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Group of Peptides That Act Synergistically with Hydrophobic Antibiotics against Gram-Negative Enteric Bacteria

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A synthetic peptide, KFFKFFKFF, consisting of cationic lysine residues and hydrophobic phenylalanine residues was found to sensitize gram-negative bacteria to hydrophobic and amphipathic antibiotics. At a concentration of 3 μg/ml, it decreased the MIC of rifampin for smooth, encapsulated *Escherichia coli* by a factor of 300. Other susceptible bacterial species included *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*, but *Pseudomonas aeruginosa* was resistant. Similar results were obtained with another synthetic peptide, IKFLKFLKFL. The fractional inhibitory concentration indices for the synergism of these peptides with rifampin, erythromycin, fusidic acid, and novobiocin were very close to those determined for the previously characterized potent outer-membrane-disorganizing agents polymyxin B nonapeptide and deacylpolymyxin B. KFFKFFKFF had direct activity against the gram-positive organism *Micrococcus* strain ML36, was strongly hemolytic, and was as active on polymyxin-resistant *E. coli* mutants as on their parent. These three attributes made KFFKFFKFF different from polymyxin derivatives and similar to cationic detergents, such as cetylpyridinium chloride. However, whereas the MIC of cetylpyridinium chloride for *E. coli* is low (0.5 to 4 μg/ml), that of KFFKFFKFF is much higher (30 to 100 μg/ml). Other groups of synthetic peptides studied included polymyxin-like peptides with an intrachain disulfide bridge. Their synergism with antibiotics was less marked. Still other peptides, including KEKEKEKEKE and KKKKKKFLFL, lacked any synergism with the probe antibiotics.

The bacterial outer membrane (OM) is an effective permeability barrier against many harmful agents including antibiotics (8). The major component of the outer layer of the OM is lipopolysaccharide (LPS), and because LPS is polyanionic, the LPS layer must be stabilized by divalent cation bridges. Certain polycations with strategically placed positive charges are able to compete with these divalent cations in a way that results in permeabilization of the OM (15). Polymyxin B nonapeptide has only a very weak direct antibacterial activity and deacylpolymyxin B has a modest one, but both make *Escherichia coli* up to 100 times more susceptible to lipophilic and amphiphilic agents such as rifampin, macrolide antibiotics, fusidic acid, and novobiocin (15).

Many other polycationic peptides, including the noncyclic derivatives of polymyxin peptides, lack both the direct antibacterial activity and the OM-permeabilizing activity (15). Still others, such as cecropin B and human defensins, resemble cationic detergents that lack any synergism with hydrophobic antibiotics but have direct antibacterial activity (15, 19). Finally, even though a large number of other small cationic bactericidal peptides of eukaryotic origin have been discovered during the past 15 years (2, 6), very little is known regarding their ability to permeabilize the OM at sub-MIC concentrations.

In this communication, we report the antibacterial and OM permeability-increasing properties of a series of novel synthetic cationic peptides. Some of them have recently been shown to bind to LPS and to inactivate its toxic effects (11, 13). Many of these bear structural resemblance to polymyxin and are in a cyclic conformation by virtue of a disulfide bridge. Others carry alternating basic and hydrophobic amino acid residues. We will show that some of these peptides are very effective permeabilizers of the OM but that their activity differs in many qualitative aspects from that of deacylpolymyxin B.

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MATERIALS AND METHODS

Synthetic peptides. Peptides were synthesized by using an automatic synthesizer (Milligen 9050; Millipore, Burlington, Mass.) and the method described in reference 13. They were purified by reversed-phase high-pressure liquid chromatography (HPLC) as described previously (13). Cysteine-containing peptides were cyclized by air oxidation with vigorous stirring as described in reference 13. The products were analyzed by HPLC, chemical analysis, and fast atom bombardment mass spectrometry.

Sources of other chemicals. Deacylpolymyxin B was prepared by using polymyxin acylase from *Pseudomonas* strain M-6-3 as previously described (20) and was a kind gift from Yukio Kimura (Mukogawa University, Nishinomiya, Japan). Sodium fusidate, erythromycin base, rifampin, novobiocin, vancomycin, tobramycin, norfloxacin, cloxacillin (Na salt), and cetylpyridinium chloride were from Sigma Chemical Co. (St. Louis, Mo.).

Bacterial strains. *E. coli* IH3080 (O18:K1) (21) was used as a representative of smooth, encapsulated *E. coli*. SC9252 and SC9253 are polymyxin-resistant *E. coli* K-12 mutants, and SC9251 is their parent (9, 18). The *E. coli* strain defective in lipid A biosynthesis and OM permeability barrier function was SM101 (*lpxA2*) and has previously been characterized in detail (16, 22). *Salmonella typhimurium* SH5014 (*rfaJ*, LPS chemotype Rb2) and *Pseudomonas aeruginosa* PAO1 were used in our previous permeabilization studies (17, 21). *Klebsiella pneumoniae* KY12854 and *Enterobacter cloacae* KY12645 are clinical isolates from bacteremic patients. Those strains, as well as *Micrococcus* strain ML36, are from our culture collection at the Department of Bacteriology, University of Helsinki, Helsinki, Finland.

Susceptibility determinations. MICs were determined in L broth as described previously (21). The inoculum was L-agar-grown cells, the inoculum size was 10^4 cells per ml, and the incubation was for 18 h at 28°C (SM101) or at 37°C (other strains). Growth was measured (A_{405}) with a Titertek Multiscan spectrometer (Labsystems, Helsinki, Finland). Uninoculated drug-containing media were used to blank the spectrophotometer. The lowest concentration of a drug which reduced the bacterial growth by ≥95% was interpreted as the MIC.

Assay for OM permeability-increasing activity. Antibiotic probes (rifampin, erythromycin, fusidic acid, and novobiocin) which penetrate very poorly the intact enterobacterial OM but which traverse the damaged OM were used. The

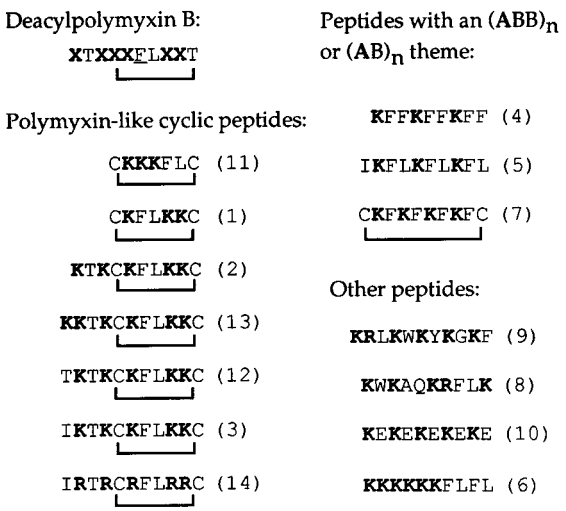

FIG. 1. Structures of deacylpolymyxin B and the synthetic peptides (1 through 14) studied. The basic amino acid residues are represented by bold letters. In the structure of deacylpolymyxin B, "X" refers to diaminobutyric acid and "F" refers to D-phenylalanine.

assay was performed as a synergistic growth inhibition assay by using checkerboard dilutions as described previously (19). Fractional inhibitory concentration (FIC) indices (1) were calculated as follows: $[(A)/MIC_A] + [(B)/MIC_B] = FIC_A + FIC_B = FIC$ index, where MIC_A and MIC_B are the MICs of drugs A and B, determined separately, and (A) and (B) are the MICs of drugs A and B when determined in combination. Drugs were interpreted to be synergistic when the FIC index was ≤0.5.

Assay for hemolytic activity. The hemolytic activity on human erythrocytes was assayed as described in reference 13.

RESULTS

Structural features of the designed peptides. The structures of the peptides studied are shown in Fig. 1. Nine of the peptides mimicked, to various degrees, the peptide portion of polymyxin B, and seven of them were in a cyclic conformation via a disulfide bridge. In addition, two linear peptides (KFFK FFKFF and IKFLKFLKFL) had an $(ABB)_n$ theme, where A is a basic amino acid residue and B is a hydrophobic one. Peptide CKFKFKFKFC had an $(AB)_n$ theme and was cyclic. Finally, one of the peptides contained a strongly cationic hexalysyl moiety linked to an apolar FLFL part, and another, $(KE)_5$, had intermittent basic and acidic residues.

Activity of the peptides against *E. coli*. All the peptides studied here lacked any potent direct activity against *E. coli*, but some had remarkable synergism with the hydrophobic and amphipathic antibiotics rifampin and erythromycin (Table 1). In this respect, the most effective peptides were KFFKFFKFF and IKFLKFLKFL. A concentration of IKFLKFLKFL as low as 1 μg/ml sensitized *E. coli* to rifampin by a factor of approximately 30. Also, CKFKFKFKFC and KRLKWKYKGKF as well as the cyclic polymyxin-like peptides KTKCKFLKKC and IRTRCRFLRRC had sensitizing activity, even though this activity was weaker than that of KFFKFFKFF and IKFLKFLK FL.

Detailed results obtained with KFFKFFKFF and KTKCK FLKKC are shown in Fig. 2A and B, respectively. A KFFK FFKFF concentration of 3 μg/ml decreased the MIC by a factor of 1,000. The highest concentration of KTKCKFLKKC tested, 100 μg/ml, achieved a 100-fold decrease in the MIC of rifampin.

FIC indices (1) can be calculated for agents that have direct antibacterial activity and can help in quantifying the degree of synergism. As can be seen from Table 2, the FIC indices for KFFKFFKFF and IKFLKFLKFL were very similar to those determined for deacylpolymyxin B, the reference peptide with a strong OM permeability-increasing property. Furthermore, the FIC indices show that both these peptides acted synergistically not only with rifampin and erythromycin but also with the other notably hydrophobic or amphipathic probe antibiotics studied (fusidic acid and novobiocin). KFFKFFKFF (10 μg/ml) decreased the MIC of fusidic acid by a factor of 300 (from 300 to 1 μg/ml) and decreased that of novobiocin by a factor of 30 (from 30 to 1 μg/ml). Both KFFKFFKFF and IKFLKFLKFL displayed a very slight degree of synergism with cloxacillin (FIC indices of 0.36 and 0.43, respectively) and no synergism with norfloxacin (0.6 and 1.0), tobramycin (0.63 and 1.0), or vancomycin (>0.63 and >0.63).

Activity of the peptides against other enteric bacteria and *P. aeruginosa*. The activity of the peptides against *K. pneumoniae*

TABLE 1. Synergism of the peptides with rifampin and erythromycin against *E. coli* IH3080

Peptide (no.)	MIC (μg/ml) at indicated peptide concn (μg/ml)											
	Rifampin						Erythromycin					
	0.3	1	3	10	30	100	0.3	1	3	10	30	100
None	10						30					
Deacylpolymyxin B	10	0.1	0.01	—[a]	—	—	10	3	1	—	—	—
KFFKFFKFF (4)	10	1	0.03	0.03	0.01	—	30	30	1	3	1	—
IKFLKFLKFL (5)	10	0.3	0.01	0.01	—	—	30	10	1	1	—	—
CKFKFKFKFC (7)	10	10	3	1	0.1	—	30	30	30	30	1	—
KKKKKKFLFL (6)	10	10	3	3	—	—	30	30	30	30	—	—
KRLKWKYKGKF (9)	3	1	1	0.3	0.1	0.01	30	30	30	10	1	1
KWKAQKRFLK (8)	10	10	3	1	1	0.3	30	30	30	30	10	10
CKKKFLC (11)	10	3	3	3	1	0.1	30	30	30	30	10	1
CKFLKKC (1)	10	10	10	3	0.3	0.1	30	30	30	30	30	1
KTKCKFLKKC (2)	10	3	3	1	0.3	0.1	30	30	30	10	3	1
KKTKCKFLKKC (13)	10	3	3	3	1	0.3	30	30	30	30	10	3
TKTKCKFLKKC (12)	10	10	10	3	1	1	30	30	30	30	10	3
IKTKCKFLKKC (3)	10	10	10	3	3	1	30	30	30	30	30	30
IRTRCRFLRRC (14)	3	3	3	1	1	0.1	30	30	30	10	1	1
KEKEKEKEKE (10)	10	10	10	10	10	10	30	30	30	30	30	30

[a] —, the peptide alone inhibited the growth.

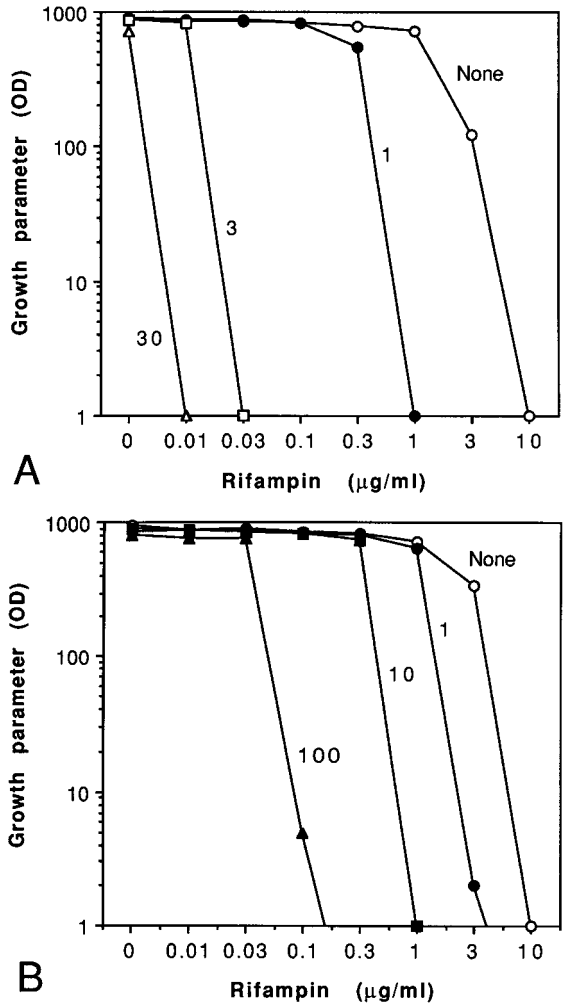

FIG. 2. Susceptibility of *E. coli* IH3080 to rifampin in the presence of increasing concentrations of KFFKFKFF (A) or the cyclic peptide KTKCK FLKKC (B), as measured in an 18-h growth inhibition assay. The peptide concentrations (micrograms per milliliter) are given beside each curve. OD, optical density.

and *E. cloacae* (Table 3) and *S. typhimurium* (data not shown) was reminiscent of that against *E. coli*. Accordingly, KFFKFF KFF and IKFLKFLKFL had strong synergism with rifampin. Unlike deacylpolymyxin B, none of the peptides exhibited any significant synergism with rifampin against *P. aeruginosa* (data not shown).

Activity of KFFKFFKFF against polymyxin-resistant strains.

TABLE 2. FIC indices[a] for the synergism of peptides 4 to 7 with hydrophobic antibiotics, as measured by using *E. coli* IH3080 as the target organism

Peptide (no.)	FIC index with:			
	Rifampin	Erythromycin	Fusidic acid	Novobiocin
Deacylpolymyxin B	0.03	0.13	0.04	0.04
KFFKFFKFF (4)	0.03	0.06	0.10	0.04
IKFLKFLKFL (5)	0.06	0.13	0.10	0.13
CKFKFKFKFC (7)	0.20	0.30	0.40	0.33
KKKKKKFLFL (6)	0.40	1.10	1.33	0.66

[a] See Materials and Methods for a definition of FIC index. FIC indices of ≤0.5 indicate synergism.

The polymyxin-resistant mutants of *E. coli* and *S. typhimurium* have an altered LPS structure and are resistant to the antibacterial and OM permeability-increasing action of many polycations, including polymyxin and polylysines (15). On the other hand, they are fully susceptible to cationic detergents (15, 19), certain cationic peptides such as cecropin B and tachyplesin (10, 19), and octapeptin, an antibiotic structurally very close to polymyxins but having a fatty acid tail two methylene units longer than that of polymyxins (7, 15). As shown in Table 4, KFFKFFKFF was as effective against the polymyxin-resistant SC9252 and SC9253 strains of *E. coli* as it was against their wild-type parent. In this respect, KFFKFFKFF clearly differed from deacylpolymyxin B, which was practically inactive against SC9252 and SC9253. As shown previously (19), the cationic detergent cetylpyridinium chloride had low MICs for both the polymyxin-resistant and -susceptible strains, but sublethal concentrations lacked any synergism with rifampin.

Other properties of KFFKFFKFF. The OM-defective *lpxA2* mutant SM101 of *E. coli* has a dramatic 30- to 100-fold increase in susceptibility to a variety of drugs against which the normal OM acts as an effective permeability barrier (16, 22). As shown in Table 4, the MIC of KFFKFFKFF for SM101 was 30 μg/ml and hence close or identical to that for wild-type smooth, encapsulated *E. coli* (IH3080) or *E. coli* K-12 (SC9251). Accordingly, the wild-type intact OM is not an effective barrier against KFFKFFKFF. We have previously shown that this is also a property of cationic detergents (19).

KFFKFFKFF was inhibitory to the gram-positive bacterium *Micrococcus* strain ML36 (Table 4). Also, cationic detergents are known to be active against both gram-negative and gram-positive bacteria. However, the MICs indicated that the activity of KFFKFFKFF was much weaker than that of cetylpyridinium chloride (Table 4). Deacylpolymyxin B was inactive against *Micrococcus* strain ML36.

Like cationic detergents, KFFKFFKFF possessed hemolytic activity. It caused hemolysis of human erythrocytes at a concentration of 40 μg/ml, while polymyxin was not hemolytic at 1,500 μg/ml.

DISCUSSION

We report here the discovery of a group of OM permeability-increasing agents. The cationic peptides KFFKFFKFF and IKFLKFLKFL were found to be almost as effective as deacylpolymyxin B and polymyxin B nonapeptide in their ability to sensitize enteric bacteria such as *E. coli*, *E. cloacae*, *K. pneumoniae*, and *S. typhimurium* to hydrophobic antibiotics. At a concentration as low as 3 μg/ml, the peptide KFFKFFKFF decreased the MIC of rifampin for smooth, encapsulated wild-type *E. coli* by a factor of 300. Very similar results were obtained by using IKFLKFLKFL. Further investigations revealed that KFFKFFKFF probably acts in a way very different from that characteristic of polymyxin derivatives. Unlike deacylpolymyxin B, KFFKFFKFF had direct activity against *Micrococcus* strain ML36, the gram-positive target organism studied, was strongly hemolytic, and was as active on polymyxin-resistant *E. coli* mutants as on their parent. These attributes make KFFKF FKFF resemble cationic detergents such as cetylpyridinium chloride. However, whereas cetylpyridinium chloride has a strong direct antibacterial action, manifested at concentrations as low as approximately 0.5 to 4 μg/ml, the MIC of KFFKFFK FF is high, approximately 30 to 100 μg/ml.

Even though cationic peptides and detergents disintegrate the OM, this is probably not their lethal target. After penetrating the OM, they most probably exert their direct antibacterial activity by damaging the cytoplasmic membrane (2, 6, 14,

TABLE 3. Synergism of the peptides with rifampin against *K. pneumoniae* and *E. cloacae*

Peptide (no.)	MIC (μg/ml) of rifampin at indicated peptide concn (μg/ml) for:							
	K. pneumoniae KY12854				*E. cloacae* KY12645			
	3	10	30	100	3	10	30	100
None	10				10			
Deacylpolymyxin B	0.3	0.1	NT[a]	NT	1	1	NT	NT
KFFKFFKFF (4)	0.3	0.1	0.03	0.01	0.1	0.1	0.03	0.01
IKFLKFLKFL (5)	0.3	0.03	0.01	—[b]	0.03	0.03	0.01	0.01
CKFKFKFKFC (7)	10	10	1	0.3	10	3	1	0.3
KKKKKKFLFL (6)	10	10	10	1	10	10	3	0.01
KRLKWKYKGKF (9)	10	10	1	1	3	3	1	1
KWKAQKRFLK (8)	10	10	10	3	10	10	3	1
CKKKFLC (11)	10	10	10	3	10	10	3	0.3
CKFLKKC (1)	10	10	10	1	10	10	10	0.3
KTKCKFLKKC (2)	10	10	3	1	10	10	3	1
KKTKCKFLKKC (13)	10	10	10	10	10	10	3	1
TKTKCKFLKKC (12)	10	10	10	10	10	10	3	1
IKTKCKFLKKC (3)	10	10	10	10	10	10	10	3
IRTRCRFLRRC (14)	10	10	3	1	10	10	1	1
KEKEKEKEKE (10)	10	10	10	10	10	10	10	10

[a] NT, not tested.
[b] —, the peptide alone inhibited the growth.

15). Therefore, the above-presented results suggest, as a working hypothesis, that KFFKFFKFF and IKFLKFLKFL have a potent OM-disorganizing action but a rather weak damaging action on the cytoplasmic membrane. This OM-disorganizing action most likely involves interaction of the peptide with the anionic and hydrophobic lipid A part of LPS. Evidence of the importance of the hydrophobic interaction comes from the finding that hydrophilic cationic peptides, such as Lys-5, possess only an extremely weak OM-disorganizing property (15).

Interestingly, an LPS-binding protein, the *Limulus* anti-LPS factor present in the hemolymph of *Limulus* crabs, possesses an amphipathic loop that consists of alternating cationic and hydrophobic residues (3, 4). Furthermore, the specific sequence of the *Limulus* anti-LPS factor interacting with lipid A has been identified with the aid of synthetic peptides within the LPS-binding loop; the interaction was found to rely on a structural $(AB)_n/(ABB)_n$ amphipathic motif (11, 12, 20). This motif is also found in another protein of *Limulus* crabs, the endotoxin-binding protein-protease inhibitor, as well as in LPS-binding mammalian proteins, such as the permeability-increasing protein of neutrophilic blood cells, the LPS-binding protein produced by hepatocytes and present in acute sera, and the LPS receptor protein CD14 (5, 20).

In our study, the cyclic peptide CKFKFKFKFC was significantly less active than KFFKFFKFF. It would be interesting to evaluate whether this is due to the cyclicity of the peptide or to the possibility that peptides with the $(ABB)_n$ motif are more suitable to interact with LPS embedded in the OM than are the peptides with the $(AB)_n$ motif.

Regarding the other group of synthetic peptides studied in this work, i.e., the polymyxin-like peptides with an intrachain disulfide bridge, we were able to show that they, too, had synergistic activity with hydrophobic antibiotics, even though this activity was clearly less potent than that of deacylpolymyxin and the $(ABB)_n$ peptides. While the affinity of KTKCK FLKKC for isolated LPS parallels that of polymyxin B (13), the concentration of this peptide required for effective synergism with hydrophobic antibiotics was approximately 30 times higher than that determined for deacylpolymyxin. However, the absence of significant toxicity of KTKCKFLKKC (13) makes this peptide potentially very valuable as an adjuvant for antibiotic therapy.

It can be anticipated that KFFKFFKFF and related peptides will find value as microbiological tools in various fields of basic and applied research. Many probes and drugs used in studies of bacterial physiology and biochemistry are hydrophobic and cannot penetrate the wild-type OM but can be expected to cross the OM damaged by KFFKFFKFF. In biotechnology, the bioconversion processes can greatly benefit from drugs that allow maximal entry of substrates inside the cell. Applications can also be found in diagnostic bacteriology, in which rapid identification procedures are based on enzyme assays of whole

TABLE 4. Activity of KFFKFFKFF, cetylpyridinium chloride, and deacylpolymyxin B against polymyxin-susceptible (SC9251) and -resistant (SC9252 and SC9253) strains and the *lpxA* mutant (SM101) of *E. coli*, as well as *Micrococcus* strain ML36

Compound	SC9251		SC9252 (PMR[a])		SC9253 (PMR)		MIC for SM101 (*lpxA*)	MIC for ML36
	MIC[b]	SC[c]	MIC	SC	MIC	SC		
KFFKFFKFF	30	1	30	1	30	1	30	10
Cetylpyridinium chloride	4	NS[d]	2	NS	2	NS	0.5	0.5
Deacylpolymyxin B	10	0.3	>100	10	>100	10	1	>100

[a] PMR, polymyxin resistant.
[b] Given in micrograms per milliliter.
[c] SC, synergistic concentration, i.e., the minimal concentration (in micrograms per milliliter) of the compound required to decrease the MIC of rifampin by a factor of ≥10. The MIC of rifampin in the absence of any compounds was 10 μg/ml for SC9251, SC9252, and SC9253.
[d] NS, no synergism found at peptide concentrations that lack direct antibacterial activity.

bacteria. Finally, the peptides described here might be valuable as therapeutic drugs that extend the narrow antibacterial spectrum of many hydrophobic and amphipathic antibiotics which are otherwise useful.

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