Published in final edited form as:

J Am Chem Soc. 2013 August 14; 135(32): . doi:10.1021/ja406231b.

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

Zvi Hayouka^a, Saswata Chakraborty^a, Runhui Liu^a, Melissa D. Boersma^c, Bernard Weisblum^b, and Samuel H. Gellman^{a,*}

^aDepartment of Chemistry University of Wisconsin, Madison, Wisconsin, 53706, USA

^bDepartment of Medicine University of Wisconsin, Madison, Wisconsin, 53706, USA

^cBiotechnology Center, University of Wisconsin, Madison, Wisconsin, 53706, USA

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 mixtures, 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 co-polymers, materials that contain mixtures of chains with many distinct subunit sequences and lengths. A number of unnatural backbones, including polystyrene¹⁵, polymethacrylate^{16,17,18}, nylon-3^{14,19,20} and polyolefins²¹⁻²³, 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 hemolytic²⁴ or displayed low antimicrobial activity.²⁵ Most of the unnatural polymers examined to date have contained

*Corresponding Author gellman@chem.wisc.edu.

Notes The authors declare no competing financial interests.

Supporting Information Experimental details for synthesis and analysis of random peptides mixtures, antibacterial assays and hemolytic assays are available free of charge via the Internet at <http://pubs.acs.org>.

stereogenic centers and been generated in stereochemically random forms. In contrast, poly- α -peptide mixtures are readily prepared in homochiral form.²⁴

The ability of a heterogeneous set of polymer chains to mimic the activity profile of a homogeneous peptide (one sequence, one chain-length, one stereochemistry) is interesting in practical terms because chemical synthesis of sequence-specific oligomers is more difficult and expensive than is co-polymerization. Thus, antibacterial random co-polymers may be more readily applicable to some real-world problems than are homogeneous peptide agents. The prospect of optimizing co-polymer 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. Achieving this goal with polymerized materials themselves, however, can be challenging because there is so much variation within a sample generated via co-polymerization of a binary monomer combination.

Here we describe a strategy intended to fill the gap between a highly diverse co-polymer mixture and a homogeneous peptide by 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. Subunit stereochemistry is easily controlled because the configuration of an α -amino acid residue is set before incorporation into the backbone. In contrast, stereogenic centers in many co-polymers explored for antibacterial activity, such as polystyrenes, polymethacrylates and polyolefins,¹⁴⁻²³ 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 co-polymerization 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 host defense peptides (Table 1).^{26,27} The hydrophobic residue in each mixture was selected from Leu, Ile and Phe, and the cationic residue was selected from Lys and 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 resin-bound chains (four-fold molar excess of amino acids relative to reactive sites on the solid phase synthesis resin; for the 1:1 combination, this means two-fold molar excess of Fmoc(ϵ -Boc)-L-Lys and two-fold molar excess of Fmoc-L-Phe at each coupling step).

The activated forms of different protected α -amino acids may not have identical reactivities; therefore, amino acid analysis was conducted for the six binary α -peptide mixtures. In control experiments, we found that the deduced proportion could vary between 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). 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.

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 activity 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 pairs (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 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 other lengths were generally similar to 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 subunit proportion was evaluated in each series at the 20-mer length: mixtures were prepared with 7:3, 3:7 and 1:9 combinations of Fmoc-L-Leu + Fmoc(ϵ -Boc)-L-Lys, or Fmoc-L-Phe + Fmoc(ϵ -Boc)-L-Lys, for comparisons with the mixtures prepared with 1:1 combinations (Figures S5 and S6). In both series, a tendency toward modestly higher MIC values (weaker activities) 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 1:9 mixtures showed very weak antibacterial activity. Overall, these comparisons indicate that the optimal antibacterial activity is observed for the 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 μ g/mL in each case) or the concentration required for 50% hemolysis (HC₅₀; 6 μ g/mL or 25 μ g/mL, respectively). Hemolytic activity was strongly diminished for the 10-mer mixtures in each series (MHC = 50 μ g/mL for L-Leu+L-Lys and 6 μ g/mL for L-Phe+L-Lys; HC₅₀ > 400 μ g/mL in both cases). However, as noted above, antibacterial activities were somewhat lower for this chain length. Hemolytic activity was strongly diminished also 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 homochiral binary peptide mixtures that feature both potent antibacterial activities and low hemolytic activity by controlling chain length, subunit identity or subunit proportion. We turned next to an evaluation of stereochemical variations, based on 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 ^LL^LK or ^LF^LK in Table 2) with the mixtures generated from D-amino acids (^DL^DK or ^DF^DK) as well as the heterochiral mixtures generated from L-Lys and either D-Leu or D-Phe (^DL^LK or ^DF^LK), and the heterochiral mixtures generated from D-Lys and

either L-Leu or L-Phe ($^L\text{L}^D\text{K}$ or $^L\text{F}^D\text{K}$). Two additional types of stereochemical variant were examined, one synthesized with a 1:1 combination of racemic amino acids ($^{\text{Rac}}\text{L}^{\text{Rac}}\text{K}$ or $^{\text{Rac}}\text{F}^{\text{Rac}}\text{K}$) and the other prepared by combining equal-weight samples of the homochiral mixtures, to generate materials designated $^L\text{L}^L\text{K}+^D\text{L}^D\text{K}$ or $^L\text{F}^L\text{K}+^D\text{F}^D\text{K}$.

The biological evaluations in the stereochemical series showed that this dimension of structural variation exerts little impact on antibacterial activities (Figures S7 and S8 and Table 2). Greater variation was observed, however, in terms of hemolytic activity (Figure 2). In both the Leu+Lys and Phe+Lys series, some stereochemical mixtures displayed significantly lower hemolytic activity 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 $^{\text{Rac}}\text{L}^{\text{Rac}}\text{K}$ mixture was significantly less hemolytic than other members of this series (Figure 2A). Even more dramatic differences were seen among the Phe+Lys mixtures. Both heterochiral mixtures, $^D\text{F}^L\text{K}$ and $^L\text{F}^D\text{K}$, displayed very weak hemolytic activity (Figure 2B). Among all materials we have examined, the heterochiral Phe+Lys mixtures were most successful at mimicking the HDP activity profile, including selectivity for prokaryotic vs. eukaryotic cells. In studies with homogeneous peptides, Shai et al.³²⁻³⁴ have observed that specific heterochiral sequences can manifest greater antibacterial vs. hemolytic selectivity than do 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 $^L\text{F}^L\text{K}$ and $^D\text{F}^D\text{K}$ mixtures were highly hemolytic. The similarity of these two samples is not surprising, because the antibacterial and hemolytic activities of host-defense peptides 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 $^L\text{F}^L\text{K}+^D\text{F}^D\text{K}$, a dramatic decline in hemolysis ensued. In contrast to the hemolysis trend, there was little difference in antibacterial activity among $^L\text{F}^L\text{K}$, $^D\text{F}^D\text{K}$ and $^L\text{F}^L\text{K}+^D\text{F}^D\text{K}$. This puzzling ‘combination effect’ on hemolytic activity was specific to the Phe+Lys series; Figure 2A shows that $^L\text{L}^L\text{K}$, $^D\text{L}^D\text{K}$ and $^L\text{L}^L\text{K}+^D\text{L}^D\text{K}$ were all very similar in hemolytic activity.

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 co-polymers,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 are all seen to influence biological activity among the binary mixtures we examined. The results highlight the importance of controlling stereochemistry for achieving an optimal profile, i.e., selectivity for prokaryotic cells, at least among binary subunit combinations. Perhaps this desirable profile can be achieved among homochiral mixtures if the composition is made more diverse, for example, by including subunits that are neither hydrophobic nor cationic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Nanoscale Science and Engineering Center at UW-Madison (DMR-0832760) and the NIH (R01GM093265). Z. H. was supported in part by a Fulbright Fellowship. We would like to acknowledge the UW-Madison peptide center facility.

REFERENCES

- (1). Diamond G, Beckloff N, Weinberg A, Kisich KO. *Curr. Pharm. Des.* 2009; 15:2377–2392. [PubMed: 19601838]
- (2). Zasloff M. *Nature.* 2002; 415:389–395. [PubMed: 11807545]
- (3). Rathinakumar R, Walkenhorst WF, Wimley WC. *J. Am. Chem. Soc.* 2009; 131:7609–7617. [PubMed: 19445503]
- (4). Boman HG. *J. Intern. Med.* 2003; 254:197–215. [PubMed: 12930229]
- (5). Hancock RE. *Lancet. Infect. Dis.* 2001; 1:156–164. [PubMed: 11871492]
- (6). Wimley WC. *ACS. Chem. Biol.* 2010; 5:905–917. [PubMed: 20698568]
- (7). Zasloff M. *J. Am. Soc. Nephrol.* 2007; 18:2810–2816. [PubMed: 17942949]
- (8). Hilpert K, Elliott MR, Volkmer-Engert R, Henklein P, Donini O, Zhou Q, Winkler DF, Hancock RE. *Chem. Biol.* 2006; 13:1101–1107. [PubMed: 17052614]
- (9). Brogden KA. *Nat. Rev. Microbiol.* 2005; 3:238–250. [PubMed: 15703760]
- (10). Yeaman MR, Yount NY. *Pharmacol. Rev.* 2003; 55:27–55. [PubMed: 12615953]
- (11). Shai Y. *Biochim. Biophys. Acta-Biomembranes.* 1999; 1462:55–70.
- (12). Tossi A, Sandri L, Giangaspero A. *Biopolymers.* 2000; 55:4–30. [PubMed: 10931439]
- (13). Axelsen PH. *Biophys. J.* 2008; 94:1549–1550. [PubMed: 18065456]
- (14). Mowery BP, Lee SE, Kissounko DA, Epand RF, Epand RM, Weisblum B, Stahl SS, Gellman SH. *J. Am. Chem. Soc.* 2007; 129:15474–15476. [PubMed: 18034491]
- (15). Gelman MA, Weisblum B, Lynn DM, Gellman SH. *Org. Lett.* 2004; 6:557–560. [PubMed: 14961622]
- (16). Kuroda K, DeGrado WF. *J. Am. Chem. Soc.* 2005; 127:4128–4129. [PubMed: 15783168]
- (17). Palermo EF, Sovadinova I, Kuroda K. *Biomacromolecules.* 2009; 10:3098–3107. [PubMed: 19803480]
- (18). Paslay LC, Abel BA, Brown TD, Koul V, Choudhary V, McCormick CL, Morgan SE. *Biomacromolecules.* 2012; 13:2472–2482. [PubMed: 22738241]
- (19). Mowery BP, Lindner AH, Weisblum B, Stahl SS, Gellman SH. *J. Am. Chem. Soc.* 2009; 131:9735–9745. [PubMed: 19601684]
- (20). Liu R, Chen X, Hayouka Z, Chakraborty S, Falk SP, Weisblum B, Masters KS, Gellman SH. *J. Am. Chem. Soc.* 2013; 135:5270–5273. [PubMed: 23547967]
- (21). Ilker MF, Nusslein K, Tew GN, Coughlin EB. *J. Am. Chem. Soc.* 2004; 126:15870–15875. [PubMed: 15571411]
- (22). Gabriel GJ, Madkour AE, Dabkowski JM, Nelson CF, Nusslein K, Tew GN. *Biomacromolecules.* 2008; 9:2980–2983. [PubMed: 18850741]
- (23). Lienkamp K, Madkour AE, Musante A, Nelson CF, Nusslein K, Tew GN. *J. Am. Chem. Soc.* 2008; 130:9836–9843. [PubMed: 18593128]
- (24). Zhou C, Qi X, Li P, Chen WN, Chang MW, Leong SS, Chan-Park MB. *Biomacromolecules.* 2010; 11:60–67. [PubMed: 19957992]
- (25). Engler AC, Shukla A, Puranam S, Buss HG, Jreige N, Hammond PT. *Biomacromolecules.* 2011; 12:1666–1674. [PubMed: 21443181]
- (26). Wang Z, Wang G. *Nucleic Acids Res.* 2004; 32:D590–592. [PubMed: 14681488]
- (27). Mishra, Biswajit; Wang, Guangshun. *J. Am. Chem. Soc.* 2012; 134:12426–12429. [PubMed: 22803960]
- (28). Yanisch-Perron C, Vieira J, Messing. *J. Gene.* 1985; 33:103–119.
- (29). Young FE, Smith C, Reilly BE. *J. Bacteriol.* 1969; 98:1087–1097. [PubMed: 4977981]

- (30). Weisblum B, Demohn V. J. Bacteriol. 1969; 98:447–452. [PubMed: 5784204]
- (31). Nicas TI, Wu CY, Hobbs JN Jr. Preston DA, Allen NE. Antimicrob. Agents. Chemother. 1989; 33:1121–1124. [PubMed: 2528940]
- (32). Shai Y, Oren Z. J. Biol. Chem. 1996; 271:7305–7308. [PubMed: 8631748]
- (33). Papo N, Oren Z, Pag U, Sahl HG, Shai Y. J. Biol. Chem. 2002; 277:33913–33921. [PubMed: 12110678]
- (34). Pag U, Oedenkoven M, Papo N, Oren Z, Shai Y, Sahl HG. J. Antimicrob. Chemother. 2004; 53:230–239. [PubMed: 14729742]
- (35). Wade D, Boman A, Drain CM, Andreu D, Boman HG, Merrifield RBP. Natl. Acad. Sci. USA. 1990; 87:4761–4765.

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 was used for each coupling step, rather than a single protected amino acid. The results of three coupling steps are illustrated. In this process, each bead of the solid support will bear many growing chains with many different sequences. The mixture contains 4^n peptide sequences if the amino acids are racemic.

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

Table 1

Antimicrobial activities for peptide mixtures with L residue configuration

20-mer Peptide Mixture	Hydrophobic: Cationic residue ratio ^b	MIC µg/mL ^a			
		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 Residue proportion in the peptide mixture, according to the amino acid analysis. The uncertainty is 3-4 % in each value. All amino acid residues have L absolute configuration.

Table 2

Antimicrobial activities for peptide mixtures with varying stereochemistry

20-mer Peptide Mixture	Res. ratio ^b	MIC $\mu\text{g/mL}^a$				MHC/ HC ₅₀ $\mu\text{g/mL}$
		E.c	B.s	S. a	E.f	
L _L L _L K	52:48	6	3	12	6	<3/6
D _L L _L K	57:43	12	3	6	12	6/50
L _L D _L K	59:41	12	3	6	12	<3/6
D _L D _L K	56:44	6	3	6	3	<3/<3
Rac _L Rac _K	59:41	12	3	12	25	12/100
L _L L _L K+ D _L D _L K		12	6	12	6	<3/<3
L _F L _L K	59:41	12	3	12	6	<3/25
D _F L _L K	54:46	12	6	25	12	50/>400
L _F D _L K	61:39	25	6	25	12	200/>400
D _F D _L K	63:37	12	6	25	12	<3/6
Rac _F Rac _K	54:46	25	25	25	25	12/200
L _F L _L K +D _F D _L 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 hydrophobic:cationic residue proportion in the peptide mixture, according to amino acid analysis. The uncertainty is 3-4 % in each value.