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Interaction of the Cyclic Antimicrobial Cationic Peptide Bactenecin with the Outer and Cytoplasmic Membrane*

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Bactenecin, a 12-amino acid cationic antimicrobial peptide from bovine neutrophils, has two cysteine residues, which form one disulfide bond, making it a cyclic molecule. To study the importance of the disulfide bond, a linear derivative Bac2S was made and the reduced form (linear bactenecin) was also included in this study. Circular dichroism spectroscopy showed that bactenecin existed as a type I β-turn structure regardless of its environment, while the reduced form and linear bactenecin adopted different conformations according to the lipophilicity of the environment. Bactenecin was more active against the Gram-negative wild type bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* than its linear derivative and reduced form, while all three peptides were equally active against the outer membrane barrier-defective mutants of the first two bacteria. Only the two linear peptides showed activity against the Gram-positive bacteria *Staphylococcus epidermidis* and *Enterococcus facaelis*. Bactenecin interacted well with the outer membrane and its higher affinity for *E. coli* UB1005 lipopolysaccharide and improved ability to permeabilize the outer membrane seemed to account for its better antimicrobial activity against Gram-negative bacteria. The interaction of bactenecin with the cytoplasmic membrane was determined by its ability to dissipate the membrane potential by using the fluorescence probe 3,3-dipropylthiacarbocyanine and an outer membrane barrier-defective mutant *E. coli* DC2. It was shown that the linear derivative and reduced form were able to dissipate the membrane potential at much lower concentrations than bactenecin despite the similar minimal inhibitory concentrations of all three against this barrier-defective mutant.

Polycationic antimicrobial peptides have been found in a variety of sources, including humans, mammals, plants, insects, and bacteria (1). The primary structures of these positively charged molecules are highly diverse, yet their secondary structures share the common feature of amphipathicity (2). α-Helical peptides, including cecropins (3) and β-sheet peptides, including defensins (4), have been studied extensively. It has been proposed (1, 2) that cationic peptides first interact with bacteria by binding to their negatively charged surfaces, and for Gram-negative bacteria they act as outer membrane permeabilizers. Their interactions with the cytoplasmic membrane have been proposed to lead to the disruption of membrane structure (5), resulting in dissipation of the transmembrane potential (6) and eventual cell death.

Recently, a few cationic peptides with only one disulfide bond forming a looped structure have been identified (7–11). One of them, bactenecin (also called dodecapeptide), was found in bovine neutrophils (12). It has 12 amino acids, including four arginine residues and two cysteine residues and is the smallest known cationic antimicrobial peptide. The two cysteine residues form a disulfide bond to make bactenecin a loop molecule. Bactenecin was previously found to be active against *Escherichia coli* and *Staphylococcus aureus*, and strongly cytotoxic for rat embryonic neurons, fetal rat astrocytes, and human glioblastoma (13). However, little is known about its antimicrobial mechanism and whether it shares the common killing mechanism of other antimicrobial peptides or if it has a distinct mode of action due to its unique compact structure (*cf.* the silk moth peptide cecropin, which is a 26-amino acid amphipathic α-helix). Its small size and only single disulfide bond also makes bactenecin an interesting candidate for research and drug development. The aim of this study was to investigate how bactenecin interacts with and kills microoganisms. Interestingly we found a rather distinct spectrum of activity for bactenecin compared with its linear form.

MATERIALS AND METHODS

Bacterial Strains and Chemicals—Bacterial strains for antimicrobial activity testing included *E. coli* UB1005 and its antibiotic supersusceptible derivative DC2 (14), *Pseudomonas aeruginosa* K799 and its antibiotic-supersusceptible derivative Z61 (15), *Salmonella typhimurium* 14028s (16), *S. aureus* ATCC25923, and clinical isolates of *Staphylococcus epidermidis* (clinical isolate), *Enterococcus faecalis* ATCC29212, and *Listeria monocytogenes* (food isolate).

Polymyxin B and 1-*N*-phenylnaphylamine (NPN)[1] were purchased from Sigma. 3,3-Dipropylthiacarbocyanine (DiS-C_3-(5)) was from Molecular Probes (Eugene, Oregon). Dansyl-polymyxin B was synthesized as described previously (18). The lipids 1-pamitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Northern Lipids Inc. (Vancouver, British Columbia, Canada).

Synthesis and Refolding of Bactenecin—Bactenecin and variants bac2S were synthesized by Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry by the Nucleic Acid/Protein Service unit at the University of British Columbia using an Applied Biosystems, Inc. (Foster City, CA) model 431 peptide synthesizer. The purchased bactenecin variants were in their fully reduced forms. After a series of trials to determine the optimal strategy, the disulfide bond was formed by air-oxidation in 0.01 M Tris buffer at room temperature for 24 h. The concentration of

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[1] The abbreviations used are: NPN, 1-*N*-phenylnaphylamine; DiS-C3-(5), 3,3-dipropylthiacarbocyanine; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; POPC, 1-pamitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; MALDI, matrix-assisted laser desorption/ionization; MIC(s), minimal inhibitory concentration(s); LPS, lipopolysaccharide.

TABLE I
Amino acid sequences of bactenecin and its derivatives

Peptide classification	Name	Amino acid sequence[a]	Size	Net charge
Native	Cyclic bactenecin	RLCRIVVIRVCR	12	+4
Linear	Lin Bac	RLCRIVVIRVCR	12	+4
	Lin Bac2S	RLSRIVVIRVSR	12	+4
	Lin Bac2S-NH_2	RLSRIVVIRVSR-NH_2	12	+5
	Lin BacR	RRLCRIVVIRVCRR	14	+6
Cyclic	BacR	RRLCRIVVIRVCRR	14	+6
	BacP	RRCPIVVIRVCR	12	+4
	BacP-NH_2	RRCPIVVIRVCR-NH_2	12	+5
	BacP3K	KKCPIVVIRVCK	12	+4

[a] One-letter amino acid code. Cysteine residues linked together with disulphide bonds are underlined

bactenecins was kept below 100 μg/ml in the oxidation buffer to minimize the formation of multimers. A reversed phase column Pep RPC HR5/5 (Amersham Pharmacia Biotech; Quebec, Canada) was used to purify the disulfide-bonded bactenecins from their multimer by-products. The column was equilibrated with 0.3% (v/v) aqueous trifluoroacetic acid and eluted with a gradient of acetonitrile in 0.3% trifluoroacetic acid at a flow rate of 0.7 ml/min. Peptide concentration was determined by amino acid analysis. Matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry (for native bactenecin only) and acid-urea polyacrylamide gel electrophoresis (19) were used to confirm that the disulfide bond was properly formed and a pure product obtained.

Circular Dichroism—A Jasco (Japan) J-720 spectropolarimeter was used to measure the circular dichroism spectra (20). The data were collected and analyzed by Jasco software. Liposomes POPC/POPG (7:3) were prepared by the freeze-thaw method to produce multilamellar vesicles as described previously (21), followed by extrusion through 0.1-μm double-stacked Nuclepore filters using an extruder device (Lipex Biomembranes, Vancouver, British Columbia, Canada), resulting in unilamellar liposomes. Peptide at a final concentration of 50 μM was added to 100 μM liposomes and incubated at room temperature for 10 min before the CD measurement.

Antimicrobial Activity—The minimal inhibitory concentration of peptides was determined by a modified 2-fold microtiter broth dilution method modified from that of Steinberg *et al.* (22). Using the classical method (23), higher concentrations of peptides tend to precipitate in the LB broth, thus the concentrations of peptides in the sequential wells are not accurate. Also the peptides stick to the most readily available (tissue-culture treated polystyrene) 96-well microtiter plates. Therefore the 2× series of dilutions was performed in Eppendoff tubes (polypropylene) before mixing with LB broth. Serial of 2-fold dilutions of peptides ranging from 640 to 1.25 μg/ml were made in 0.2% bovine serum albumin, 0.01% acetic acid buffer in the Eppendoff tubes. Ten μl of each concentration was added to each corresponding well of a 96-well microtiter plate (polypropylene cluster; Costar Corp., Cambridge, MA). Bacteria were grown overnight and diluted 10^{-5} into fresh LB broth or Todd Hewitt broth for *Streptococcus*. LB medium contained 10 g/liter tryptone and 5 g/liter yeast extract, with no salt. Todd Hewitt contained 500 g/liter beef heart infusion, 20 g/liter bacto-neopeptone, 2 g/liter bacto-dextrose, 2 g/liter sodium chloride, 0.4 g/liter disodium phosphate, 2.5 g/liter sodium carbonate. One-hundred μl of broth containing about 10^4–10^5 colony-forming units/ml of tested bacteria was added to each well. The plate was incubated at 37 °C overnight. The MIC was taken as the concentration at which greater than 90% of growth inhibition was observed.

Dansyl-Polymyxin B Displacement Assay—E. coli UB1005 LPS was prepared according to the phenol-chloroform-petroleum ether extraction method (24). The dansyl-polymyxin B displacement assay (25) was used to determine the relative binding affinity of peptides for LPS.

Membrane Permeabilization Assays—The ability of peptides to permeabilize the outer membrane was determined by the NPN assay of Loh *et al.* (26). Cytoplasmic membrane permeabilization was determined by using the membrane potential sensitive cyanine dye DiS-C_3-(5) (27). The mutant *E. coli* DC2 with increased outer membrane permeability was used so that DiS-C_3-(5) could reach the cytoplasmic membrane. Fresh LB medium was inoculated with an overnight culture, grown at 37 °C, and mid-logarithmic phase cells (A_{600} = 0.5–0.6) were collected. The cells were washed with buffer (5 mM HEPES, pH 7.2, 5 mM glucose) once, then resuspended in the same buffer to an A_{600} of 0.05. The cell suspension was incubated with 0.4 μM DiS-C_3-(5) until DiS-C_3-(5) uptake was maximal (as indicated by a stable reduction in fluorescence due to fluorescence quenching as the dye became concentrated in the cell by the membrane potential), and 100 mM KCl was added to equilibrate the cytoplasmic and external K^+ concentration. One ml of cell culture was placed in a 1-cm cuvette, and the desired concentration of tested peptide was added. The fluorescence reading was monitored by using a Perkin-Elmer model 650–10S fluorescence spectrophotometer (Perkin-Elmer Corp.), with an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The maximal increase of fluorescence due to the disruption of the cytoplasmic membrane by certain concentration of cationic peptide was recorded. A blank with only cells and the dye was used to subtract the background. Control experiments[2] titrating with valinomycin and K^+ showed that the increase in fluorescence was directly proportional to the membrane potential and that a buffer concentration of 100 mM KCl prevented any effects of the high internal K^+ concentration and corresponding opposing chemical gradient.

RESULTS

Bactenecin and Its Linear Derivative—The amino acid sequence of bactenecin and its linear derivative are shown in Table I. The linear derivative (Lin-Bac2S) with two cysteine residues replaced by two serine residues, was made to determine the importance of the disulfide bond in bactenecin's antimicrobial activity. The reduced form of bactenecin was also included in this study as a linear version of bactenecin. The identity of these peptides was confirmed by MALDI mass spectrometry. The MALDI data showed the molecular mass of the reduced bactenecin as 1486 ± 1 dalton and oxidized bactenecin as 1484 ± 1 dalton, in agreement with formation of one disulfide bond in the latter. Linear reduced bactenecin did not reform its disulfide bonds spontaneously within the lifetime of these experiments, as confirmed by its gel electrophoretic mobility (which was altered by disulfide bond formation).

Circular Dichroism—CD spectrometry (Fig. 1*A*) showed that linear, reduced bactenecin and linear Bac2S were present in 10 mM sodium phosphate buffer as unordered structures, which had a strong negative ellipticity near 200 nm. The CD spectrum of native bactenecin (Fig. 1*A*) demonstrated a negative ellipticity near 205 nm, typical of that seen for a type I β-turn structure (29) and resembling oxyribonuclease and nuclease, which are short polypeptides with a disulfide bond (28). In 60% TFE buffer, in the presence of liposomes and 10 mM SDS, the native bactenecin retained a similar structure (Fig. 1, *B–D*). However, the reduced form and Lin-Bac2S exhibited clearly distinct structures from those observed in the aqueous solution. In 60% trifluoroethanol (considered a helix-inducing solvent), these two peptides tended to form an α-helical structure (Fig. 1*B*), whereas and in the presence of liposomes or 10 mM SDS (a membrane-mimicking detergent), a β-sheet structure was evident (Fig. 1, *C* and *D*).

Antimicrobial Activity—The MIC of bactenecin and its derivatives against a range of bacteria was determined (Table II) by using a modified broth dilution method. Bactenecin was active against all Gram-negative bacteria tested. It was relatively inactive (MIC = 64 μg/ml) against the Gram-positive bacte-

[2] M. Wu, E. Maier, R. Benz, and R. E. W. Hancock, submitted for publication.

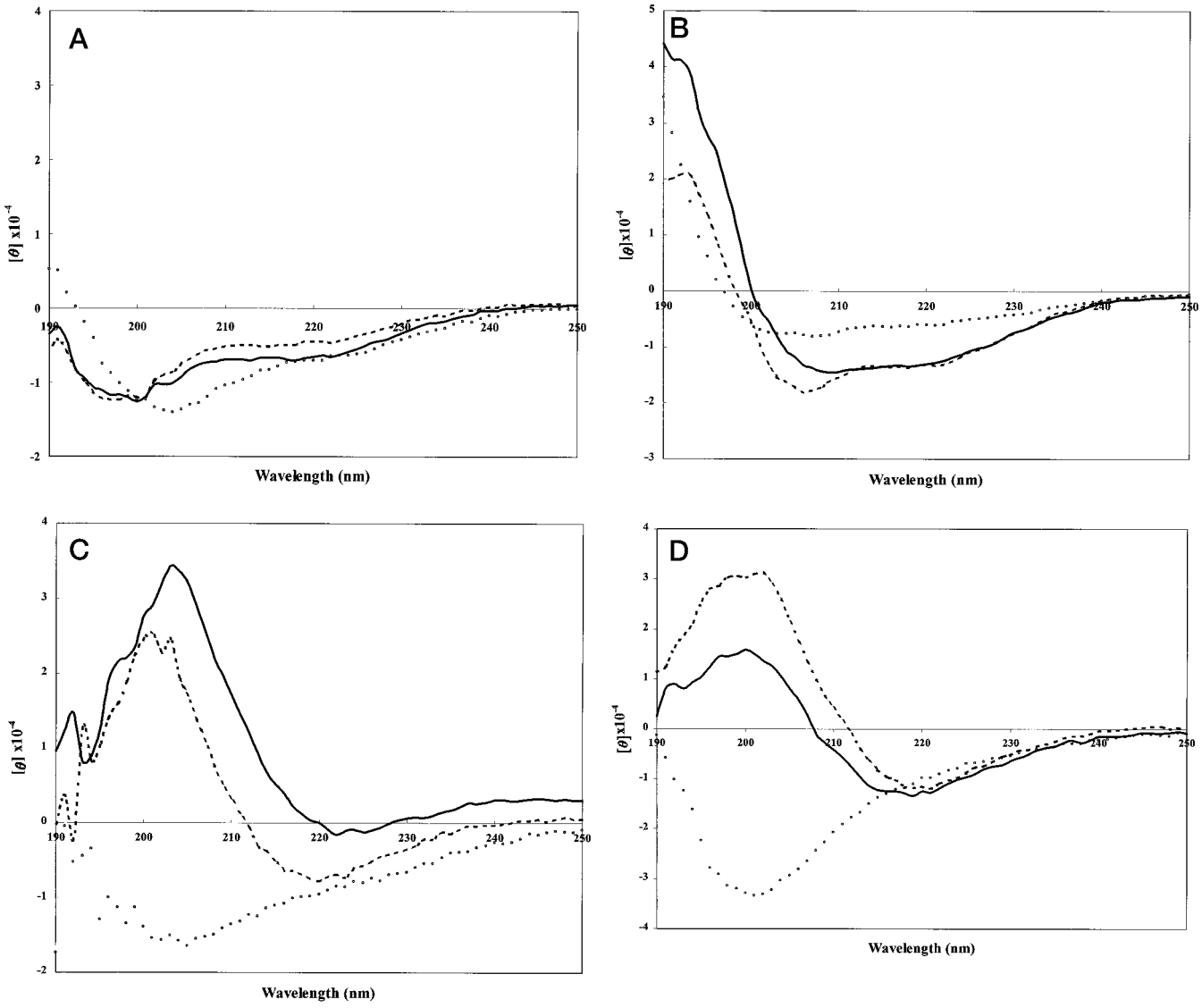

FIG. 1. **CD spectra of bactenecin, its linear (reduced) form Lin Bac and a linear variant Lin bac2S in media of various lipophilicity.** The concentrations of peptides and liposomes were 50 and 100 μM, respectively. CD measurements were taken in 10 mM sodium phosphate buffer (pH 7.0) in the absence (*A*) and the presence (*B*) of POPC/POPG. *C* shows the spectra in the presence of 60% (v/v) TFE, and D shows the spectra in the presence of 10 mM SDS. *Open circles*, bactenecin; *solid line*, reduced bactenecin; *dashed line*, Lin-Bac2S.

TABLE II

Differential activity of native cyclic and linear bactenecins against Gram-negative bacteria, outer membrane-altered, antibiotic supersusceptible mutants DC2 and Z61, and selected Gram-positive bacteria

Species and strains	Relevant phenotype	MIC		
		Bactenecin (oxidized)	Lin Bac	Lin Bac2S
			$\mu g/ml$	
E. coli UB1005	Parent of DC2	8	>64	32
E. coli DC2	Antibiotic-sensitive	2	2	2
P. aeruginosa K799	Parent of Z61	4	>64	>64
P. aeruginosa Z61	Antibiotic-sensitive	0.5	0.5	0.5
S. typhimurium	Wild type	8	>64	>64
S. epidermidis	Clinical isolate	>64	8	8
E. faecalis	Wild type	>64	8	8

rium *S. aureus*, in contrast to a previous report (12). The linear variant Lin-Bac2S and reduced bactenecin (Lin-Bac) were inactive against wild type Gram-negative bacteria. *P. aeruginosa* Z61 and *E. coli* DC2 are outer membrane barrier-defective mutants, that have more permeable outer membranes than their parent strains, allowing potentially easier access of the peptides to the cytoplasmic membrane. All three bactenecins exhibited equivalent activity against these two mutants. Linearization by reduction or changing cysteine to serines dramatically changed the antimicrobial activity for two Gram-positive species *S. epidermidis* and *Enterococcus facaelis*. For other antimicrobial peptides with disulfide bonds, reduction of these disulfides generally results in complete loss of antimicrobial activity (30–32). In contrast, accompanying the linearization was a shift in spectrum of activity from Gram-negative selective to Gram-positive selective, which corresponded to the sub-

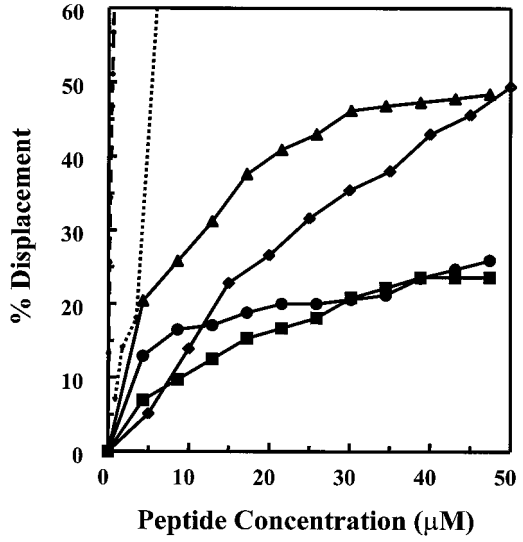

FIG. 2. **Binding of peptides to LPS as assessed by their ability to displace dansyl-polymyxin B from *E. coli* UB1005 LPS.** Dansyl-polymyxin B was added to 1 ml of 3 μg/ml LPS to a final concentration of 1 μM, which saturated the binding sites on LPS, and the fluorescence sensitivity was adjusted to 90%. The peptides and Mg^{2+} were titrated in, resulting in a decrease in fluorescence due to the competitive displacement of dansyl-polymyxin from the LPS, resulting in a reduction in fluorescence. Symbols: *triangles*, cyclic bactenecin; *squares*, Lin-Bac2S; *closed circles*, linear (reduced) bactenecin (Lin Bac), *dashed line*, polymyxin B; *dotted line*, indolicidin (from Ref. 20); *diamonds*, $MgCl_2$.

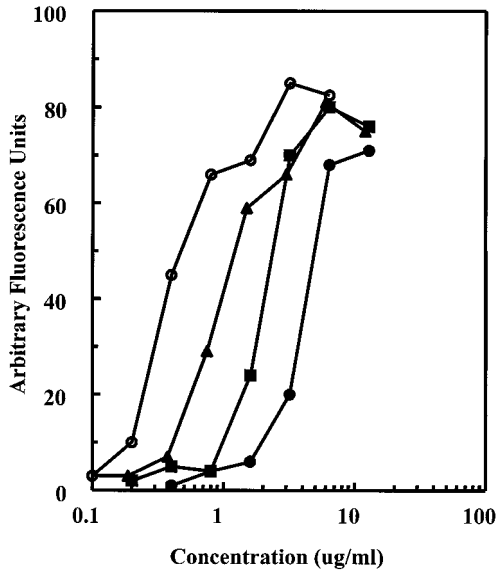

FIG. 3. **Peptide-induced outer membrane permeabilization assessed by the NPN uptake in *E. coli* UB1005.** Mid-log phase *E. coli* cells were collected and incubated with NPN in the presence of various concentrations of native cyclic bactenecin (oxidized), linear reduced bactenecin (Lin Bac), and Lin Bac2S. NPN was taken up into cells when the outer membrane was disrupted by the peptides. The uptake of NPN was measured by the increase of fluorescence. Symbols: *triangles*, cyclic bactenecin; *squares*, Lin-Bac2S; *closed circles*, linear (reduced) bactenecin; *open circles*, polymyxin B.

stantially different structures adopted in liposomes.

The Binding of Bactenecins to Purified E. coli UB1005—The MIC results indicated that the interaction with the outer membrane might be critical in the explaining the difference in antimicrobial activity against Gram-negative bacteria among three bactenecin forms. The first step of cationic peptide antimicrobial action has been shown to involve the binding of the cationic peptide to the negatively charged surface of the target cells (1). In Gram-negative bacteria, this initial interaction occurs between the cationic peptides and the negatively charged LPS in the outer membrane (20, 33, 34). Such binding can be quantified using the dansyl-polymyxin B displacement assay. Dansyl-polymyxin B is a fluorescently tagged cationic lipopeptide, which is nonfluorescent in free solution, but fluoresces strongly when it binds to LPS. When the peptides bind to LPS, they displace dansyl-polymyxin B, resulting in decreased fluorescence, which can be assessed as a function of peptide concentration (Fig. 2). Bactenecin was a relatively weak LPS binder compared with polymyxin B and similar to the peptide indolicidin (13 amino acids with a net charge of +2; Ref. 20), but it was still better than Mg^{2+}, the native divalent cation associated with LPS. Most importantly, it seemed that native cyclic bactenecin bound to LPS far better than its linear derivatives, which partially explained the difference in activities against Gram-negative bacteria.

Effect on Outer Membrane Permeability—Antimicrobial peptides bind to LPS, displacing the native divalent cations. Due to their bulky nature they disrupt the outer membrane and self-promote their own uptake across the outer membrane (33, 34). In order to determine whether better binding ability resulted in better outer membrane permeabilization, a NPN assay was performed. NPN is a neutral hydrophobic probe that is excluded by an intact outer membrane, but is taken up into the membrane interior of an outer membrane that is disrupted by antimicrobial peptide action. NPN fluoresces weakly in free solution but strongly when it enters the membrane. Fig. 3 showed that polymyxin B permeabilized the outer membrane to a 50% of maximal increase in fluorescence arbitrary units at 0.4 μg/ml, while bactenecin, Lin-Bac2S, and linear bactenecin caused half-maximal permeabilization at 0.8, 2, and 4.5 μg/ml, respectively. Bactenecin was thus better than the linearized derivatives at permeabilizing the outer membrane of *E. coli* UB1005.

Effect on the Inner Membrane Potential Gradient—It has been proposed that the antibacterial target of cationic peptides is at the cytoplasmic membrane. Cationic peptides are generally able to interact electrostatically with the negatively charged headgroups of bacterial phospholipids and then insert into the cytoplasmic membrane, forming channels or pores that are proposed to lead to the leakage of cell contents and cell death. However there is very little data for peptides pertaining to measurement of the disruption of the cytoplasmic membrane permeability barrier, despite ample evidence that membrane disruption can occur in model membrane systems (35). Although, some authors have utilized measurements of the accessibility of a normally membrane-impermeable substrate to cytoplasmic β-galactosidase, this assay suffers from using a bulky substrate (ortho-nitrophenyl galactoside) (36, 37). To circumvent this, we have developed an assay involving the membrane potential-sensitive dye diS-C_3-(5) to measure the disruption of electrical potential gradients in intact bacteria. The use of the *E. coli* mutant DC2 permitted us to perform this assay in the absence of EDTA (required by previous workers who have used similar assays in *E. coli* (38, 39)). The fluorescent probe diS-C_3-(5), which is a caged cation, distributes between cells and medium depending on the cytoplasmic membrane potential. Once it is inside the cells, it becomes concentrated and self-quenches its own fluorescence. If peptides form channels or otherwise disrupt the membrane, the membrane potential will be dissipated, and the DiS-C_3-(5) will be released into the medium causing the fluorescence to increase, as can be detected by fluorescent spectrometry. In these assays, 0.1 M KCl was added to the buffer to balance the chemical potential of K^+ inside and outside the cells. Therefore the MICs of bactenecin, reduced bactenecin, and bac2S in the

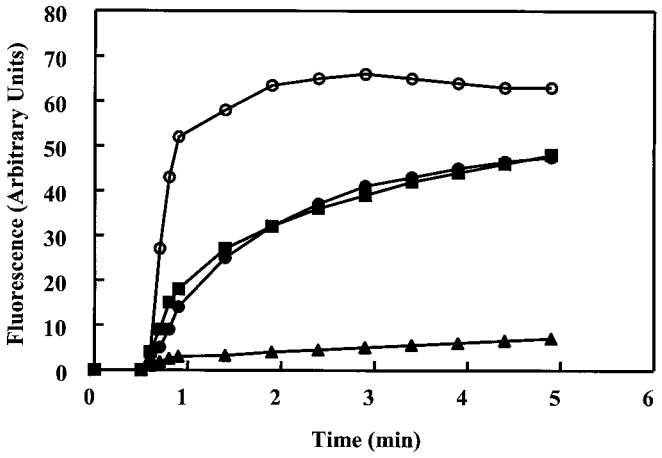

FIG. 4. **Peptide-induced inner membrane permeabilization assessed by the diS-C_3-(5) assay.** Mid-log phase cells were collected and resuspended in buffer (5 mM HEPES, 5 mM glucose) to an A_{600} of 0.05. A 0.4 μM final concentration of diS-C_3-(5) was incubated with cell suspensions until no more quenching was detected, then 0.1 M KCl was added. The desired peptide concentration (8 μg/ml for the bactenecins and 1 μg/ml for CEMA) was added to a 1-cm cuvette containing 1 ml of cell suspension. The fluorescence change (in arbitrary units) was observed as a function of time. Symbols: *triangles*, cyclic bactenecin; *squares*, Lin-Bac2S; *closed circles*, linear (reduced) bactenecin; *open circles*, CEMA.

presence of 0.1 M KCl were determined and shown to be 8–16 μg/ml (*i.e.* 4–8-fold higher than in low salt). Despite these similar MICs for the three peptides *versus E. coli* DC2, the influence of these peptides on the membrane potential was quite different (Fig. 4). The linear bactenecins at around their MIC (8 μg/ml) caused a rapid increase in fluorescence that was similar to that seen for a control α-helical peptide CEMA at its MIC of 1 μg/ml. However despite a similar 30-s delay prior to initiation, the kinetics were somewhat slower with CEMA causing a maximal depolarization of the cytoplasmic membrane (increase in fluorescence) within 2 min, whereas the linear bactenecins caused only 50% maximal depolarization of the cytoplasmic membrane in this period of time. In stark contrast to both the linear bactenecins and CEMA, native cyclic bactenecin at 8 μg/ml caused a very modest depolarization within the first 5 min (14% of that observed with reduced bactenecin, reaching a maximum of 30% in 1 h).

Structure-Activity Relationships—A series of peptides related to bactenecin were made (Table I) in an attempt to decipher important features of these peptides contributing to antimicrobial activity. Included in this series were peptides that differed in charge due either to amidation of the carboxyl terminus (Lin-Bac2S-NH_2 and BacP-NH_2) or addition of arginines to the NH_2 and COOH terminus (Lin-BacR and cyclic BacR), contained an added proline residue in the bactenecin ring to promote cyclization (BacP), or contained a substitution of three lysines for arginines (BacP3K). In total, four linear peptides (denoted Lin-Bac for clarity) and five cyclic peptides were investigated. Antimicrobial activity was assessed for the bacteria studied above in addition to two Gram-positive pathogens, *S. aureus* ATCC25923 and a food isolate of *L. monocytogenes* (Table III). The latter Gram-positive bacterium was reasonably susceptible to cyclic bactenecin; however, the linear bactenecin and Lin-Bac2S were 8-fold more active (but not particularly active against *S. aureus*).

Among the linear peptides, an increase in positive charge tended to result in increased activity against Gram-negative bacteria for both Lin-Bac2S-NH_2 (+4) and Lin-BacR (+5). However neither of these peptides had activities (except against *E. coli*) equivalent to that of cyclic bactenecin. The increase in positive charge of the peptides also tended to result in an increase in activity against the Gram-positive bacteria (*cf.* BacR *versus* Bac, Lin-Bac2S-NH_2 *versus* Lin-Bac2S, BacP-NH_2 *versus* BacP). Clearly amidation of the carboxyl terminus was very favorable to antimicrobial activity against both Gram-negative and Gram-positive bacteria. Overall, good Gram-positive activity tended to require the peptides to be linear, although the cyclic peptide BacP-NH_2 had reasonable activities against the Gram-positive bacteria *S. aureus* and *L. monocytogenes*.

The substitution of an arginine with a proline residue in the ring structure in BacP (and moving the arginine in place of the leucine residue at position 2) resulted in a loss of activity against all bacteria except *E. coli*. This indicated that the three-dimensional structure of the peptide was important, since the net charge was identical to that of bactenecin, the overall hydrophobicity very similar, and the substitution of Arg for Leu in position 2 was not detrimental in BacR. Interestingly, the further substitution of three arginines for three lysine residues in BacP3K resulted in a very weakly active peptide, suggesting that these two basic residues may not be equally effective in promoting bactenecin activity.

DISCUSSION

The α-helical and β-structured classes are two groups of antimicrobial polycationic peptides that have been well studied. Although their precise antimicrobial mechanism is somewhat unclear, it has been proposed that the outer and the cytoplasmic membranes of Gram-negative bacteria are their primary and final targets respectively (1). They have been proposed to kill bacteria by first electrostatically interacting with the surface of the bacterial cytoplasmic membrane (after self-promoted uptake across the outer membrane for Gram-negative bacteria). Then under the influence of a membrane potential, they are proposed to insert into the membrane and form channels to leak internal constituents. However much of this mechanism is based on data from model membrane studies.

Bactenecin belongs to a group of cationic peptides with only one disulfide bond. In this study, it was shown that bactenecin was active against the wild type Gram-negative bacteria *E. coli*, *P. aeruginosa*, and *S. typhimurium*, whereas the linear derivative and reduced form were virtually inactive against these bacteria but had gained activity against certain Gram-positive bacteria. For other disulfide-bonded peptides such as the β-sheet defensins (30), the protegrins (31), and the tachyplesins (32), the loss of ability to form a disulfide bond results in a complete loss of structure and activity. Thus the observation that bactenecin, when linearized, undergoes a dramatic shift in activity spectrum (Table II) and in structure (Fig. 2) is unprecedented and surprising.

Furthermore, cyclic bactenecin behaved in a fundamentally different fashion to the linear bactenecins and the α-helical 28-amino acid peptide CEMA (34), with respect to cytoplasmic membrane permeabilization (depolarization of the membrane potential gradient). Previous studies used artificial liposomes to study the interaction of antimicrobial peptides with membranes. In this study, live cells of *E. coli* DC2, an outer membrane hyperpermeable mutant, were used in conjunction with a fluorescent dye, diS-C_3-(5), which was released from cells when the membrane potential is disrupted, leading to fluorescence dequenching. Despite their equivalent MIC value against *E. coli* DC2, the pattern of interaction of bactenecin and its linear variants with the cytoplasmic membrane was quite different. Whereas CEMA and linear bactenecin and Lin-Bac2S caused rapid depolarization of the membrane, cyclic bactenecin caused only a slow and minor change in membrane potential. Thus we

TABLE III
Structure-activity relationships amongst cyclic and linear bactenecins

Bacteria	MIC								
	Bac	Lin-Bac	Lin-Bac2S	Lin-Bac2S-NH_2	Lin-BacR	BacR	BacP	BacP-NH_2	BacP3K
	$\mu g/ml$								
E. coli	8	>64	32	2	4	2	4	2	16
P. aeruginosa	4	>64	>64	16	16	4	>32	16	>64
S. typhimurium	8	>64	>64	32	32	4	16	16	32
S. aureus	64	>64	16	4	>64	64	32	4	>64
S. epidermidis	>64	8	8	1	4	8	>32	32	>64
E. facaelis	>64	8	8	4	4	32	>32	>64	>64
L. monocytogenes	8	1	1	0.25	0.5	0.125	16	1	16

conclude that cyclic bactenecin kills cells in a completely different way to the other antimicrobial peptides, which have been proposed to act on the cytoplasmic membrane of bacteria. Although the actual mechanism of killing was not investigated in this study, we propose that bactenecin is able to cross the cytoplasmic membrane of Gram-negative bacteria and act on a target inside cells (*e.g.* negatively charged nucleic acids).

In contrast, it would appear that the linear bactenecins are acting in the same way on the cytoplasmic membrane of bacteria as do other larger peptides. The linear variants dissipated the cytoplasmic membrane potential at the MIC and showed partial activity on membranes (data not shown), even at concentrations as low as 0.125 μg/ml (less than 1% of the MIC). However, while it is straightforward to imagine how 28-mer peptides like CEMA might be able to span a biological membrane to form a channel by a barrel-stave mechanism (1, 5, 6), it is not so simple to understand how a 12-mer peptide containing 50% polar residues could span such a membrane.

Both the cyclic and linear versions of bactenecin, as well as Bac2S, were equally active against outer membrane permeability defective mutants of *E. coli* and *P. aeruginosa*. This observation indicated that the disulfide bond was important for interaction with the outer membrane as confirmed here. Bactenecin had a better binding ability for LPS and also permeabilized the outer membrane better, explaining its better activity *versus* wild type Gram-negative bacteria. Computer modeling of bactenecin with InsightII software (Biosym Technologies Inc., San Diego, CA) indicated that bactenecin was a loop molecule with a hydrophobic ring and a positively charged face constructed from the COOH- and NH_2-terminal portions of the molecule (2). Such a conformation, which was consistent with the CD spectral studies (Fig. 2) which indicated that bactenecin existed as a rigid β-turn loop molecule regardless of its environment, may make bactenecin a more amphipathic molecule than the unstructured linear and reduced forms, which exist in solution as random structures. This could explain why bactenecin interacted better with the negatively charged LPS than its linear and reduced form. It is also worth mentioning that bactenecin would also be too small to span the membrane and form pores or channels unless a multimer is involved.

The original report of the isolation of bactenecin suggested it was active against both *E. coli* and *S. aureus* (12), whereas we demonstrated that cyclic bactenecin has very little activity against the latter bacterium. Therefore, we are tempted to speculate that Romeo *et al.* (12) were working with a mixture of linear and cyclic bactenecin or that their preparations were partly or completely amidated (since amidation of two of our peptides improved activity against *S. aureus* by 4–8-fold). Unfortunately, despite two attempts to synthesize amidated bactenecin, we were unable to obtain a preparation sufficiently pure enough to permit identification of the desired product.

Our studies of structure activity relationships revealed certain factors that were important in the activity of the linear and cyclic bactenecins against bacteria. The most obvious correlations observed were the improvement in activity against Gram-negative bacteria with cyclization (due to disulfide bond formation) and with increased positive charge. In addition while cyclization tended to decrease activity against Gram-positive bacteria, while increasing the positive charge by addition of two arginines or by amidation of the COOH-terminal carboxyl, led to an improvement in activity against Gram-positive bacteria. Despite the small size of these peptides, we observed MICs against important bacterial pathogens that are equal to or better than much larger peptides, and we suggest that these peptides offer a potentially fruitful basis for isolation of antibiotic peptides for clinical use.

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