CYCLIC ANTIBIOTIC PEPTIDE DESIGN: STRUCTURE AND MEMBRANE INTERACTION

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INTRODUCTION

The appearance and proliferation of microbial antibiotic resistance demands the development of new classes of antibiotics with novel modes of action. Reports of bacteria that have developed resistance to established drugs, including the "antibiotic of last resort" vancomycin, are appearing with increasing frequency.[1] In an effort to address this threat of microbial antibiotic resistance, researchers have pursued several strategies, one being the development of peptide-based antibiotics. Many vertebrates, including humans, produce antibiotic peptides as part of their innate immune response. These antibiotic peptides exhibit a fast and lethal mode of action that is quite different from the mode of action of other synthetic antibiotics, making peptide antibiotics attractive therapeutic targets.[2] This has helped to stimulate new research into active peptide antibiotics.

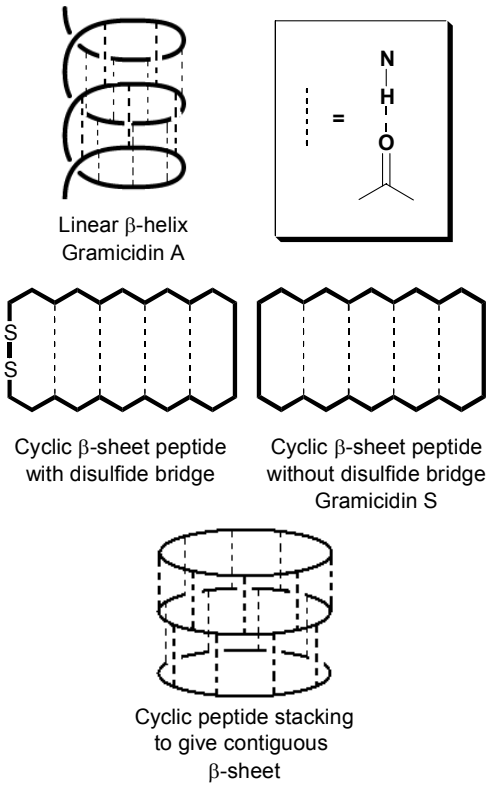

Figure 1. Structural motifs of antibiotic peptides

Over 400 natural antimicrobial peptides have been isolated and characterized. Based on chemical structure, these peptides may be classified into two main groups: linear and cyclic. The mode of action for the majority of these peptides (both linear and cyclic) is believed to involve membrane disruption, leading to cell leakage.[3] The linear peptides, such as magainins and melittins, exist mainly as α-helical amphipathic structures (containing segregated hydrophobic and hydrophilic moieties), or as β-helices as found in gramicidin A (GA). Cyclic peptides, which mainly adopt an amphipathic β-sheet structure, can be further divided into two subgroups: those containing disulfide bonds, such as tachyplesin, and those that do not, such as gramicidin S.[4] (Figure 1)

Nearly all known natural cyclic peptides display high antibacterial activity. However, many are also highly hemolytic and thus lack the selectivity required for a human antibiotic.[5] Efforts to develop cyclic peptides as antibiotics *in vivo* have been directed toward the development of analogs that possess greater selectivity for bacterial cells over erythrocytes. In order to design analogs capable of selective

permeation of only bacterial membranes, researchers require an intimate knowledge of protein secondary structure as well as the nature of amino acid interactions with lipid membranes. This review will discuss various types of cyclic antibiotic peptides, how their structures determine antibiotic function, and how they self-assemble to form tubular structures capable of selectively penetrating bacterial membranes.

CYCLIC PEPTIDES CONTAINING DISULFIDE BRIDGES

Examples of antibiotic peptides exhibiting constrained conformations are present in nature. Cyclic β-sheet peptides can be constrained by disulfide cross-links or backbone cyclization. Tachyplesins, polyphemusins, bactenecins, and protegrins are examples of disulfide-constrained antibiotic peptides.[2] Tachyplesin I is a 17-residue cyclic peptide that forms a rigid antiparallel β-sheet structure connected by two disulfide bridges and a type II β-turn (Figure 2). These crosslinks preform the peptide secondary structure in aqueous solution and in acidic phospholipid membranes, unlike α-helical peptides which adopt secondary structure only after membrane binding.[5] However, tachyplesin is not perfectly amphipathic, as positively charged arginine residues can be found on both sides of the sheet.[6] This peptide is believed to act by membrane permeation via electrostatic and hydrophobic interactions, but the exact mechanism is not known.[6] Matsuzaki and coworkers studied the antibiotic efficacy of the unconstrained peptide by irreversibly cleaving the disulfide linkages.[7] Activity and membrane permeability studies of both cyclic and acyclic peptides using calcein fluorescence methods showed that the acyclic version had weaker antibiotic activity. However, subsequent investigations revealed that organization of negatively charged lipid bilayers was more strongly perturbed by the acyclic peptide, suggesting that the two analogs work through different mechanisms (Figure 3).[7] It was hypothesized that a small number of the cyclic peptides bound to the negatively charged lipid bilayer may aggregate and then permeate to form anion-selective pores. Eventually the unstable pores

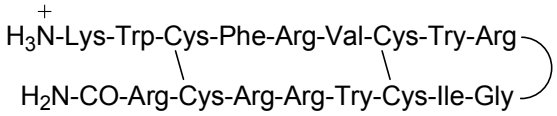

Figure 2. Tachyplesin I

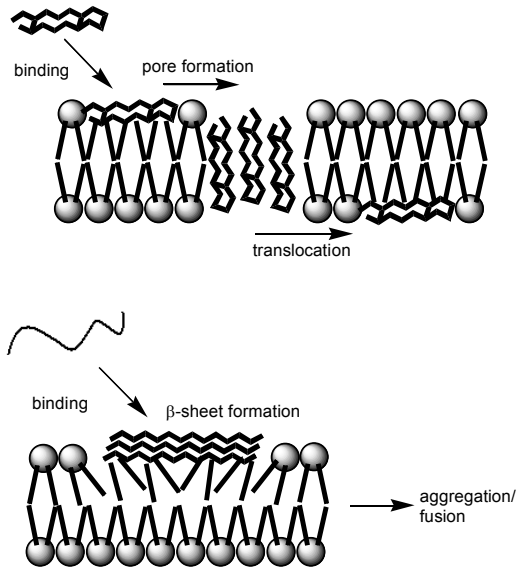

Figure 3. Models of tachyplesin-lipid bilayer interactions. (Top) Natural cyclic tachyplesin. (Bottom) Acyclic tachyplesin.

disintegrate, and peptide molecules translocate to both the inner and outer leaflets of the bilayer, presumably disrupting the integrity of the membrane. Even though the acyclic version also binds well to the outer leaflet of lipid membranes, the formation of interchain β-sheets prevents pore formation and translocation across the membrane. It is believed that the β-sheet destabilizes the bilayer by disordering the acyl chain region of the lipids and by neutralizing the charge on the membrane surface. The net effect is that the disulfide bridges allow the peptide to disrupt the lipid bilayer by constraining the conformation of the peptide and preventing the formation of inter-peptide β-sheets.

Hancock and coworkers performed experiments to determine the role of amphipathicity on membrane activity for polyphemusin I.[8] In the native peptide, seven positively charged amino acids in the 18-residue polyphemusin are distributed throughout the β-sheet structure and separated by hydrophobic blocks. These hydrophobic blocks may partition deeply into the hydrophobic portion of the membrane, immersing the charged residues into the hydrophobic region as well. If this is the case, then as the peptide reorganizes in order to sequester the hydrophilic residues, it may permeate the membrane and disrupt the lipid organization. Hancock and coworkers prepared three derivatives which increased the molecule's amphipathicity by repositioning the charged residues without significantly modifying the secondary structure. The results indicated that increasing amphipathicity reduced membrane activity, suggesting that analogs would remain tightly bound to the surface of the membrane owing to their increased amphipathicity.

CYCLIC PEPTIDES LACKING DISULFIDE BRIDGES – GRAMICIDIN S

Gramicidin S (GS) is a backbone cyclized decapeptide, *cyclo*-(Val-Orn-Leu-D-Phe-Pro)$_2$, with an amphipathic antiparallel β-sheet secondary structure supported by two type II' β-turns (Figure 4). GS is an extremely powerful antibiotic that is also very hemolytic and therefore can only be used in topical applications. While the affinity and location of GS in phospholipid membranes are not presently understood, the synthesis and application of analogs with lower hemolytic activity while maintaining bacterial potency are in progress.[9]

Hodges and coworkers have performed a number of systematic structure-activity studies with GS analogs.[10] Modifications of the ring size directly affect the β-sheet integrity, in turn affecting membrane binding. Investigations with ring sizes ranging from 4 to 14 residues showed that only decapeptides retained both Gram-negative and Gram-positive

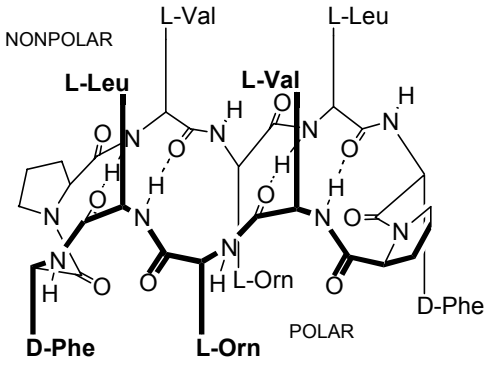

Figure 4. Antiparallel β-sheet structure of gramicidin S.

activity, indicating a particular β-sheet structure is required for function. Nearly all the ring size modifications lowered hemolytic activity. A systematic study was also performed to determine the optimal balance of hydrophobic and hydrophilic domains required for both high bacterial activity and low hemolytic activity.[5] A 14-residue peptide was employed, systematically substituting each single amino acid with its enantiomer to disrupt the amphipathic nature of the molecule. Observed amphipathicities indicated that hemolytic activity was related to high amphipathicity; thus reducing peptide amphipathicity or overall hydrophobicity of the peptide lowered hemolytic activity. Decreasing amphipathicity (to a certain threshold) also increased Gram-negative activity. For both Gram-negative and Gram-positive bacteria, it appeared that only peptides with the appropriate binding affinity to the outer membrane or layer could penetrate the inner cytoplasmic membrane. Tightly bound peptides do not reach the inner membrane, and weakly bound peptides do not immerse into the outer membrane.

NOVEL SELF-ASSEMBLING ANTIBIOTIC PEPTIDES

Approach to Tubular Design

Many naturally occuring peptide antibiotics adopt a tubular shape that appears to be essential for their activity. In an effort to mimic this behavior, researchers have undertaken several different approaches to create tubular strucutres including (1) aggregating rods to form a barrel-shaped framework; (2) coiling of a linear molecule into a helix; (3) rolling a two-dimensional sheet-like structure; and (3) stacking of disc-shaped subunits.[11] Of these approaches, the stacking of disc-shaped subunits provides the most design flexibility and synthetic convergence. Instead of assuming an intramolecular β-sheet secondary structure, these cycles self-assemble intermolecularly to form a contiguous β-sheet in the form of a hollow, open-ended tube. Following examples from other researchers,[12,13] Ghadiri and coworkers successfully synthesized an organic nanotube composed of cyclic peptide subunits in 1993. The self-assembly process of Ghadiri's octapeptide *cyclo*[-(D-Ala-Glu-D-Ala-Gln)$_2$-] could be proton-

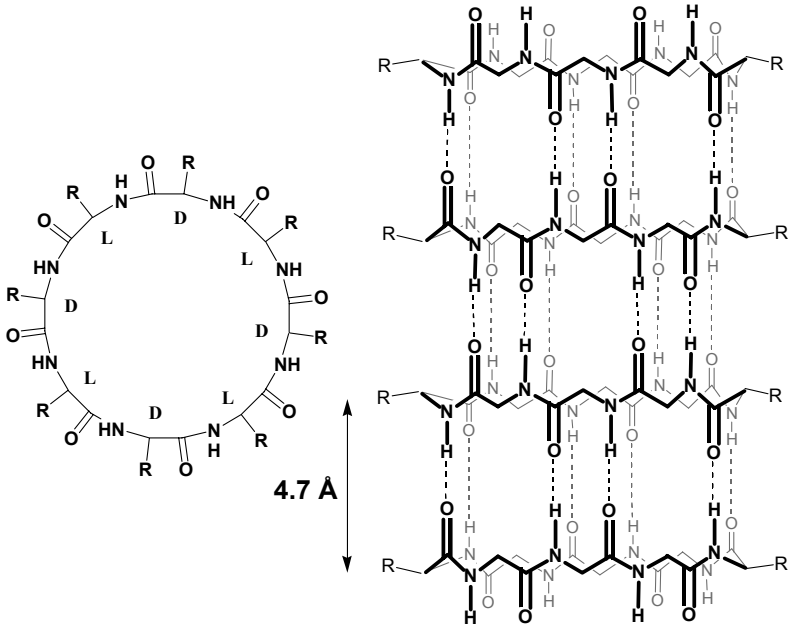

Figure 5. Octapeptide with alternating D,L residues self-assemble to form tubular structures.

triggered in aqueous solutions.[14] The deprotonation of the glutamic acid side chain at alkaline pH formed a charged species, which solvated the subunits. Ring stacking was prevented by electrostatic repulsion. Reprotonation allowed the formation of a hydrogen-bonded network through subunit stacking. An intersubunit distance of 4.7Å was determined by electron diffraction and molecular modeling, supporting a hydrogen-bonded packing arrangement of flat cyclic peptides with side chains in the plane of the peptide backbone (Figure 5). Fourier-transform infrared (FT-IR) spectroscopy showed characteristics of β-sheet formation through backbone-backbone hydrogen bonding. The IR spectrum of the nanotube closely resembled that of gramicidin A, a linear α-helical peptide of alternating D and L amino acids known to form β-helical transmembrane ion channels.

Dimerization Studies

To obtain a greater understanding of the stacking interaction, Ghardiri and coworkers employed a simpler model. *N*-Methylation of the backbone amide functionalities limited the self-assembly process to two subunits (Figure 6).[15] The enthalpy change in nonpolar organic solvent for the dimerization of *cyclo*[(-Phe-D-*N*-MeAla-)$_4$-] was found to be ΔH°_{298} = –11.0 kcal/mol. This supports the conjecture that the enthalpic contribution of intermolecular hydrogen bonding is the major driving force for self-assembly in nonpolar solvents. The change in entropy was found to be ΔS°_{298} = –23.7 cal/K mol. If ring stacking were to continue, the free energy stabilization would be additive.

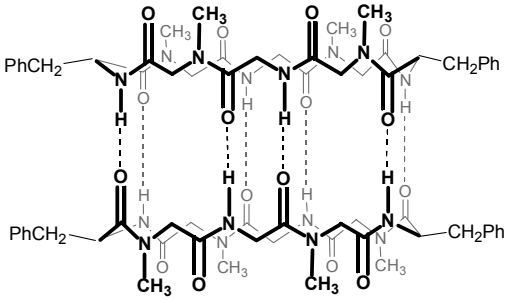

Figure 6. *N*-Methylated dimer.

A second dimer having four-fold symmetry (not shown) was employed as a model system in order to study the relative stabilities of parallel and antiparallel β-sheets. It was found that of the diastereomeric complexes, the antiparallel β-sheet structure is more stable than the parallel structure.[16] An additional study with a dimer having only 2-fold symmetry around the C_2 axis was used to determine if side chain - side chain interactions play a role in the β-sheet arrangement (Figure 7). An equal mixture of the two possible antiparallel β-sheet diastereomers **A** and **B** was

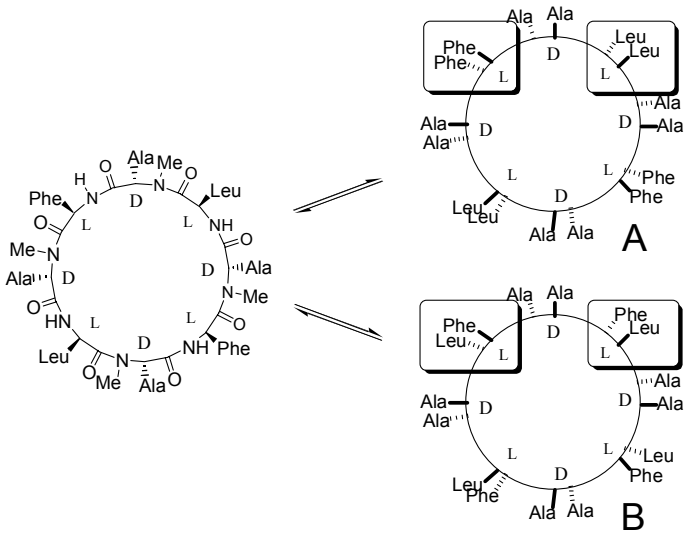

Figure 7. Two possible diastereomers of dimer for peptides with 2-fold symmetry.

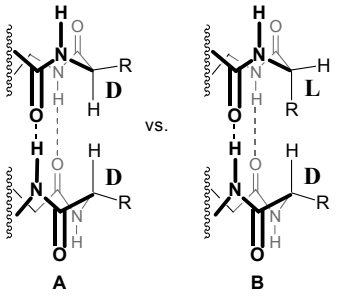

Figure 8. Homochiral vs. heterochiral stacking.

formed, indicating that side chain interactions do not contribute significantly to structure stability.[16] A final factor affecting the self-assembly process is the relative sheet register, which describes the spatial relationship of the hydrogen-bonded residues. Dimer A in Figure 8 has a homochiral relationship with D-residues juxtapositioned above D-residues. A heterochiral arrangement (Figure 8B) is destabilized by intersubunit side chain-backbone steric interactions.[17] The characterization of the dimers provided a clear picture of the self-assembled structures and was applied to the design of nanotubes with specific properties thought to be required for antibiotic activity.

Nanotube Membrane Channels

While all of the synthetic cyclic peptides discussed so far are biologically inspired, the first examples by Matsazuki, Hancock, and Hodges were synthesized by single amino acid substitutions on natural peptides, whereas Ghadiri and coworkers have synthesized their cyclic peptides from the bottom up. The eight-residue cyclic peptide *cyclo*[-(Trp-D-Leu)$_3$-Gln-D-Leu-] was designed to study both the self-assembly process in lipid bilayers and ion conductance.[18] The highly hydrophobic indole side chain of tryptophan promotes subunit incorporation into the lipid bilayer of unilamellar vesicles. In the case of gramicidin A (GA), two monomers assemble in the bilayer to form a membrane-spanning end-to-end dimer. Once GA penetrates the outer leaflet of the membrane, it cannot diffuse back out. Consequently, at low concentrations, only a portion of the vesicle population demonstrates proton transport. The rate of the ion channel formation is concentration-dependent. The self-assembled nanotubes exhibit a similar phenomenon. Dye studies showed that upon addition of channel-forming subunits, the imposed pH gradient collapsed. At low concentrations, proton transport was limited, indicating that some vesicles did not have enough subunits to form a membrane-spanning channel. Further ion conductance studies determined that the rates of K^+ and Na^+ transport are nearly three times faster than those of GA.

By increasing the number of residues in the cyclic peptides, Ghadiri and coworkers were able to increase the pore diameter of the self-assembled nanotubes.[19] A 10-residue cyclic peptide, *cyclo*[Gln-(D-Leu-Trp)$_4$-D-Leu], having a pore diameter of 10 Å, was used to study glucose transport across lipid bilayers.[20] Using an enzyme-coupled assay, they observed the diffusion of glucose from unilamellar vesicles by monitoring the production of NADPH. The first-order rate profile supported a transmembrane channel diffusion process. A decapeptide was shown to transport L-glutamic acid in a similar fashion.[21] These studies established that self-assembled pore structures can be exploited for efficient size-selective molecular transport across lipid membranes.

One simple design for a functional transmembrane channel orients the central axis of the tube approximately perpendicular to the plane of the lipid membrane. Polarized attenuated total reflectance, grazing angle reflection – absorption, and transmission FT-IR techniques estimated the *cyclo*[-(Trp-D-Leu)$_3$-Gln-D-Leu-] nanotube tilt angle to be 7˚ from the lipid bilayer normal, comparable to the orientation of gramicidin A in lipid bilayers.[22]

Antibacterial Activity

Complete characterization of structure and membrane activity of the self-assembled peptide nanotubes allowed Ghadiri and coworkers to explore potential applications. Due to resemblances to both gramicidin A and α-helical antibiotic peptides, the stacked peptide nanotubes were hypothesized to also display antibiotic activity, with the advantage that the peptide nanotubes would offer greater design flexibility. A series of 22 six- and eight-residue amphipathic cyclic D,L-α-peptides were synthesized to test this theory.[23] Each peptide contained one positively charged, basic residue to bind to negatively charged bacterial membranes. Orientation studies as mentioned above showed that the nanotubes were oriented at 70 ± 5˚ tilt angle from the membrane normal, which is consistent with the carpet-like mode of membrane permeation of linear α-helical peptides.[24]

In vitro assays were first conducted for Gram-negative and Gram-positive antibacterial activity and for hemolytic activity (Table 2). Results indicated that a strong basic residue (Arg or Lys) was essential for activity, as the negative charge of acidic residues likely experience repulsion at the bacterial membrane. Single amino acid substitutions of the subunit peptide are multiplied throughout the tubular structure, resulting in a wide variety of activity. No living bacteria were detected after 5 min exposure to minimum inhibitory concentrations (MICs) of the subunits. Subunit 4 exhibited the strongest activity against Gram-negative *E. coli*. Subunits 1, 2, and 3 exhibited the broadest spectrum of bacterial activity and were investigated for *in vivo* toxicology and bacterial activity in mice. Mice infected with lethal doses of methicillin-resistant *S. aureus* (MRSA) were administered doses of peptide 2. Control mice died within 48 hours, but as many as 67% (depending on type of injection) of mice receiving peptide 2 survived over the seven-day study. This class of peptides shows considerable potential in the treatment of MRSA infections.

Table 2. Minimum inhibitory concentrations (MIC) for selective D,L cyclic peptides in vitro.

	MIC (μM)		
Peptide	*S. aureus* Gram(+)	*E. coli* Gram(-)	Hemolysis HD_{50}
1 (KQRWLWLW)	6	80	45
2 (RRKWLWLW)	6	15	50
3 (KKLWLW)	10	17	80
4 (RRLWLW)	35	5	90

CONCLUSIONS

Although antibiotic peptides exhibit broad spectrum of bacterial activity, many are not selective for bacterial over mammalian cells. Without a knowledge of the exact mode of action of the single, cyclic peptides, it is difficult to design selective antibiotic peptides. However, natural antibiotic cyclic peptides and their synthetic analogs have demonstrated what is fundamentally necessary for activity and selectivity: an organized structure possessing a delicate balance of amphipathicity. Instead of trying to synthesize a single peptide with the exact balance, Ghadiri has synthesized peptide subunits that only have what are considered to be the necessary components for activity (hydrophobic and basic amino acids). The subunits assemble themselves in the membrane based in the environment. The non-covalent forces allow the subunits to arrange and rearrange to form the most stable supramolecular isomer. This design for antibiotic peptides may be a step toward combating the developing strains of drug-resistant bacteria.

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