Structure-Function Studies of Amphiphilic Antibacterial Peptides

Roberto Bessalle,[†] Alfred Gorea,[‡] Itamar Shalit,[‡] Jörg W. Metzger,[§] Chhabil Dass,[⊥] Dominic M. Desiderio,[∥] Mati Fridkin*,[†]

Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel, Clinical Microbiology Research Laboratory, Infectious Disease Unit, Sourasky Medical Center, Tel-Aviv, Israel, Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-7400 Tübingen, Germany, and Departments of Neurology and Biochemistry and the Charles B. Stout Neuroscience Mass Spectrometry Laboratory, University of Tennessee—Memphis, 800 Madison Ave, Memphis, Tennessee 38163

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The synthesis of 11 peptides, ranging in composition from 9 to 17 amino acid residues, by solid-phase methodology was accomplished with the purpose of studying how the amphiphilic and hydrophobic character, the size of the molecule, and the charge distribution modulate the antibacterial activity. It was found that peptides composed of 16 and 17 amino acid residues, with high hydrophobic (mainly due to Trp or Phe) and hydrophilic (due to Lys) character distributed along opposite amphiphilic faces, showed considerable antibacterial activity against clinically isolated bacteria together with Gram positive and Gram negative ATCC bacterial strains. However, the hemolytic capacity of the peptides was also significant. Decreasing the hydrophobic character of the molecule by replacing Trp or Phe with Leu residues while maintaining the basic contribution of Lys drastically reduced the hemolytic activity and only slightly decreased the bioactivity. Peptides composed of 9–10 amino acid residues with high hydrophobic and basic nature possess antibacterial activity but, in general, are less active than the larger counterpart peptides. By replacing all Trp residues of a short peptide by Leu residues, the activity was considerably reduced. Circular dichroism studies and antibacterial assays showed that shorter peptides with very low helical content, and thus deprived of amphiphilic character, still have appreciable bioactivity. This observation, coupled with the fact that due to their small size they cannot span the bacterial outer lipid bilayer, may suggest different mechanisms of action for long-chain vis-a-vis short-chain peptides.

Introduction

Many of the natural antibacterial peptides isolated from mammalian, amphibian, and insect origin assume a unique amphiphilic secondary structure upon interaction with the outer phospholipid bilayer of the bacteria. This characteristic may be induced by α-helical, e.g. magainin,[1] cecropin,[2] sarcotoxin,[3] or β-sheet, e.g. defensin,[4] molecular organization. It seems logical to postulate that the amphiphilic structure was selected in nature as a part of a common defensive tool in combating invading microorganisms. In this respect, it is worth noting that peptides homologous to defensins were recently discovered in certain invertebrates,[4] suggesting that they might be ancestral components of the host defense system.

The mode of action of amphiphilic antibacterial peptides is not yet entirely clear. Recent studies suggest their involvement in formation of ion channels with consequent disruption of bacterial phospholipid bilayers and eventual cell death.[5-7]

To evaluate the significance of amphiphilicity to bioactivity and to understand structure–activity relationships of natural antimicrobial peptides, extensive synthetic efforts have been directed toward modification of several structural features. Thus for example, analogs with improved antibacterial activity, and low cytotoxicity, were found for cecropins[8-10] by synthesizing model peptides that mimic certain structural molecular characteristic of the peptides. It was concluded that high antibacterial activity, for this class of compounds, requires a positively-charged helical amphipathic N-terminal segment that is connected to a hydrophobic helical C-terminal segment by a flexible nonhelical hinge region. These studies led to the synthesis of a series of hybrid peptides, including cecropin A-melittin segments with enhanced bioactivity.[11] Augmentation of the helical content of magainin 2 (mag-2) increases its bioactivity.[12] Several amphiphilic, helical, and cationic model peptides related to the prepiece moieties of mitochondrial protein precursor were found to be active against Gram-positive bacteria.[13]

The present study evaluates the significance of amphiphilicity and length of the peptide chain to antibacterial potency. For this purpose we synthesized several model amphiphilic peptides based on a rational choice of different degrees of hydrophobic and basic characters and different sizes. These peptides are termed as "modelins" and abbreviated as "mod". The activity of the peptides against a wide spectrum of bacteria was assessed.

Results and Discussion

Rationale for the Design of Modelins. It is widely accepted that one of the major targets of the antimicrobial bioactive peptides is the bacterial outer phospholipid bilayer. The initial interaction between the peptide and the microbe is presumably of an electrostatic nature involving the positively charged side-chain residues of the amphiphilic peptide and the highly negatively charged bacterial surface. Once the interaction occurs, it is not known whether the peptide lies parallel to the cell surface with the hydrophobic residues imbedded into the phospholipids bilayer while the polar residues exposed to the

* To whom requests for reprints should be addressed.
† The Weizmann Institute of Science.
‡ Sourasky Medical Center.
§ University of Tübingen.
⊥ Department of Neurology and the Charles B. Stout Neuroscience Mass Spectrometry Laboratory, University of Tennessee—Memphis.
∥ Departments of Neurology and Biochemistry and the Charles B. Stout Neuroscience Mass Spectrometry Laboratory, University of Tennessee—Memphis.

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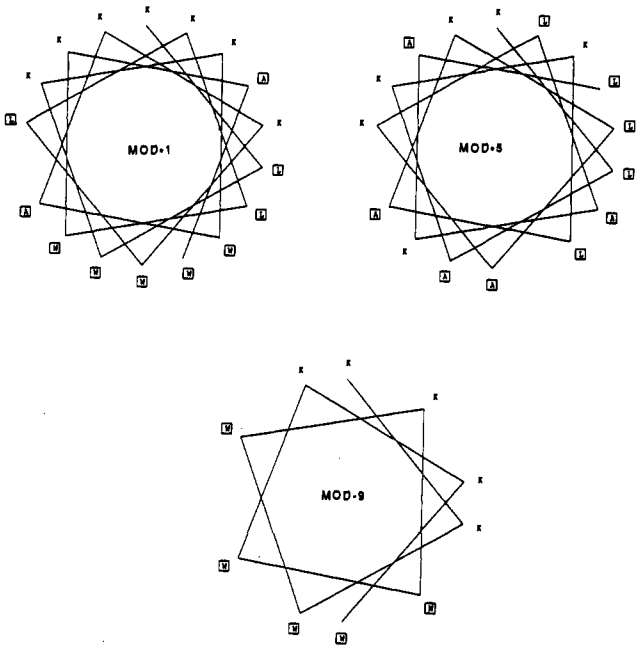

Figure 1. Schiffer–Edmunson[16] helical wheel projections of modelins 1, 5, and 9. (□) Hydrophobic amino acid residues.

outside and/or if the peptide penetrates into the bilayer as a monomer (or as an already defined assembled structure) forming inside specific structural aggregates composed of two or more species. Thus, either way, disruption of the physiological equilibrium of the bacterial membrane occurs, with a concomitant cell death. According to this theoretical mechanism, it is quite obvious that modifications of amphiphilicity or polar and hydrophobic character of the molecule might affect bioactivity. These modifications may provide important information concerning the structure–activity relationship. Furthermore, to span completely the bacterial outer phospholipid bilayer, a chain of approximately 20 amino acid residues is needed, provided that the peptide is 100% α-helical.[14,15] However, a study involving shorter peptides may shed additional light upon mode of peptide–bacteria interactions and mechanism of bacterial killing.

Modelins were synthesized mainly with the purpose of studying the structure–activity relationship of a series of model peptides having different degrees of hydrophobicity and varied basic character of the amino acids distributed throughout an "ideal" amphiphilic secondary structure and assuming 100% helicity. Thus, full helicity is required for a modelin to be completely amphiphilic. The graphical display of the amphiphilic alpha helical character of three synthetic modelins, based on the Schiffer–Edmundson helical wheel representation,[16] is depicted in Figure 1.

We chose to investigate peptides composed of 16 and 17 amino acid residues (here defined as long peptides), because initial studies with several similarly designed peptides of 21 and 23 amino acid residues reveal that the two groups possess nearly the same antibacterial activities (data not shown). Peptides of 9 and 10 amino acid residues (defined as "short peptides") were also studied.

Design of Modelins. Modelins were designed by Macromodel, a computer modeling software for macromolecules with three-dimensional graphics capabilities.[17]

Structural Relationship of Modelins. Table I shows the primary sequences of modelins synthesized. Magainin 2 is used as an antibacterial internal reference. The peptides synthesized are described in the following section.

Modelin 1 (mod-1) comprises seven Lys residues on the polar face of the amphiphilic structure and 10 hydrophobic residues composed of two Ala, three Leu, and five Trp on the opposite face. All of these amino acids possess the potential to form α-helices upon binding with phospholipid bilayers.[18] The amphiphilic helical peptide mod-1 served as a basis for the synthesis of several analogs by the following methods.

(1) Changing the hydrophobic character of the amino acids positioned at the hydrophobic face of the amphiphilic surface.[19] Thus, (a) all Trp residues were replaced by the less hydrophobic Leu and Ala residues whereas Leu^{11} was replaced by Ala (mod-2). (b) All Trp, except the first one at the carboxylic terminus, were replaced by Phe residues, which are slightly less hydrophobic than Trp and more hydrophobic than Leu and Ala (mod-3).

(2) Changing the hydrophilic residues exposed at the polar face of the amphiphilic peptide. (a) All Lys residues were replaced by the less charged, at physiological pH, His residues (mod-4). (b) Shortening mod-2 by one amino acid residue (Lys^{8}) afforded a 16-mer (mod-5).

(3) Increasing the content of Lys residues of mod-1 from 7 to 10. This change was accomplished by replacing Ala^{7} and Leu^{11,16} for Lys residues (this augments the hydrophilic surface face area) and also by replacing Leu^{2} and Trp^{6,14} for Ala residues. Both changes decreased the hydrophobic moment of the molecule (mod-6).

(4) Shortening mod-1 to its first 10 residues beginning at the carboxy terminus (mod-7).

(5) Shortening mod-6 to its first nine residues from the carboxy terminus (mod-8).

An additional three model peptides were designed and synthesized as follows:

(6) An amphiphilic 10-mer peptide in which the hydrophilic face is formed by five Lys and the hydrophobic face by either five Trp (mod-9) or five Leu (mod-10) residues.

(7) A 9-mer peptide composed of three Lys, four Trp, and two Ala residues were designed with a random distribution of its amino acid moieties with the aim to achieve a molecule devoid of any amphiphilic character (mod-11).

Synthesis, Purification, Molecular Mass Determination, and Amino Acid Sequence Confirmation. Modelins were synthesized by the solid-phase methodology, employing manual procedures and using a scheme based on t-Boc chemistry and HF-labile side-chain protecting groups. HBTU/HOBT and DCC/HOBT were used as coupling reagents.

Peptide purification was accomplished by gel filtration on Sephadex-15 followed by high-performance liquid chromatography (HPLC) to yield homogeneous products (≥97%) by the criterion of UV absorbance at 210 nm. Amino acid analyses of the pure peptides after acid hydrolysis confirmed the theoretical composition. The molecular masses were validated by fast atom bombardment/mass spectrometry (FAB-MS) and/or ion spray mass spectrometry (IS-MS) (Table II).

Circular Dichroism. In order to determine the content of α-helicity of modelins, a circular dichroism study was performed in 50 mM potassium phosphate and 50% trifluoroethanol (TFE) (in 50 mM potassium phosphate) at pH 7 as already described.[20] Table III shows the helical content for some modelins, as calculated according to Wu et al.[21] When the peptides were dissolved in buffered

Table I. Sequences of Modelins

modelins	sequences	size[a]
mod-1	H-Lys-Leu-Trp-Lys-Lys-Trp-Ala-Lys-Lys-Trp-Leu-Lys-Leu-Trp-Lys-Ala-Trp-OH	17
mod-2	H-Lys-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Ala-Lys-Ala-Leu-OH	17
mod-3	H-Lys-Leu-Phe-Lys-Lys-Phe-Ala-Lys-Lys-Phe-Leu-Lys-Leu-Phe-Lys-Ala-Trp-OH	17
mod-4	H-His-Leu-Trp-His-His-Trp-Ala-His-His-Trp-Leu-His-Leu-Trp-His-Ala-Trp-OH	17
mod-5	H-Lys-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Ala-Lys-Leu-Ala-Lys-Ala-Leu-OH	16
mod-6	H-Lys-Ala-Trp-Lys-Lys-Ala-Lys-Lys-Lys-Trp-Lys-Lys-Leu-Ala-Lys-Lys-Trp-OH	17
mod-7	H-Lys-Lys-Trp-Leu-Lys-Leu-Trp-Lys-Ala-Trp-OH	10
mod-8	H-Lys-Trp-Lys-Lys-Leu-Ala-Lys-Lys-Trp-OH	9
mod-9	H-Lys-Lys-Trp-Trp-Lys-Trp-Trp-Lys-Lys-Trp-OH	10
mod-10	H-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-OH	10
mod-11	H-Trp-Lys-Lys-Trp-Leu-Trp-Ala-Lys-Trp-OH	9

[a] Number of amino acid residues.

Table II. Mass Spectral Data of Modelins

	$[M + H]^+$			$[M + H]^+$	
peptide	calcd	found	peptide	calcd	found
mod-1[a]	2327.9	2328	mod-7[b]	1386.7	1386
mod-2[a]	1836.4	1836	mod-8[b]	1215.6	1216
mod-3[b]	2171.8	2171	mod-9[a]	1590.0	1591
mod-4[b]	2390.7	2386.2[c]	mod-10[a]	1224.7	1225
mod-5[a]	1708.3	1708	mod-11[a]	1331.6	1332
mod-6[a]	2184.8	2185			

[a] FAB-MS.[27,28] [b] Ion spray MS.[29] [c] Multiply charged quasimolecular ion, $(M + 3H)^{3+}$.

Table III. Structural Data from Circular Dichroism of Magainin 2 and Modelins

	% α-helix			% α-helix	
peptide	50 mM potassium phosphate, pH 7	50% CF_3CH_2OH[a]	peptide	50 mM potassium phosphate, pH 7	50% CF_3CH_2OH[a]
mag-2	0	43	mod-6	0	24
mod-1	0	39	mod-7	0	<5
mod-2	0	51	mod-8	0	<5
mod-3	0	60	mod-9	0	50
mod-4	0	8	mod-10	0	14
mod-5	0	61	mod-11	0	<5

[a] In phosphate buffer, pH 7.

Table IV. Percent Hemolysis of Human Red Blood Cells[a]

	concentration (μg/mL)			
peptide	25	50	100	200
mag-2	0	1	2	2
mod-1	10	18	31	55
mod-3	5	15	26	40
mod-4	0	0	0	0
mod-5	1	1	1	2
mod-8	0	0	0	0
mod-11	1	1	4	5

[a] Mellitin at concentrations of 2.5, 5, 10, and 20 μg/mL exerted 2, 8, 26, and 64%, respectively.

water, no helicity was observed. In the presence of 50% TFE, however, the longer peptides, with the exception of mod-4, displayed 40–60% of helicity whereas the shorter peptides were found not to be helical with the exception of mod-9, which displayed 50% helicity. Modelins 4, 7, 8, and 11 did not show any significant helicity.

Cytotoxicity. The hemolytic activity toward human erythrocytes was evaluated for some selected modelins (Table IV). Modelins 1 and 3, which are very hydrophobic, were the only peptides found to possess high cytotoxicity (e.g. at a concentration of 100 μg/mL, 31% and 26%, respectively). Modelin 11 produced 4% of hemolysis at the same concentration. The other modelins (Table IV) and magainin 2 did not have high cytotoxicity. Melittin, a highly hemolytic peptide, was used as a standard and

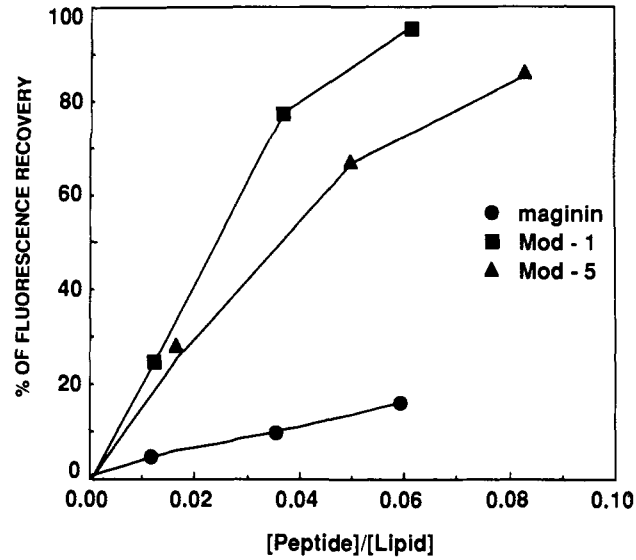

Figure 2. Dissipation of diffusion potentials induced by modelin 1, modelin 5, and magainin 2 in soybean vesicles. Peptides were added to 1 mL of buffer containing a constant concentration of vesicles (phospholipid concentration = 38 μM) preequilibrated with the fluorescent dye diS-$_2$-5 and valinomycin (see Materials and Methods).

exerted 64% hemolysis at 20 μg/mL (a concentration 10 times lower than the highest concentration of modelins used).

Membrane Permeability Studies: Valinomycin-Mediated Diffusion Potential Assay. Two representative, highly active modelins, i.e. 1 and 5, were tested along with magainin 2 for their ability to dissipate the diffusion potential in sonicated small unilamellar vesicles (SUV) prepared from soybean lecithin. Peptides at increasing concentrations were mixed with the SUV (at constant concentration), pretreated with the fluorescent dye 3,3′-diethylthiodicarbocyanine iodide (diS-C_2-5) and valinomycin. Peptide–lipid molar ratios ranging from ~0.0015:1 to ~0.06:1 were employed. Maximal activity for each peptide/lipid molar ratio tested was determined by monitoring the fluorescence recovery until a plateau was observed (usually after 15 min) (Figure 2). Each point represents the mean of three separate experiments with a standard deviation of about 5%. The results demonstrate a high, perturbing activity for modelins 1 and 5 and substantial, though significantly lower, for magainin 2. Association of modelin 5 with SUV appears to be completed in a few minutes and particulary at higher molar ratios of peptide–lipid (Figure 3). Nearly quantitative fluorescence recovery is observed in about 4 min.

Bioactivity. Table V shows the in vitro antibacterial activity of mag-2 (used as an internal antibacterial

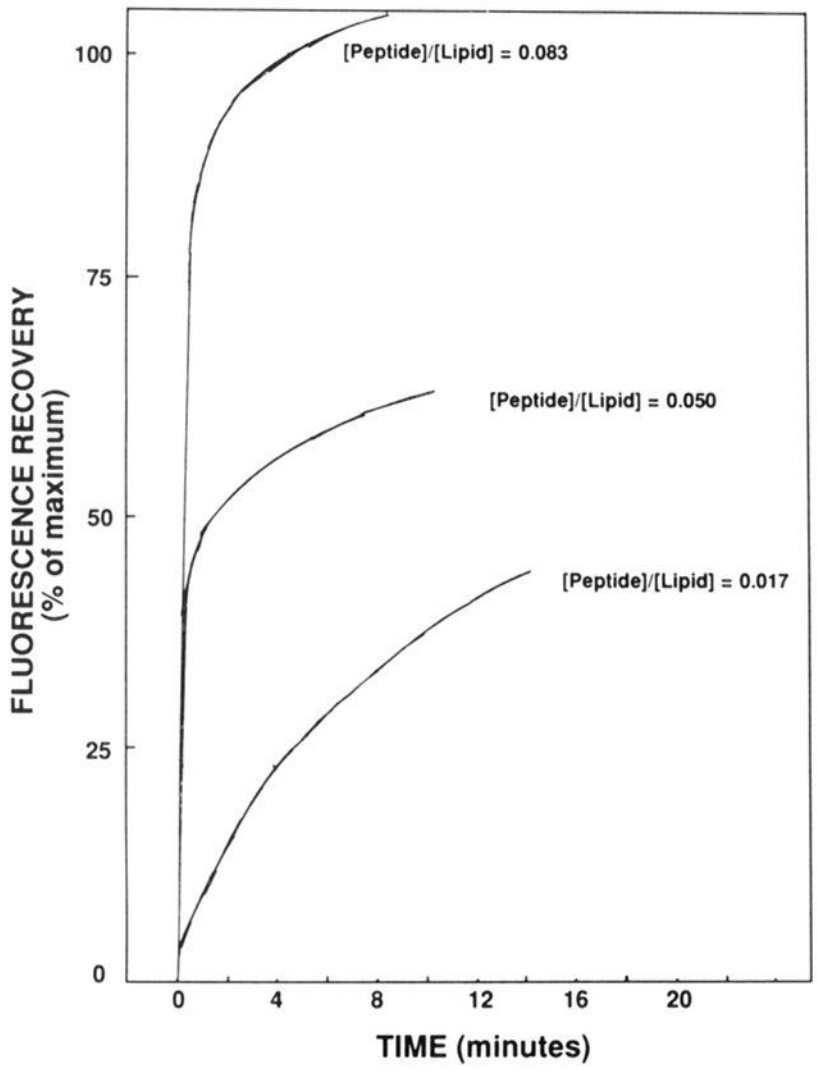

Figure 3. Time dependence of dissipation of diffusion potentials induced by modelin 5 in soybean vesicles as a function of the molar ratio of peptide to lipid.

Table V. In Vitro Susceptibilities of ATCC Strains of *E. coli*, *P. aeruginosa*, and *S. aureus* to Modelins

	MIC (μg/mL)		
peptide	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 29213
mag-2	100	100	>200
mod-1	6.25	3.12	50
mod-2	25	50	200
mod-3	6.25	12.5	
mod-4	25	12.5	50
mod-5	6.25	3.12	12.5
mod-6	200	>200	200
mod-7	25	12.5	12.5
mod-8	25	12.5	25
mod-9	50	50	12.5
mod-10	>200	>200	>200
mod-11	50	12.5	25

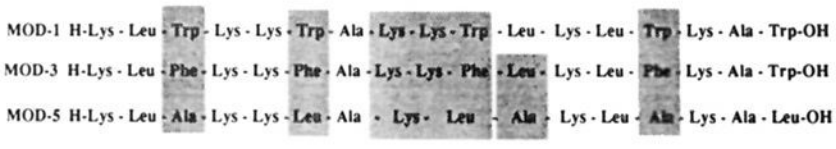

Figure 4. Comparison of the primary structure of mod-1, mod-3, and mod-5. Domains of major substitutions are highlighted.

standard) and modelins against three ATCC strains (*E. coli*, *P. aeruginosa*, and *S. aureus*). Modelins 1, 3, and 5 (Figure 4) are the most active peptides of the series and are considerably more active than magainin 2. Modelin 1 and 3 are 17 amino acid residues long, and mod-5 is 16 amino acid residues long (Table I). The three peptides contain Lys as a basic amino acid, and Trp, Phe, and Leu as the main hydrophobic residues for modelins 1, 3, and 5, respectively. The differences in hydrophobicity did not affect, in general, the bioactivity of modelins 1, 3, and 5 against a series of ATCC and clinically isolated bacterial strains (Tables V–VIII). The peptides exhibited an MIC

Table VI. Comparative Antimicrobal Activity of Modelins 1, 3, 4, 5, and 7 against Selected Clinical Isolates of Gram-Negative Bacteria

	MIC (μg/mL)				
microorganism	mod-1	mod-3	mod-4	mod-5	mod-7
Shigella flexneri	3.12	3.12	12.5	25	≤0.39
Salmonella anatum	12.5	12.5	>100	25	12.5
Proteus mirabilis	>100	>100	>100	>100	>100
Enterobacter cloacae	≤0.39	6.25	≤0.39	3.12	≤0.39
Enterobacter cloacae ROS	25	12.5	50	12.5	50
Klebsiella pneumoniae	12.5	12.5	>100	12.5	>100
Acinetobacter anitratas	3.12	6.25	≤0.39	3.12	≤0.39

Table VII. Antimicrobal Activity of Modelins 1, 3, 5, 7, and 11 against Different Strains of *Pseudomonas aeruginosa*

	MIC (μg/mL)				
strains	mod-1	mod-3	mod-5	mod-7	mod-11
1	6.25	6.25	3.12	25	50
2	3.12	12.5	6.25	12.5	12.5
3	12.5	6.25	3.12	50	50
4	12.5	12.5	3.12	50	50
5	12.5	12.5	6.25	50	50
6	>100	25	>100	>100	>100
7	12.5	6.25	6.25	50	50
8	12.5	12.5	50	50	50
9	>100	>100	>100	>100	>100
10	25	50	>100	50	>100
11	>100	25	6.25	50	50
ATCC 25916	3.12	3.12	6.25	25	50

Table VIII. Activity of Modelins 1, 3, 5, 7, and 11 against Different Strains of *Staphylococcus aureus*

	MIC (μg/mL)				
strains	mod-1	mod-3	mod-5	mod-7	mod-11
1	12.5	3.12	≥100	≥100	50
2	6.25	25	≥100	≥100	25
3	25	25	≥100	≥100	≥100
4	12.5	25	≥100	≥100	50
5	12.5	50	≥100	≥100	50
6	12.5	25	≥100	≥100	50
7	25	25	≥100	≥100	25
8	25	25	≥100	≥100	50
9	25	50	≥100	≥100	50
10	25	25	≥100	≥100	50
11	25	25	≥100	≥100	50
ATCC 28953	25	25	≥100	≥100	≥100

(minimal inhibitory concentration) of 6.25 μg/mL against *E. coli* (ATCC 25922), 12.5–25 μg/mL against *E. cloacae* ROS, 3.12 μg/mL against *P. aeruginosa* (ATCC 27853), and 12.5–50 μg/mL against *S. aureus* (ATCC 29213). Similar values were found against several clinically isolated Gram-negative strains (Table VI), and various strains of *P. aeruginosas* (Table VII) and *S. aureus* (Table VIII), with the exception of mod-5 (MIC of ≥100 μg/mL against *S. aureus* compared to mod-1 and mod-3).

Modelins 2 and 4 showed lower antibacterial activity than modelins 1, 3, and 5, but still better than magainin 2 toward all of the strains tested (Tables V–VIII). Mod-2 and mod-4 showed similar MIC values for *E. coli* (25 μg/mL) and *P. aeruginosa* (12.5–50 μg/mL) and lower activity (MIC ≥ 50 μg/mL) for *P. aeruginosas* and *E. cloacae*.

At the physiological pH of 7.4, which is approximately the pH used in our in vitro assays, the His residues of mod-4 (where all Lys residues of mod-1 were replaced by His residues) are only partially protonated ($pK_a \approx 6.7$) whereas Lys residues of mod-1 are fully protonated ($pK_a \approx 10$). Thus, the high content of a positive charge seem to be associated with the potency. However, too many

positive charges (i.e. mod-6) inhibit the antibacterial activity MIC ≥200 μg/mL; Table V).

The peptides with smaller size (modelins 7–9 and 11, Table V) showed considerable activity against three of the bacterial strains. Mod-10 was not at all active up to an MIC of 200 μg/mL. In general, the MIC values found for modelins 7–9 and 11 are about 12.5–50 μg/mL. Mod-7 was also tested against some Gram-negative bacteria (Table VI). High antibacterial activities were found for the clinically isolated strains *S. flexneri*, *Enterobacter cloacae* (MIC ≤0.39 μg/mL), and *Acinetobacter anitraters* (MIC 1.56 μg/mL).

The conclusions of all the above observations are not straightforward. A basic assumption in the design of modelins was, as mentioned before, that the peptides assume complete helicity in a hydrophobic membrane-like milieu (50% trifluoroethanol). It was found, however (Table III), that the peptides possess only partial helicities. This fact creates obvious difficulties if bioactivity is to be interpreted only with respect to amphiphilicity. This fact is especially pertinent, with regard to modelins 4, 7, 8, and 11. The four peptides possess substantial antibacterial capacity but reveal low helical content (Table III). This observation may suggest that amphiphilicity is not the sole important requirement for manifestation of bioactivity. The observation that the shorter-chain, nonhelical peptides (modelins 7, 8, 9, and 11) showed significant bioactivities against microorganism in spite of the fact that they cannot span the bacterial lipidic bilayer may suggest that they operate via different pathways, e.g., through formation of an "ordered" aggregate during or following penetration of the lipidic surface.

On the basis of our model peptides, we propose several pertinent structural requirements, which we believe are important for exertion of antibacterial activity. Peptides that possess a certain degree of amphiphilicity and contain highly charged residues (Lys and perhaps Arg) and prominent hydrophobic residues might display antibacterial activity and also hemolytic activity. For longer peptides (16–17-mer), in which the hydrophobic character of the nonpolar residues is lowered, the hemolytic activity is reduced but the bioactivity is still preserved (i.e. modelin 1, vis-a-vis modelins 3 and 5). For shorter peptides (9–10-mer), lowering the hydrophobic character of the nonpolar residues may result in total loss of the activity. For instance, when Trp residues of mod-9 are replaced by Leu (mod-10), the antibacterial activity is drastically reduced (Table V). Decreasing the size of the peptide could result in a less cytotoxic peptide, but usually reduction in bioactivity is observed. The longer peptides (16–17-mer) are generally more active than the shorter ones (9–10-mer). The relative proportion between the hydrophobic and basic character is also important. Our model peptides show that, in order to achieve high bioactivity, 30–45% of the amino acid residues should be basic, whereas the rest can be hydrophobic. Mod-6, a highly basic peptide (≈60% basic residues), lost its antibacterial activity (≥200 μg/mL) as compared to modelins 1, 3, and 5 (Table V). It is worth mentioning here that Andreu et al.[11] have recently reported that shortened cecropin A–melittin (CA–M) hybrids (15-, 18-, and 20-mer) possess bioactive properties similar to the parent larger molecules (35–37-mer). The peptides include the first seven amino acid residues corresponding to the amino terminal segment of cecropin A [CA(1–7) Lys-Trp-Lys-Leu-Phe-Lys-Lys]. This sequence is not antibacterial per se but it is required for optimal activity. It is very similar in nature to modelins, i.e. a highly charged and hydrophobic peptide.

The comparison between modelins and magainin, which in this study served as both an amphiphilic model peptide and as an internal antibacterial standard, deserves further attention. Thus, modelins 1 and 5 manifested a marked perturbation effect on vesicles of soybean lecithin, while magainin 2 exhibited a substantial, but much smaller, influence. This finding may be explained in view of the over-whole positive charge of the peptides and the zwitterionic nature of the soybean vesicles. At physiological conditions, modelins 1 and 5 carry seven and six positively charged moieties of lysine and one negatively charged C-terminal residue out of 17 and 16 amino acid residues, respectively. Magainin 2, on the other hand, possesses only four complete positive charges of lysine and a partially protonated histidine residue, and two negatively charged residues (Glu-19 and the C-terminal moiety). It might therefore be expected that association of peptides with the rather neutral phospholipid vesicles and their perturbation will be relative to the electrostatic forces involved. Magainin was reported to penetrate vesicles composed of acidic phospholipids. Consequently, it was proposed that it exerts its antibacterial action by similar permeation through the bacterial negatively charged surfaces and, thereby, modulation of membranal potential and permeability and of cellular functions.[22] In view of the above, it seems that the mode of action of magainin and of modelins on bacteria is fundamentally rather similar. Quantitative differences, however, may stem from the intensity of interaction between peptides and cells. In this respect, it is worth mentioning here that we could significantly augment the antibacterial activity of magainin by positive-charge chain extension.[23]

In conclusion, results of the present structure–activity study indicate that it is possible to modulate antibacterial activity of peptides by means of amino acid substitution which alter their basic and hydrophobic characteristics. Substantial bioactivity can be preserved by chain shortening and by maintaining a certain proportion between positively-charged and hydrophobic moieties. Availability of short active peptides may facilitate understanding of the mechanisms of action, the design of novel potent derivatives, and the use of peptides in the actual treatment of primarily topical bacterial infections.

Materials and Methods

Synthesis of Peptides. Synthesis of peptides, employing the solid-phase strategy,[24] was carried out manually on a chloromethylated polystyrene/2% divinylbenzene resin (Chemalog, South Plainfield, NJ)[25] or with PAM resin (Bachem, Torrance, California). Protected amino acid derivatives were purchased from Bachem (Bubendorf, Switzerland). α-Amino groups of amino acids were protected by *tert*-butyloxycarbonyl. Side-chain protecting groups were as follows: serine, *O*-benzyl; glutamic acid, γ-benzyl; lysine, N^{ϵ}-2-chlorobenzyloxycarbonyl; histidine, N^{im}-benzyloxycarbonyl; tryptophan, *N*-formyl; arginine N^{w}-tosyl. All coupling stages were performed with a 3-fold excess of protected amino acid derivatives with an equimolar mixture of *N*,*N*′-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) or 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/HOBT as reagents. Deprotection and cleavage from resin was achieved by the anhydrous HF procedure,[26] followed when necessary by treatment with ethanolamine to affect removal of *N*-formyl protecting groups from Trp residues.[25] The product was purified to homogeneity by initial chromatography on Sephadex G-15, using 0.1 N acetic

acid as the eluent, followed by HPLC (LiChrosorb RP-8; 7 μm, 240–10 mm, Merck, Darmstadt, Germany), employing a linear gradient of acetonitrile (10–80%) in 0.1% aqueous trifluoroacetic acid. Magainin 2 was purchased from Sigma (St. Louis, MO).

Amino Acid Analysis, Sequence, and Mass Spectrometry. Amino acid analysis was performed on Dionex automatic amino acid analyzer following exhaustive acid hydrolysis. Sequence determination was accomplished using an Applied Biosystem 470A gas-phase microsequencer, coupled to an Applied Biosystem 120 PTH analyzer. Fast atom bombardment mass spectrometry (FAB-MS) analysis was performed on a VG 7070-HF (Manchester, UK) hybrid mass spectrometer outfitted with a Digital PDP-11/24 minicomputer-based VG-11-250 M^+ data system. Glycerol was used as a liquid matrix and Cs^+ ions of 35 keV as the primary beam.[27,28] Ion spray mass spectra was performed on a Sciex API III triple–quadrupole mass spectrometer equipped with an Ionspray ion source (Sciex, Thornhill, Toronto, Canada).[29]

Hemolytic Activity Assay. Three milliliters of freshly packed human erythrocytes, obtained from healthy volunteers, were washed three times with isotonic phosphate buffered saline (PBS, pH 7.4) and diluted to a final volume of 20 mL in the same buffer. Aliquots (190 λ) of cell suspension were placed in eppendorf tubes, and solutions (10 λ) of different concentrations of the tested peptides, in PBS, were added. Following gentle mixing, while incubated for 30 min at 37 °C, the tubes were centrifuged at 4000 rpm for 5 min. Aliquots (100 λ) of supernatants were taken and diluted to 1 mL with PBS. Absorptions at 576 nm were measured. Hemolysis exerted by 0.1% Triton X-100 was considered 100%. All experiments were duplicated.

Far-Ultraviolet Circular Dichroism Spectra. Peptides ((2.5–5) × 10^{-5} M) were dissolved in either 50 mM potassium phosphate buffer, pH 7.0, or 50% (v/v) trifluoroethanol (Merck, Darmstad, Germany) in the same buffer. A quartz cell of 1.0-cm pathlength was employed. Scans were performed at 23 °C over a wavelength range of 240–200 nm on a JASCO Model J-500C spectrophotometer with a JASCO NP-500 data processor. Calculation of the percent of helicity content ($\%_h$) was performed according to Wu et al.[21] as follows:

$$\%_h = \frac{([\theta]_{222} - [\theta]^0_{222})}{[\theta]^{100}_{222}}$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm. Values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0 and 100% helix content at this wavelength, are estimated as 2000 and 30 000 deg·cm²/dmol, respectively.

Antibacterial Studies. Stock solutions of peptides were prepared from pure peptide powders. Compounds were dissolved in water, filter sterilized (0.45-μm Acrodisc filter, Gelman Sciences) and stored at −70 °C. No change in antimicrobial activity was detected in any compound during a period of 1 month. Chromatographic controls revealed that all of the peptides studied proved to be chemically stable at corresponding experimental conditions.

The in vitro antimicrobial activity of peptides was tested by the microbroth dilution technique using Tryptic Soy Broth (Difco Laboratories, Detroit, MI) as a growth medium. Bacterial inoculum was 5 × 10^5 CFU/mL and microplates were incubated at 37 °C for 18–20 h.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUV) were prepared from soybean lecithin by sonication. Briefly, dry lipid and cholesterol were dissolved in $CHCl_3$/MeOH (2:1, v/v) such that the mixture contained 10% (w/w) cholesterol. The solvents were evaporated under a stream of nitrogen, and the lipids (at a concentration of 7.2 mg/mL) were resuspended in K^+ buffer (50 mM K_2SO_4, 25 mM HEPES–SO_4^{2-}, pH 6.8) by vortex mixing. The resulting lipid dispersion was sonicated (10–20 min) in a bath-type sonicator (G1115SP1 sonicator, Laboratory Supplies Company Inc., New York) until the turbidity had cleared. The lipid concentration of the solution was then determined by phosphorus analysis.[30] Vesicles were visualized as follows: a drop containing vesicles was deposited onto a carbon-coated grid and negatively stained with uranyl acetate. The grids were examined using JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo). Vesicles were shown to be unilamellar with an average diameter of 20–40 nm.[31]

Diffusion Potential Dissipation Experiments: Fluorometric Detection of Membrane Pores. Pore-mediated diffusion potential assay were performed, following previously described methodology.[32–34] In a typical experiment, 4 μL of a liposome suspension, prepared in K^+ buffer, were diluted in 1 mL of isotonic K^+-free buffer (50 mM Na_2SO_4, 25 mM HEPES–SO_4^{2-}, pH 6.8) in a glass tube to which the fluorescent, potential-sensitive dye 3,3′-diethylthiodicarbocyanine iodide (diS-C_2-5; Mr = 492; Sigma, St. Louis, MO) was then added. A 1-μL sample of 10^{-7} M valinomycin solution was added to the suspension in order to slowly create a negative diffusion potential inside the vesicles, leading to a quenching of the dye's fluorescence. Once the fluorescence had stabilized, 3–10 min later, the tested peptides were added. The subsequent dissipation of the diffusion potential, reflected as an increase in fluorescence, was monitored on a Perkin-Elmer LS-5 spectrofluorometer, with excitation set at 620 nm and emission at 670 nm, with gain adjusted to 100%. The percentage of fluorescence recovery, F_t, is defined as follows:

$$F_t = (I_t - I_o/I_f - I_o) \times 100$$

where I_o = the initial fluorescence, I_f = the total fluorescence observed before the addition of valinomycin, and I_t = the fluorescence observed after adding the peptide, at time t.

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