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Interplay among Subunit Identity, Subunit Proportion, Chain Length, and Stereochemistry in the Activity Profile of Sequence-Random Peptide Mixtures

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Supporting Information

ABSTRACT: Fmoc-based solid-phase synthesis methodology was used to prepare peptide mixtures containing one type of hydrophobic residue and one type of cationic residue. Each mixture was random in terms of sequence but highly controlled in terms of length. Analysis of the antibacterial and hemolytic properties of these mixtures revealed that selective antibacterial activity can be achieved with heterochiral binary mixtures but not homochiral binary mixture, if the proper amino acid residues are used.

Host-defense peptides (HDPs) are produced by eukaryotes as part of the innate immune response to bacterial infection.[1−5] The mode of HDP action varies among different examples, and a particular HDP may have more than one antibacterial mechanism; however, many HDPs share the ability to disrupt bacterial membranes.[2,6−12] HDPs display a characteristic selectivity, favoring attack on prokaryotic membranes relative to eukaryotic membranes.[3,5] This selectivity is thought to arise from the net cationic charge common to HDPs, since the external surfaces of prokaryotic cells typically have a larger net negative charge than do the external surfaces of eukaryotic cells.[13] HDPs are rich in hydrophobic residues, which presumably mediate disruptive interactions with the hydrophobic interior of a lipid bilayer.[5,14]

The broad molecular diversity among HDPs suggests that their prokaryotic-selective activity is not tightly coupled to specific features of amino acid sequence or peptide conformation.[6,14] This situation has inspired recent evaluation of several families of sequence-random hydrophobic−cationic copolymers, materials that contain mixtures of chains with many distinct subunit sequences and lengths. A number of unnatural backbones, including polystyrene,[15] polymethacrylate,[16−18] nylon-3,[14,19,20] and polyolefins,[21−23] have been reported to display antibacterial behavior with varying levels of hemolytic activity. The only evaluation of sequence-random poly(α-amino acid) materials for this purpose, however, identified antibacterial polymers that were strongly hemolytic[24] or displayed low antimicrobial activity.[25] Most of the unnatural polymers examined to date have contained stereogenic centers and been generated in stereochemically random forms. In contrast, poly(α-peptide) mixtures are readily prepared in homochiral form.[24]

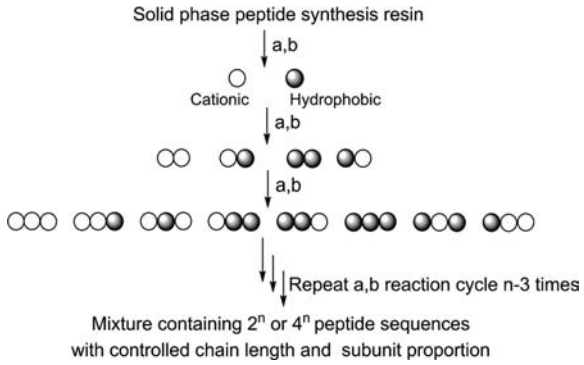

Figure 1. Synthesis of sequence-random peptide mixtures used for this research, where (a) is coupling of a binary combination of Fmoc-protected amino acids that have hydrophobic (filled sphere) or cationic (open sphere) side chains (after deprotection in the latter case) and (b) is Fmoc deprotection. Standard Fmoc-based solid-phase synthesis methods were employed, but a mixture of protected amino acids rather than a single protected amino acid was used for each coupling step. The results of three coupling steps are illustrated. In this process, each bead of the solid support bears many growing chains with many different sequences. If the amino acids are racemic, the mixture contains 4^n peptide sequences, where n is the number of coupling steps.

The ability of a heterogeneous set of polymer chains to mimic the activity profile of a homogeneous peptide (one sequence, one chain length and one stereochemistry) is interesting in practical terms because chemical synthesis of sequence-specific oligomers is more difficult and expensive than copolymerization. Thus, antibacterial random copolymers may be more readily applicable to some real-world problems than are homogeneous peptide agents. The prospect of optimizing copolymer properties would be enhanced if one could gain a clearer understanding of relationships between activity and molecular parameters, including chain length and the identity, proportion, and stereochemistry of subunits. However, achieving this goal with polymerized materials themselves can be challenging because there is so much variation within a sample generated via copolymerization of a binary monomer combination.

Here we describe a strategy intended to fill the gap between a highly diverse copolymer mixture and a homogeneous peptide by

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using solid-phase synthesis to generate peptide oligomer mixtures with more limited diversity than can be achieved via a random copolymerization process. At each coupling step we use a combination of protected α-amino acids. This approach leads to a product mixture in which the subunit sequence is random but the length is much more effectively controlled than is possible for a mixture of chains produced via a true polymerization process. The subunit stereochemistry is easily controlled because the configuration of an α-amino acid residue is set before it is incorporated into the backbone. In contrast, stereogenic centers in many copolymers explored for antibacterial activity, such as polystyrenes, polymethacrylates, and polyolefins,[15−18,21−23] are created during the polymerization process with little or no control. Our unconventional use of solid-phase synthesis facilitates an examination of the ways in which subunit identity, subunit proportion, chain length, and stereochemistry influence the antibacterial and hemolytic activities of peptide mixtures. Ultimately, such information may be useful in tailoring authentic copolymerization processes to generate heterogeneous materials with improved properties.

We began by surveying six binary cationic−hydrophobic residue combinations based on L-α-amino acids commonly found in HDPs (see Table 1).[26,27] The hydrophobic residue in each mixture was Leu, Ile, or Phe, and the cationic residue was Lys or Arg. A mixture of 20-mers was prepared for each pairing by conducting 20 successive coupling steps with a 1:1 molar combination of the two protected α-amino acids [e.g., Fmoc(ε-Boc)-L-Lys + Fmoc-L-Phe] (Figure 1). As is standard in solid-phase synthesis, we used an excess of protected α-amino acid reagents at each coupling step to promote extension of all of the resin-bound chains (4-fold molar excess of amino acids relative to reactive sites on the solid-phase synthesis resin; for the 1:1 combination of L-Lys and L-Phe, this meant a 2-fold molar excess of Fmoc(ε-Boc)-L-Lys and a 2-fold molar excess of Fmoc-L-Phe at each coupling step).

Table 1. Antimicrobial Activities for 20-mer Peptide Mixtures with the L Residue Configuration

		MIC (μg/mL)[a]			
mixture	hyd:cat ratio[b]	*E.c.*	*B.s.*	*S.a.*	*E.f.*
LK	52:48	6	3	12	6
IK	54:46	>200	3	>200	100
FK	59:41	12	3	12	6
LR	62:38	>200	25	50	100
IR	69:31	>200	100	>200	>200
FR	70:30	>200	50	>200	50
magainin 2		100	>200	>200	>200

[a]MIC results for *E. coli* (*E.c.*), *B. subtilis* (*B.s.*), *S. aureus* (*S.a.*), and *E. faecium* (*E.f.*). [b]Ratio of hydrophobic to cationic residues in the peptide mixture as determined by amino acid analysis. The uncertainty in each value is 3−4%. All of the amino acid residues had the L absolute configuration.

The activated forms of different protected α-amino acids may not have identical reactivities; therefore, amino acid analysis of the six binary α-peptide mixtures was conducted. In control experiments, we found that the deduced proportion could vary among independent analyses of a given sample by 3−4% in subunit proportion, and we observed a comparable level of variation between nominally identical mixtures synthesized at different times (Figure S1 in the Supporting Information). Table 1 shows that the amino acid proportions of some binary mixtures deviated significantly from the 1:1 proportion of the starting materials. In general, pairings that contained L-Lys as the cationic subunit displayed proportions close to 1:1, while pairings that contained L-Arg tended toward a 2:1 proportion favoring the hydrophobic subunit.

The antibacterial activities of the six binary α-peptide mixtures were assessed by measuring minimum inhibitory concentration (MIC) values for a panel of four bacteria, including laboratory strains of *Escherichia coli*[28] and *Bacillus subtilis*[29] and clinical strains of *Staphylococcus aureus* (methicillin-resistant)[30] and *Enterococcus faecium* (vancomycin-resistant)[31] (Figure S2 and Table 1). Mixtures containing L-Arg as the cationic component showed lower activities relative to mixtures containing L-Lys. This trend may arise from the generally lower proportion of cationic residues in the L-Arg mixtures relative to the L-Lys mixtures (Table 1); however, exploration of different proportions for the Leu + Lys and Phe + Lys mixtures (discussed below) suggested that variation in this parameter has only a modest impact on MIC (Figure S2). Therefore, we suspect that L-Arg is less effective than L-Lys as a cationic subunit in terms of antibacterial activity. Among the three mixtures containing L-Lys, the identity of the hydrophobic residue played an important role: the L-Ile + L-Lys mixture was much less active toward three of the four bacteria than were the L-Leu + L-Lys and L-Phe + L-Lys mixtures (Table 1 and Figure S2).

Subsequent experiments focused on the L-Leu + L-Lys and L-Phe + L-Lys mixtures because they displayed the most potent antibacterial properties. In each series, the 20-mer mixture was compared with 10-mer, 15-mer, 25-mer, and 30-mer mixtures. For L-Leu + L-Lys, the 10-mer mixture showed significantly higher MIC values (lower activity) for all four bacteria, but the values for the other lengths were generally similar to that for the 20-mer mixture. For L-Phe + L-Lys, modest declines in MIC were observed relative to the 20-mer mixture for both shorter and longer mixtures (Figures S3 and S4). Variation in the subunit proportion was evaluated for each series at the 20-mer length: mixtures were prepared with 7:3, 3:7, and 1:9 combinations of Fmoc-L-Leu or Fmoc-L-Phe with Fmoc(ε-Boc)-L-Lys for comparison with the mixtures prepared with 1:1 combinations (Figures S5 and S6). In both series, a tendency toward modestly higher MIC (weaker activity) was observed for the 7:3 and 3:7 combinations relative to 1:1; this tendency was somewhat more pronounced in the L-Phe + L-Lys series. Both of the 1:9 mixtures showed very weak antibacterial activity. Overall, these comparisons indicate that the optimal antibacterial activities are observed for mixtures generated from 1:1 starting material combinations at the 20-mer length.

The 1:1 20-mer mixtures generated from L-Lys with either L-Leu or L-Phe proved to be highly hemolytic, as judged by the minimum hemolytic concentration (MHC) (<3 μg/mL in each case) or the concentration required for 50% hemolysis (HC_{50}) (6 or 25 μg/mL, respectively). The hemolytic activity was strongly diminished for the 10-mer mixtures in each series (MHC = 50 μg/mL for L-Leu + L-Lys and 6 μg/mL for L-Phe + L-Lys; HC_{50} > 400 μg/mL in both cases). However, as noted above, the antibacterial activities were somewhat lower for this chain length. The hemolytic activity was also strongly diminished for mixtures in which the subunit proportion favored L-Lys over the hydrophobic subunit, but in these cases there was an even sharper decline in antimicrobial activity than was observed for the 10-mer 1:1 mixtures.

The results obtained with combinations of cationic and hydrophobic L-amino acids suggest that it is difficult to identify

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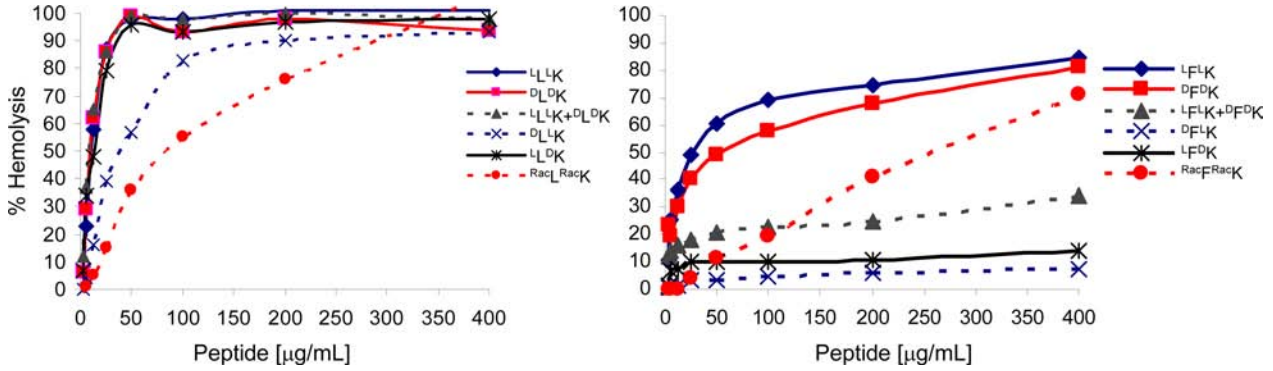

Figure 2. Hemolytic activities of Leu + Lys and Phe + Lys mixtures with different stereochemical profiles.

homochiral binary peptide mixtures featuring both potent antibacterial activity and low hemolytic activity by control of the chain length, subunit identity, or subunit proportion. We turned next to an evaluation of stereochemical variations using the subunit identity, subunit proportion, and chain length parameters that seemed most effective in the L-amino acid studies. Thus, the stereochemical studies involved mixtures containing 20-mers generated with a 1:1 combination of amino acids, either Leu + Lys or Phe + Lys. We compared the homochiral mixtures generated from L-amino acids (designated $^{L}L^{L}K$ and $^{L}F^{L}K$ in Table 2) with the homochiral mixtures generated from D-amino acids ($^{D}L^{D}K$ and $^{D}F^{D}K$) as well as the heterochiral mixtures generated from L-Lys and either D-Leu or D-Phe ($^{D}L^{L}K$ or $^{D}F^{L}K$) or D-Lys and either L-Leu or L-Phe ($^{L}L^{D}K$ or $^{L}F^{D}K$). Two additional types of stereochemical variant were examined, one type synthesized with 1:1 combinations of racemic amino acids ($^{Rac}L^{Rac}K$ and $^{Rac}F^{Rac}K$) and the other type prepared by combining equal-weight samples of the homochiral mixtures ($^{L}L^{L}K$ + $^{D}L^{D}K$ and $^{L}F^{L}K$ + $^{D}F^{D}K$).

The biological evaluations in the stereochemical series showed that this dimension of structural variation exerts little impact on the antibacterial activity (Figures S7 and S8 and Table 2).

Table 2. Antimicrobial Activities for Peptide Mixtures with Varying Stereochemistry

		MIC (μg/mL)[a]				
mixture	hyd:cat ratio[b]	*E.c.*	*B.s.*	*S.a.*	*E.f.*	MHC/HC$_{50}$[c]
$^{L}L^{L}K$	52:48	6	3	12	6	<3/6
$^{D}L^{L}K$	57:43	12	3	6	12	6/50
$^{L}L^{D}K$	59:41	12	3	6	12	<3/6
$^{D}L^{D}K$	56:44	6	3	6	3	<3/<3
$^{Rac}L^{Rac}K$	59:41	12	3	12	25	12/100
$^{L}L^{L}K$+ $^{D}L^{D}K$		12	6	12	6	<3/<3
$^{L}F^{L}K$	59:41	12	3	12	6	<3/25
$^{D}F^{L}K$	54:46	12	6	25	12	50/>400
$^{L}F^{D}K$	61:39	25	6	25	12	200/>400
$^{D}F^{D}K$	63:37	12	6	25	12	<3/6
$^{Rac}F^{Rac}K$	54:46	25	25	25	25	12/200
$^{L}F^{L}K$+ $^{D}F^{D}K$		12	6	12	12	<3/>400

[a]MIC results for *E. coli* (*E.c.*), *B. subtilis* (*B.s.*), *S. aureus* (*S.a.*), and *E. faecium* (*E.f.*). [b]Ratio of hydrophobic to cationic residues in the peptide mixture as determined by amino acid analysis. The uncertainty in each value is 3–4%. [c]MHC: Minimum hemolytic concentration; HC$_{50}$: concentration required for 50% hemolysis, both values are in μg/mL.

However, greater variation in the hemolytic activity was observed (Figure 2). In both the Leu + Lys and Phe + Lys series, some stereochemical mixtures displayed significantly lower hemolytic activities relative to the homochiral 20-mer mixtures. The most promising stereochemical profile varied with the identity of the hydrophobic residue. All of the Leu + Lys mixtures were fairly strong inducers of hemolysis, but the $^{Rac}L^{Rac}K$ mixture was significantly less hemolytic than other members of this series (Figure 2A). Even more dramatic differences among the Phe + Lys mixtures were seen. Both of the heterochiral mixtures, $^{D}F^{L}K$ and $^{L}F^{D}K$, displayed very weak hemolytic activities (Figure 2B). Among all of the materials we examined, the heterochiral Phe + Lys mixtures were most successful at mimicking the HDP activity profile, including selectivity for prokaryotic over eukaryotic cells. In studies with homogeneous peptides, Shai and co-workers[32−34] observed that specific heterochiral sequences can manifest greater antibacterial versus hemolytic selectivity than homochiral stereoisomers, but it was not obvious that heterochiral peptide mixtures could be superior to homochiral mixtures.

The Phe + Lys stereochemical variation series yielded a curious observation. Both the $^{L}F^{L}K$ and $^{D}F^{D}K$ mixtures were highly hemolytic. The similarity of these two samples is not surprising because the antibacterial and hemolytic activities of HDPs and their enantiomers are generally indistinguishable.[35] (Such observations provided early evidence that the antibacterial mechanism involves interaction with the lipid bilayer rather than a specific protein target.) Figure 2B shows that when the two homochiral mixtures were combined to generate $^{L}F^{L}K$ + $^{D}F^{D}K$, a dramatic decline in hemolysis ensued. In contrast to the hemolysis trend, there was little difference in antibacterial activity among $^{L}F^{L}K$, $^{D}F^{D}K$, and $^{L}F^{L}K$ + $^{D}F^{D}K$. This puzzling "combination effect" on the hemolytic activity was specific to the Phe + Lys series; Figure 2A shows that the hemolytic activities of $^{L}L^{L}K$, $^{D}L^{D}K$, and $^{L}L^{L}K$ + $^{D}L^{D}K$ were very similar.

We have used solid-phase peptide synthesis methodology in an unusual way to isolate the impact of distinct structural parameters on the antibacterial and hemolytic properties of sequence-random cationic–hydrophobic peptide mixtures. This effort was motivated by widespread and growing interest in cationic–hydrophobic copolymers,[14−23] which may represent useful alternatives to host-defense peptides and other sequence-specific oligomers for antimicrobial applications. Subunit identity, subunit proportion, chain length, and stereochemistry were all seen to influence the biological activity among the binary mixtures we examined. The results highlight the importance of controlling the stereochemistry to achieve an optimal profile (i.e., selectivity for prokaryotic cells), at least among binary subunit combinations. Perhaps this desirable profile can be achieved

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among homochiral mixtures if the composition is made more diverse, for example, by including subunits that are neither hydrophobic nor cationic.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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