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Reverse Engineering Truncations of an Antimicrobial Peptide Dimer to Identify the Origins of Potency and Broad Spectrum of Action

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Antimicrobial peptides hold promise against antibiotic resistant pathogens. Here, to find the physicochemical origins of potency and broad spectrum antimicrobial action, we report the structure−activity relationships of synthetic intermediates (peptides A−D) of a potent lysine branched dimeric antibacterial peptide ΔFd. Our studies show that a tetracationic character in a weak helical fold (peptide C) elicits potent but narrow spectrum antimicrobial activity [Minimum inhibitory concentrations (MICs) *E. coli* 10 μM, *S. aureus* > 100 μM]. In contrast, a hexacationic character in a strong, amphipathic helix (ΔFd) confers potent and broad spectrum action [MICs *E. coli* 2.5 μM, *S. aureus* 5 μM]. While ΔFd caused rapid and potent permeabilization of the *E. coli* membranes, the less helical intermediates (peptides A−D) showed slow and weak to no responses. Two seminal findings that may aid future drug design are (a) at identical helicity, increasing charge enhanced outer membrane permeabilization, and (b) at identical charge, increasing helicity stimulated rate of outer membrane permeabilization and kill kinetics besides enhancing potency leading to broad spectrum action.

Introduction

Due to widespread resistance to almost all conventional antibiotics, there is an urgent need to discover or design novel antibiotics.[1] Naturally occurring antimicrobial[2] and host defense peptides are being developed as new anti-infective therapeutic agents.[3] Peptide antibiotics offer several advantages over their nonpeptide counterparts. These include (a) their lower tendency to induce resistance in bacteria (b) specific action against bacterial membranes, (c) the ability to target both the surface and the intracellular milieu of bacteria, and (d) broad spectrum action against both Gram negative and Gram positive bacteria. However, the utility of these naturally occurring peptides has been curtailed by drawbacks like high cost of production, poor potency, instability, and poor selectivity.[4] Therefore, to improve their performance, efforts have been directed toward understanding the physicochemical code governing the activity of these peptides.[5−7]

A number of studies have elucidated the influence of individual biochemical parameters like charge, hydrophobicity, and conformation on the potency and selectivity of antimicrobial peptides.[8−10] However, the composite physicochemical code governing their antimicrobial properties like spectrum of action, kill kinetics, and membrane permeabilizing abilities has remained largely undefined. As action of antimicrobial peptides (AMPs[a]) is a multipronged composite of several parameters, elucidation of this code could help in designing better antibiotics. Such information is necessary to generate antibiotic peptides that harbor the minimum functional core with no extraneous features which enhance the cost of production without a concurrent enhancement of their antibiotic profile.

We have recently reported the seminal role of a lysine branched dimeric motif in the *de novo* design of a potent, broad spectrum antimicrobial peptide ΔFd.[11] This branched dimeric peptide has two cationic decapeptide amphipathic helices (Ac-G-ΔF-R-K-ΔF-H-K-ΔF-W-A) linked to the α and ε amino groups of a lysyl residue (Figure 1A). ΔFd is characterized by the presence of α,β-didehydrophenylalanine (ΔF), a nonproteinogenic amino acid which constrains the peptides harboring it in a helical conformation[12] and renders them relatively more resistant to proteases than their phenylalanine-containing counterpart. The chemical structures of phenylalanine (F) and its α,β-didehydrophenylalanine analogue (ΔF) are shown in parts B and C of Figure 1, respectively. ΔFd shows potent, broad-spectrum activity against both Gram negative and Gram positive bacteria and exhibits strong selectivity for bacterial cells.[11]

To (a) identify the minimum functional module of ΔFd and (b) dissect the origins of strong potency and broad spectrum of action in ΔFd, we have examined the conformational and antibiotic properties of four synthetic intermediates of ΔFd. These intermediates have allowed us to unravel the effects of charge and helicity on (a) antibiotic potency, (b) spectrum of action, (c) kill kinetics, and (d) membrane permeabilization. Even though it is well-known that the antimicrobial action of a peptide is governed by several factors including charge, helicity, and amphipathicity,[6,13] the hierarchy and interplay of these factors in the diverse actions of an antimicrobial peptide are not known. Approaches that rely upon site specific substitutions are sequence of a peptide, to understand structure

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[a] Abbreviations: AMPs, antimicrobial peptides; CD, circular dichroism; CFU, colony forming units; ΔF, α,β-didehydrophenylalanine; MRE, mean residue ellipticity; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; NPN, 1-*N*-phenylnaphthylamine; PI, propidium iodide; SDS, sodium dodecyl sulfate.

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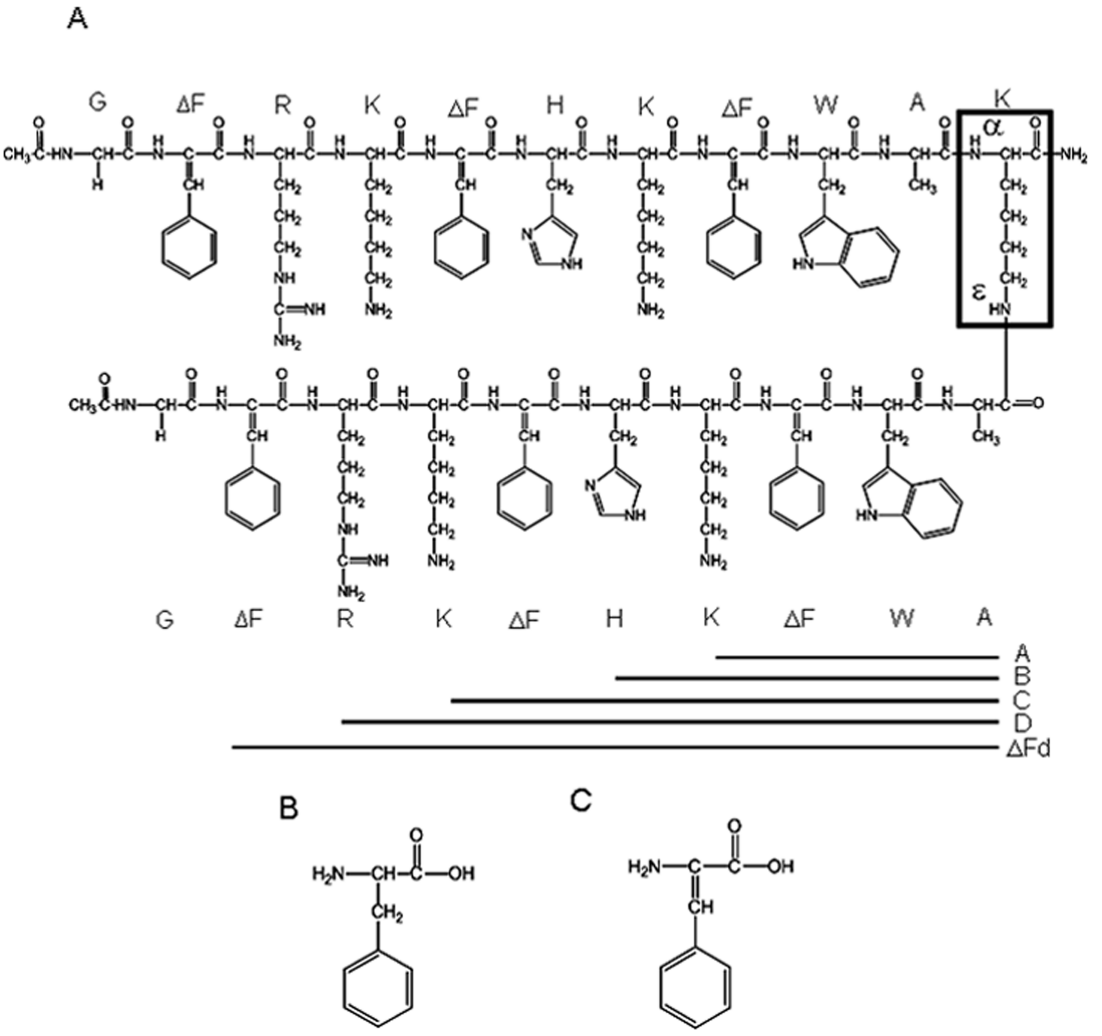

Figure 1. (A) Chemical structure of lysine branched dimer ΔFd. Letters denote single letter codes for amino acid residues. It may be noted that the two amino groups (marked as α and ε) of the boxed central lysine make amide bonds with the carboxy terminal alanyl residue of each peptide chain. Solid lines demarcate the truncation boundaries of the dimers A through D. Acetyl-glycyl cap is common to all peptides shown. Chemical structures are shown for (B) phenylalanine and (C) α,β-didehydrophenylalanine. All chemical structures have been made using ChemDraw Ultra 11.0.

activity relationships,[14−17] often do not allow firm conclusions, as they affect more than one property of a peptide by a single change. Here, we have used a reverse engineering approach to identify how features of antimicrobial activity such as potency, spectrum of action, kill kinetics, and permeabilization of the bacterial membrane evolve in a potent antimicrobial peptide with progressive changes in its physicochemical characteristics like charge and helicity.

Results

Peptide Design. To determine the minimum peptide length required for the antibiotic activity of ΔFd, we generated its sequentially N-terminally truncated analogues viz peptide A (5 residues/chain), peptide B (6 residues/chain), peptide C (8 residues/chain), and peptide D (9 residues/chain) (Table 1). As N-capping motifs and the nature of N terminal residue have been shown to affect peptide structure and activity,[18,19] all the peptides listed above were uniformly glycine capped and acetylated at the N terminus. The chemical structure of ΔFd with the truncation boundaries of its shortened dimer analogues has been shown in Figure 1A.

The resulting peptides differed in physicochemical properties such as length, charge, and the number of α,β-didehydrophenylalanine (ΔF) residues (Table 1). Peptides A and B differed in length but had the same charge (+2) and number of ΔF residues (2 ΔFs). Peptides C and D with 4 ΔFs each had a charge of +4 and +6, respectively. The full-length dimer ΔFd had 6 ΔFs and a charge of +6. The RPHPLC retention times of peptides A−D and ΔFd were: 34, 45, 45, 39, and 39 min respectively (Supporting Information Figure 1).

Circular Dichroism Based Structures of Dimers of Increasing Lengths. Circular dichroism (CD) spectroscopy was used to determine the secondary structure of the peptides in the membrane mimetic solution of sodium dodecyl sulfate (SDS). The CD spectra of dimers A (11 mer) through ΔFd (21 mer) (Figure 2A) indicate a progressive increase in nucleation of stable secondary structures.

Dimer A (11 mer, 1 ΔF/chain), with very weak ellipticity at ~280 nm, appears to be nearly random. Dimer B (13 mer, 1 ΔF/chain,) with significantly enhanced ellipticity of the 280 nm band, appears to be acquiring a more stable β-turn structure.[12] This is likely to be an effect of the increase in

Table 1. Physicochemical Properties of the Peptides Used in This Study

Peptide	Sequence[a]	Molecular Weight (Expected)	Molecular Weight (Observed)	Charge	No. of ΔFs
Peptide A	Ac-G-K-ΔF-W-A-K-NH_2 Ac-G-K-ΔF-W-A	1404	1403	+2	2
Peptide B	Ac-G-**H**-K-ΔF-W-A-K-NH_2 Ac-G-**H**-K-ΔF-W-A	1678	1679	+2	2
Peptide C	Ac-G-**K-ΔF**-H-K-ΔF-W-A-K-NH_2 Ac-G-**K-ΔF**-H-K-ΔF-W-A	2224	2224	+4	4
Peptide D	Ac-G-**R**-K-ΔF-H-K-ΔF-W-A-K-NH_2 Ac-G-**R**-K-ΔF-H-K-ΔF-W-A	2536	2534	+6	4
ΔFd	Ac-G-**ΔF**-R-K-ΔF-H-K-ΔF-W-A-K-NH_2 Ac-G-**ΔF**-R-K-ΔF-H-K-ΔF-W-A	2826	2826	+6	6

[a] The progressive increments in chain length are indicated by the residues in boldface. The N terminus acetyl glycyl cap is common to all peptides.

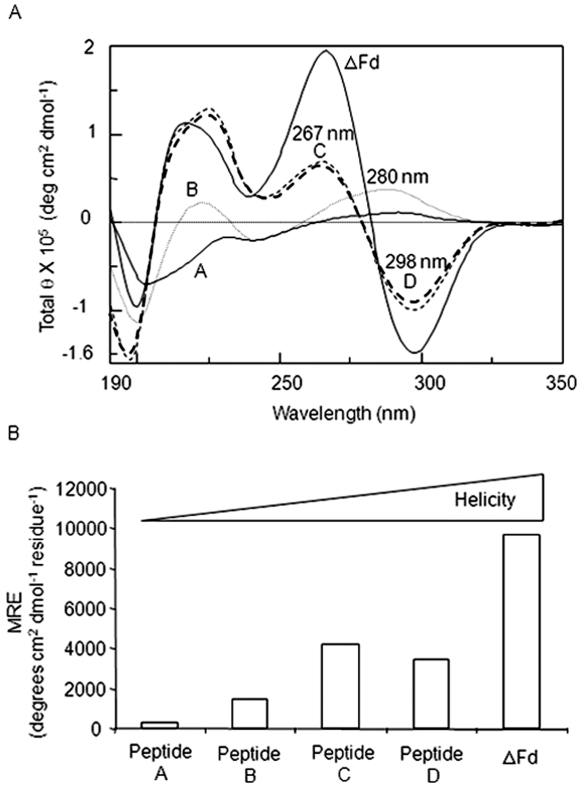

Figure 2. (A) Circular dichroism based conformational analysis of dimeric peptides A−D and ΔFd (6 μM each) in 20 mM SDS/10 mM sodium phosphate buffer pH 7.5. (B) Histogram showing mean residue ellipticity (MRE) values for the dimeric peptides A−D and ΔFd (for 267 nm band).

chain length as a consequence of introducing one histidyl residue per chain in dimer B. Interestingly, dimers C (17 mer) and D (19 mer), each with two ΔF residues per chain, showed an excitonic couplet at 267 nm (+) and 298 nm (−). This couplet is a signature of the formation of right handed 3_{10} helices in ΔF containing peptides.[20] The nearly overlapping spectra of dimers C (8 mer/chain) and D (9 mer/chain) suggest that the introduction of an additional ΔF residue to each chain of dimer B (1 ΔF/6 mer chain) caused the transition from β-turn (dimer B) to 3_{10} helices (dimers C and D).

Table 2. Antibacterial Activities of the Peptides Studied[a]

	E. coli ML35p			*S. aureus* ATCC 700699		
peptide	MIC (μM)	MBC (μM)	MBC/MIC	MIC (μM)	MBC (μM)	MBC/MIC
peptide A	>100			>100		
peptide B	>100			>100		
peptide C	10	15	1.5	>100		
peptide D	5	10	2	>100		
ΔFd	2.5	5	2	5	7.5	1.5

[a] Standard error measurement (SEM) values of OD_{600} for maximum growth and maximum CFU/mL were ≤1% of mean and 0.6% of mean, respectively. All SEM values for end point OD_{600} (MIC) and CFU/mL (MBC) of two independent experiments (each done in triplicates) have been shown in Experimental Section.

It may be noted that the excitonic couplet seen with dimers C and D is unequal, with 1.5 times greater negative intensity centered at 298 nm than the positive intensity centered at 267 nm. In moving from dimers C/D (2 ΔFs/chain) to ΔFd (3 ΔFs/chain), we observe (a) the 267−298 nm couplet becomes significantly stronger in intensity and (b) the positive ellipticity (267 nm) becomes a little stronger than the negative ellipticity (298 nm). It was observed that without any change in the status of charge, the introduction of the third ΔF residue per chain in ΔFd resulted in a 3-fold increment in molar ellipticity of the helix associated excitonic couplet (Figure 2A). Thus, it is clear that in the present series, the dimer ΔFd makes the strongest helix (Figure 2B).

Antibacterial Activities of Dimers of Increasing Lengths. The antibacterial activity of ΔFd and its truncated analogues (peptides A−D), was tested against *Escherichia coli* ML35p and *Staphylococcus aureus* ATCC 700699 (Table 2).

The antibacterial effect of the peptides against *E. coli* ML35p was found to increase in potency with chain length (peptides A−ΔFd) and the aforementioned features associated with increasing chain length. The poorly structured shorter dimers A and B (charge: +2, 2 ΔFs) did not inhibit the growth of either the Gram negative *E. coli* or the Gram positive *S. aureus* [minimum inhibitory concentration (MIC) > 100 μM]. Peptide C (charge: +4 and 4 ΔFs, weak 3_{10} helical structure) was quite potent and bactericidal against *E. coli* [MIC: 10 μM; minimum bactericidal concentration (MBC): 15 μM] but failed to act against *S. aureus* (MIC > 100 μM).

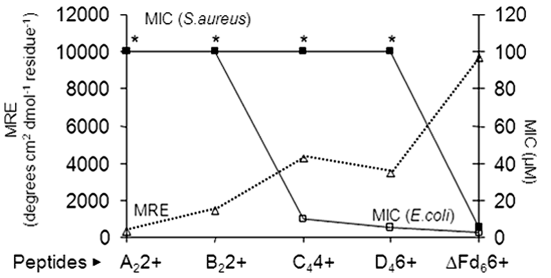

Figure 3. Plot showing inverse relationship between mean residue ellipticity (MRE) (for 267 nm band) and MIC for peptides A−D and ΔFd tested against *E. coli* ML35p and *S. aureus* ATCC 700699. The number of positive charges and the number of ΔF residues in each peptide are indicated by superscript and subscript, respectively. *: MICs shown as 100 μM are in reality >100 μM.

Like peptide C, peptide D also had 4 ΔF residues but differed in having a higher charge (+6). Even though the CD spectra of peptides C and D (Figure 2A) were nearly overlapping in all respects, peptide D showed a significantly improved antibacterial activity against *E. coli* (MIC: 5 μM; MBC: 10 μM) over peptide C (Table 2). Thus, it can be concluded that the improved potency of dimer D may be the result of its two additional positive charges. However, both dimers C and D failed to act against *S. aureus* up to a concentration of 100 μM and hence were not broad spectrum in antibiotic action. In sharp contrast, the introduction of the third ΔF residue to each chain resulting in ΔFd was associated with dramatic improvements in antibacterial potency and spectrum of action. The strong helical nature of ΔFd (Figure 2A) led not only to further potentiation of action against Gram negative *E. coli* (MIC: 2.5 μM; MBC: 5 μM) but also a broadened spectrum of action with potent bactericidal action against the Gram positive *S. aureus* (MIC: 5 μM, MBC: 7.5 μM). The results suggest that antibacterial potency and spectrum of action are a function of increasing peptide length, charge and helicity. The inverse correlation between helicity and MIC against *E. coli* and *S. aureus* is shown in Figure 3.

In summary, two trends were apparent from the above results: (a) as seen in case of peptide C, +4 charges, 4 ΔFs and weak 3_{10} helical structure represent the minimum requirements for antibacterial activity, and (b) a broad spectrum of action is conferred by the presence of +6 charges, 6 ΔFs and a strong helical fold (as seen in case of ΔFd).

Comparative Kill Kinetics of Dimers C, D, and ΔFd. In addition to its potency, the suitability of a peptide as an antimicrobial agent is assessed also by the kinetics or the rate at which it effects its killing action. At 4 times the respective MIC, each dimer showed characteristic distinctions in the rate of *E. coli* killing. At 5 min, while peptides C and D showed a 2-fold decrease in colony forming units (CFU)/mL (from 2×10^5 to 1×10^5 CFU/mL) (50% *E. coli* cells killed), ΔFd showed a 25 fold decrease in CFU/mL (from 2×10^5 to 8×10^3 CFU/mL) (96% *E. coli* cells killed) (Figure 4A). An approximate estimation suggests that 95% killing was achieved by the three dimers in 40 min (peptide C), 20 min (peptide D), and 5 min (ΔFd). Thus, among the three dimers, the strongest helix ΔFd displayed the fastest kill kinetics, which is suggestive of a good correlation between peptide helicity and kill kinetics (Figure 4B). Although different in charge, peptides C (+4) and D (+6) exhibited comparable molar ellipticities (Figure 2A) and killed bacteria at comparable rates (Figure 4A). However, kill kinetics increased significantly in case of ΔFd (charge: +6), which had the

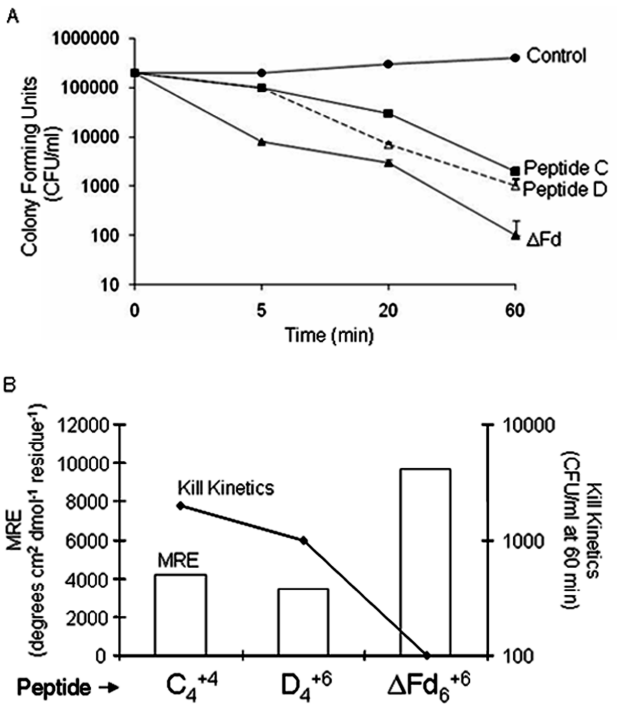

Figure 4. (A) Kill kinetics of ΔFd (10 μM), peptide C (40 μM), and peptide D (20 μM) (at 4× MIC each) against *E. coli* ML35p. (B) Plot showing inverse relationship between mean residue ellipticity (MRE) (for 267 nm band) and rate of cell death (kill kinetics) caused by peptides C, D, and ΔFd against *E. coli* ML35p measured as colony forming units (CFU)/mL. The number of positive charges and the number of ΔF residues in each peptide are indicated by superscript and subscript, respectively.

same charge but higher helicity than peptide D. These results suggest that even though both charge and helicity are determinants of antibacterial potency, helicity seems to play a more dominant role than charge in imparting a higher rate of bacterial killing to ΔFd.

Outer Membrane Permeabilization by Dimers C, D, and ΔFd. Outer membrane permeabilization refers to disruption of membrane integrity which facilitates the uptake of exogenous molecules.[21] This is an important step in the mode of action of antibacterial peptides, as these have been shown to gain entry into the bacteria via a "self-promoted uptake" mechanism involving displacement of native Ca^{2+} and Mg^{2+} ions from the lipopolysaccharide (LPS) layer of the bacterial membrane.[22] To determine the physicochemical factors influencing membrane permeabilization, we studied the outer and inner membrane permeabilizing abilities of the three dimers (C, D, and ΔFd). We used 1-*N*-phenyl-naphthylamine (NPN) as a fluorescent probe (NPN fluoresces strongly only in a hydrophobic environment like the interior of a membrane)[22] to identify the kinetic traits of outer membrane permeabilization associated with the three dimers. These dimers, examined at 10 μM each, showed a characteristic time dependent rise in fluorescence intensity due to increased membrane permeabilization and, consequently, uptake of NPN fluorescence (Figure 5A).

The rise in fluorescence signal was gradual and shallow (peptide C), more rapid and high intensity (peptide D), and most rapid with high intensity (ΔFd). Viewed in context of the charge and helicities of these peptides, two clear trends in the degree and rate of permeabilization are evident. First, among the three peptides, peptide C (charge: +4) showed the

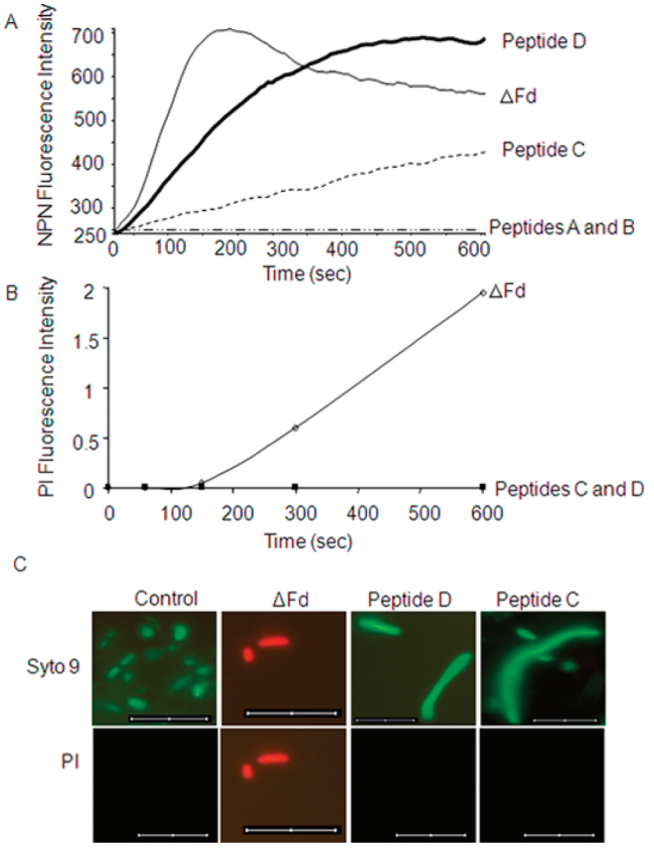

Figure 5. (A) Outer membrane permeabilization of *E. coli* ML35p by peptides (10 μM each) measured by increase in NPN fluorescence for 10 min. (B) Inner membrane permeabilization of *E. coli* ML35p by peptides (10 μM each) measured by increase in PI fluorescence for 10 min. (C) Varied cell morphologies induced by the full length dimer ΔFd and its shorter versions D and C. Fluorescence microscopic images of *E. coli* ML35p treated with peptides at the respective MICs of each: ΔFd (2.5 μM), peptide D (5 μM), peptide C (10 μM) for 18 h, and stained by PI/Syto9. Control represents cells not treated with peptide. The same section has been viewed under Syto filter (top panel) and PI filter (bottom panel). Scale bars (10 μm) have been indicated as white bars.

least degree and rate of outer membrane permeabilization. Second, even though the degree of permeabilization was the same for peptide D (charge: +6) and ΔFd (charge: +6), the rate of permeabilization was higher for the more helical ΔFd. These results suggest that while the degree of permeabilization is influenced by charge, the rate of permeabilization appears to be determined by the helicity of the peptide.

Inner Membrane Permeabilization by Dimers C, D, and ΔFd. Kinetics of bacterial inner membrane permeabilization by the dimers was studied by measuring the uptake of the fluorescent probe propidium iodide (PI). This dye enters only membrane compromised cells and fluoresces upon binding to nucleic acids.[23] Figure 5B shows that ΔFd, the strongest helix and the most potent antibiotic in the present series, showed strong ability to permeabilize the inner membrane. However, unlike outer membrane permeabilization (Figure 5A), the permeabilization of inner membrane by ΔFd showed a lag period of ~2.5 min. A closer look at the trends of the outer versus the inner membrane permeabilization induced by ΔFd suggests that the time (~230 s) at which the outer membrane permeabilization signal shows a small fall coincides with the time (~150−300 s) when inner permeabilization shows the first rise (Figure 5B). In contrast, the shorter dimers C and D failed to permeabilize the inner membrane. Consistent with these observations, the fluorescence microscopy based monitoring of Syto 9 (freely permeable to both wild type and membrane compromised cells) and PI (permeable only into membrane compromised cells) also indicated that only the ΔFd treated *E. coli* were PI permeable (Figure 5C). It was interesting to observe that in contrast to ΔFd and peptide D, peptide C gave rise to a thin filamentous phenotype (Figure 5C) suggestive of inhibition of cell division. Further studies are warranted to understand the underlying mechanism of filamentation induced by this peptide in *E. coli*.

Discussion

The *de novo* designed, lysine branched dimeric peptide ΔFd is a potent broad spectrum antimicrobial {MIC (*E. coli*): 2.5 μM; MIC (*S. aureus*): 5 μM} without undue hemolytic activity.[11] To (a) define the minimum functional antimicrobial peptide module of ΔFd and (b) to find the origins of its potency and broad spectrum of action, we designed a series of N-terminally truncated analogues of ΔFd (peptides A−D) (Table 1). These truncated shorter dimers differed in various physicochemical factors like charge and helicity and thus allowed us to investigate the roles of individual factors as well as their interplay in regulating antibiotic activity. In the context of antimicrobial peptides, such a reverse engineering approach allows us to delineate the hierarchy and interplay of the important biological parameters without being impeded by confounding variables arising as a consequence of amino acid substitution strategies. The guiding principles of antibiotic peptide design that have emerged from the present study are discussed below.

Antibiotic Potency Improves with Charge and Helicity. The shorter peptide dimers A (11 mer) and B (13 mer), with 2 ΔFs were neither rich in charge (+2) nor in folding propensity (Figure 2). The lack of antibiotic activity (MIC > 100 μM) in these two dimers indicates the specific requirements of charge and conformation for antibiotic activity. Peptide C (17 mer, charge: +4, 4 ΔFs) marked the beginning of the formation of a folded structure (as evidenced by CD spectroscopy, Figure 2) and the simultaneous introduction of two additional positive charges. Interestingly, this was the shortest peptide in the present series to show antibiotic activity against *E. coli* (MIC: 10 μM; MBC: 15 μM). However, it was narrow spectrum in action and did not show activity against *S. aureus* (MIC > 100 μM). This led us to infer that in the present series, a charge of +4 and the presence of a weak helical fold represented the minimum threshold requirements for antibiotic activity against *E. coli*. The hexacationic peptide D (19 mer, charge: +6, 4 ΔFs) was structurally at par with peptide C (Figure 2). However, peptide D [MIC (*E. coli*): 5 μM; MBC: 10 μM] was more potent than peptide C against *E. coli* and lacked activity against *S. aureus* (MIC > 100 μM). This suggested that in moving from peptide C to peptide D, the 2-fold potentiation of MIC was due to the introduction of two additional positive charges. ΔFd (21 mer, charge: +6, 6 ΔFs) formed the strongest helix (Figure 2) and showed the highest potency against *E. coli* (MIC: 2.5 μM, MBC: 5 μM). Figure 6A is a three-dimensional graphical representation of the influence of charge and helicity on antibiotic potency, as defined by the MIC. The graph shows that in all the dimers, from peptide C to ΔFd, antibiotic potency against *E. coli* shows a strong correlation with both charge and helicity. Previous studies have shown that an increment of charge[8] or

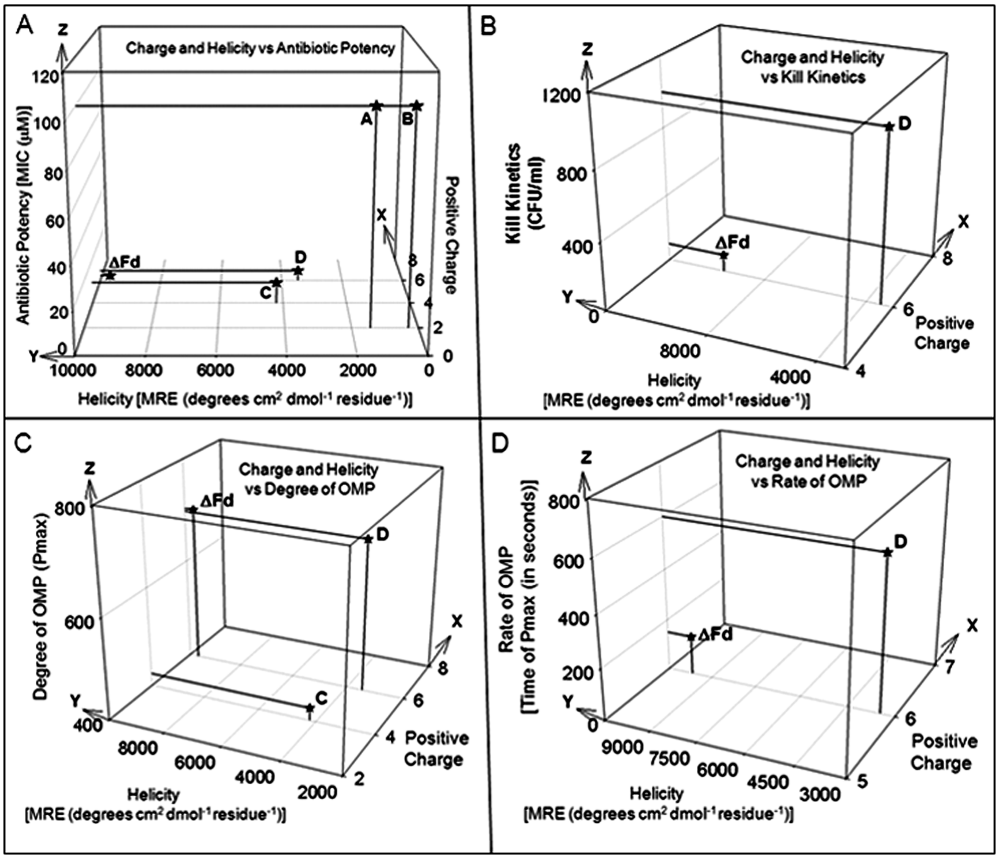

Figure 6. Multiparameter physicochemical correlates of antibacterial activity against *E. coli*. Combined influence of charge and helicity (MRE) on (A) antimicrobial potency, (B) kill kinetics (CFU/mL) (C) degree (P_{max}), and (D) rate (time of P_{max} in s) of outer membrane permeabilization (OMP). MRE: mean residue ellipticity; MIC: minimum inhibitory concentration; P_{max}: permeabilization maximum (maximum permeabilization achieved in the duration of the experiment).

helicity[16,24] in isolation improves antimicrobial potency. Our results suggest that the interplay of both these physicochemical factors in the same template may be necessary to elicit potent antimicrobial activity.

In the Background of Constant Charge, Increment in Helicity Broadens the Spectrum of Action and Enhances the Kill Kinetics. While potency against *E. coli* was influenced by both charge and helicity, spectrum of action was largely governed by the helicity of the cationic peptide. As seen previously in Figure 3, unlike the parent peptide ΔFd, none of the shorter dimers was broad spectrum in action. It may be noted that although both peptide D (weak helix) and the full length ΔFd (strong helix) are hexacationic, peptide D targets only *E. coli* (MIC (*E. coli*): 5 μM; MIC (*S. aureus*): > 100 μM), whereas ΔFd acts against both *E. coli* and *S. aureus* (MIC (*E. coli*): 2.5 μM; MIC (*S. aureus*): 5 μM). This suggests that even though charge is essential for antimicrobial activity, it must be introduced in a conformational context for the peptide to become broad spectrum in action.

It may however be noted that in addition to differences in helicity, peptide D (4 ΔFs) and ΔFd (6 ΔFs) differ also in hydrophobicity because ΔFd has two additional ΔF residues over peptide D (Table 1). As the positive influence of hydrophobicity on antimicrobial activity is well documented,[5,25] it seems reasonable to suppose that the improved antibacterial activity of ΔFd, in comparison to the less hydrophobic peptide D, could be attributed, in part, to its increased hydrophobic character. Previously, we have dissected the role of helicity in ΔFd by constructing its less helical counterpart, D-Lys-ΔFd.[11] ΔFd and D-Lys-ΔFd are isomeric in sequence, charge, and hydrophobicity (Supporting Information Table 1) but differ only in their helicity.[11] In contrast to ΔFd {MIC (*E. coli*): 2.5 μM; MIC (*S. aureus*): 5 μM}, D-Lys-ΔFd showed a narrow spectrum of antibiotic action {MIC (*E. coli*): 5 μM; MIC (*S. aureus*): 55 μM}.[11] Thus, even though the helical ΔFd and the nonhelical D-Lys-ΔFd share the same charge and hydrophobicity, the reduced helicity of D-Lys-ΔFd is solely responsible for its narrow spectrum of antibiotic action. Therefore, in terms of spectrum of antibiotic action, D-Lys-ΔFd (which is nonhelical like peptide D), does not benefit from its increased hydrophobicity over peptide D. This suggests that the narrow spectrum of action and kill kinetics of peptide D result primarily from its reduced helicity and not hydrophobicity. Although in the context of our present study, the influence of hydrophobicity cannot be completely ruled out, our results suggest that helicity may exert overriding effects on hydrophobicity in influencing spectrum of antibiotic action. The inverse relationship between MIC and MRE (Figure 3), observed in our current study, confirms the strong requirement of helicity for the design of broad spectrum antimicrobial peptides. It is well-known that Gram positive and Gram negative bacterial membranes differ in composition due to presence or absence of components like lipopolysaccharide (in gram negative) or teichoic acids (in gram positive), which are also believed to act as receptors for antibacterial peptides.[26,27] Further studies are required to determine whether conformation of antimicrobial peptides influences their interaction with these receptors and if it is this interaction that defines their spectrum of action.

In addition to potency of killing, another important parameter of the performance of an antibiotic is the rate at which it kills the target bacterium. A faster killing rate of an antibiotic is expected to result in a faster clearance of bacterial load in a patient. Thus, in peptides of comparable antibiotic potencies, a faster acting antibiotic would be more desirable. While the physicochemical code governing antibiotic potency has been investigated extensively, the factors affecting the rate of bacterial killing have so far remained largely undefined. In this study, we have found that helicity has more prominent role than charge in imparting faster kill kinetics to a peptide. This conclusion is based on the observations that (a) peptides C (charge: +4) and D (charge: +6) with comparable helicities, albeit different charges, exhibit nearly similar kill kinetics and (b) both peptide D (weakly helical) and ΔFd (strongly helical) are hexacationic and yet the kill kinetics of peptide D is an order of magnitude slower than is the case with ΔFd (Figure 4). In addition, we have previously shown that against *E. coli*, the nonhelical D-Lys-ΔFd exhibits significantly poorer kill kinetics than ΔFd.[11] Taken together, the results suggest that helicity has a stronger influence on bacterial kill kinetics than charge and hydrophobicity. Figure 6B is a graphical representation of the combined influence of charge and helicity on kill kinetics. The graph shows that the identically charged peptides D and ΔFd (charge: +6), owing to their differing helicities, exhibit differing kill kinetics. Therefore, strong helicity in a peptide confers not only potent, broad spectrum antimicrobial activity but also makes it a rapidly killing antimicrobial agent.

Charge Determines Degree while Helicity Governs the Rate of Outer Membrane Permeabilization. The permeabilization of bacterial membranes by antibacterial peptides represents the first line of attack on the bacterial intruders causing disruption of transmembrane potentials crucial to survival of bacteria.[27] This membrane permeabilizing action of AMPs enables them to sensitize bacteria to nonpeptide antibiotics for which the LPS layer acts as a formidable barrier.[28] Thus, it is important to delineate the physicochemical parameters of a peptide that facilitate membrane permeabilization. We studied the degree and rate of outer membrane permeabilization of *E. coli* by measuring the uptake of the fluorescent dye NPN (Figure 5A). The results (Figure 6C) show the influence of charge and helicity on the maximum permeabilization (P_{max}). The graph shows that among the three peptides, peptide C (charge: +4, moderate helicity) showed slow, minimal permeabilization and peptides D (charge: +6, moderate helicity) and ΔFd (charge: +6, maximal helicity) both permeabilized the *E. coli* outer membrane maximally. The different helicities of peptides D and ΔFd did not influence the P_{max} achieved by these peptides (Figure 6C). Therefore, in contrast to the pivotal role of helicity in potency of an antimicrobial peptide (Figure 6A), it appears that charge plays a more important role than helicity in the degree of permeabilization achieved by such a peptide.

However, while peptides D and ΔFd exhibited the same P_{max}, the rate of attainment of P_{max} was three times faster for ΔFd than peptide D (Figure 5A). Figure 6D shows the influence of charge and helicity on the rate of bacterial outer membrane permeabilization. The graph clearly shows that rate of permeabilization is influenced by helicity and not charge. The hexacationic peptide D (less helical) and ΔFd (strongly helical) showed maximum permeabilization at 600 and 150 s, respectively. The slower rate of permeabilization by the less helical peptide D suggests the important role of helicity in enhancing the rate of membrane permeabilization. Interestingly, this trend of the rate of membrane permeabilization mirrors the rate of bacterial killing (Figure 6B). This suggests that a correlation exists between the helical content of an antimicrobial peptide, rate of membrane permeabilization, and the rate of bacterial killing. Friedrich et al. (1999), have shown that the highly helical CP29 exhibits better kill kinetics and membrane permeabilizing activities as compared its less helical analogue CP26 (both Cecropin−melittin (CEME) hybrid peptide analogues).[29] While individually, charge[30] and helicity[31] have been shown to influence membrane permeabilization, our findings specifically pinpoint that charge may be the determinant of P_{max}, while helicity may determine the rate of outer membrane permeabilization.

Inner Membrane Permeabilization May Not Be Essential for Antibacterial Activity. The requirements of inner membrane permeabilization were found to be more stringent than that of outer membrane permeabilization. This is substantiated by the fact that only the full length ΔFd permeabilized the inner membrane (Figure 5B). In contrast, peptides C and D were unable to permeabilize the inner membrane even after 18 h (Figure 5C). Antimicrobial peptides such as Buforin II, which have the ability to inhibit bacterial growth, also fail to permeabilize the bacterial inner membrane to PI.[24] Viewed in conjunction with the inability of the potent dimers C and D to permeabilize the inner membrane, this suggests that inner membrane permeabilization may not be a stringent requirement for antimicrobial activity. As peptide D (4 ΔFs, charge: +6) is much less helical and hydrophobic when compared to ΔFd (6 ΔFs, charge: +6), the selective acquisition of the ability to permeabilize the inner membrane by ΔFd can be intuitively attributed to: (a) hydrophobicity or (b) helicity. To find which of these parameters was the most relevant, we examined the inner membrane permeabilization properties of D-Lys-ΔFd. As shown in Supporting Information Figure 2, while the degree of inner membrane permeabilization achieved by ΔFd and D-Lys-ΔFd was identical, ΔFd (6 ΔFs) showed a distinct edge over the nonhelical D-Lys-ΔFd (6 ΔFs) in the rate of permeabilization. The results suggest that helicity exerts overriding effects on hydrophobicity to influence membrane permeabilization. Further, our data (Supporting Information Figure 2) suggests that while the extent of inner membrane permeabilization is influenced by hydrophobicity, the rate of inner membrane permeabilization is governed primarily by helicity.

The inner membrane permeabilization by ΔFd showed a lag period of ~150 s. This coincided with the time at which the outer membrane permeabilization by ΔFd shows a fall in intensity (Figure 5A). This sequential phenomenon seems to suggest that ΔFd may be migrating from the outer to the inner membrane at ~150 s and causing heightened permeabilization of the inner membrane (Figure 5A and B). However, the fact that ΔFd is most potent may suggest that peptides that permeabilize both the outer and inner bacterial membranes may be more effective antimicrobials than those that permeabilize the outer membrane alone. It is interesting to note that dimers C and D can potentially be used to selectively permeabilize the outer membrane without influencing the integrity of the inner membrane. In this way, they are expected to sensitize Gram negative bacteria and provide an adjuvant effect to the action of antibiotics like novobiocin and erythromycin.[21]

In summary, our results show that in a dimeric scaffold, a tetracationic character in a weak helical fold is sufficient to

elicit potent but narrow spectrum antibiotic action (*E. coli* (Minimum inhibitory concentration (MIC): 10 μM); *S. aureus* (MIC > 100 μM)). However, in the same dimeric scaffold, a hexacationic character embedded in a strong, amphipathic helix is necessary for potent and broad spectrum action (*E. coli* (MIC 2.5 μM) and *S. aureus* (MIC 5 μM)). We report that: (a) at identical helicity, incremental changes in charge enhance outer membrane permeabilization, (b) at identical charge, incremental changes in helicity enhance the rate of outer membrane permeabilization, initiate inner membrane permeabilization, enhance antibiotic potency, and kill kinetics and lead to broad spectrum of action across the Gram negative−Gram positive divide. Taken together, our results demonstrate how the interplay of charge and helicity regulates the activity of antibacterial peptides.

Conclusions

The term "antimicrobial activity" is not restricted to potencies alone but is a comprehensive term, encompassing kill kinetics, spectrum of action, and membrane perturbing effects. In the present study, we have observed that each of these parameters is governed by a fine balance of different physicochemical factors. The results suggest that the components of the physicochemical code, e.g. charge and helicity, governing antibacterial activity of AMPs, do not act alone but in concert to influence antibiotic action. This emphasizes the need to study these not just in part but also in unison with each other. Such studies are necessary to understand the interactions between these factors so that a more comprehensive design is achieved. These results can serve as guidelines to generate improved analogues of existing antimicrobial peptides or *de novo* design "tailor-made" templates which are more competent to fight life threatening bacterial infections.

Experimental Section

General. All solvents and reagents were used as received unless specified. Reverse phase high performance liquid chromatography (RPHPLC) peptide purification was performed on C18 PRC-ODS column (Shimadzu, 2 cm × 15 cm, 15 μm; detection: 214 and 280 nm). The purity of the final compounds was determined by RPHPLC (column: Novapak C18, 4 μm, 4.6 mm × 250 mm; flow rate: 1 mL/min; solvent system: water−acetonitrile, 0.1% TFA; gradient: 5% acetonitrile to 75% acetonitrile in 70 min (1% acetonitrile/minute); detection: 214 nm (Supporting Information Figure 1)). The peptides were found to have >95% purity. OD_{600} was measured in a microtiter plate reader (VERSA max tunable, Molecular Devices, Sunnyvale, CA). Fluorescence assays were performed on Perkin-Elmer LS 50 fluorimeter, and fluorescence microscopic analysis was done on Nikon Fluorescence microscope. Circular dichroism spectroscopic analysis was performed on Jasco J-810 spectropolarimeter.

Materials. Fmoc (Fluorenylmethoxycarbonyl) amino acid derivatives and Rink Amide methylbenzhydrylamine (MBHA) resin for peptide synthesis were from Nova Biochem; *N*,*N*′-diisopropylcarbodiimide (DIPCDI), piperidine, dimethylformamide (DMF), dichloromethane (DCM), *N*-hydroxybenzotriazole (HOBt), isobutylchloroformate (IBCF), trifluoroacetic acid (TFA), triisopropylsilane (TIS), D,L-threo-β-phenylserine, sodium hydroxide (NaOH), citric acid, glucose, HEPES, and bovine serum albumin (BSA) were from Sigma Chemical Company; *N*-methylmorpholine (NMM) and 1-*N*-phenyl-naphthylamine (NPN) were from Aldrich; sodium chloride, acetic anhydride and tetrahydrofuran (THF) were from Qualigens; acetic acid, ethyl acetate, sodium acetate, and sodium sulfate were from Merck, India; silica gel thin layer chromatography (TLC) plates (60F-254) were from Merck, Germany; Mueller Hinton (MH) broth and Bacto agar were from Difco; propidium iodide (PI) and Syto 9 were from Molecular Probes.

Preparation of Fmoc-X-DL-threo-β-phenylserine. Fmoc-X-DL-threo-β-phenylserine (X = Gly, Lys (Boc), Ala) were synthesized in a salt coupling using a mixed anhydride method. Fmoc amino acid (15 mmol) (dissolved in 15 mL of sodium refluxed and distilled THF) was activated at −15 °C for 10 min with IBCF and NMM (15 mmol each). A solution of 15 mmol of DL-threo-β-phenylserine made in 1 equiv of NaOH (15 mL) was added to the mixed anhydride, and the reaction mixture was stirred at room temperature overnight. Following evaporation of THF, citric acid was added to the aqueous solution to attain pH ∼ 2. The precipitate obtained was dissolved in 100 mL of ethyl acetate and transferred to a separating funnel. Following the removal of the lower aqueous layer, the ethyl acetate layer was washed extensively with water to remove citric acid up to its last traces while monitoring the pH of washings on a pH paper. The ethyl acetate layer was washed with brine and allowed to pass through a bed of anhydrous sodium sulfate. Evaporation of ethyl acetate on a rotary evaporator gave solid dipeptide acids.

Preparation of Fmoc-X-ΔPhe Azalactone. Fmoc-X-DL-threo-β-phenylserine was mixed with recrystallized anhydrous sodium acetate (obtained by fusing the salt and allowing it to cool in a desiccator) in freshly distilled acetic anhydride and stirred overnight. The thick slurry obtained was mixed with ice and stirred in a cold room. Following trituration, the yellow dipeptide azalactone was filtered on sinter funnel and dried to constant weight. The authenticity and purity of the azalactones was assessed by thin layer chromatography (TLC), mass spectroscopy, and UV−visible spectroscopy.

Peptide Synthesis. Peptides were synthesized as C terminal amides using standard Fmoc (fluorenylmethyloxycarbonyl) chemistry on Rink Amide MBHA (methylbenzhydrylamine) resin in the manual mode using DIPCDI/HOBt as coupling agents. Fmoc-Lys(Fmoc)-OH was used to make a branching core in the dimers. The side chain protections used were: 2,2,-5,7,8-pentamethylchroman-6-sulfonyl, Pmc (Arg), and *t*-butyloxycarbonyl, Boc (Lys, Trp). Couplings were done in DMF at 4-fold molar excess at concentrations ∼500 mM. Removal of Fmoc was by 20% piperidine in DMF. Both coupling of amino acid and Fmoc deprotection were monitored by Kaiser test.[32] ΔPhe was introduced into peptides as Fmoc-X-ΔPhe azalactone (X = Gly, Lys (Boc), Ala) dipeptide block,[20] which was allowed to couple overnight in DMF. At the completion of assembly of the peptides, their amino termini were acetylated using 20% acetic anhydride in DCM. After acetylation of peptides, the resin was washed extensively with DMF, DCM, and methanol and dried in a desiccator under vacuum. Peptides were cleaved by stirring the resin in a cleavage mixture (95% TFA, 2.5% water, and 2.5% TIS) for 2 h. The suspension was filtered using a sinter funnel, TFA was rotary evaporated, and peptide was precipitated in cold dry ether. Ether was filtered through a sinter funnel, and peptide on the funnel was dissolved in 5% acetic acid and lyophilized.

Peptide Purification and Mass Spectrometry. Crude peptide was purified by reverse phase high performance liquid chromatography (RPHPLC) using water−acetonitrile gradient (HPLC conditions: C18 PRC-ODS column (Shimadzu, 2 cm × 15 cm, 15 μm; flow rate: 5 mL/min); gradient: 5−75% ACN, 0.1% TFA in 70 min; detection at 214 and 280 nm). The identity of peptides was confirmed by electrospray ionization mass spectrometry at ICGEB, New Delhi (Supporting Information Figure 3).

Antibiotic Susceptibility Testing. The minimum inhibitory concentrations (MICs) were determined against *Escherichia coli* ML35p and the vancomycin/methicillin resistant *Staphylococcus aureus* ATCC 700699 according to the modified MIC method for cationic antimicrobial peptides.[33] Overnight grown bacterial cells were diluted in Mueller Hinton (MH) broth to a cell density of 10^5 CFU/mL. Then 100 μL of this culture was aliquoted into the wells

of a 96-wells flat bottom microtiter plate (Costar) and 11 μL of 10× stock of each peptide (in 0.2% BSA and 0.01% acetic acid) was added. This mixture was incubated at 37 °C in a rotary shaker incubator (Kuhner, Switzerland) set at 200 rpm. After 18 h of incubation, OD_{600} was measured in a microtiter plate reader. MIC is defined as the lowest concentration of a drug that inhibits measurable growth of an organism after overnight incubation. Peptide concentrations were determined spectrophotometrically at 280 nm (ε_{280}: 19000 $M^{-1}cm^{-1}$ for ΔF, 5050 $M^{-1}cm^{-1}$ for tryptophan). Each experiment was done in triplicate and was repeated at least twice. Standard error measurements (SEM) values have been calculated for end point OD_{600} (for each of the MIC values determined) from triplicates of two independent experiments. The OD_{600} values (±SEM) for *E. coli* were: ~0.7 ± 0.007 (untreated control), 0.011 ± 0.0048 (ΔFd), 0.009 ± 0.002 (peptide C), and 0.008 ± 0.0025 (peptide D). The OD_{600} values (±SEM) for *S. aureus* were: ~0.7 ± 0.006 (untreated control), 0.068 ± 0.003 (ΔFd).

Minimum bactericidal concentrations (MBCs) were determined in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI)[34] against *E. coli* ML35p and *S. aureus* ATCC 700699 by plating 100 μL from each clear well of the MIC experiment on MH Agar plates in triplicates. After incubation for 18 h, MBC was identified as the lowest concentration that did not permit growth of 99.9% bacteria on the agar surface. Standard error measurements (SEM) values have been calculated for end point CFU/mL (for each of the MBC values determined) from triplicates of two independent experiments. The CFU/mL values (±SEM) for *E. coli* were: ~10^5 ± 632 CFU/mL (untreated control, at the start of the experiment), 109 ± 3 CFU/mL (ΔFd), 115 ± 1 CFU/mL (peptide C), and 108 ± 3 CFU/mL (peptide D). The CFU/mL values (±SEM) for *S. aureus* were: ~10^5 ± 614 CFU/mL (untreated control, at the start of the experiment), 100 ± 1 CFU/mL (ΔFd).

Determination of Kill Kinetics. Overnight grown *E. coli* ML35p cells were diluted in MH broth to a cell density of 10^5 CFU/mL. One mL of this cell suspension was incubated with each peptide at 4X MIC (peptide C (40 μM), peptide D (20 μM), ΔFd (10 μM), or water (in case of control)) and incubated at 37 °C, 200 rpm in a rotary shaker incubator (Kuhner, Switzerland). At different time points, a 100 μL aliquot was withdrawn, diluted, and plated on MH agar plates. The plates were kept at 37 °C for 20 h and colonies counted.

Outer Membrane Permeabilization Assay. The outer membrane permeabilization activity of the peptides was determined by the NPN (*N*-phenylnapthylamine) assay.[22] Midlog phase *E. coli* ML35p cells were harvested (4000 rpm, 4 °C, 10 min), washed, and resuspended in 5 mM glucose/5 mM HEPES buffer pH 7.2. Then 10 μL of 250X concentration of peptides in water was added to a cuvette containing 2.5 mL of cells and 10 μM NPN (50 μL from a 500 μM stock in acetone). Excitation wavelength: 350 nm (slit width: 5 nm); emission wavelength: 420 nm (slit width: 10 nm). The uptake of NPN as a measure of outer membrane permeabilization was monitored by the increase in fluorescence of NPN for 10 min.

Inner Membrane Permeabilization. Midlog phase *E. coli* ML35p cells were harvested (4000 rpm, 4 °C, 10 min), washed, and resuspended in 5 mM glucose in 5 mM HEPES buffer pH 7.2. Then 10 μL of 250X concentration of peptides in water was added to a cuvette containing 2.5 mL of cells and 2.7 μM propidium iodide (PI). Excitation wavelength: 535 nm (slit width: 5 nm); emission wavelength: 617 nm (slit width: 10 nm). The uptake of PI as a measure of inner membrane permeabilization was measured by the increase in fluorescence of PI for 10 min.

PI and Syto 9 Uptake Based Fluorescence Microscopic Analysis of Cell Permeabilization by Peptides. Overnight grown *E. coli* ML35p cells were subcultured (OD_{600}: 0.35), harvested by spinning (4000 rpm, 10 min), washed, and resuspended in 5 mM HEPES pH 7.2 to get 10^8 cfu/mL. Then 15 μL of *E. coli* suspension was incubated (37 °C, 200 rpm) in 135 μL of 5 mM HEPES buffer pH 7.2 containing peptides at their MICs overnight. The samples were incubated with PI (2.7 μM) and Syto 9 (6 μM) for 15 min. A smear was made, heat fixed, and visualized under a fluorescence microscope. Cells without peptide served as control.

Circular Dichroism (CD) Spectroscopy. CD experiments were performed on a spectropolarimeter with a 1 mm path length cuvette. Spectra were acquired between 190 and 340 nm at 25 °C (scan speed 200 nm/min, response time 4 s, bandwidth 1 nm) in 20 mM SDS/10 mM sodium phosphate buffer pH 7.5. Five spectra were collected and averaged.

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Supporting Information Available: Physicochemical properties of ΔFd and D-Lys-ΔFd, reverse phase HPLC (RPHPLC) chromatograms, inner membrane permeabilization by peptide D, ΔFd and D-Lys-ΔFd, and electrospray ionization mass spectra of the peptides studied. This material is available free of charge via the Internet at http://pubs.acs.org.

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