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Influence of preformed α-helix and α-helix induction on the activity of cationic antimicrobial peptides

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Abstract: One prominent class of cationic antibacterial peptides comprises the α-helical class, which is unstructured in free solution but folds into an amphipathic α-helix upon insertion into the membranes of target cells. To investigate the importance of α-helicity and its induction on interaction with membranes, a series of peptides was constructed based on a hybrid of moth cecropin (amino acids 1–8) and bee melittin (amino acids 1–18) peptides. The new peptides were predicted to have a high tendency to form α-helices or to have preformed α-helices by virtue of construction of a lactam bridge between glutamate and lysine side-chains at positions *i* and *i* + 4 at various locations along the primary sequence. In two examples where the use of lactam bridge constraints induced and stabilized α-helical structure in benign (aqueous buffer) and/or hydrophobic medium, there was a decrease in antibacterial activity relative to the linear counterparts. Thus the preformation of α-helix in solution was not necessarily beneficial to antimicrobial activity. In the one case where the lactam bridge did result in increased antibacterial activity (lower minimal inhibitory concentration values) it did not increase α-helical content in benign or hydrophobic medium. Broadly speaking, good activity of the peptides against *Pseudomonas aeruginosa* correlated best (r^2 = 0.88) with a helican parameter which was calculated as the induction of α-helix in a membrane-mimicking environment divided by the α-helix formation under benign conditions. Interestingly, the activity of the lactam bridge peptide constructs correlated in part with alterations in bacterial outer or cytoplasmic membrane permeability.

Abbreviations: HOBt, N-hydroxybenzotriazole; HBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMM, N-Methylmorpholine; NMP, 1-Methyl-2-pyrrolidinone; OFm, 9-Fluoroenylmethyl.

Cationic antimicrobial peptides are ubiquitous in nature, forming part of the defenses against invading microorganisms of many species of life (1, 2). They fall into four broad structural classes, the most common of which is the so-called α-helical class. These peptides, typified by cecropins from silk moths, melittin from bees and magainins from frogs, are largely unstructured in free solution but, upon interacting with target membranes, they insert into the membrane, fold into an amphipathic α-helix and have been proposed to associate to form multi-state channels which permit cell contents to leak out, resulting in cell death. Because of their simple structures and compositions and the somewhat predictable tendency of specific amino acids to participate in α-helix formation, the α-helical class is one of the best studied with respect to structure/activity relationships (3–8). Such studies have shown that amino acids which interrupt helix formation tend to reduce activity, whereas those which improve amphipathicity according to helical wheel projections tend to improve activity. Through rational improvement of these peptides, specific molecules in this class have been designed which are currently being considered for or are actually undergoing clinical trials as human therapeutics (9, 10).

Although we know that these cationic peptides undergo a structural transition upon entry into membranes (4, 5, 11), we are uncertain as to whether this transition plays a direct role in membrane insertion and subsequent killing. To address this question we have built a series of related α-helical peptides with designs based on the general theme of cecropin-melittin hybrids in which we have inserted a lactam bridge to preform at least one turn of the α-helix. In this manuscript we show that preformation of α-helix in benign medium by incorporation of a single lactam bridge constraint generally leads to reduced antimicrobial activity compared with the parent molecules.

Experimental Procedures

Peptide synthesis and purification

All peptides were prepared by solid-phase peptide synthesis using a benzhydrylamine hydrochloride resin on an ABI 430A synthesizer. The side-chains of glutamic acid and lysine residues involved in lactam formation were protected as OFm and 9-fluorenylmethoxycarbonyl derivatives, respectively. Lactam bridges were formed on the resin by deprotection of the desired side-chains with 20% piperidine in NMP followed by cyclization with 5-fold excess of HBTU/HOBt/NMM in NMP (12). The peptides were cleaved from the resin by reaction with HF (10 mL/g resin) containing 10% anisole for 1 h at −5 to 0°C. The crude peptides were purified by reversed-phase high-performance liquid chromatography (RPC) on a SynChropak RP-4 preparative C4 column (250 × 21.2 mm internal diameter, 6.5 mm particle size, 300 Å pore size) (SynChrom, Lafayette, IN) with a linear AB gradient of 0.1% B/min with a flow rate of 5 mL/min, where solvent A was 0.05% trifluoroacetic acid (TFA) in water and solvent B was 0.05% TFA in acetonitrile. The purity of the peptides was determined by RPC. Mass analysis of the peptides was performed on a Fisons VG Quattro triple quadropole mass spectrometer (Manchester, UK) fitted with an electrospray ionization source operating in the positive ion mode. Several 10-μL injections of the peptide samples (usually in aqueous acetonitrile containing 0.05% TFA at an approximate concentration of 50 pmol/μL) were made into a carrier solution composed of water/acetonitrile (1:1, v/v) containing 0.05% TFA at a rate of 10 μL/min into the electrospray source. The quadropoles were scanned from 600 to 1400 mass over charge ratio at 10 sec/scan. Data were acquired in the multichannel acquisition mode with 10 to 15 scans typically being summed to produce a spectrum. The peptides were further characterized by amino acid analysis. For amino acid analysis, peptides were hydrolyzed in 6 N HCl containing 0.1% phenol for 1 h at 160°C in sealed evacuated tubes. The concentration of peptide solutions was determined by amino acid analysis. Amino acid analysis was performed on a Beckman model 6300 amino acid analyzer (Beckman, San Ramon, CA).

Bacterial strains and growth conditions

Pseudomonas aeruginosa K799 and *Escherichia coli* UB1005 were wild-type strains, whereas *P. aeruginosa* Z61 and *E. coli* DC2 were outer membrane altered, antibiotic supersusceptible mutants derived from these wild-type strains (13). *Staphylococcus epidermidis* and *Candida albicans* were clinical isolates obtained from Dr. T. Chow, University of British Columbia. *E. coli* strain ML-35 was used for measuring inner membrane permeability (14) and was β-galactosidase constitutive and lacking the lactose permease. Bacteria were grown at 37°C in Luria broth (1% tryptone, 0.5% yeast extract). Minimal inhibitory concentrations (MICs) of the peptides against bacteria were determined by the broth-dilution method in microtitre trays (15).

Inner membrane permeability

The ability of cationic peptides to unmask the β-galactosidase activity of *E. coli* ML-35 was measured with ortho nitrophenyl galactoside (ONPG) as a substrate (14). In this strain ONPG

cannot cross the inner membrane unless some agent such as peptide permeabilizes it. Cells grown to an OD_{600} of 1.0 were harvested and gently resuspended in 10 mM sodium phosphate buffer, pH 7.5 containing 100 mM NaCl in a volume sufficient to dilute the cells to an OD_{600} of 0.3 (approximately 3×10^8 cfu/mL). The cell suspension (0.6 mL) was then transferred to a cuvette and ONPG added to a final concentration of 1.5 mM. After achieving a stable baseline, peptide was added to a final concentration of 2 μg mL^{-1}, and the increase in absorbance at 405 nm recorded. As controls, the peptide solutions were replaced with an equivalent volume of 0.1% trifluoroacetate. The rate of ONPG uptake was determined as the equilibrium hydrolysis rate over the first 4 min.

Outer membrane permeability

The extent of uptake of 1-N-phenylnaphthylamine (NPN) across the outer membrane into cells (13, 16) was determined by the level of enhanced fluorescence of NPN as it penetrated into the membrane interior of *E. coli* UB1005 cells. This was determined at a variety of concentrations of peptide, and the concentration leading to half-maximal increase in fluorescence (I_{50}) was determined.

Circular dichroism analyses

Circular dichroism spectra were measured on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) equipped with a Jasco DP-500N data processor. A Lauda water bath (model RMS, Brinkmann Instruments, Rexdale, ON) was used to control the temperature of the cell. Constant N_2 flushing was used. The instrument was routinely calibrated with an aqueous solution of *d*-10-(+)-camphorsulfonic acid at 290 nm. Ellipticity was reported as mean residue molar ellipticity ([θ]), in deg·cm^2·$dmol^{-1}$ and calculated from the equation:

$$[\theta] = \theta_{obs}(mrw)/10lc$$

where θ_{obs} was the ellipticity measured in degrees, mrw was the mean residue weight (molecular weight divided by the number of amino acid residues), *c* was the peptide concentration in grams per milliliter and *l* was the optical path length of the cell in centimeters. CD spectra were the average of four scans obtained by collecting data at 0.1-nm intervals from 255 to 190 nm.

Results and Discussion

This work was initiated in an attempt to understand how the folding of cationic antimicrobial peptides is related to their antibacterial activity. Previous data had suggested that the α-helical family of peptides, including cecropin-melittin hybrids, adopts a random configuration in free solution but fold into an α-helical configuration upon entering a membrane-like environment (3, 7). However, the actual process of interaction of a cationic peptide with a bacterial cell is quite complex, and it was not known at what stage the folding event occurred and whether it was required for or promotes a critical step in this interaction pathway (1). The mechanism of interaction of cationic peptides with Gram-negative bacterial cells involves the following events: initial interaction with divalent cation binding sites on the surface lipopoly-saccharide (LPS) of the outer membrane; distortion, consequent permeabilization of and self-promoted uptake across the outer membrane; electrostatic interaction with the cytoplasmic membrane surface, membrane thinning and insertion of cationic peptides so they span this membrane; formation of channels leading to extrusion of essential substances and death (1, 9, 11, 14). This complex process involves at least two binding steps and passage across or insertion into two cell envelope membranes, although it is extremely rapid with up to 7 logs of killing of bacteria within 2 min at concentrations of 2- to 4-fold the MIC (Gough, M., and R.E.W. Hancock, unpublished results). Therefore, the exact location at which this folding takes place is difficult to dissect biochemically. For this reason, we attempted here to address this question by asking whether prefolding of the peptides was advantageous.

All peptides were based on a cecropin (1–8)/melittin (1–18) hybrid peptide CEME (6, 13) which was previously shown to have good antibacterial activity. Several analogs of cecropin and melittin hybrids have been synthesized and studied (6–8). The general conclusions based on these studies were that optimal hybrids should contain an N-terminal amphipathic and C-terminal hydrophobic domain linked by a hinge region (8), in which the N-terminal segment of the analogs must contain a helical amphipathic region of 11 residues or more (3, 17) including an aromatic residue at position 2 (Fig. 1) (3). The presence of a hinge region provides conformational flexibility because of the presence of Gly and/or Pro residues (18).

Taking these factors into account, four parent peptides were designed (Fig. 1). The peptides 490 and 493 had identical C-termini (amino acids residues 15–26) and differed by only four residues in the N-terminus. The introduction of two Gly residues at positions 16 and 17 imparted increased flexibility to the hinge region. Peptides 490 and 491 were related by having identical N-terminal segments (residues 1–14). However the C-terminal segment of 491 was changed

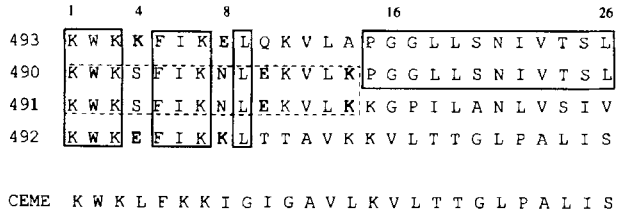

Figure 1. Sequence of linear and lactam-bridged cationic peptides. Bold, residues indicate the position of the lactam bridge/ion pair. Regions of identical sequence are boxed.

by the inclusion of an additional positively charged residue (P15K), the introduction of a Pro residue at position 17 and the subtle increase in the overall amphipathicity of the molecule (S20A and S25I). The sequence of peptide 492 was substantially different from the other sequences and most closely resembled CEME.

For all four sequences a glutamic acid and lysine residue spaced *i* to *i* + 4 apart were inserted into the sequence in the N-terminal segment at positions 4 and 8 (492, 493) or positions 10 and 14 (490, 491). This allowed for inclusion of a lactam bridge in the "lac" series of peptides or an ion pair in the "lin" series of peptides on the hydrophilic face of the helix (Fig. 2). Both lactam bridges and ion pairs have proven effective in inducing and stabilizing helical structure in peptides (19–23). For peptide 493 lac, the orientation of the lactam bridge (N-terminal to C-terminal direction) was reversed from Glu to Lys, as used in all other peptides, to Lys to Glu. Lactam bridges, which are spaced *i* to *i* + 4 residues apart and oriented Lys to Glu, have been shown to be helix destabilizing in aqueous conditions but readily adopt a helical structure in 50% trifluoroethanol (TFE) (12). In contrast Glu to Lys lactam bridges are generally stabilizing in both aqueous and hydrophobic media.

Structure of the peptides

Only three of the peptides demonstrated substantial formation of α-helix in benign buffer, namely 491 lac, 492 lac and 490 lin (Table 1, Fig. 3). All other peptides gave rise to CD spectra which were a mixture of random and helical conformations as indicated by the minima around 200 nm. Peptide 490 lac, which had a lactam bridge in exactly the same position as 491 lac, and peptide 493 lac, which was the most closely related peptide to 490 lac, showed very low levels of α-helix in benign buffer. Upon interaction with the membrane-mimicking environment provided by TFE (24–26) all peptides showed a significant increase in α-helical content. However, a substantially wide range of helical content, as determined by the method of Chen *et al.* (27), was observed for the eight peptides. Peptides 491 lac and 492 lac, which adopted the greatest amount of helical structure in benign conditions, were almost fully helical in 50% TFE (92% and 93%, respectively). However, 490 lac and 493 lac were only 46% and 45% helical under identical conditions, and both peptides were less helical than their linear counterparts in 50% TFE. For peptide 490 lac this low helical content is explainable because the lactam bridge is adjacent to the flexible hinge region (P15 G16 and G17) of the peptide (Fig. 1). Peptide 492 lin, although unstructured in benign conditions, showed the greatest increase in helical content between benign and 50% TFE (78% helix induction, Table 1), suggesting that this sequence has the greatest propensity to adopt a helical conformation. In general, the difference between the α-helical content in benign buffer and in TFE can be considered as a measure of the relative ability to undergo structural transition upon contact with membranes (Table 1). As an indication of the ability to undergo this transition, we introduced the helican parameter which takes the percent α-helix induced in TFE (% α-helix in 50% TFE – % α-helix in benign media) divided by the percent α-helix in benign medium (Table 1). This helican parameter was then compared with antibacterial activity (see below).

Antibacterial activity

Three of the linear derivatives 491 lin, 492 lin and 493 lin had MICs that were similar to or better than CEME (13, 17), despite the reduction of net positive charge (from +5 to +4) in peptide 493 lin and the inclusion of an additional negatively charged glutamate and positively charged lysine in all of these peptides (Fig. 1). Presumably the positioning of these added residues was more favorable in 493 lin than in 490 lin (also with a net charge of +4) because 490 lin showed antibacterial activities that were two to four times lower (i.e. MIC 2- to 4-fold higher) than those observed for 493 lin (Table 2). Peptide 490 lin also showed the weakest activity against the fungus *C. albicans*, whereas peptide 492 lin showed by far the best activity.

The reduced antibacterial activity of 490 lin was compensated for by inclusion of the lactam bridge between residues 10 and 14 resulting in 2- to 4-fold lower MICs. However, in the other three peptides, the lactam bridge actually led to reduced antibacterial activity of between 2- and 8-fold (with the relative magnitudes of increase in MIC being 491 > 492 > 493). In all cases, activity against *C. albicans* was abolished in the lactam-bridged peptides.

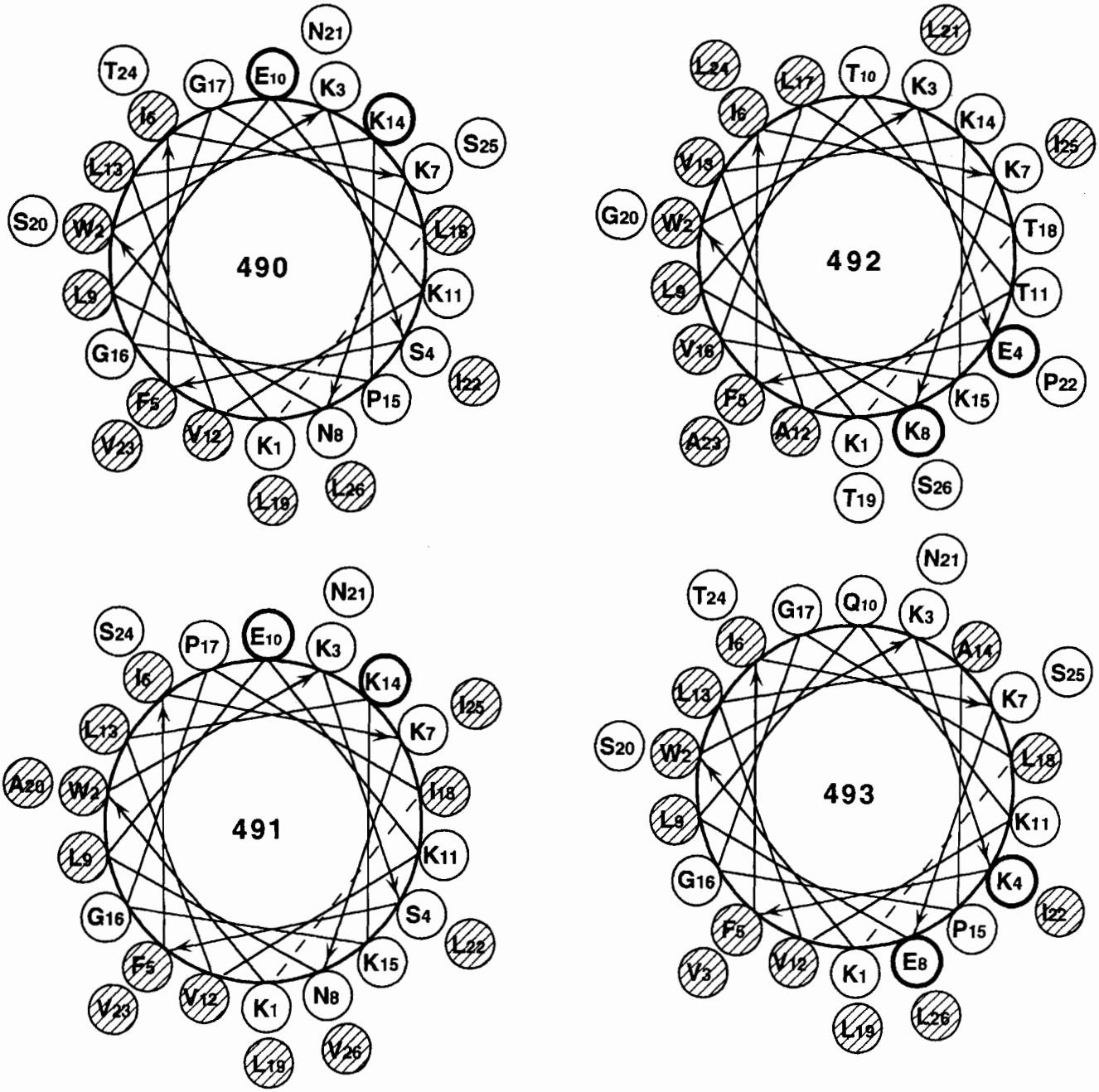

Figure 2. Helical wheel projection of the peptide sequences. Hatched circles correspond to hydrophobic residues. Bold circles correspond to residues involved in the lactam bridge.

Interaction with cell membranes

Cationic peptides are taken up across the outer membrane of Gram-negative bacteria by self-promoted uptake (1, 13, 14). This involves the interaction of the peptides with divalent cation binding sites on LPS to disrupt the outer membrane barrier and promote peptide uptake. This can be followed by measuring the uptake of a hydrophobic fluorescent probe, NPN (13, 14, 16). An increase in ability to disrupt the membrane was assessed by a decrease in the concentration of peptide required to give half-maximal uptake of NPN in *E. coli*. Of the four lactam-bridged peptides, peptide 490 lac, which showed the lowest helical content in benign medium compared with its linear counterpart, had the greatest ability to permeabilize the outer membrane with an I_{50} of 0.6 μg/mL (Table 3). For peptides 491 lac and 492 lac, which had the most α-helical structures in benign medium, their ability to permeabilize the outer membrane was greatly reduced (I_{50} equal to 5.6 and 4.0 mg/mL, respectively). For the linear peptides this trend was not observed.

The actual site of action of cationic peptides is proposed to be at the cytoplasmic membrane, where the peptide inserts, forms channels and disrupts the permeability barrier (11, 14). This can be measured by enhanced access of the

Table 1. **Nascent and solvent-induced α-helix formation in linear and lactam-bridged cationic peptides**

Peptide	α-Helix (%)		Helix induction[c]	Helican parameter[d]
	Benign[a]	50% TFE[b]		
490 lin	29	51	22	0.75
490 lac	6	46	40	6.67
491 lin	8	50	42	5.25
491 lac	37	92	55	1.48
492 lin	16	94	78	4.87
492 lac	66	93	27	0.41
493 lin	9	55	46	5.11
493 lac	10	45	44	4.40

a. Benign medium is 50 mM KH_2PO_4, 50 mM KCl, pH 7.0.
b. The benign medium is mixed 1:1 (v/v) with TFE.
c. % Helix induction is % α-helix in 50% TFE minus the % α-helix in benign medium.
d. The Helican parameter is the % helix induction divided by the % α-helix in benign medium.

chromogenic substrate ONPG to cytoplasmic β-galactosidase in a strain lacking a cytoplasmic membrane permease. The lactam derivatives were about 1.4-fold better able to permeabilize the inner membrane of *E. coli* to ONPG than were the equivalent linear peptides. The 490 series however was an exception because the parent molecule 490 lin was obviously less effective than the other peptides at increasing ONPG hydrolysis, whereas the lactam derivative showed a 2.5-fold increase in ONPG uptake compared with 490 lin. The 492 sequence was by far the most active sequence with both 492 lin and 492 lac being the most active peptides in their respective linear and lactam-bridged peptide groups. Similarly, 492 lac was substantially more active than 492 lin.

We hypothesized that the combination of low α-helix in benign medium and high α-helix induction in hydrophobic medium (TFE) would correlate with antibacterial activity. Therefore, we introduced the helican parameter, which is a measure of this transition. The original design of the parent peptides was made to conform to an Edmundson helical wheel projection in which the hydrophobic residues were confined to one face of the wheel (i.e. to one side of the helix) and the hydrophilic and charged residues were confined to the other. Despite this, these peptides, with the exception of 490, had little α-helix in solution in agreement with analogous α-helical peptides (4). The overall design of peptides 490 lin and 493 lin included a flexible Pro-Gly-Gly region (491 lin had Pro-Gly) because kinked α-helical peptides are common in nature (1) and because previous studies (7, 8) suggested that this flexible region favored improved activity. All the parent peptides, based loosely on the cecropin/melittin hybrid CEME, contained a glutamate acidic residue. Despite this, three of the linear peptides (491 lin, 492 lin and 493 lin) had activities that were equivalent to those observed for CEME (13, 16). Associated with these peptides was a high helican parameter value. Peptide 490 lin, which was notably less potent in antibacterial activity, had the lowest helican parameter value. In addition, several other observations correlated with this. Peptide 490 lin had the weakest activity against the Gram-positive bacterium *S. epidermidis* and the fungus *C. albicans*, neither of which have an outer membrane, which suggests that interaction with cytoplasmic membranes might be perturbed. Moreover, this peptide had the weakest ability to influence cytoplasmic membrane permeability, which is consistent with 490 lin having the weakest ability to form channels in the cytoplasmic membrane of bacteria and fungi.

In contrast 492 lin showed the best antibacterial activity especially against Gram-positive bacteria (MICs vs. *Bacillus subtilis* and *Enterococcus faecalis* were 4-fold better than the next best peptide 493 lin) and the fungus *C. albicans*. Correlated with this, 492 lin had a very high helican induction parameter and the greatest ability to permeabilize inner membranes. There was no obvious correlation between outer membrane permeability and activity, or between absolute α-helical content in TFE or water and activity. Thus, we would conclude from these data that the helican parameter influences the ability to form channels (i.e. permeability of membranes to ONPG) and consequently activity.

Only two peptide pairs (with and without lactam bridges) showed a large difference in helix induction (% α-helix in TFE – % α-helix in benign conditions) when comparing the lactam-bridged derivative with its linear counterpart. The 490 and 492 peptide pairs show an almost 3-fold and 2-fold difference in α-helix induction (Table 1). If α-helix induction is important for antimicrobial activity the linear peptide 492 should be more active than 492 lac, and the lactam-bridged peptide 490 should be more active than 490 lin. In other words, the helican parameter should be high for the most active peptide in each pair (low MIC values) and low for the least active peptide in each pair. Consistent with this, 492 lin was substantially more active than peptide 492 lac and 490 lac was substantially more active than its linear counterpart (Table 2). Where the helican parameters were similar within a pair of peptides (comparing 493 lin and 493 lac), the MIC values were also similar. Overall good activity of the peptides against *P. aeruginosa* correlated best (r^2 = 0.88 by linear regression) with a helican parameter which was calculated as the induction of α-helix in a membrane-mimicking environment divided by the α-helix formation under benign conditions.

The lactam bridge in peptides 491 and in particular 492 induced α-helical structure in benign medium from 8 to 37% and 16 to 66 %, respectively (Table 1). In peptide 491 lac the peptide could be induced to an almost fully α-helical peptide in 50% TFE (92% α-helix compared with 50%

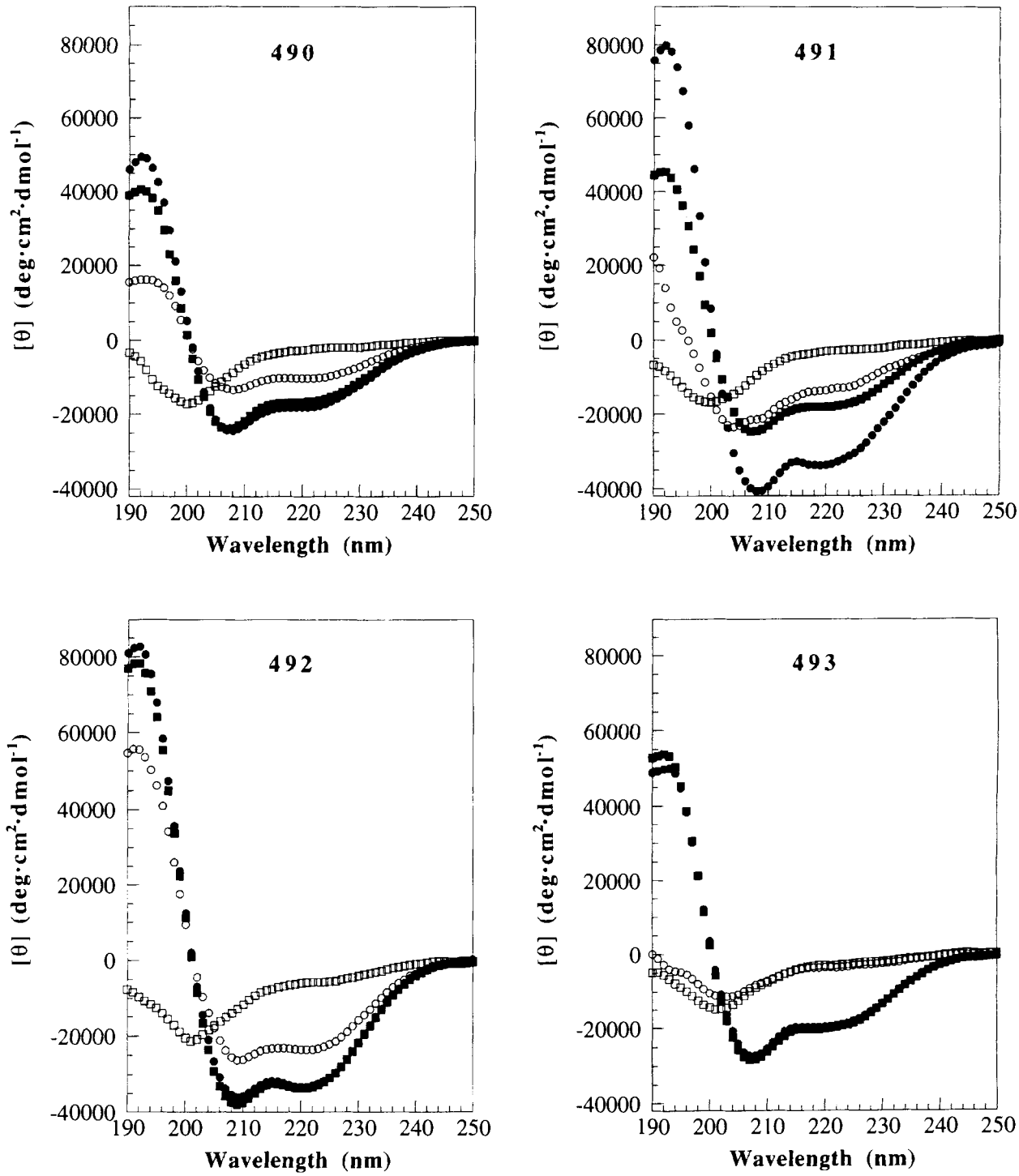

Figure 3. CD spectra of the lactam-bridged and linear peptides. The number at the top of the spectra indicates the sequence of the peptides (Fig. 1). For each spectra, □ refers to the linear peptide and ○ refers to the lactam-bridged peptide in benign conditions (50 mM KH_2PO_4, 50 mM KCl, pH 7.0) whereas ■ and ● refer to the linear and lactam-bridged peptides in 50% TFE.

α-helix for 491 lin). These results show that *i* to *i* + 4 lactam bridges can stabilize helical structure in these peptides in benign and hydrophobic medium as demonstrated previously. However, in both cases this increased α-helical content in benign medium resulted in higher MIC values. In addition, both of these peptides had a decreased ability to disrupt outer membrane permeability. These results further support the hypothesis that a low α-helical content is desirable for the peptides before insertion into the hydrophobic environment of the membrane.

In three cases, the inclusion of a lactam bridge actually reduced antimicrobial activity, although different magnitudes

***Table 2.* Antibacterial activities of linear and lactam-bridged peptides**

	MIC (mg/mL)					
Peptide	*P. aeruginosa* K799	*P. aeruginosa* Z61	*E. coli* UB1005	*E. coli* DC2	*S. epidermidis*	*C. albicans*
490 lin	25	6.2	3.1	3.1	50	200
490 lac	6.2	3.1	1.5	1.5	12.5	>200
491 lin	6.2	3.1	1.5	0.8	50	100
491 lac	50	12.5	12.5	6.2	>100	>100
492 lin	6.2	1.5	1.5	0.8	12.5	12.5
492 lac	50	6.2	3.1	1.5	50	>100
493 lin	6.2	3.1	1.5	0.8	12.5	50
493 lac	12.5	6.2	1.5	1.5	12.5	>100

of effects were observed. The greatest decrease in activity was observed for 491 lac compared with its parent peptide 491 lin. Associated with this 2- to 8-fold increase in MIC was a 1.6-fold decrease in the ability to permeabilize outer membranes. Thus this peptide might actually be becoming stalled during the process of crossing the outer membrane (because it had similar ability to permeabilize the inner membrane).

Conclusions

Our results with cecropin-mellitin hybrids demonstrates that insertion of lactam bridges into the sequence generally resulted in reduced antibacterial activity. However, the lactam-bridged peptides did have an enhanced ability to permeabilize the cytoplasmic membrane. The data presented here seem to suggest that the helican parameter (low α-helix in benign medium and the ability to adopt an α-helix in a membrane-like environment) correlates with antibacterial activity.

***Table 3.* Ability of peptides to permeabilize the outer and cytoplasmic membranes in E. coli**

Peptide	Outer membrane permeabilization to NPN I_{50} (μg/mL)	Inner membrane permeabilization to ONPG (units)
490 lin	2.5	1.0
490 lac	0.6	2.5
491 lin	3.5	1.7
491 lac	5.6	1.9
492 lin	3.5	2.6
492 lac	4.0	3.7
493 lin	3.0	1.7
493 lac	3.0	2.4

a. 1 unit of activity is equal to 0.7 μmol ONPG hydrolyzed per min per mg cells.

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