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Structure−Activity Relationships of Piscidin 4, a Piscine Antimicrobial Peptide

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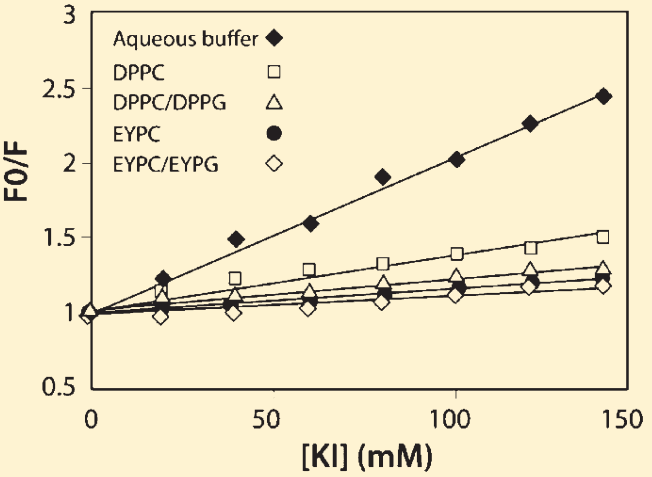
ABSTRACT: Piscidin 4, an antimicrobial peptide recently isolated from mast cells of hybrid striped bass (*Morone chrysops* female × *Morone saxatilis* male), is unusual in that it is twice as long (44 amino acids) as the typical members of the piscidin family. We previously showed that native piscidin 4 had a modified amino acid at position 20, but synthetic piscidin 4 (having an unmodified Trp at position 20) had similar potent activity against a number of both human and fish bacterial pathogens. In this study, the structure and membrane topology of synthetic piscidin 4 were examined using liposomes as model bilayers. Circular dichroism analyses revealed that it had a disordered structure in aqueous solution and folded to form a relatively weak α-helical structure in both membrane-mimetic trifluoroethanol solutions and liposome suspensions. Fluorescence data (piscidin 4 embedded in liposomes) and leakage experiments indicated that piscidin 4 interacted strongly with the hydrophobic part of the liposome. Binding of piscidin 4 to liposomes induced significant blue shifts of the emission spectra of the single Trp residue (Trp^{20}). Quenching of Trp^{20} by water-soluble quencher (either acrylamide or I^-) indicated that the fluorescence of Trp^{20} decreased more in the presence of liposomes than in buffer solution, thus revealing that Trp^{20} is less accessible to the quenchers in the presence of liposomes. The relative leakage abilities of piscidin 4 (1 μM) with liposomes were in the following order: DPPC (100%) ≥ EYPC (94%) > DPPC/DPPG (65%) > EYPC/EYPG (0%). This high activity against DPPC and EYPC liposomes was contrary to our data suggesting that piscidin 4 has a much weaker tendency to form an α-helix than other piscidins, such as piscidin 1. However, the structural similarity of protozoan membranes to EYPC liposomes might explain our discovery of the potent activity of piscidin 4 against the important skin/gill parasite ich (*Ichthyophthirius multifiliis*), but its negligible hemolytic activity against vertebrate membranes (hybrid striped bass or human erythrocytes). It also suggests that other conformation(s) in addition to the α-helix of this peptide may be responsible for its selective activity. This differential toxicity also suggests that piscidin 4 plays a significant role in the innate defense system of hybrid striped bass and may be capable of functioning extracellularly.



Vertebrates and invertebrates produce in some cases antimicrobial polypeptides (AMPs) that show a cytolytic effect in vitro on bacteria, fungi, parasites, protozoa, and enveloped viruses, presumably contributing to the innate immune system of the host against invading microorganisms. A large variety of AMPs have been isolated from fish.[1−3] Most are linear, amphipathic, antimicrobial cationic polypeptides and include pardaxin from the skin secretion of the Moses sole (*Pardachirus marmoratus*),[4] pleurocidin from the skin mucus of winter flounder (*Pleuronectes americanus*),[5] misgurin from the loach (*Misgurnus anguillicaudatus*),[6] and chrysophsins from red sea bream (*Chrysophyrs major*).[7] Other AMPs have been isolated from hagfish, including *Epatretus burgeri*[8] and *Myxine glutinosa*.[9] On the other hand, bass hepcidin, a cyclic peptide restricted by disulfide bonds, has been purified from hybrid striped bass (*Morone chrysops* × *Morone saxatilis*).[10] There is also genomic evidence of the presence of several other types of AMPs in fish, including cathelicidins in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*)[11,12] and defensins in zebrafish (*Danio rerio*) and pufferfish (*Takifugu* sp. and *Tetraodon* sp.).[13] In addition, the genes for hepcidin,[14] LEAP-2,[3] and NK-lysin[15] have recently been identified in channel catfish (*Ictalurus punctatus*).

One of the most common groups of AMPs in fish consists of piscidins, a family of peptides with a highly conserved, histidine-rich, phenylalanine-rich N-terminus and a more variable C-terminus.[1] Piscidins have potent, broad-spectrum antimicrobial activity against viruses, bacteria, fungi, and parasites.[1,16−18] As they were first isolated from mast cells of the commercially cultured hybrid striped bass (white bass, *M. chrysops*, female, × striped bass, *M. saxatilis*, male),[1]

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there is immunochemical[19] and genomic[20,21] evidence of the widespread presence of piscidins in higher teleosts (i.e., order Perciformes), including 11 species in eight families (Moronidae, Sciaenidae, Sparidae, Latidae, Siganidae, Belontidae, Cichlidae, and Percichthyidae).[19,21,22]

Conformational analysis of piscidin 1 by nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy indicated that the peptide takes an amphipathic α-helical conformation.[23−25] Piscidins are more hemolytic than magainin 2, a peptide antibiotic from the aquatic frog *Xenopus laevis*, but less hemolytic than mellitin, a peptide from bee venom that has relatively weak antibacterial activity.[1] Piscidin 3 is the least hemolytic of the piscidins described to date; its most significant structural distinction seems to be a glycine substituted for histidine at position 17, which would tend to disrupt the amphipathic α-helix[26] and lower its amphipathicity; this often correlates with reduced hemolytic activity.[27]

Recently, we isolated and characterized a novel type of piscidin 4, an antimicrobial peptide from hybrid striped bass (white bass, *M. chrysops*, female, × striped bass, *M. saxatilis*, male), which is twice the length of other members of the piscidin family.[28] This peptide has a molecular mass of 5329.25 Da, has 44 residues (FFRHLFRGAKAIFRGARQGXRAHKVVSRYRNRDVPETDNNQEEP), and exhibits considerable (to >65%) N-terminal sequences homology with piscidins 1−3. Piscidin 4 has a modified amino acid at position 20 that, on the basis of mass spectrometry data, is probably a hydroxylated tryptophan. Synthetic piscidin 4 (with an unmodified tryptophan at position 20) has antibacterial activity similar to that of the native peptide against a number of fish and human pathogens, including multidrug resistant bacteria. A Schiffer-Edmunson plot suggested that piscidin 4 had the potential to adopt an amphipathic α-helix. However, the details of the interactions, especially the conformational changes induced by phospholipid bilayers, are unknown.

Many cytolytic peptides act on biomembranes that are amphipathic in nature.[29] In the amphipathic environments, the peptide is forced to adopt particular secondary structures such as α-helix, β-structure, and 3_{10}-helix. The putative molecular targets for the AMPs are reported to be represented by bacterial phospholipid bilayers with a net negative charge.[30−32] Consequently, most AMPs preferentially bind to acidic phospholipid membranes, which mimic the bacterial membranes, rather than zwitterionic phospholipid membranes.[30,31,33−38]

The unusually long sequence of piscidin 4 prompted us to examine some of its biophysical properties so that we could determine if its structure−activity relationships varied from those of smaller piscidins, for which a number of studies have been recently published.[17,18,23−25,39] To investigate the membrane interaction of piscidin 4, in this study, we used liposomes composed of zwitterionic and/or acidic phospholipids. Synthetic P4 contains Trp at position 20, located in a hydrophobic cluster, thus allowing us to use fluorescence spectroscopy to study its association with artificial membranes. Analysis of the emission spectra and fluorescence quenching of piscidin 4 was used to characterize peptide−membrane interactions and to investigate the topology of this peptide in phospholipid bilayers. We also measured the conformational states of piscidin 4 in its interaction with membranes using CD spectroscopy. Furthermore, the hemolytic activity and antiparasitic activity of piscidin 4 were tested with erythrocytes and *Ichthyophthirius multifiliis* (ich) trophonts. The conformation and association of piscidin 4 into phospholipid bilayers provided insight into the mode of its action and the effect of different lipids on its function and selectivity.

MATERIALS AND METHODS

General. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho(1′-*rac*-glycerol) (DPPG), egg yolk L-α-phosphatidylcholine (EYPC), and egg yolk L-α-phosphatidylglycerol (EYPG) were purchased from Avanti Polar Lipids (Alabaster, AL). EYPC and EYPG are natural phospholipids that contain unsaturated fatty acid chains, giving the liposomes more fluidity than those composed of DPPC or DPPG. The latter are synthetic phospholipids that contain saturated fatty acid chains and form membranes that are more rigid. EYPC and DPPC liposomes mimic the outer leaflet of eukaryotic membranes, while EYPC/EYPG (3:1) and DPPC/DPPG (3:1) liposomes mimic the outer surface of bacterial membranes. Calcein was purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents were of the highest grade available.

Peptide Synthesis. Piscidin 4, piscidin 1, and magainin 2 (all with a free carboxy terminus) were synthesized using Fmoc chemistry on a Rainin Symphony instrument that provides on-instrument cleavage of the peptide from the resin. After synthesis, peptides were purified via analytical reverse phase HPLC using a YMC C_{18} column (4 mm × 50 mm, 3 μm particle size, 120 Å pore size support) using an acetonitrile (ACN) gradient with elution at a rate of 1 mL/min, where buffer A was 0.05% trifluoroacetic acid (TFA) in water and buffer B was 80% ACN in 0.05% TFA in water. Peptides were detected by their absorbance at 210 nm. Mass spectrometry of an aliquot of each purified peptide was conducted on a Micromass TofSpec SE mass spectrometer that was operated in positive ion mode and equipped with a nitrogen laser (337 nm), a reflectron, delayed extraction, and a postacceleration detector. The purified peptides were lyophilized from a 0.05% TFA/ACN solution and stored at −70 °C while being desiccated under argon gas until they were reconstituted in solvent.

Determination of Peptide Concentrations. Concentrations of piscidin 1 and piscidin 4 were determined by the dried weights of powder as follows. Because piscidin 1 and piscidin 4 have a high pI, it is possible that they contain a significant amount of counterions (e.g., TFA) after purification and lyophilization. When a peptide containing several basic residues is weighed, the additional mass of the TFA molecules should be included. Thus, we added the mass of the counterions (TFA) in accordance with the number of basic residues and the N-terminus of each piscidin and calculated the masses of the piscidin 4 and piscidin 1 peptide preparations using this correction.

Determination of Hydrophobicity. Piscidin 4 and piscidin 1 were eluted with an isocratic gradient of 7% ACN in 0.1% TFA for 10 min followed by a linear gradient from 7 to 70% ACN in 0.1% TFA for 45 min at a flow rate of 1 mL/min on a C_4 reversed phase column [Jupiter 5 μm, 300 Å, 4.6 × 250 mm (Phenomenex, Torrance, CA)]. The eluate was monitored at 214 nm using a Waters 486 UV detector, and the elution time was recorded for each peptide.

Hemolysis Assay. Hybrid striped bass and human erythrocytes were collected by venipuncture, diluted 1:1 (v/v) in Alsever's solution, and washed thrice in phosphate-buffered saline (PBS) [50 mM sodium phosphate and 150 mM NaCl (pH 7.4)] via centrifugation for 1 min at 2700*g* and 4 °C. The pellet was resuspended to a 10% suspension (v/v) in PBS, and 50 μL of the suspension was added to replicate wells of a 96-well polypropylene microtiter plate. Twofold serial dilutions of piscidin 4 or magainin 2 were prepared in PBS and 0.2% BSA, and 50 μL of each dilution was added to duplicate wells. The 100% hemolysis and baseline values were obtained by incubating RBCs with 0.1% Triton X-100 and PBS,

respectively. Melittin (Sigma, catalog no. M4171) was used as an additional positive control. After incubation for 10 min at room temperature, the plate was read at 490 nm in a plate reader (Elx800, Bio-Tek Instruments, Inc., Winooski, VT). The relative optical density, as compared with that of the 100% hemolysis and baseline values, was defined as percent hemolysis.

Antiparasitic Assay. We tested antiparasitic activity against *I. multifiliis* (ich) trophonts as described previously.[40] Ich is a ciliate protozoan that infects the skin and gill epithelium of virtually every freshwater fish.[41] The parasite has three stages, and the trophont is the stage that feeds in the skin and gill epithelium and thus would likely be in the most intimate contact with piscidin 4. Briefly, trophonts were scraped off the skin of anesthetized fish and used immediately. Filter-sterilized aquarium water (80 μL) was added to duplicate test wells of a 96-well flat-bottom polystyrene microtiter plate, followed by 10 μL of each test sample. Three trophonts in 10 μL of aquarium water were then added and observed every min for the first 15 min, every 10 min for the next 30 min, every 15 min for the next 75 min, and every hour for the following 4 h for death (indicated by lysis and/or complete lack of motility).

Preparation of Liposomes. Small unilamellar liposomes were prepared as follows. Briefly, the phospholipid (6.85 mg, ~8.6 μM) was dissolved in chloroform (1 mL) and dried by being run under a stream of nitrogen gas in a conical glass tube. The lipid residues were subsequently maintained under a reduced pressure overnight. The dry lipids were then suspended in 3 mL of 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.4) containing 0.1 M NaCl by continuous vortexing at room temperature for 30 min. The suspension was then sonicated at 50 °C for 30 min with 10 min intervals using a probe-type sonicator (Fisher Sonic Dismembrator model 300). The liposomes produced (lipid concentration of ~3.0 mM) were immediately used for the CD and titration measurements.

Large unilamellar vesicles (LUVs) with encapsulated calcein were prepared by the extrusion method.[72] The desired mixtures of phospholipids were dried in glass tubes under nitrogen and then lyophilized overnight to yield lipid films. The dry lipid films were suspended in leakage buffer [20 mM TES buffer containing 150 mM NaCl (pH 7.4)] and 70 mM calcein and then vortexed occasionally to disperse the lipids. The suspension was frozen and thawed in liquid nitrogen for 10 cycles and extruded 10 times through 0.1 μm polycarbonate membrane filters in an Avanti mini extruder apparatus (Avanti Polar Lipids). After extrusion, unencapsulated calcein was removed from the LUVs with encapsulated calcein by gel filtration on an 18 cm Sepharose 4B column equilibrated with leakage buffer. Fractions containing LUVs were used for calcein release measurements. The CD and fluorescence studies were conducted at 25 °C. Leakage measurements were performed at 41 °C with DPPC or DPPC/DPPG (3:1) liposomes.

CD Spectra. CD was determined as we have described previously.[31] CD spectra were recorded on a JASCO J-700 spectropolarimeter connected with a quartz cell with a path length of 1 mm. The spectra were recorded from 250 to 195 nm with peptide in either TES buffer, TFE (50 or100%), or liposomes. Spectra in 5 mM TES buffer (pH 7.4) were recorded at a peptide concentration of 50 μM. For measurements of CD spectra of peptides in liposomes, the peptide was dissolved directly in 5 mM TES buffer (pH 7.4) containing 1.0 mM liposomes. To compensate for light scattering due to liposomes, the CD spectrum of liposomes alone was subtracted from that of peptide in the presence of liposomes. All measurements were taken at 25 °C, and the data were expressed in terms of mean residue molar ellipticity (degrees square centimeters per decimole). The percentage of α-helix content in the peptide was estimated using the formula of Chen et al.[42]

Fluorescence Spectra. All fluorescence measurements were performed in quartz cuvettes with a path length of 1 cm. Fluorescence spectra were recorded on a PTI C61 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) with both excitation and emission band passes set at 5 nm. Spectroscopic titration of piscidin 4 with liposomes was performed as described previously.[31] Appropriate aliquots of liposomes (3.0 mM) were successively added to a solution (1.0 mL) of peptide (5 μM) in 5 mM TES buffer (pH 7.4). After each addition of liposomes, the mixture was incubated at 25 °C for 10 min to achieve equilibration. The tryptophan residue of piscidin 4 was excited at 280 nm, and emission spectra were recorded from 300 to 400 nm, averaging five scans. Spectra were recorded as a function of the lipid:peptide molar ratio and corrected for the contribution of light scattering in the presence of liposomes. Blue shifts were calculated as the differences in wavelength of the maxima in emission spectra of lipid/peptide and peptide-only samples.

Quenching of Trp Fluorescence by Acrylamide and KI. Quenching of the tryptophan fluorescence of piscidin 4 was measured via titration with aliquots of water-soluble quencher (either acrylamide or I^-) to the peptide in the absence or presence of liposomes as described previously.[31] The peptide:lipid molar ratio was 1:100. To reduce absorbance by acrylamide, excitation of tryptophan at 295 nm was used instead of that at 280 nm.[43] The quenching data were analyzed with the Stern−Volmer equation:

$$F_o/F = 1 + K_{sv}[Q] \quad (1)$$

where F_o and F are the fluorescence intensities at 350 nm in the absence and presence of a quencher at concentration [Q], respectively. K_{sv} is the Stern−Volmer quenching constant, which is a measure of the accessibility of the tryptophan residue to the quencher.

Leakage of Calcein from Liposomes. To determine the kinetics of membrane perturbation by piscidin 4 and if there was a preferential targeting of membranes with certain compositions, we measured the ability of piscidin 4 to induce leakage of a fluorescent dye (calcein) from DPPC/DPPG or EYPC/EYPG (3:1, w/w) liposomes, or from DPPC or EYPC liposomes. Leakage of calcein from liposomes was assessed by the procedure of Kim et al.[72] A liposome susupension (25 μL, final concentration of 95 μM) containing encapsulated calcein and 50 μL of an appropriately diluted peptide solution in TES buffer were added to 20 mM TES buffer (pH 7.4) to give a final volume of 1.0 mL. The increase in the fluorescence of calcein, when leaking out of liposomes, was monitored at an emission wavelength of 520 nm with an excitation wavelength at 490 nm. The change in fluorescence intensity was measured for 3 min after the addition of peptide. Complete release of calcein was achieved by adding 10 μL of 10% (v/v) Triton X-100. The percentage of dye release was calculated as follows:

$$\text{dye leakage } (\%) = 100(F - F_0)/(F_t - F_0) \quad (2)$$

where F is the fluorescence intensity caused by the peptide and F_0 and F_t are those with buffer alone and with 0.1% Triton X-100, respectively.

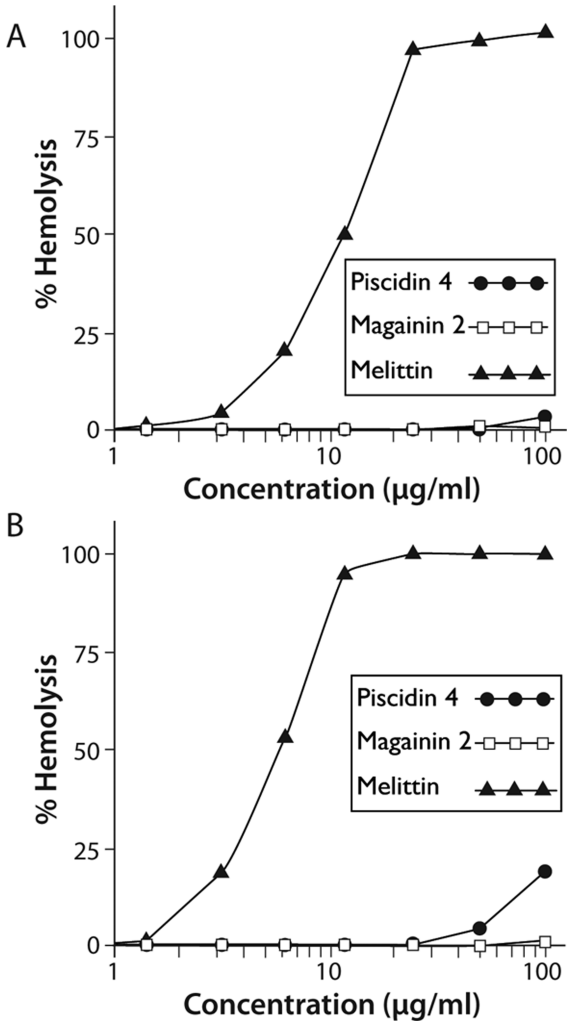

Figure 1. Hemolytic activity of piscidin 4, magainin 2, and melittin against erythrocytes of (A) humans and (B) hybrid striped bass.

RESULTS

Hemolytic Activity. The hemolytic activity of piscidin 4 against both hybrid striped bass and human erythrocytes was much lower than its effective antibacterial concentrations reported previously[28] and was similar on a weight basis to that of the relatively nonhemolytic magainin 2. As expected, melittin was highly hemolytic (Figure 1).

CD Study. A Schiffer—Edmunson plot suggested that piscidin 4 had the potential to form an amphipathic α-helix,[28] and its interaction with artificial lipid membranes supported this model. CD spectra were recorded in TES buffer and in the presence of 100% trifuoroethanol (TFE), 50% TFE, or liposomes comprised of DPPC, DPPC/DPPG (3:1), EYPC, or EYPC/EYPG (3:1) (Figure 2). In buffer solution, piscidin 4 exhibited a negative maximum around 200 nm, indicating that it formed mainly a random structure. However, in 100 or 50% TFE solutions (α-helix-promoting solvent), piscidin 4 had a double-minimum peak around 205 and 222 nm, indicative of α-helical structure; the helical content was 23 and 17%, respectively. In all liposomes, the peptide adopted a mean percentage helicity that was similar to or slightly lower than that in TFE. Interestingly, the α-helical conformation appeared to be slightly more pronounced in the

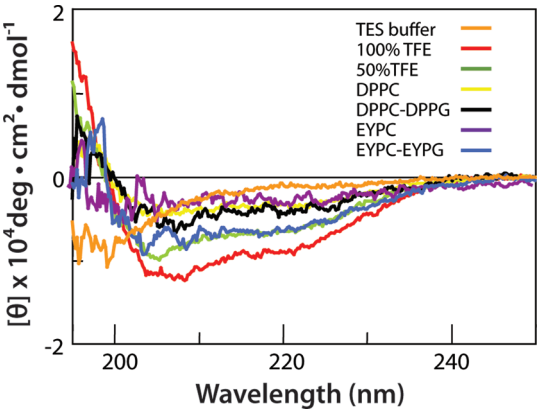

Figure 2. Circular dichroism spectra of piscidin 4 in TES buffer, 100% TFE, 50% TFE, and DPPC, DPPC/DPPG (3:1), EYPC, and EYPC/EYPG (3:1) liposomes. Peptide and lipid concentrations were 0.05 and 1 mM, respectively.

presence of EYPC/EYPG liposomes (17%) than in liposomes composed of DPPC (9%), DPPC/DPPG (11%), or EYPC (7%). The secondary structure of piscidin 4 (7−11% helical) suggested the presence of a partial helical structure, although the piscidin 4 helical percentages in all liposomes were very low.

Fluorescence Study. In an attempt to discern the location of piscidin 4 in lipid bilayers, its interactions with liposomes were investigated by fluorescence spectroscopy. The fluorescence spectrum of piscidin 4 in buffer showed an emission maximum at 345 nm, meaning that the Trp residue was exposed to the aqueous environment[44] (Figure 3). Upon addition of either DPPC (neutral) or DPPC/DPPG (acidic) liposomes, similar blue shifts (to 321 nm) were observed with an increasing lipid concentration, which accompanied an increase in the maximal fluorescence intensity (Figure 4). The increasing fluorescence intensity indicates a more rigid environment around the Trp residue.[44] The emission maxima of piscidin 4 in the presence of either EYPC or EYPC/EYPG liposomes also shifted to a shorter wavelength (Figure 4). The blue shift was most substantial with EYPC/EYPG liposomes. In the presence of DPPC, DPPC/DPPG, or EYPC liposomes, the blue shifts in the Trp emission spectra were all saturated at a lipid:peptide molar ratio of 60:1 (i.e., 300 μM lipid was required for full incorporation of 5 μM peptide into the lipid bilayer) and were maximally shifted by ∼24, ∼24, and ∼23 nm, respectively. However, in the presence of EYPC/EYPG liposomes, the blue shift was saturated at a lipid:peptide molar ratio of 20:1 (i.e., only 100 μM lipid was required for full incorporation of 5 μM peptide into the lipid bilayer) and was maximally shifted by ∼30 nm, suggesting the membrane affinity of piscidin 4 was highest for EYPC/EYPG liposomes.

Quenching of Trp Emission by Acrylamide and Iodide. Fluorescence quenching, where a compound (quencher) decreases fluorescence emission intensity, is affected by the accessibility of the quencher to the Trp residue. Quenching of Trp fluorescence can be measured by the difference in the fluorescence intensity of a peptide with and without liposomes. The fluorescence of Trp decreased in a concentration-dependent manner upon addition of either KI or acrylamide to the peptide solution in the presence and absence of liposomes. However, in the presence of liposomes, a smaller decrease in fluorescence intensity was evident, indicating that the

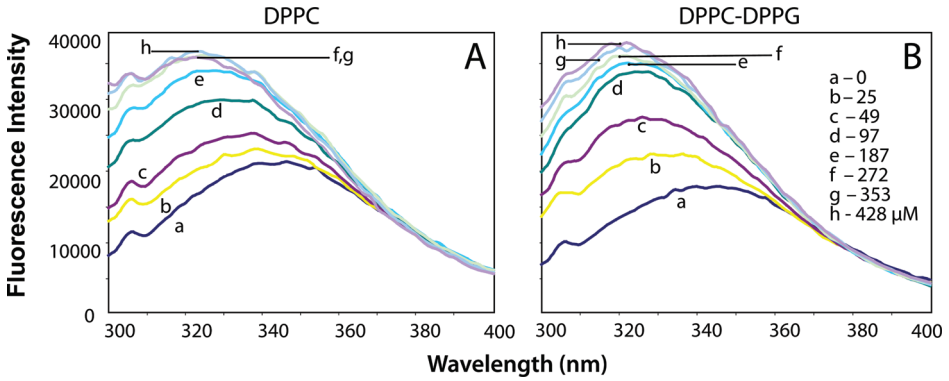

Figure 3. Fluorescence spectra of piscidin 4 in the presence of (A) DPPC and (B) DPPC/DPPG (3:1) liposomes. The peptide concentration was 5 μM. Lipid concentrations of (a) 0, (b) 25, (c) 49, (d) 97, (e) 187, (f) 272, (g) 353, and (h) 428 μM were used. The shoulder visible at the lower wavelengths is due to Raman scattering of the buffer.

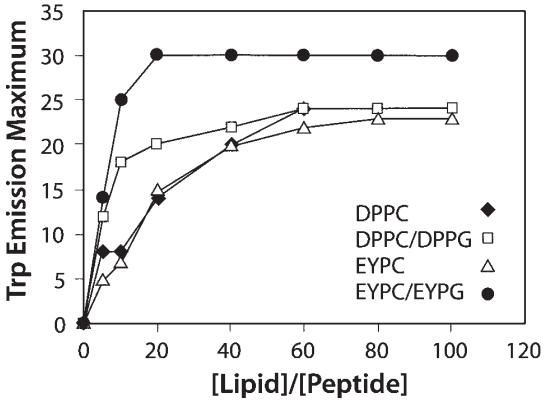

Figure 4. Blue shifts in Trp emission maxima of piscidin 4 in DPPC, DPPC/DPPG (3:1), EYPC, or EYPC/EYPG (3:1) liposomes.

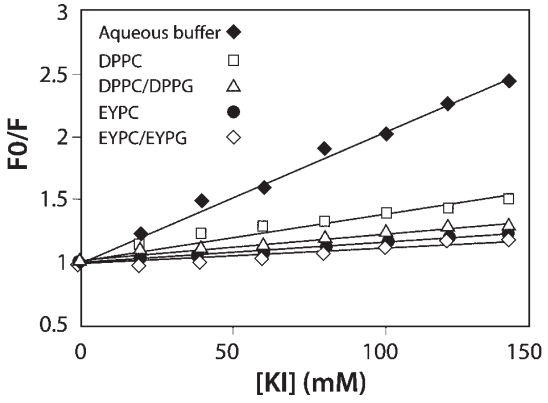

Figure 5. Stern–Volmer plots for the quenching of the fluorescence of the Trp residue of piscidin 4 by KI in an aqueous buffer or in the presence of DPPC, DPPC/DPPG (3:1), EYPC, or EYPC/EYPG (3:1) liposomes. The final concentrations of piscidin 4 and liposomes were 5 and 500 μM, respectively, in a total volume of 1 mL of 5 mM TES buffer.

Table 1. Comparison of the Interaction of Piscidin 4 with Various Liposomes

property	EYPC	EYPC/EYPG	DPPC	DPPC/DPPG
composition	natural	natural	semisynthetic	semisynthetic
flexibility	more	more	less	less
charge	neutral	negative	neutral	negative
membrane target	eukaryote	bacteria	eukaryote	bacteria
CD spectra (% helicity)	7	17	9	11
leakage (% at 1 μM)	94	0	100	65
blue shift (nm)	23	30	23	24
quenching (K_{sv} value)	2	1	4	3

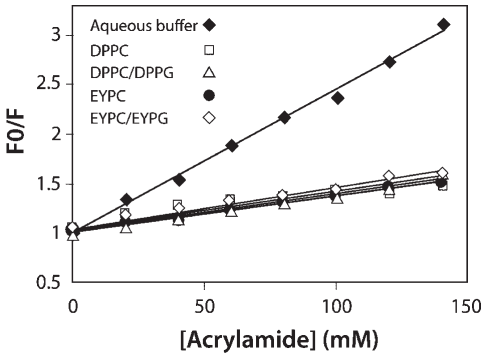

Figure 6. Stern–Volmer plots for the quenching of the fluorescence of the Trp residue of piscidin 4 by acrylamide in an aqueous buffer or in the presence of DPPC, DPPC/DPPG (3:1), EYPC, or EYPC/EYPG (3:1) liposomes. The final concentrations of piscidin 4 and liposome were 5 and 500 μM, respectively, in a total volume of 1 mL of 5 mM TES buffer.

Trp in piscidin 4 was less accessible to the quencher in the presence of all liposome preparations. Compared with measurements in the absence of liposomes, the values for K_{sv} quenching with acrylamide were very similar with all liposome preparations, but slightly different from those for I^- quenching. While the values for K_{sv} quenching with I^- decreased in the order DPPC > DPPC/DPPG > EYPC > EYPC/EYPG, the values were not much different [and were similar to other data (Table 1)].

The slopes of the Stern–Volmer plots of piscidin 4 showed a 4-fold decrease in K_{sv} with acrylamide quenching and a 3–8-fold decrease in K_{sv} with KI quenching in the liposome preparations, compared to that in the absence of liposomes (Figures 5 and 6 and Table 2). The lowest slope of the Stern–Volmer plots of piscidin 4 for I^- was with EYPC/EYPG liposomes (K_{sv} = 1.32 M^{-1}), being about $^1/_8$ of that in the absence of liposomes (K_{sv} = 10.32 M^{-1}); the slope of the Stern–Volmer plot for acrylamide was similar. Lower K_{sv} values mean that there was less

accessibility of acrylamide or I^- to Trp, meaning that the peptide was in the hydrophobic region of the bilayers.

Leakage of Calcein from Liposomes. To test whether bacterial membranes could be preferentially targeted by piscidin 4, membrane perturbation was examined by measuring calcein release using piscidin 1 as a reference. Calcein has excitation and emission wavelengths of 490 and 520 nm, respectively. Dye leakage profiles using DPPC, DPPC/DPPG, EYPC, and EYPC/EYPG liposomes are shown in Figure 7. Piscidin 4 had the strongest leakage ability with DPPC and EYPC liposomes. The relative leakage abilities of 1 μM piscidin 4 with liposomes were in the following order: DPPC (100%) ≥ EYPC (94%) > DPPC/DPPG (65%) > EYPC/EYPG (0%) (Table 3). The leakage ability of piscidin 4 was similar or weaker than those of piscidin 1 for DPPC or EYPC/EYPG liposome preparations, except EYPC and DPPC/DPPG liposomes, where piscidin 4 caused more leakage at a similar micromolar concentration (piscidin 4, 94 and 65% leakage at 1 μM EYPC and DPPC/DPPG liposomes, respectively; piscidin 1, 65 and 16% leakage at 1 μM EYPC and DPPC/DPPG liposomes, respectively) (Table 3).

Table 2. Stern−Volmer Quenching Constants [K_{sv} (M^{-1})] of KI and Acrylamide for Piscidin 4 (5.0 μM) in the Presence or Absence of Liposomes (500 μM)[a]

		acylamide		KI	
	blue shift (nm)	K_{sv} (M^{-1})	% quenched	K_{sv} (M^{-1})	% quenched
buffer	0	15.02	100	10.32	100
DPPC	24	3.58	24	3.59	35
DPPC/DPPG (3:1)	24	4.01	27	2.03	20
EYPC	23	3.58	24	1.67	16
EYPC/EYPG (3:1)	30	4.14	28	1.32	13

[a] Note that the K_{sv} in acrylamide and the blue shift value of EYPC/EYPG liposomes were not matched.

Hydrophobicity. On the C_4 column, piscidin 4 eluted at 35 min while piscidin 1 eluted at 37.8 min (Table 3).

Antiparasitic Activity. For piscidin 4, the PC_{min} (minimal protozoacidal concentration), the lowest concentration at which at least one parasite died, was 25 μg/mL (3.4 μM), while PC_{100} (100% protozoacidal concentration), the lowest concentration at which all parasites died, was 50 μg/mL (6.8 μM) (Table 3).

DISCUSSION

We previously reported that piscidin 4 was a constituitively expressed AMP from hybrid striped bass gill that had potent, broad-spectrum activity against both Gram-positive and Gram-negative bacteria.[28,45] Piscidin 4 is a member of the piscidin family, which appears to consitiute the most common AMPs present in fish.[28]

Secondary structure predictions suggested that the 11-amino acid N-terminus is the only segment of piscidin 4 that is likely to form an α-helix. In contrast, the C-terminus (amino acids 33−44) is highly acidic (pI 3.39) and forms a random coil.[28] Piscidin 4 also has a large hydrophobic region comprising the Phe^2, Leu^5, Phe^6, Ala^9, Ile^{12}, Phe^{13}, Ala^{16}, Trp^{20}, and Val^{34} residues that are most likely configured into helix and loop regions. The distribution of hydrophobic and charged residues has a crucial influence on the antimicrobial activity of AMP.[46] The presence of several positive charges, combined with a clearly demarcated delineation of hydrophobic and hydrophilic side chains in a linear α-helical structure in the N-terminus, provides some evidence that piscidin 4 may act against bacteria in a manner similar to those of other AMPs by forming pores that permeabilize the bacterial membrane. This presumption was supported by the membrane interaction studies. However, the relative importance of the α-helical conformation in its activity is unclear (see below).

While our CD study suggested that piscidin 4 had an unordered structure in buffer solution, evidence of a low percentage of the peptide forming an α-helix was observed in the presence of TFE and all four liposome preparations (Figure 2). We used TFE as strong helix-inducing agent to mimic the hydrophobic environment of the

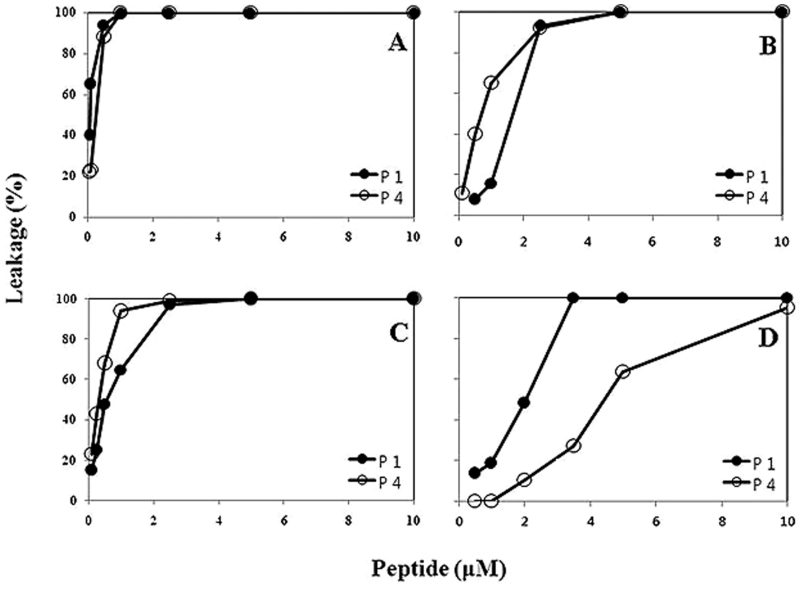

Figure 7. Release of encapsulated calcein from (A) DPPC, (B) DPPC/DPPG (3:1), (C) EYPC, or (D) EYPC/EYPG (3:1) liposomes as a function of the peptide concentration of piscidin 4 (○) or piscidin 1 (●). The concentration of the liposomes was 70 μM.

Table 3. Relative Activities of Piscidin 4 versus Piscidin 1 against Various Membrane Targets[a]

membrane target	piscidin 4	piscidin 1
PC_{min} for ich (μg/mL) [μM]	25 [3.4]	6.3 [1.7][b]
PC_{100} for ich (μg/mL) [μM]	50 [6.8]	12.5 [3.4][b]
% human RBC hemolysis at 100 μg/mL (13.6 μM)	1.4	100[c]
% EYPC leakage at 1 μM	94	65
CD spectrum (% helicity)	23 (100% TFE)	40 (75% TFE)[d]
hydrophobicity	−1.21[e]	0.39[e]
hydrophobic moment	0.58[e]	0.70[e]
RP-HPLC retention time (min)	35	37.8

[a] PC_{min} (minimal protozoacidal concentration) is the lowest concentration at which at least one parasite died. PC_{100} (100% protozoacidal concentration) is the lowest concentration at which all parasites died. The mean hemolytic activity of piscidin 4 for three replicates was 0.6% + 0.3% + 3.2% = 1.4%. [b] Data from ref 40. [c] Data from ref 1. Piscidin 2 is also 100% hemolytic at 100 μg/mL. [d] Data from ref 39. [e] Data from ref 28.

membrane.[47] Piscidin 4 adopted 17% helical content in 50% TFE and 23% helical content in 100% TFE, suggesting that it might be not form much helix in a hydrophobic environment and that α-helicity is not closely related to its interaction with the hydrophobic core of the bilayer. The amount of helicity appeared to be slightly higher with negatively charged liposomes than with neutral liposomes and slightly higher with liposomes consisting of natural, unsaturated, acidic phospholipids (EYPC/EYPG) than those consisting of synthetic, saturated, acidic phospholipids (DPPC/DPPG), but these variations were not greatly different (Table 1). Because piscidin 4 adopted a low degree of α-helical conformation with all artificial membrane preparations (including those with neutral lipids), its interaction is probably not purely mediated by electrostatic interaction and α-helical structure. Because we studied the CD experiments under lipid phase transition temperatures (e.g., 41 °C for DPPC and DPPG), it may have affected the result of the CD experiment. With an increase in the temperature above the phase transition temperature in a CD experiment with a cecropin−melittin hybrid peptide, a much better defined α-helix could be observed and decreased β-sheet content was observed.[48,49] Therefore, our results obtained from the CD experiments at the lipid phase transition temperature could be lower or weaker than that obtained above the lipid phase transition temperature.

Other piscidins are also predicted to adopt an amphipathic α-helical conformation upon binding to the lipid-containing cell membrane of a pathogen.[1,24,25,39] However, piscidin 4 adopts a much weaker α-helical structure (23% α-helix in 100% TFE) than piscidin 1 (40% α-helix in 75% TFE).[39] Piscidin 4 may be less helical than piscidin 1 because piscidin 4 contains more Gly and Pro. Lacking a side chain, Gly imparts greater flexibility, while a Pro residue can act as a helix breaker, causing rigidity and a bend in the peptide.[50] These properities make it more difficult for a peptide to maintain secondary structure. In addition, the acidic C-terminal region might inhibit the electrostatic interaction of piscidin 4 with acidic liposomes. The low percent helicity with all liposomes further suggests that piscidin 4 may form additional secondary structures. A secondary structure prediction program (EMBOSS, Garnier method) suggested not only an α-helix at residues 1−11 but also a β-sheet segment for residues 12−14 and 24−28, a turn segment for residues 29−33, and a randon coil for residues 34−44.[28] These predicted results suggested that piscidin 4 contained a low helical content, and it was similar to the result suggested by its CD spectra. Therefore, the results obtained from the CD spectra and EMBOSS prediction are well-matched. Because these predicted structures are based upon modeling and not experimental data, further studies are needed to substantiate them.

Piscidin 4 had significantly less hemolytic activity than either piscidins 1−3[1] or melittin (Figure 1). Hemolytic activity is often used as a potential indicator of possible host cytotoxicity of AMP and suggests that piscidin 4 might be more likely to function extracellularly than other piscidins because it would presumably be less toxic to host cells. The weaker hemolytic activity of piscidin 4 compared to piscidin 1 might be due to the C-terminal acidic random coil region of piscidin 4 inhibiting the insertion of piscidin 4 into the hydrophobic region of the erythrocyte membrane. Alternatively, it might be due to the less ordered secondary structure of piscidin 4 than piscidin 1 inhibiting the insertion of piscidin 4 into the erythrocyte membrane. The calculated hydrophobic moment, hydrophobicity, and helicity of piscidin 4 are relatively lower than those of piscidin 1, and on the basis of the retention time in RP-HPLC, the hydrophobicity of piscidin 4 is also relatively lower than that of piscidin 1 (Table 3). The HPLC data are collected in an environment that is not conducive to optimizing the secondary structure of the peptides; therefore, while these data allow us to appreciate hydrophobicity, they do not necessarily provide insights into amphipathicity, and the latter is particularly important for antimicrobial potency. Usually, antimicrobial peptides with high hemolytic activity show strong leakage ability against neutral membranes, and this ability is closely related to the hydrophobicity of the peptide.[51] However, while pisicidin 4 exhibited weak hemolytic activity (against vertebrate erythrocytes), it had strong leakage activity (against EYPC liposomes). This apparent contradiction may be due to the fact that the total number of hydrophobic residues in the piscidin 4 sequence is greater than in the more hemolytic piscidin 1, despite the fact that the hydrophobicity of piscidin 4 is relatively low compared to that of piscidin 1, despite the higher hydrophobicity of piscidin 1 (Table 3). Nevertheless, the specific reason for this discrepancy is unclear.

The dye release experiments showed that the membrane perturbing effect of piscidin 4 was similar or weaker that those of piscidin 1 in DPPC or EYPC/EYPG liposomes. However, in EYPC and DPPC/DPPG liposomes, piscidin 4 exhibited especially strong leakage ability and was surprisingly more potent than piscidin 1 (Figure 7). These leakage data were somewhat contradictory to the CD results and hemolytic activity. The leakage efficiency of an AMP is usually directly related to its α-helicity, as well as the surface charge density of the liposomes.[52] However, the piscidin 4 leakage response showed no such obvious relationship. At low peptide:lipid ratios, AMPs typically cause low or barely detectable dye leakage, while at a P:L ratio above a threshold value, they cause major leakage, leading to a sigmoidal dose−response curve.[53] These responses suggest that an AMP must accumulate on the membrane to induce

leakage. The nonsigmoidal curves almost observed with piscidin 4 suggest that there was no accumulation of peptide on the liposome surface and the tested concentration of piscidin 4 might be higher than the threshold concentration (i.e., the threshold concentration of piscidin 4 in EYPC liposomes might be less than 1 μM). Thus, the charge of the lipid headgroups and specific secondary structure (especially α-helix) might not be much related to the interaction of piscidin 4 with these membranes, and there might be other reasons for the strong leakage ability of piscidin 4 with EYPC.

This might relate to the differential toxicity of piscidin 4 for protozoan membranes compared to vertebrate (human and piscine erythrocyte) membranes and is further suggested by the relatively high potency of piscidin 4 for the protozoan parasite ich compared to its low toxicity for erythrocyte membranes. Protozoan membranes are most similar in composition to EYPC. While vertebrate erythrocyte membranes are also chemically similar to EYPC, their membrane fluidity is more like that of DPPC. Protozoan membranes consist of >70% neutral phospholipids (phosphatidylcholine and phosphatidylethanolamine), ∼15% acidic phospholipids (phosphatidylglycerol, phosphatidylinositol, and phosphatidylserine), and ∼5% sphingomyelin but do not contain sterols.[54−56] The human erythrocyte outer leaflet consists of ∼40% neutral phospholipids (phosphatidylcholine and phosphatidylethanolamine), ∼30% sphingomyelin, and ∼10% sterols (cholesterol in mammals).[51,57] The sterol in erythrocytes increases the degree of order of the acyl chain[58] and might make it more rigid than the protozoan membrane. The efficient blocking of the membrane intercalation of AMP also appears to be achieved by the combination of sphingomyelin and cholesterol.[37,59] Similarly, DPPC (saturated phospholipid) forms a more rigid membrane than EYPC (unsaturated phospholipid). Thus, while piscidin 4 might bind to the outer leaflet of the erythrocyte membrane (mimicked by DPPC) (as suggested by the CD, Trp fluorescence, and quenching data), it might not permeate deeply into the erythrocyte membrane because of the ordered membrane acyl chain, and stabilized and rigid bilayer structure formed by sterol.

The fluorescence data for piscidin 4 in the presence of liposomes demonstrated a dose-dependent blue shift, which might have been due to either the Trp residue being translocated into a highly polar amino acid cluster or it being deeply embedded in the lipid bilayer. The former possibility is unlikely because, as shown in the Schiffer−Edmunson plot, when piscidin 4 assumes an α-helical structure (Figure 4), the Trp residue appears to be located at the interface of a hydrophilic and a hydrophobic cluster. These findings indicate that the hydrophobic face of the amphiphilic α-helical structure of the peptide is embedded in the lipid bilayer.

In the study of Trp fluorescence, upon addition of all liposomes, similar blue shifts were observed with an increasing lipid concentration, which accompanied an increase in the maximal fluorescence intensity. These results suggest that piscidin 4 can be bound to liposomes regardless of the charge of the lipid headgroups, and piscidin 4 showed even more blue shift in the presence of EYPC/EYPG liposomes.

In the presence of DPPC, DPPC/DPPG, or EYPC liposomes, the blue shifts in the Trp emission spectra were all saturated at a lipid:peptide molar ratio of 60:1 (i.e., 300 μM lipid was required for full incorporation of 5 μM peptide into the lipid bilayer) and were maximally shifted by ∼24, ∼24, or ∼23 nm, respectively. However, in the presence of EYPC/EYPG liposomes, the blue shift was saturated at a lipid:peptide molar ratio of 20:1 (i.e., 100 μM lipid was required for full incorporation of 5 μM peptide into the lipid bilayer) and maximally shifted by ∼30 nm. The saturation of the blue shift at a peptide:lipid ratio of 1:20 (100 μM EYPC/EYPG) suggests that the Trp indole amino group can fully interact with the phospholipid headgroups without Trp−Trp interaction or aggregation, because if there were peptide aggregation or Trp−Trp interaction, there would not be a concentration-dependent increase in the fluorescence intensity and a blue shift with the addition of liposomes. Compared to the saturated concentration in the other liposomes, smaller lipid concentrations were required to obtain the maximal effect on piscidin 4 fluorescence when EYPC/EYPG liposomes were present in the bilayer, indicating higher affinity of piscidin 4 for liposomes containing unsaturated acidic phospholipids.[37] These results suggest that the quantum yield of the membrane-bound Trp residues is a function of the amount of membrane-bound peptide per liposome.

In the binding of a synthetic gastrin isomer (an amphiphatic pentapeptide related to gastrin) to dimyristoylphosphatidylcholine (DMPC, a 40 Å thick liposome), a 20 nm blue shift suggested that the peptide was completely in a hydrophobic environment,[60] while in the binding of somatostatin to POPG vesicles, a 12 nm blue shift suggested that the peptide does not completely penetrate into the central part of the bilayer but rather remains in the headgroup region of the phospholipid.[61] Thus, the 23−30 nm blue shifts of piscidin 4 indicate that the Trp residue is probably in a completely hydrophobic environment, deeply buried in EYPC/EYPG liposomes. This result agrees with the greatest helicity occurring with EYPC/EYPG liposomes (Figure 2). In membrane proteins, Trp is usually found in the interfacial region because of its strong preference for the interface.[62] However, piscidin 4 is not a membrane protein but rather is a soluble protein. For it to disrupt a bacterial membrane, it must permeate the membrane; in this process, the Trp residue enters the membrane. Evidence of this is shown in Figures 5 and 6, where the Trp is quenched in the titration experiment. Another example of Trp quenching is shown in the studies of temporin L.[37]

The quenching data also support the presence of Trp in the apolar environment. Both acrylamide and KI were used as quenchers. Acrylamide, being neutral and water-soluble, is very sensitive to the degree of accessibility of tryptophan to the solvent containing the acrylamide and has the advantage in that no electrostatic interactions take place with the headgroups of negatively charged phospholipids.[37,63] The slight difference from acrylamide in quenching by KI might be due to the electrostatic interaction of K^+ ion with acidic residues in the C-terminus of piscidin 4, as well as neutralization of repulsion with acidic headgroups in the liposomes.

The first 11 amino acids of piscidin 4 can penetrate the membrane up to 16.5 Å, and this is totally in the hydrophobic region of the membrane because the thickness of the DPPC or EYPC liposome bilayer is ∼40 Å[37] and that of the headgroup is ∼5 Å.[64] Only if the 11-amino acid N-terminus were perpendicularly inserted into the membrane would there be no blue shift because the Trp residue is located at position 20. Thus, if only the 11-amino acid N-terminus penetrated the lipid bilayer and there were a blue shift, the Trp residue would probably be in the hydrophobic region of the outer leaflet area (5−20 Å) and piscidin 4 would be lying parallel to the surface of the membrane. If piscidin 4 can penetrate perpendicularly into the membrane, it would need to penetrate into the membrane up to 30 Å; at present, we have no evidence of this. Our fluorescence and quenching experiments suggest a change in Trp's position from within the buffer to inside the membrane, as shown by the blue shift. Thus, we suggested that Trp localizes to and faces the hydrophobic region of the membrane. However, if piscidin 4 inserts perpendicularly into the membrane, Trp might not be located inside the membrane because the Trp residue is located at position 20 from

the N-terminus and at position 25 from the C-terminus. Thus, if Trp was located in the hydrophobic region as we suggested, horizontal binding is more likely to explain Trp's position; therefore, we suggested that piscidin 4 might be embedded parallel to the membrane. What we mean by "lying parallel" is the position shown in the model of membrane interaction for the amphipathic peptide KLAL.[65] In KLAL, at a low negative surface charge, hydrophobic interactions between the hydrophobic face of the helix and the lipid acyl chains drive insertion of the peptide into the inner nonpolar membrane region, causing disturbance in the arrangement of the acyl chains. In the latter case, piscidin 4 would not rest on the surface of the membrane but rather would lie embedded in the membrane parallel to the outside layer.

Several models have been proposed to explain the mechanism via which AMPs disrupt membrane integrity. In the barrel-stave model, there is transmembrane pore formation. This model requires a peptide to be of sufficient length to traverse the hydrophobic core of the bilayer and implies direct contact between peptides upon channel formation. In the wormhole model, there is development of peptide−lipid associations in toroidal pores. The toroidal-pore model addresses the fact that many peptides are simply too small to span an unperturbed phospholipid bilayer when in an α-helical conformation, where ∼22 amino acids are required to traverse the bilayer, which is typically 32−38 Å wide. In the carpet mechanism, a single layer of peptides forms on the membrane surface. The carpet model requires accumulation of the peptide at the bilayer surface like a carpet; when a threshold concentration of monomers is reached, the membrane is permeated and disintegrated in a detergent-like manner without the formation of discrete channels.[66] Thus, the in-plane diffusion model does not require self-association. The insertion of in-plane peptides into lipid bilayers leads to disturbances in the bilayer packing within a diameter of approximately 100 Å and a reduction in the average bilayer thickness. Diffusion of the peptides within the membrane causes regions of instability to overlap, disturbances of lipid packing to accumulate, and transient openings to occur. Support for such a model is further derived from the observation that short peptides such as mastoparan (14 residues), cecropin−melittin hybrids, some as short as 15 amino acids, and synthetic peptides (8−14 residues) are also capable of exhibiting channel-like activity, although these peptides are too short to reach through the membrane.[67]

Recent CD and NMR spectroscopy studies with an amidated form of piscidin 1 have suggested that it most likely permeabilizes membranes via formation of toroidal pores rather than the barrel-stave mechanism.[39] In another recent study using solid state NMR of both amidated and free forms of both piscidin 1 and piscidin 3 in the presence of hydrated, oriented lipid bilayers, all piscidin analogues were found to form an α-helical structure, lying parallel to the surface of the membrane. The overall structures of these piscidins were strongly amphipathic, and on the basis of the NMR data, the authors concluded that piscidins 1 and 3 interacted with a membrane via either a carpet or in-plane mechanism of penetration.[23,24]

If only an 11-amino acid region in piscidin 4 can penetrate the membrane, it cannot span the bilayer, making either the toroidal-pore, carpet, or in-plane diffusion model more likely. A sufficient sequence length is needed to traverse the hydrophobic core of the bilayer to form transmembrane pores by the "barrel-stave" model,[66] making it difficult for short peptides to fit this model. Toroidal-pore and in-plane diffusion models are more compatible with AMPs that are too short to span the phospholipid bilayer in an α-helical conformation, such as mastoparan, the cecropin−melittin hybrid, and trichogin, which have fewer than 15 amino acids.[66,67] However, the blue shift and quenching experiments suggest that the Trp residue in piscidin 4 is located between the end of the headgroup of the phospholipids (∼5 Å) and the center of the bilayer (∼20 Å), indicating that it might be embedded in the bilayer by lying parallel, making the in-plane diffusion model more likely. Also, because the Trp at position 20 is embedded in the liposome membrane, it is unlikely that the 20 amino acids in the N-terminus would be emedded by spanning the depth of the membrane in all four liposome preparations. Further studies, especially NMR data, are needed to confirm these findings.

Peptide−lipid interaction is often evaluated by a peptide's hydrophobicity and hydrophobic moment. The amphiphilicity of a peptide is revealed by its hydrophobic moment.[68] The mean residue hydrophobicity, hydrophobicity measured by RP-HPLC retention time, mean helical hydrophobic moment, and helicity of piscidin 4 are lower than those of piscidin 1 (Table 3), and their different hemolytic, antiparasitic, and leakage activities suggest that they have different mechanisms of action. Other elements of secondary structures may be present in piscidin 4 and thus may be the basis of a different mechanism of action for this longer peptide.

Kini and Evans[69] were one of the first to propose that cytolytic peptides must adopt a specific conformation to interact with a cell membrane, with the cationic region being involved in binding to the cell surface, while the hydrophobic region determined its lytic ability. All our data suggest that there is a conformational change in piscidin 4 that allows the hydrophilic part of the peptide to interact with the acidic moiety of membrane phospholipids by lying parallel, with its hydrophobic segment then penetrating into the hydrocarbon moiety of the membrane. It seems likely that the amphiphilic α-helical structure and an appropriate hydrophobicity of piscidin 4 cause an expansion and disturbance of the membrane structure, causing increased membrane permeability or transient openings and cell death, which is compatible with an in-plane diffusion model. Chekmenev et al.[70] recently suggested that piscidin 1 binds to membranes through an "in-plane dynamic" model and then diffuses laterally along the membrane's surface before it disrupts membrane composition. These results are similar to those from our suggested membrane binding model for piscidin 4 via an "in-plane diffusion" model with the membrane then lying embedded in the membrane parallel to the outside layer. The latter is also like the amphipathic peptide KLAL with a low negative surface charge.[65] Our results are also similar to those for piscidin 1 and its analogues[71,72] and temporins.[37]

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■ ABBREVIATIONS

NMR, nuclear magnetic resonance; CD, circular dichroism; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho(1′-*rac*-glycerol); EYPC, egg yolk L-α-phosphatidylcholine; EYPG, egg yolk L-α-phosphatidylglycerol; TFA, trifluoroacetic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TFE, trifluoroethanol; PBS, phosphate-buffered saline; ACN, acetonitrile.

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