaceticus* AC II; *P. aeruginosa* ATCC 27853.

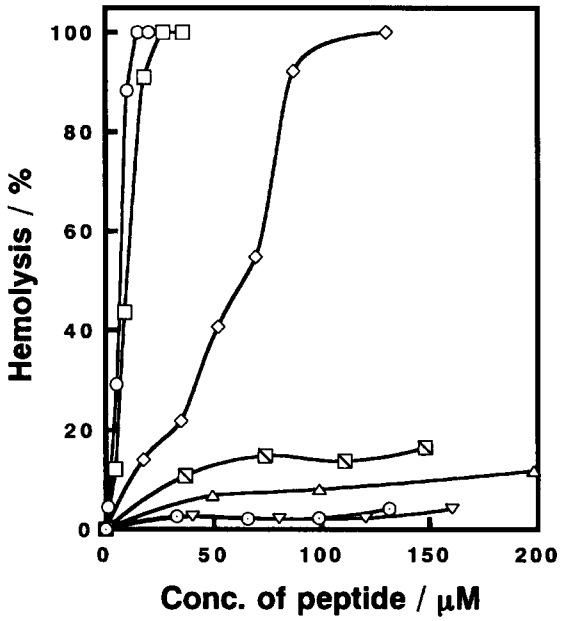


Fig. 3. Dose-response curves of hemolytic activity induced by model peptides. The number of red blood cell is 2×10^7/ml. Designations are as follows: ○, **1**; □, **2**; ◇, **3**; ▽, **5**; △, **6**; □, **7**; ⊙, **8**.

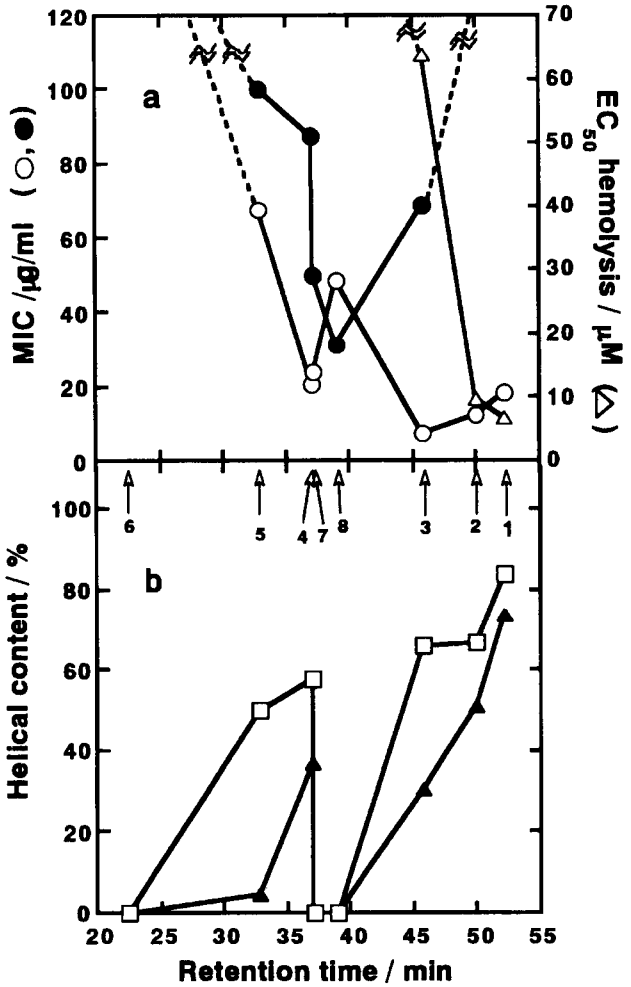


Fig. 4. The relationship between antimicrobial activity against Gram-positive and negative bacteria, and hemolytic activity (a) and the retention time and helix-forming ability (b) of model peptides. Designations are as follows: ○, Gram-positive bacteria; ●, Gram-negative bacteria; △, hemolytic activity; ▲, α-helical content in the presence of egg PC; □, in the presence of egg PC–egg PG (3 : 1). The numbers under boundary line between Figs. 4a and 4b show the examined model peptides as described in Table 1.

Escherichia coli is shown.[26] Thus, in the present study we also evaluated the relative peptide hydrophobicity in terms of the peptide elution time, expressed by linear TFA–water to TFA–acetonitrile gradients on C18 RP-HPLC, and tried to correlate it to the biological activity.

Figure 4 shows the relationships among the retention time, biological activity and helix-forming ability. As for the antimicrobial assay method, we employed the agar-plate dilution method, because, although the values of MICs were seriously affected against Gram-negative bacteria by the type of assay, the tendency of the activity remained unaltered among the tested three assay methods. The results clearly show that the retention behavior of model peptides on RP-HPLC can be correlated with the activity and selectivity for mammalian cells, as well as Gram-positive and -negative bacteria. Hemolysis needs the strongest hydrophobicity; the retention time is more than 45 min in Fig. 4. In this range, the hemolytic activity drastically decreases with the reducing retention time. For Gram-positive bacteria, those peptides showing antibacterial activity were eluted over a wide range of retention times (35—55 min), meaning that strong hydrophobicity is not necessary to exhibit this activity. The maximum inhibitory level may be located at about 45 min of retention time. The peptides showing the activity for Gram-negative bacteria were eluted in a narrower range (from 35 to 45 min) than those for Gram-positive bacteria, and the

maximum inhibitory level may appear at about 40 min. It should be noted that the relatively strong hydrophobicity negatively acts on antimicrobial activity. The retention time corresponding to the least hydrophobicity needed for both antimicrobial activities is about 30 min. The negative charge is generally responsible for the decrease of hydrophobicity. It is interesting to note that the peptides have an optimal hydrophobicity for activity against both Gram-positive and Gram-negative bacteria, and that the optimum region for Gram-negative bacteria is present at lower hydrophobicity than the optimum region for Gram- positive bacteria. This means that the boundary region for a change in activity for both bacteria exists between peptides **7** and **8**; when the antimicrobial activity is compared with both peptides, the former is more active against Gram-positive bacteria and less active against Gram-negative bacteria than the latter (Table 3).

The introduction of D-amino acids in $4'_3$ led to decreases in the hydrophobicity, helical content and hemolytic activity, but to an increase in the antimicrobial activity against Gram-positive bacteria. Furthermore, the activity against Gram-negative bacteria was observed in three D-amino acid-substituted analogs according to the agar-plate assay system. It is clear that the helix formation needed to produce the amphiphilic structure is not necessary to show antimicrobial activity. It should be noted that although the peptides showing the highest activity against Gram-positive bacteria are able to take the α-helical structure, those peptides having the highest antimicrobial activity for Gram-negative bacteria took no helical structure, indicating that the helical structure possessed by peptides may be a disadvantage to Gram-negative bacteria. Previously, we designed and synthesized three analogs of 4_3, [Pro^6]4_3, [Pro^{2,6}]4_3, and [Pro^{2,6,10}]4_3 that replaced Ala residue(s) with Pro, which is well known to be a strong helix-breaker.[14] The helical contents of Pro-analog peptides in the presence of acidic liposome decreased with an increase of Pro residues. Moreover, a reduction of the helical content was correlated with the antimicrobial activity against Gram-positive bacteria, *S. aureas* and *B. subtilis*. However, the results of the present study suggest that the reduced activity in the Pro-containing 4_3 peptides may be related to a decrease in the hydrophobicity, rather than a decrease in the amphiphilicity due to a decrease in the helicity. In this connection, the hydrophobicity scale of Pro is much smaller than that of Ala.[18]

We have previously designed and synthesized five amphiphilic α-helical model peptides (Hel series) with systematically varied hydrophobic-hydrophilic balances, among which highly hydrophobic peptides showed strong hemolytic activity, but no antimicrobial activity. Peptides consisting of Lys and Leu have an ideal helical structure that is strictly separated into polar and non polar faces, when they take the α-helical structure. Naturally occurring amphiphilic α-helical antimicrobial peptides often contain one or more helix-breaking amino acids, and are always less perfectly amphiphilic than the idealized polypeptides.[2,4] Imperfect amphiphilicity and the resulting lower binding affinity of the natural antimicrobial peptides are probably selected so as to minimize indiscriminate host organism cell lysis. As shown in the present study the peptides with short chains composed of about 12 residues have similar levels of antimicrobial activity to naturally occurring peptides longer than about 25—30 residues. The natural peptides often have one or more helix breaker amino acid(s) near to the middle of their sequence. The respective separate parts may work independently to exhibit antimicrobial activity. This seems to be supported by numerous experimental results showing that the small fragmented peptides or chimera peptides among insect antimicrobial peptides and proteins (for instance, lit[17,27,28]) have often been found to have the same antimicrobial activity as the longer, original one. In connection to this, NMR-based studies using amphiphilic model helices of eighteen amino acid residues have shown that the incorporation of an adjacent pair of D-amino acids in the central part causes a local change in the structure and flexibility while keeping the separate helical structure.[29]

Our present results are completely compatible with the those by Oren and co-workers; that is, the α-helical amphiphilic structure is required for cytolytic ability against mammalian cells, but is not necessary for bacterial cell lysis.[10] Additionally, in the range exhibiting cytolytic activity, the peptides have different hydrophobicity regions in which they show their optimum activity against both bacteria and erythrocytes. In the highest hydrophobicity region the peptides display high levels of hemolytic activity. In the medium hydrophobicity levels the peptides are able to display high levels of activity against Gram-positive bacteria. In the lowest hydrophobicity regions the peptides are able to display high levels of activity against Gram-negative bacteria. These differences of action modes may be explained by the abilities of the binding to membranes and the disturbance of lipid matrices, as discussed in detail by Dathe et al.[8] It is well known that the structure of biological membranes is considerably different in mammalian and bacterial cells: 1) the outer leaflet of an erythrocyte-membrane is mainly composed of neutral phospholipids; 2) Gram-positive bacteria contain negatively charged compounds such as teichoic and teichuroic acids; and 3) Gram-negative bacteria composed of their inner and outer membranes have a high degree of negatively charged lipopolysuccharides on the exterior surface of the outer membranes, although the inner membrane is comparable to that of mammalian cells. Thus, the content of negative charges in bio-membranes may be an important factor in determining the selectivity of the biologically active peptides: a) cytotoxic peptides acting on neutral membranes of erythrocyte need strong hydrophobicity, but positive charges are not necessary; b) the peptides acting on Gram-negative bacterial membranes containing a high degree of negative charge need to possess much more positive charge, resulting in a decrease in the hydrophobicity of the peptide and; c) the peptides acting on Gram-positive bacterial membranes need a medium level of hydrophobicity and positive charge between erythrocytes and the Gram-negative bacteria. In this connection, Matsuzaki et al.,[30] have clearly shown, using model membranes, that the selective toxicity

between bacterial membranes and erythrocyte membranes on an channel-forming amphiphilic antimicrobial peptide, magainin, depends on the lipid composition in the target membranes.

Amphiphilic helical peptides with various hydrophobic-hydrophilic balances in their molecule can be designed and synthesized as shown in previous studies. It is also easy to design an antimicrobial peptide having the proper and ideal hydrophobicity, by a proper distribution of positive charge, and the introduction of D-amino acid, and/or helix breaker residue in the amphiphilic helical structure. The hydrophobicity can be easily evaluated by RP-HPLC. This may be useful in designing clinically effective antimicrobial peptides with highly potent activity and high bacterial selectivity.

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