Plicatamide, an Antimicrobial Octapeptide from *Styela plicata* Hemocytes*

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Plicatamide (Phe-Phe-His-Leu-His-Phe-His-decDOPA), where decDOPA represents decarboxy-(E)-α,β-dehydro-3,4-dihydroxyphenylalanine, is a potent antimicrobial octapeptide from the blood cells of the solitary tunicate, *Styela plicata*. Wild type and methicillin-resistant *Staphylococcus aureus* (MRSA) responded to plicatamide exposure with a massive potassium efflux that began within seconds. Soon thereafter, treated bacteria largely ceased consuming oxygen, and most became nonviable. Native plicatamide also formed cation-selective channels in model lipid bilayers composed of bacterial lipids. Methicillin-resistant *S. aureus* treated with plicatamide for 5 min contained prominent mesosomes as well as multiple, small dome-shaped surface protrusions that suggested the involvement of osmotic forces in its antimicrobial effects. To ascertain the contribution of the C-terminal decDOPA residue to antimicrobial activity, we synthesized several analogues of plicatamide that lacked it. One of these peptides, PL-101 (Phe-Phe-His-Leu-His-Phe-His-Tyr-amide), closely resembled native plicatamide in its antimicrobial activity and its ability to induce potassium efflux. Plicatamide was potently hemolytic for human red blood cells but did not lyse ovine erythrocytes. The small size, rapid action, and potent anti-staphylococcal activity of plicatamide and PL-101 made them intriguing subjects for future antimicrobial peptide design.

Phe-Phe-His-Leu-His-Phe-His-decDOPA (plicatamide)† is a modified octapeptide found in the hemocytes of *Styela plicata*.

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Peptide Purification

Native plicatamide was purified from freshly harvested hemocytes (blood cells) of *S. plicata* as described recently (1). We determined their peptide content either by performing quantitative amino acid analysis or by doing analytical reverse phase-HPLC on a C18 column and then computing and comparing the area under the curve (AUC) at 215 nm with the AUC of an appropriate standard previously subjected to quantitative amino acid analysis.

Peptide Synthesis

The synthetic peptides used in our initial experiments were custom-synthesized by Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry at Research Genetics (Huntsville, AL) and purified to homogeneity by reverse phase-HPLC. Mushroom tyrosinase (6680 units/mg) was purchased from Sigma, and all other reagents were of analytical grade. Tyrosine amide (Sigma) was used to prepare PL-103 and -102 (Table I) by converting the C-terminal tyrosine of PL-101 and -102 to dopamine (DOPA, 3,4-dihydroxyphenylalanine) in PL-103; and DOPA-amide in PL-104. Of these octapeptides, PL-101 most closely simulated the antimicrobial properties of native plicatamide. This report will describe the effects of plicatamide on staphylococci.

MATERIALS AND METHODS

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synthesizer using FastMoc chemistry and purified by reverse phase-HPLC as described above.

Antimicrobial Assays

Radial Diffusion Assay—The assay has been described elsewhere (4). Our Gram-positive test organisms were Staphylococcus aureus 9390918-3, MRSA ATCC 33591, a methicillin-resistant S. aureus strain, and Listeria monocytogenes, strain EGD. In some experiments we also tested Escherichia coli, ML-35p, and Pseudomonas aeruginosa, MR3007, a strain that was resistant to many conventional antibiotics. Native plicatamide was serially 3.16-fold diluted with 0.01% acetic acid that contained 0.1% human serum albumin to minimize its nonspecific adsorption to plastic tubes. Organisms were grown to mid-logarithmic phase at 37 °C in trypticase soy broth. After they were washed with 10 mM phosphate buffer, pH 7.4, 4 × 10^7 bacterial colony-forming units (CFU) were incorporated into 10 ml of the underlay gel mixture. Unless otherwise stated, the underlay gels also contained 1% w/v agarose (Sigma A-6013), 10 mM sodium phosphate buffer, pH 7.4, and 0.3 mg/ml trypticase soy broth powder. Some underlay gels were supplemented with 100, 175, or 250 mM NaCl. A 6 × 6 array of sample wells, each 3.2 mm in diameter and 1.2 mm deep, was punched in the underlay gel. These allowed 8-μl aliquots of each dilution to be introduced. After the plates had incubated for 3 h at 37 °C, a nutrient-rich overlay gel (60 mg/ml trypticase soy broth powder in 1% w/v agarose) was poured, and the incubation was continued overnight to allow surviving organisms to form microcolonies. The diameters of completely clear zones were measured to the nearest 0.1 mm and expressed in units (1 unit = 0.1 mm), after first subtracting the well diameter. Because a linear relationship exists between the zone diameter and the log_{10} of the peptide concentration, the X intercept of this line was determined by a least mean squares fit and was considered to represent the minimal effective concentration (MEC).

Colony Counting Assays—Stationary or mid-logarithmic phase bacteria were prepared as described above and incubated with antimicrobial peptides at 37 °C in an agarose-free liquid medium containing 100 mM NaCl, 10 mM sodium phosphate, or Tris buffer, pH 7.4, and such other additions as are described in the text. Aliquots (20 μl) were removed at intervals, diluted appropriately, and transferred to nutrient agar plates with a Spiral Plater (Spiral Biotech, Rockville, MD). Colonies were counted after overnight incubation at 37 °C.

Broth Microdilution Assays—These assays used cation-adjusted, Mueller Hinton II Broth (BD Biosciences) and were performed according to the guidelines of the National Committee for Clinical Laboratory Standards (5), except that the 10° stock plicatamide was prepared and serially diluted in acidified water (sterile 0.01% acetic acid) instead of in Mueller Hinton II Broth.

Potassium Release

Test organisms were incubated overnight in 50 ml of trypticase soy broth at 37 °C, washed three times with buffer (100 mM NaCl, 10 mM Tris acetate, pH 7.4), and resuspended in this buffer at ~2.5 × 10^9 CFU/ml. Experiments were done at 37 °C in stirred polypropylene tubes surrounded by a 50-ml water-jacketed reaction vessel (Kimbler/Kontes, Vineland, NJ). The tube contained 6 × 10^6 CFU of washed, stationary phase bacteria in 100 mM NaCl, 10 mM Tris acetate, pH 7.4, in a final volume of 250 μl. An Orion SensorLink PCM-700 pH/ISE meter, fitted with a MI-442 potassium electrode (Milton Roy, Rochester, NY) was used to monitor the pH of the bacterial suspension. A 50-μl aliquot of each dilution to be introduced was removed to the beginning of Teflon tubing with 0.25-mm inner diameter. The design of the vessel allowed 8-μl aliquots to be introduced into the vessel. After being sampled, the vessels were reassembled and the lines were flushed with buffer. The peptide was added and the vessel was sealed with a V-clamp. The vessel was exposed at room temperature to 42.5 μg/ml native plicatamide in PBS (100 mM NaCl and 10 mM sodium phosphate, pH 7.4) containing 1% w/v trypticase soy broth. At intervals, 1-ml aliquots were removed, centrifuged briefly at 2000 g, and immediately resuspended in 1 ml of freshly made 2% glutaraldehyde in PBS. After 30 min on ice, the fixed organisms were washed in PBS.

For scanning EM, 10% of the above bacteria were adhered for 30 min to mixed cellulose ester membrane filters with 0.025-μm pores (Millipore, Bedford, MA). The filters were washed twice with 10 mM sodium phosphate, pH 7.4, and dehydrated through a graded ethanol series into hexamethyldisilane. After carbon coating, the samples were viewed on a Cambridge Stereoscan Electron Microscope.

The remaining bacteria were washed in PBS, post-fixed for 45 min at room temperature in 1% osmium tetroxide, dehydrated through ethanol to propylene oxide, and embedded in Epon 812. After staining with uranyl acetate at 60 °C for 15 min, and then by lead citrate, the sections were viewed on a JEOL CX II microscope.

Planar Lipid Bilayers

Solvent-containing phospholipid bilayer membranes were formed by placing a small bubble of 15 mg/ml lipid solution in n-heptane onto the end of Teflon tubing with 0.25-mm inner diameter. The design of the chamber allowed 50 μl of solution to be rapidly introduced immediately adjacent to the membrane lipid bilayer (7). The chamber was composed of Avanti Polar Lipids (Alabaster, AL) and stored at ~20 °C. Agar salt bridges connected the electrodes to the solutions, and voltage clamp conditions were employed in all experiments. The cis-side (i.e., the side to which peptide was added) was taken as ground. All stated voltages refer to the voltage of the trans-side. Current was recorded with an Axomicroelectrode amplifier with a 10-GΩ high impedance head stage and stored on videotape for later playback and analysis. Membrane capacitance and resistance were monitored to ensure the formation of reproducible membranes. The peptide stock solution (2 mg/ml) was stored at 4 °C, and the working solutions were prepared immediately before use. The bath solution contained 100 mM KCl and 10 mM Tris-HCl buffer, pH 7.4, or 10 mM MES-Tris buffer, pH 5.5 and pH 6.5, or 10 mM Tris citrate buffer pH 7.4.

RESULTS

Antimicrobial Activity of Plicatamide—Fig. 1a summarizes a series of radial diffusion assays done in underlay gels containing 100 mM NaCl at pH 7.4 and pH 5.5. Both native plicatamide and PL-101 (Phe-Phe-His-Leu-His-Phe-His-Tyr-amide) were more effective microbicides at neutral pH. Although native plicatamide and PL-101 had similar potency against the two Gram-positive organisms, S. aureus and L. monocytogenes, the native plicatamide was 2–3-fold more potent against the Gram-negative test strains, E. coli and P. aeruginosa. Fig. 1b shows that PL-101 was considerably more active than either PL-102 (Phe-Phe-His-Leu-His-Phe-His-Tyr-tyr-amide) or the two DOPA-containing peptides PL-103 and PL-104. The sequences of these peptides are shown in Table I.

Composition of Native Plicatamide Preparations—In several of our preparations of native plicatamide, FTIR analyses re-
revealed additional bands, characteristic of lipids and/or phospholipid, in addition to the expected absorption bands for a peptide (Fig. 2c). A mixture of synthetic PL-101 and palmitoylloleoylphosphatidylglycerol provided a similar FTIR spectrum (Fig. 2b), whereas PL-101 gave a typical peptide spectrum (Fig. 2a). Because the antimicrobial data shown in Fig. 1a were obtained with a preparation of plicatamide that contained only the expected peptide bands, we consider it unlikely that any co-purified lipids were responsible for the antimicrobial properties of our other preparations.

![Figure 1](Image)

**Fig. 1.** *Antimicrobial activity.* Two-stage radial diffusion assays (mean ± S.E., n = 3) compares the activity of native plicatamide and PL-101 at pH 7.4 and pH 5.5. *b* compares the activities of synthetic PL-101, -102, -103, and PL-104, in radial diffusion assays performed at pH 6.5. The peptide sequences are provided in Table I.

| Peptide    | Residues 1–7 | Residue 8 |
|------------|--------------|-----------|
| Plicatamide| FFHLHFP      | dcDOPA    |
| PL-101     | FFHLHFP      | Tyrosine amide |
| PL-102     | FFHLHFP      | Tyrosine acid |
| PL-103     | FFHLHFP      | DOPA acid |
| PL-104     | FFHLHFP      | DOPA amide |

Colony counting experiments revealed that native plicatamide killed MRSA and *S. aureus* very rapidly (Fig. 4). The peptide was equally effective in medium with or without nutrients, and we found little difference in the susceptibility of mid-logarithmic and stationary phase staphylococci to plicatamide (data not shown). Furthermore, staphylocidal activity was not impaired by including 10 μg/ml catalase in the medium, nor did inclusion of 1 mM Ca2+ or 1 mM Mg2+ impair it (data not shown).

**Effect on Bacterial Membrane Integrity**—We assessed the membrane integrity of plicatamide-treated staphylococci by measuring their loss of cytoplasmic potassium (Fig. 5). To ensure adequate amounts (100–200 nmol) of total K+, bacterial concentrations of ~7.5 × 10^7 CFU/ml were used. We also measured viability by removing aliquots at intervals for colony counting. The virtually immediate and substantial efflux of K+ from plicatamide-treated MRSA is consistent with an antimicrobial mechanism that targets their cell membrane. Native plicatamide induced a similar efflux of K+ from *S. aureus* and synthetic PL-101 induced K+ efflux from both *S. aureus* and MRSA (data not shown).

**Model Membrane Bilayers**—We also examined the effects of plicatamide on planar bilayer membranes prepared from *E. coli* lipids dissolved in n-heptane. The untreated membranes were stable between ±100 mV and displayed low (<10 picosiemens) conductance. At pH 7.4, plicatamide concentrations below 5
59%/H9262 g/ml caused short spikes of increased conductance, whereas concentrations between 5 and 10%/H9262 g/ml sometimes induced substantial conductivity (Fig. 6a), with an essentially linear integral current-voltage response (Fig. 6b). Plicatamide concentrations above 10%/H9262 g/ml typically increased conductance very quickly before destroying the membrane. When these various concentrations of plicatamide were added at pH 5.5, no increased conductivity resulted (data not shown).

The plicatamide-modified membranes were cation-selective, manifesting a current reversal potential of −15.5 ± 3.5 mV for a 10-fold KCl gradient. They showed relatively little selectivity for potassium versus sodium (2.1 ± 1 mV for bi-ionic system at 100 mM, and 10.7 ± 3.2 mV for 100 mM KCl, 1 M NaCl system).

Although adding 1–10 mM CaCl2 had no effect on plicatamide-induced currents, adding 1 mM ZnCl2 blocked current flow by up to 75% (data not shown). Because we have found that plicatamide binds zinc, we attribute the inhibitory effect of this cation to its interaction with plicatamide, rather than to nonspecific stabilization of the membrane. It is noteworthy that ZnCl2 blocks pore formation by two histidine-rich polypeptides: aerolysin from Aeromonas hydrophila (8, 9) and a histidine-rich analogue of staphylococcal α-hemolysin (10).

Oxygen Consumption—Exposing MRSA to plicatamide or PL-101 quickly decreased their consumption of oxygen by 87.1%, from a basal rate of 5.1 to 0.66 nmols/min/10^8 CFU, within 60 s after 10%/H9262 g/ml native plicatamide was added (Fig. 7). Synthetic PL-101 was almost as effective, reducing O2 consumption by 81.8% to 0.93 nmols/min/10^8 CFU. Untreated control organisms continued to consume O2 at the basal rate until the chamber became anaerobic.

Microbroth Dilution Assays—We also performed conventional, National Committee for Clinical Laboratory Standards

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930918-3, and L. monocytogenes of plicatamide exceeded 100 in our radial diffusion and colony count experiments, the MIC and PL-101. In contrast to its prominent antimicrobial effects manifested evident in Fig. 10.

Man erythrocytes, acting with almost the same potency as widely considered to represent gold standards in testing antimicrobials, we decided to investigate the cause of this apparent discrepancy. Initially, we suspected that the Mueller-Hinton broth used in National Committee for Clinical Laboratory Standards-type assays might not support plicatamide mediated staphylocladic activity. However, when we exposed mid-logarithmic or stationary phase S. aureus to 2 or 5 µg/ml of plicatamide in Mueller-Hinton broth, the colony counts fell by >2–3 logs after 30 and 120 min of incubation (data not shown). A few additional experiments revealed that the few organisms that survived exposure to plicatamide could repopulate the culture, thereby masking the antimicrobial properties of plicatamide, at least for microbroth dilution assays. This effect is illustrated in Fig. 8.

Effects on Bacterial Ultrastructure—MRSA treated with plicatamide showed many alterations. After only 5 min, striking changes were observed by scanning electron microscopy, wherein multiple small dome-shaped bulges, often arranged in linear and clustered arrays (Fig. 9), deformed their surfaces. These abnormalities became more marked as the duration of exposure to plicatamide increased (Fig. 10). In many bacteria, large amounts of cytoplasm extruded beyond the confines of the cell wall. Transmission electron microscopy of plicatamide-treated bacteria revealed fixed prominent mesosomes, even in cells fixed as early as 5 min after exposure to plicatamide (Fig. 11). Many plicatamide-treated MRSA contained electron dense material between their plasma membrane and cell wall, representing partially contained “eruptions” of cytoplasm akin to the more flamboyant manifestations evident in Fig. 10.

Hemolytic and Cytotoxic Properties—Plicatamide-lysed human erythrocytes, acting with almost the same potency as melittin on a weight/volume basis (Fig. 12). However, in marked contrast to melittin, plicatamide was not hemolytic for sheep red blood cells, even when applied at 80 µg/ml. Moreover, although melittin induced hemolysis over a broad pH range, the hemolytic properties of plicatamide were markedly diminished as acidity increased. Furthermore, whereas melittin was exceptionally cytotoxic for human cervical ME-180 epithelial cells, plicatamide was relatively nontoxic for these cells under the same conditions (data not shown).

DISCUSSION

Plicatamide (Fig. 13) is an interesting peptide for many reasons, not the least of which is that it violates conventional notions about antimicrobial peptides. Typically, one expects such peptides to be cationic and amphipathic molecules with 16–40 residues (11–15). A few smaller antimicrobial peptides with 11–13 residues have been described. These include the bacterencin dodecapeptides of bovine or ovine neutrophils (16, 17), bovine indolicidin (18, 19), and tigerinins, antimicrobial peptides isolated from the skin secretions of a frog, Rana tigrina (20). Plicatamide contains eight residues, and it is only modestly cationic at pH 7.4, and when it was rendered more cationic (at pH 5.5) its activity decreased.
It is remarkable that three of the smallest known antimicrobial peptides (5-S-GAD, halocyamine A, and plicatamide) should all contain a DOPA moiety. Although this could be a coincidence, it may also be an indication that this residue plays an important functional role. Although PL-103 and PL-104, the DOPA-containing synthetic analogues of plicatamide examined here, were unimpressive microbicides, we have yet to prepare an exact synthetic replica of plicatamide.

Another possible function of DOPA and dcDOPA might be to impart adhesive properties that help retain the peptide at sites of injury or infection. Byssal threads and plaques, the major adhesive structures of marine mussels (Mytilus spp.), invariably contain DOPA (26). If a Lewis base and an oxidase such as polyphenol oxidase are both present, DOPA can be converted to a DOPA quinone, whose spontaneous tautomerization forms α,β-dehydro-DOPA (27, 28).

Tunicates are protochordates-invertebrates that belong to the phylum chordata. Although the functional biochemistry of tunicate hemocytes has received relatively little attention, much is known about the microbicidal mechanisms of mammalian white blood cells, especially polymorphonucleated granulocytes (PMN). Mammalian PMN employ two principal strategies to kill microorganisms. One strategy involves using an array of antimicrobial peptides and proteins (29), and the other depends on the production of oxidants by phagocytic metabolism (30). The principal oxidants of human PMN are produced by an NADPH oxidase complex (31, 32) and include H2O2, OH (hydroxyl radical), and “downstream” products such as chloramines and hypochlorous acid formed by interactions between H2O2 and myeloperoxidase (33). In the PMN of rodents and some other mammals, copious amounts of nitric oxide are formed by an inducible nitric-oxide synthase (34).

Although it is not known if tunicate hemocytes have NADPH oxidase or inducible nitric-oxide synthase activity, phenol oxidase is released from the hemocytes of H. roretzi after foreign cells (e.g. yeast) are encountered. H. roretzi phenol oxidase, a metalloenzyme that requires copper ions for full activity (35), was reported to be antibacterial in the presence of DOPA and H. roretzi hemolymph. Because certain hemocytes (“morula cells”) of the colonial ascidian Botryllus schlosseri also contain...
phenoloxidase (36), this enzyme may be widely distributed in tunicates. The possibility that the DOPA moiety of plicatamide endows the molecule with an ability to participate in oxidative microbical reactions deserves consideration, especially because its histidines and modified DOPA residue endow plicatamide with the ability to bind transition metals.  

Whereas direct measurements of potassium efflux have seldom been applied to antimicrobial peptides, many investigators have used membrane potential sensitive carboxy cyanine dyes to follow their effects on bacterial membrane potential. For example, in a recent study of S. aureus and S. epidermidis, Hancock and co-workers (37) compared the kinetics of killing (by colony membrane) are unlikely to play a major role in its activity. The decreased activity of plicatamide under acidic conditions (pH 5.5), when its net barrier to the diffusion of plicatamide. The possibility that the DOPA moiety of plicatamide phenoloxidase (36), this enzyme may be widely distributed in tunicates. The possibility that the DOPA moiety of plicatamide was not especially cytotoxic and had little hemolytic activity for sheep erythrocytes, both plicatamide and PL-101 were extremely hemolytic for human red blood cells. We are currently trying to design active oligopeptide analogues of plicatamide with an improved cytotoxicity/hemolysis profile. If successful, these efforts could lead to practical applications.

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Plicatamide

From the standpoint of humans, the rapid and potent effects of plicatamide and PL-101 on staphylococci are also of interest. Infections caused by glycopeptide-resistant staphylococci and enterococci are becoming increasingly common, and additional agents that are effective against VanA strains of enterococci and “GISA”-type S. aureus are urgently needed (47). Although plicatamide was not especially cytotoxic and had little hemolytic activity for sheep erythrocytes, both plicatamide and PL-101 were extremely hemolytic for human red blood cells. We are currently trying to design active oligopeptide analogues of plicatamide with an improved cytotoxicity/hemolysis profile. If successful, these efforts could lead to practical applications.
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