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Mimicry of Antimicrobial Host-Defense Peptides by Random Copolymers

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The eukaryotic innate immune response to bacterial infection includes the production of peptides that kill prokaryotic invaders.[1] These "host-defense" peptides can be grouped into several structural classes, and their mechanisms of antibacterial action are varied. Many host-defense peptides are thought to act by disrupting bacterial membranes. Members of one widely studied class are induced by target membranes to adopt α-helical folding patterns.[2] These conformations are globally amphiphilic: discrete patches of lipophilic and hydrophilic side chains are projected from opposite sides of the helix (Figure 1A). Examples include cecropins[3] (from insects), magainins[4] (from amphibians) and cathelicidins[5] (from mammals). The antibacterial activity of these helix-forming peptides appears to depend on the overall spatial segregation of lipophilic and cationic side chains rather than on the specific identities of the side chains, a characteristic that has inspired the exploration of analogues containing nonproteinogenic α-amino acid residues[6] or subunits other than α-amino acid residues.[7] All of these oligomers have been synthesized in step-by-step fashion, so that the sequence of hydrophilic and lipophilic subunits would give rise, upon adoption of a specific and regular conformation, to a globally amphiphilic molecular surface (Figure 1A). Host-defense peptide mimics have considerable therapeutic potential as complements to conventional antibiotics because it is difficult for bacteria to evolve resistance to the membrane-disruption mode of action;[1] however, the cost of producing sequence-specific oligomers represents a significant stumbling block to their use.[1c]

Here we show that functional host-defense peptide mimics can be created on the basis of a conformational hypothesis that is quite different from classical helix-induction. Instead, we propose that flexible, sequence-random oligomers or polymers containing cationic and lipophilic subunits can be induced by a bacterial membrane surface to adopt irregular conformations that result in global amphiphilicity (Figure 1B). This hypothesis represents a significant expansion in our understanding of structure−activity relationships among antibacterial oligomers and polymers. In addition, this hypothesis has important practical consequences because it is far easier to prepare random copolymers than to synthesize sequence-specific oligomers. We show that materials generated via ring-opening copolymerization of β-lactams (±)-**1** and (±)-**2** (Scheme 1) match or exceed the growth-inhibiting effects of host-defense peptides toward several bacteria, including human pathogenic strains resistant to conventional antibiotics. These polymers can be tuned to display very low lytic activity toward human red blood cells ("hemolysis") while retaining antibacterial potency, a profile that is characteristic of host-defense peptides.

β-Lactam **1** can be prepared in large quantities via reaction of chlorosulfonyl isocyanate (CSI) with cyclohexene,[8] and an analogous CSI reaction is the key step for synthesis of previously unknown β-lactam **2**. Copolymerization[9] of **1** and **2** under recently developed conditions[10] followed by acid-catalyzed Boc deprotection provides polyamides **3**, members of the nylon-3 family that are

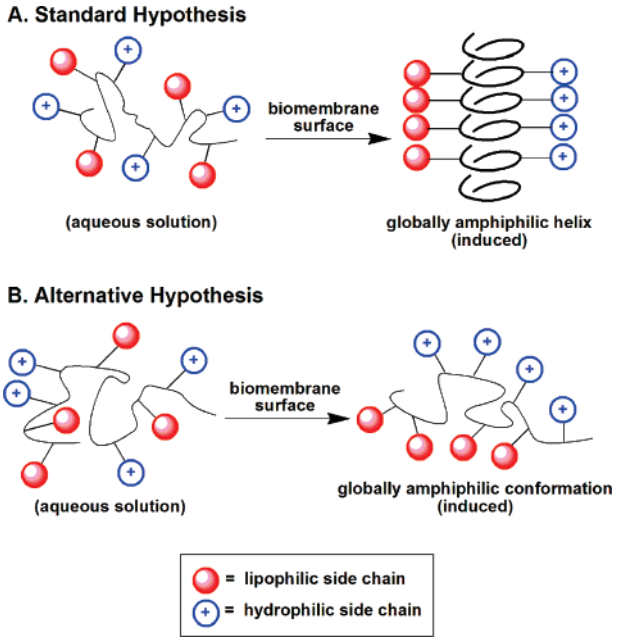
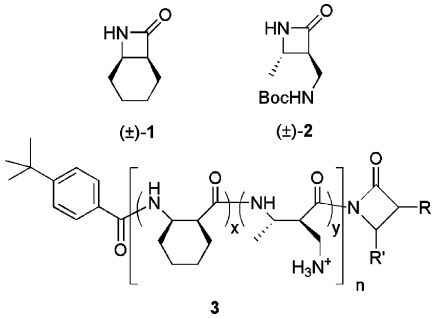


Figure 1. Complementary hypotheses that can explain the antibacterial activity of host-defense peptides and synthetic oligomers and polymers that are designed to mimic these peptides. (A) The standard hypothesis for peptides such as magainins or cecropins involves induction of a globally amphiphilic helix folding pattern upon interaction with a bacterial membrane. The globally amphiphilic conformation is proposed to be responsible for disruption of the bacterial membrane. Variations on this hypothesis, all involving the induction of regular conformations, have been invoked to explain the activity of many unnatural antibacterial oligomers. (B) An alternative hypothesis, which can explain the activity reported here for random copolymers, involves induction of globally amphiphilic but irregular conformations in the presence of a bacterial membrane.

Scheme 1. Monomers (±)-**1** and (±)-**2**, and Polymers **3**



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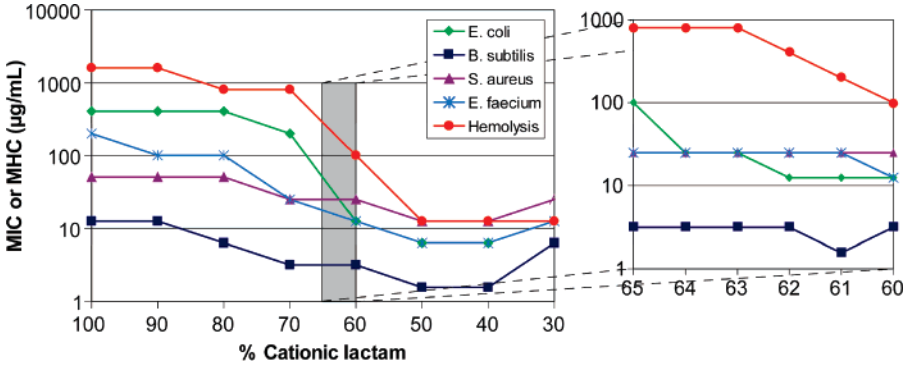


Figure 2. Antibacterial and hemolytic activities of polymers $\mathbf{3}_y$ as a function of y (i.e., the proportion of cationic subunits derived from β-lactam **2**). The region with the greatest selectivity for bacteria relative to human red blood cells, between 60 and 65% cationic subunit, is shown in the expansion at the right. The minimum inhibitory concentration (MIC) is defined as the lowest polymer concentration that completely inhibits bacterial growth. The minimum hemolytic concentration (MHC) is defined as the lowest polymer concentration at which hemolysis is detected. The lines connecting the points are intended merely to guide the eye.

Table 1. Activities of Polymer $\mathbf{3}_{60}$ and Selected Peptides against Four Bacteria and Human Red Blood Cells

	MIC (μg/mL)				
polymer	*E. coli*	*B. subtilis*	*S. aureus*	*E. faecium*	MHC (μg/mL)
polymer $\mathbf{3}_{60}$	12.5	3.1	25	12.5	100
magainin 2	100	200	>400	>400	>400
cecropin A	0.78	400	>400	>400	>400
cecropin B	1.6	400	>400	>400	>400
magainin-Ala_3	6.2	6.2	25	25	25

cationic at neutral pH. The ability to incorporate amine-containing side chains is important because most host-defense peptides bear multiple positive charges, which are thought to attract them to the negatively charged outer surfaces of bacteria.[1] Gel permeation chromatography (GPC) of the polyamides before deprotection indicates polydispersity indices (PDI) in the range 1.3−1.7. The samples in series **3** contained an average of 15−20 subunits, according to GPC and MALDI-TOF mass spectrometric analysis.

We examined the effect of polymer composition by evaluating the homopolymer formed from **2**, designated $\mathbf{3}_{100}$, and materials prepared from β-lactam mixtures with **1**:**2** proportions ranging from 10:90 to 70:30, designated $\mathbf{3}_{90}$ through $\mathbf{3}_{30}$. Figure 2 shows how variation in subunit proportion affects the minimum inhibitory concentration (MIC) toward four bacteria, *Enterococcus faecium*,[11] *Staphylococcus aureus*,[12] *Escherichia coli*,[13] and *Bacillus subtilis*,[14] and the minimum hemolytic concentration (MHC) toward human red blood cells. The *E. faecium* and *S. aureus* strains we used are clinical isolates resistant to standard chemotherapeutics (vancomycin and methicillin, respectively). Homopolymer $\mathbf{3}_{100}$ shows weak antibacterial activity (MIC ≥ 50 μg/mL for three species) and very low hemolytic activity (MHC > 1000 μg/mL). Incremental introduction of the hydrophobic subunit derived from β-lactam **1** leads to improvement in antibacterial activity (lower MIC values) until the **1**:**2** proportion reaches 50:50. Hemolytic activity remains very weak (MHC ≥ 800 μg/mL) until the **1**:**2** proportion reaches 40:60 (i.e., $\mathbf{3}_{60}$), and polymers with ≤50% of the cationic subunit are quite hemolytic.

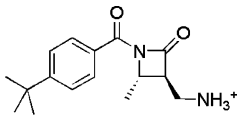
On the basis of the trends in Figure 2, we conducted a more careful analysis of $\mathbf{3}_{60}$, which displays favorable antibacterial activity without excessive hemolytic activity. Table 1 compares MIC and MHC data for $\mathbf{3}_{60}$ and three widely studied host-defense peptides, magainin 2, cecropin A, and cecropin B. Also shown are results for a magainin 2 derivative (Ser8→Ala, Gly13→Ala, Gly18→Ala; C-terminal amide) that displays substantially improved antibacterial activity relative to the natural sequence.[15] The results for $\mathbf{3}_{60}$ represent averages for five independently synthesized batches, with at least six assays for each sample. GPC analysis of these five polymer samples indicated that they contained an average of 18 residues (M_n varied between 3000, corresponding to ∼16 residues, and 3800, corresponding to ∼20 residues); PDI for the five samples fell in the range 1.3−1.4. MALDI MS data for the five samples were consistent with these GPC-based conclusions. The physical properties and biological activities of the five samples of $\mathbf{3}_{60}$ show that the polymerization reaction provides reproducible materials.

Table 1 shows that the three host-defense peptides are not active against the clinically derived strains of *S. aureus* and *E. faecium* we evaluated. The magainin derivative, on the other hand, is quite active against these pathogenic bacteria, consistent with the original report.[15] Polymer $\mathbf{3}_{60}$ is comparable to this modified magainin in activity against the pathogens as well as against *B. subtilis* (a nonpathogenic species that is related to *B. anthracis*) and *E. coli* (nonpathogenic strain). Neither polymer $\mathbf{3}_{60}$ nor the magainin derivative achieves the high activities of the cecropins against *E. coli*, which is the only Gram-negative species in our panel. Overall, the MIC data indicate that random copolymer $\mathbf{3}_{60}$ is comparable in antibacterial activity to representative host-defense peptides, especially for Gram-positive species. Polymer $\mathbf{3}_{60}$ is superior to the magainin derivative in terms of hemolytic activity (MHC = 100 vs 25 μg/mL), but both are significantly more hemolytic than the natural host-defense peptides.[16] Model studies involving large unilamellar vesicles (LUVs) with varying lipid content showed that $\mathbf{3}_{60}$ very effectively disrupts LUVs that mimic bacterial membranes but not LUVs that mimic red blood cell (RBC) membranes.[17] Overall, these results are consistent with the hypothesis that polymer $\mathbf{3}_{60}$ selectively targets bacterial cells relative to RBCs, behavior that is a hallmark of host-defense peptides.[1,2]

The data in Figure 2 suggest that hemolytic activity is very sensitive to copolymer composition when the cationic subunit proportion is 50−70%. We compared $\mathbf{3}_{60}$ to copolymers $\mathbf{3}_{61}$, $\mathbf{3}_{62}$, $\mathbf{3}_{63}$, $\mathbf{3}_{64}$, and $\mathbf{3}_{65}$ (Figure 2, expansion on right side) to identify an optimal balance of MIC and MHC. The MIC for *E. coli* rose from 12.5 to 100 μg/mL as the proportion of cationic subunit derived from **2** rose from 60% to 65%, and the MHC rose from 100 to 800 μg/mL, but the antibacterial activities for the three Gram-positive species showed little variation within this set. Polymer $\mathbf{3}_{63}$, with an MHC/MIC ratio of 32 for the pathogenic bacteria, demonstrates that substantial membrane selectivity can be achieved with this system. These results show that biological activities can be tuned, in some cases independently, via easily implemented modifications in polymer structure. The biological impact of other structural

variables, including the identity of the monomers, average size, and N-terminal capping group, remains to be explored.

A β-lactam unit embedded within an imide occurs at the C-termini of polymers **3**. Since some β-lactams exert antibacterial effects, we evaluated the activity of β-lactam imide (±)-**4**, generated by acylating the ring nitrogen and deprotecting the side chain of **2**, against our panel of bacteria. In addition, we evaluated monomer **1**. Neither compound displayed significant antibacterial activity up to a concentration of 400 μg/mL, and imide **4** decomposes rapidly. These observations suggest that the antibacterial activities of polymers **3** do not arise from the C-terminal imide/lactam unit.



(±)-**4**

Our results show that random copolymers can mimic the cell-type selectivity manifested by host-defense peptides. Specifically, the favorable activity profile displayed by copolymers **3** with **1**:**2** proportions near 40:60 suggests that the nontraditional hypothesis illustrated in Figure 1B constitutes a valid basis for design of host-defense peptide mimics. Most efforts to develop discrete oligomers with antibacterial properties, containing either natural or unnatural backbones, have started with a specific target conformation, usually a helix (Figure 1A).[6,7] Shai et al.[18a] and Dathe et al.[19] have shown that partial destabilization of the α-helical conformation, by incorporation of a few D-residues, can diminish hemolytic activity without impairing antibacterial activity in α-amino acid peptides, but these heterochiral peptides retain the ability to form an α-helix.[20] Subsequent work by Papo and Shai, however, suggests that non-helical conformations can result in antibacterial activity.[18b] Recently, Wang et al. have shown that a human cathelicidin-derived sequence containing several D-residues adopts a specific but irregular conformation that is globally amphiphilic in the presence of detergent micelles.[21a] Related results were previously obtained by Jelinek et al. for a designed 12-mer peptide containing four D-residues.[21b] Our polymer results, along with the earlier heterochiral peptide data,[18,19,21] support the "carpet" mechanism of action.[22]

Previous attempts to mimic host-defense peptides with heterochiral polymers, which are not expected to display specific conformational propensities, have led to materials that display either excessive hemolysis or limited antibacterial activity.[23] The results reported here are consistent with the hypothesis that a polar polymer backbone is important for minimizing hemolytic activity.[23a] In this regard, it is noteworthy that polymers **3** have a backbone rich in secondary amides, as do proteins. However, unlike proteins or conventional peptides, polymers in this nylon-3 family are not susceptible to degradation by proteases.[24] Our findings support a new conceptual framework for development of antibacterial materials that are selective for disruption of prokaryotic relative to eukaryotic cell membranes and that are inexpensive to prepare in large quantities.

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Supporting Information Available: Polymer and peptide synthesis procedures and characterization data, LUV experimental results, and bioassay protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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