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The Design of Bacteria Strain Selective Antimicrobial Peptides Based on the Incorporation of Unnatural Amino Acids

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1. Introduction

An intensive international research effort is currently underway to develop new classes of compounds that exhibit novel mechanisms of antibacterial activity (Bush, 2004) (Klevens et al., 2007) due to the dramatic and continued evolution of drug resistant strains of bacteria (Hartl, 2000) (Shlaes, Projan, & Edwards, 2004) (Y. Huang, Huang, & Chen, 2010) (Godballe, Nilsson, Petersen, & Jenssen, 2011) (J. Song, 2008) (Findlay, Zhanel, & Schweizer, 2010). Both natural and synthetic antimicrobial peptides (AMPs) because of their novel mechanisms of antibiotic activity, coupled with the inherent difficulty for bacteria to develop resistance to them (Hartl, 2000) (Shlaes et al., 2004) (Y. Huang et al., 2010) (Godballe et al., 2011) (J. Song, 2008) (Findlay et al., 2010) exhibit a very high potential as new therapeutic agents. Several AMPs are currently in development as topical antibiotics (Zhang & Falla, 2009).

AMPs have evolved as a host defense mechanism against invading micro-organisms in most organisms (Hartl, 2000) (Shlaes et al., 2004) (Y. Huang et al., 2010) (Godballe et al., 2011) (J. Song, 2008) (Findlay et al., 2010) . In addition to antibacterial properties AMPs are believed to be key players in innate immunity (Devine & Hancock, 2002; Y. M. Song et al., 2005). AMPs are normally highly positively charged (+3 to +9) small peptides consisting of 5-50

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amino acid residues (Hancock, 1998). These peptides are amphipathic molecules with well-defined hydrophobic and hydrophilic regions (Toke, 2005; Zasloff, 2002). As of January 2009, more than 1330 (Wang, Li, & Wang, 2009) natural and synthetic antimicrobial peptides have been characterized. These peptides have exhibited, a diversity of biological activity (Khandelia, 2008).

The inherent selectivity of AMP's for prokaryotic vs eukaryotic cells is believed to be derived from the difference in the chemical compositions of their respective membranes (Dennison, Wallace, Harris, & Phoenix, 2005). Hancock and co-workers (Powers & Hancock, 2003) have extended this hypothesis to propose that the differences in membrane composition between different strains of bacteria are responsible for the diversity in the potency and selectivity exhibited by a particular AMP against different strains of bacteria. Bacterial cells contain a high percentage of negatively charged phospholipids while mammalian cells contain a much higher concentration of zwitterionic phospholipids (Papo & Shai, 2003) (Zhang & Falla, 2009) (Findlay et al., 2010) (Godballe et al., 2011) (Y. Huang et al., 2010) (Yeaman & Yount, 2003) (Leontiadou, Mark, & Marrink, 2006) (Y. Huang et al., 2010) (Hancock & Lehrer, 1998). Other differences also exist including membrane composition (sterols, lipopolysaccharide, peptidoglycan etc) (Yeaman & Yount, 2003), structure, and transmembrane potential and polarizability. These differences are in part responsible for the observed selectivity of some AMPs for bacterial vs. mammalian cells.(Hancock & Lehrer, 1998; Yeaman & Yount, 2003) Melo and co-workers have characterized the physical properties of bacterial membranes that are conducive for AMP interactions (Melo, Ferre, & Castanho, 2009).

The membranes surrounding different types of bacteria are also different. The lipid bilayer of Gram positive bacteria is covered by a porous layer of peptidoglycan, while the structure of Gram negative bacteria is more complex with two lipid membranes containing lipopolysaccharides and porins (Dennison et al., 2005; Giangaspero, Sandri, & Tossi, 2001). There is a developing preponderance of evidence in the literature supporting the concept that the selectivity and potency of a specific AMP is determined in a large measure by the chemical composition of the target membrane.(Powers & Hancock, 2003; Yeaman & Yount, 2003)

In our laboratory we have focused on developing antimicrobial peptides that contain unnatural amino acids to control the conformational and physicochemical properties (Hicks, Bhonsle, Venugopal, Koser, & Magill, 2007) of the resulting peptide. Our original skeletal design of an unnatural AMP incorporated placement of three L-Tic-L-Oic dipeptide units into the polypeptide backbone to induce an ordered structure onto the peptide (Hicks et al., 2007). The use of the conformationally restrained amino acids (Tic an Oic) reduces the local flexibility of the peptide backbone and thus reduces the total conformational space that may be sampled by the peptide during lipid binding. The AMPs developed in our laboratory, the Tic-Oic units are connected via an amino acid spacer with defined properties of charge and hydrophobicity resulting in peptides with well-defined physiochemical properties while maintaining sufficient conformational flexibility to allow the peptide to adopt different conformations on interacting with membranes of different chemical composition (Hicks et al., 2007; Russell et al., 2010; Venugopal et al., 2010). The basic skeleton of the AMPs under investigation is given in Fig. 1. The amino acid sequences for the AMPs used in this investigation are given in Table 1.

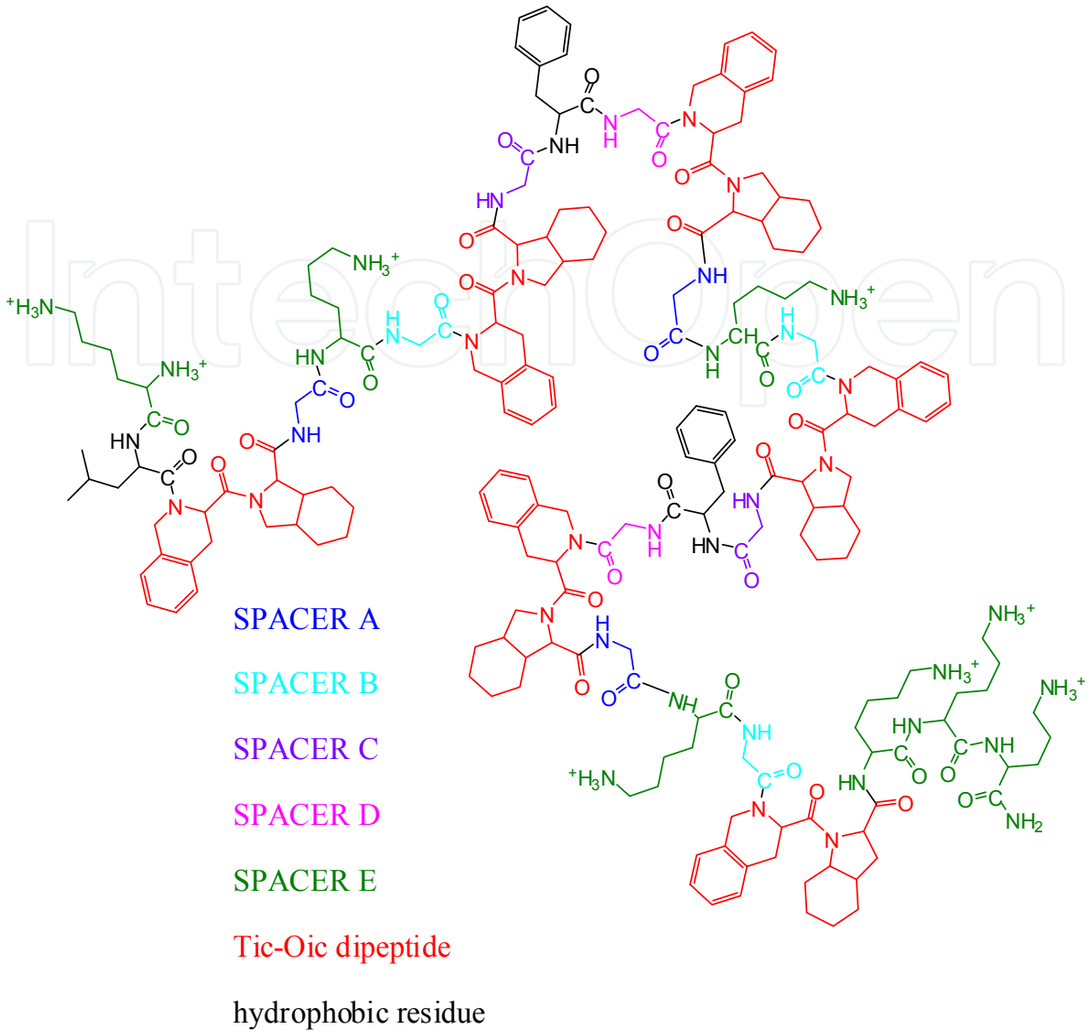

Fig. 1. The basic skeleton of the new analogs containing six Tic-Oic dipeptide units and the five SPACER residues.

In this report the in vitro antibacterial activity of a new series of AMPs incorporating six Tic-Oic dipeptide units as well as four additional Spacers, A, B, C and D on either side of the intervening hydrophobic and charged residues. These spacers define the overall conformational flexibility of the peptide backbone. A fifth Spacer, E, which defines the distance between the polypeptide backbone and the positively charged side chain amine group. Spacer E is involved in determining the overall surface charge density of the peptide as well as defining the distance between the membrane surface and the polypeptide backbone (Hicks et al., 2007). By varying the number of -CH_2- groups in Spacer E from 1 to 4 in the side chain of the basic residues, the distance between the positive charge and the peptide backbone will decrease resulting in a) less side chain flexibility during binding –this flexibility is more important in the binding with zwitterionic lipids than with anionic lipids; and b) the positive charge density will reside closer to the peptide backbone. The additional Tic-Oic dipeptide units and spacers were incorporated in hopes of improving the organism selectivity of these AMPs.

Compound Number/ Amino Acid Sequence

22	H_2N-KL-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-KR-$CONH_2$
70	H_2N-KL-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-KKKK-$CONH_2$
71	H_2N-Orn-L-Tic-Oic-Orn-Tic-Oic-F-Tic-Oic-Orn-Tic-Oic-F-Tic-Oic-Orn-Tic-Oic-Orn-Orn-Orn-Orn-$CONH_2$
72	H_2N-Dpr-L-Tic-Oic-Dpr-Tic-Oic-F-Tic-Oic-Dpr-Tic-Oic-F-Tic-Oic-Dpr-Tic-Oic-Dpr-Dpr-Dpr-Dpr-$CONH_2$
73	H_2N-Dab-L-Tic-Oic-Dab-Tic-Oic-F-Tic-Oic-Dab-Tic-Oic-F-Tic-Oic-Dab-Tic-Oic-Dab-Dab-Dab-Dab-$CONH_2$
74	H_2N-KL-Tic-Oic-GK-Tic-Oic-F-Tic-Oic-GK-Tic-Oic-F-Tic-Oic-GK-Tic-Oic-KKKK-$CONH_2$
75	H_2N-KL-Tic-Oic-K-Tic-Oic-GF-Tic-Oic-K-Tic-Oic-GF-Tic-Oic-K-Tic-Oic-KKKK-$CONH_2$
76	H_2N-KL-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-Oic-KKKK-$CONH_2$
77	H_2N-KL-Tic-Oic-K-Tic-Oic-FG-Tic-Oic-K-Tic-Oic-FG-Tic-Oic-K-Tic-Oic-KKKK-$CONH_2$
78	H_2N-KL-Tic-Oic-KG-Tic-Oic-F-Tic-Oic-KG-Tic-Oic-F-Tic-Oic-KG-Tic-Oic-KKKK-$CONH_2$
79	H_2N-KL-Tic-Oic-KG-Tic-Oic-FG-Tic-Oic-KG-Tic-Oic-FG-Tic-Oic-KG-Tic-Oic-KKKK-$CONH_2$

Table 1. Amino acid sequences of the compounds understudy

2. Biological activity

Antimicrobial peptides (AMP) have evolved in almost every class of living organism as a defense mechanism against invading micro-organisms (Yeaman & Yount, 2003) (Dennison et al., 2005). The exact mechanism of lipid-induced cytotoxicity of these peptides is currently debated in the literature (Powers & Hancock, 2003) with AMPs divided into two mechanistic classes, membrane-disruptors and non-membrane-disruptors (Powers & Hancock, 2003) (Brogden, 2005). However, there is a general agreement that the first target for either membrane-disruptor or non-membrane-disruptor AMPs is the negatively charged membranes of bacterial cells (Powers & Hancock, 2003). There are several different structural classes of membrane-disruptor AMPs but, for the purpose of this investigation we focused on linear amphipathic helical peptides. In many cases these peptides exhibit characteristics of a random coil conformation in aqueous or in organic solvents, however on binding to micelles or membranes they adopt an ordered amphipathic secondary structure (Hicks et al., 2003). This structural class of membrane-disruptors can be divided into two sub-classifications based on biological activity: 1) cell selective and 2) non-selective (Y. M. Song et al., 2005). As the name implies cell selective AMPs exhibit potent activity against bacterial cells while remaining inactive against mammalian cells, non-selective AMPs exhibit activity against bacterial as well as mammalian cells. We have previously reported the synthesis and biological evaluation of a series of potent (low μM to nm in vitro activity, and low acute toxicity 125 mg/kg in mice) bacteria selective membrane disruptors based on the incorporation of three Tic-Oic dipeptide units (Hicks et al., 2007) (Venugopal et al., 2010) (Bhonsle, Venugopal, Huddler, Magill, & Hicks, 2007). The in vitro antibacterial activity exhibited by the analogs containing six dipeptide units against seven strains of bacteria is given in Table 2. The in vitro antibacterial activity will be discussed as a function of the Spacers. The activity of Spacers A and C will be discussed together as will Spacers B and D. Spacer E will be discussed separately.

Bacteria Strain	Compound Number										
	22	**70**	***71***	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**
Acinetobacter baumanii ATCC 19606	**40.5**	**9.30**	**9.77**	186	162	**4.50**	146.00	139	146	144	139
Acinetobacter baumanii WRAIR	162	150	156	186	162	144	146.00	139	146	144	139
Staphylococcus aureus ATCC 33591(MRSA)	162	150	156	186	162	**17.90**	146.00	139	146	144	139
Brucella melitensis 16M	162	150	156	186	162	144	**73**	139	146	144	139
Bacillus anthracis AMES	**40.5**	150	156	186	162	**8.97**	**0.50**	139	146	**2.20**	139
Francisella tularensis SCHUS4	**81**	150	156	186	162	144	146.00	139	146	144	139
Burkholderia mallei	162	150	156	**84**	**81**	144	146	139	146	144	139

Table 2. In Vitro MIC values (μM) for the compounds understudy against drug resistant strains and Gram Negative Select Agents.

2.1 Spacers A and C

In this series of analogs, Spacer A is the residue preceding each internal Lys residues (N-terminal side of the Lys) and Spacer C is the residue preceding each internal Phe residue (N-terminal side of the Phe) as listed in Table 3.

Compound **22** exhibited good in vitro activity against Acinetobacter baumanii ATCC 19606, and Bacillus anthracis AMES and moderate activity against Francisella tularensis SCHUS4 and poor activity against the other bacteria strains. Replacement of the Lys-Arg residues at the C-terminus with four Lys residues in compound **74** changes in vitro activity dramatically.

Compound number	Spacer A	Spacer C
22	Gly	none
70	none	none
74	Gly	none
75	none	Gly
76	Gly	Gly

Table 3. Spacers A and C

The activity against Acinetobacter baumanii ATCC 19606 and Staphylococcus aureus ATCC 33591(MRSA) are increased by a factor of 9, and the activity against Bacillus anthracis AMES, is increased by a factor of 5, compared to compound **22**. Deleting Spacer A (Gly residue) of compound **74** and introducing Spacer C (Gly residue) in Compound **75** again dramatically changes the activity compared to both compounds **22** and **74**. The activity against Acinetobacter baumanii ATCC 19606 is now very poor. However compound **75** exhibited the highest activity against Bacillus anthracis AMES (80 fold increase over compound **74**) and Brucella melitensis 16M. Activity against the other bacteria strains was very poor. Incorporating both Spacers B and C (both Gly residues) in compound **76** reduced the activity to a level that this compound is no longer therapeutically useful. Removing both Spacers in compound **70** resulted in a compound selective for Acinetobacter baumanii ATCC 19606 and inactive against the remaining bacteria strains. This data indicates that combined use of Spacer A and C coupled with increasing the density of the positive charge at the C-terminus of the peptide produces organism selectivity.

2.2 Spacers B and D

In this series of analogs Spacers B is the residue following each internal Lys residues (C-terminal side of the Lys) and Spacer D is the residue following each internal Phe residues (C-terminal side of the Phe) as listed in Table 4.

Compound Number	Spacer B	Spacer D
77	none	Gly
78	Gly	none
79	Gly	Gly

Table 4. Spacers D and E

Incorporation of Spacers D (Gly residues in both cases) in compounds **77** and **79** reduced to the activity of these two compounds against all seven strains of bacteria to poor making these compounds no longer therapeutically useful. Incorporation of Spacer B (Gly residue) only in compound **78** exhibited selective and very good activity against Bacillus anthracis AMES.

2.3 Spacer E

In this series of analogs, Spacer E replaces the charged Lys residues with charged residues with progressively shorter side chains as shown in Table 5. Replacement of the Lys residues with Orn residues (one less carbon atom in the side chain) in compound **71** dramatically reduces the activity against Staphylococcus aureus ATCC 33591(MRSA) and Bacillus

anthracis AMES, while maintaining the activity against Acinetobacter baumanii ATCC 19606 as compared to compound **74**.

Compound Number	Spacer E
71	Orn
72	Dpr
73	Dab
74	Lys

Table 5. Spacer E

Replacement of the Lys residues with either Dpr residues (three carbon less) or Dab (two carbons less) residues in compounds **72** and **73** respectively dramatically reduces the activity against all of the bacteria strains except Burkholderia mallei. Clearly, the length of the side chain of Spacer E, and the resulting charge density, plays a major role in determining organism potency and selectivity against these seven strains of bacteria.

3. Characterization of peptide lipid interactions

The observed differences in bacteria strain selectivity for these compounds are believed to be derived directly from the conformational and physicochemical properties presented by these AMPs to the surface of the bacteria membrane. Circular Dichroism (CD), isothermal calorimetry (ITC) and calcein fluorescence leakage experiments were conducted to provide insight into the mechanisms of binding of a series of antimicrobial peptides to simple membrane models for bacterial and mammalian cells. LUVs and SUVs consisting of 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC) were selected as a simple model for the zwitterionic membranes of mammalian cells and membrane models consisting of (4:1) 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC) / **1-**Palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (Sodium Salt)) (POPG) were selected as a simple model for the anionic membranes of bacteria cells (Bringezu et al., 2007) Hicks, 2007 #19}.

3.1 Calcein leakage studies

It is well documented that AMPs containing three Tic-Oic dipeptide units exhibited potent and selective antibacterial activity (Hicks et al., 2007; Venugopal et al., 2010) **and t**hese AMPs were designed to be members of the general class of AMPs known as membrane disruptors (Shlaes et al., 2004) (Kamysz, 2005) (Zhang, Harris, & Falla, 2005) (Toke, 2005) (Godballe et al., 2011) (Ryge, Frimodt-Moller, & Hansen, 2008). Calcein leakage studies clearly indicated that the analogs containing three Tic-Oic dipeptides units are in fact membrane disruptors (Russell et al., 2010). The first step in this investigation was to confirm that the analogs containing six Tic-Oic dipeptide units are also membrane disruptors. Peptide induced calcein leakage from LUVs monitored through fluorescence, is a well documented technique for probing AMP activity to confirm membrane disruption (Andrushchenko, Aarabi, Nquyen, Prenner, & Vogel, 2008) (Medina, Chapman, Bolender, & Plesniak, 2002). Peptides at a concentration of 4μM were introduced to solutions of either POPC or 4:1 POPC/POPG LUVs containing encapsulated 70 mM calcein (Figure 2) and the resulting induced fluorescence due to leakage of calcein was monitored over a 90 minute time period.

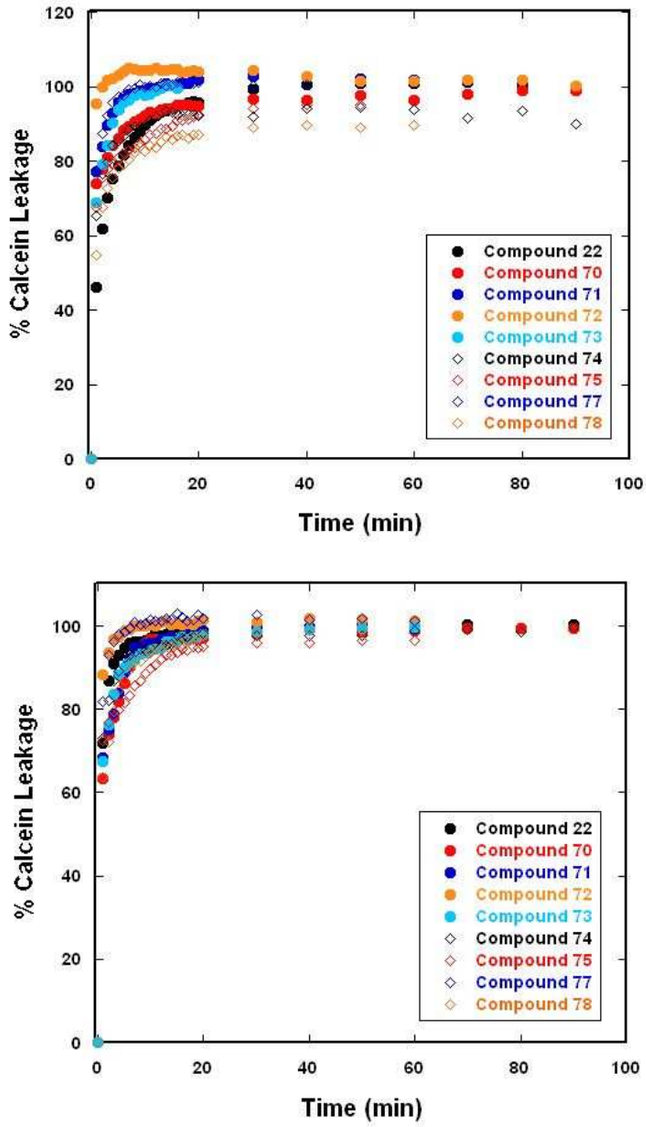

Fig. 2. Induced leakage of calcein from TOP) POPC LUVs and BOTTOM) 4:1 POPC/POPG.

The induced calcein leakage data indicates that all AMPs in this investigation interact in a very similar fashion with both zwitterionic (POPC) and anionic (4:1 POPC/POPG) LUVs. This data also strongly suggests that the mechanism of action of these AMP involves some type of membrane disruption. However, this data doesn't explain the observed organism selectivity. The observed induced calcein induce leakage behavior of the analogs containing six Tic-Oic dipeptide units is very different from that observed for the corresponding three Tic-Oic dipeptide containing analogs. The analog containing three Tic-Oic dipeptide units corresponding to compound **74** has the amino acid sequence: Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKKK-$CONH_2$ (compound **23**) (Russell et al., 2010). For compound **23** different concentrations of peptide (4 – 20 μM) were introduced to solutions of either POPC or 4:1 POPC/POPG LUVs and their induced fluorescence, due to leakage of calcein was monitored over a 90 minute time period. It can be seen in Fig. 3, compound **23** interacts with

POPC LUVs, inducing calcein leakage, in a non linear concentration dependent manner (Russell et al., 2010). However, compound **23** induces calcein leakage from 4:1 POPC/POPG LUVs in a concentration dependent manner. This data indicates the analogs containing three Tic-Oic dipeptide units interact with zwitterionic POPC LUV and anionic 4:1 POPC/POPG LUVs via different mechanisms (Russell et al., 2010). This data also indicates that the incorporation of three additional Tic-Oic dipeptide units, and thus increasing the overall length of the peptide, changes the mechanism of binding with both types of LUVs.

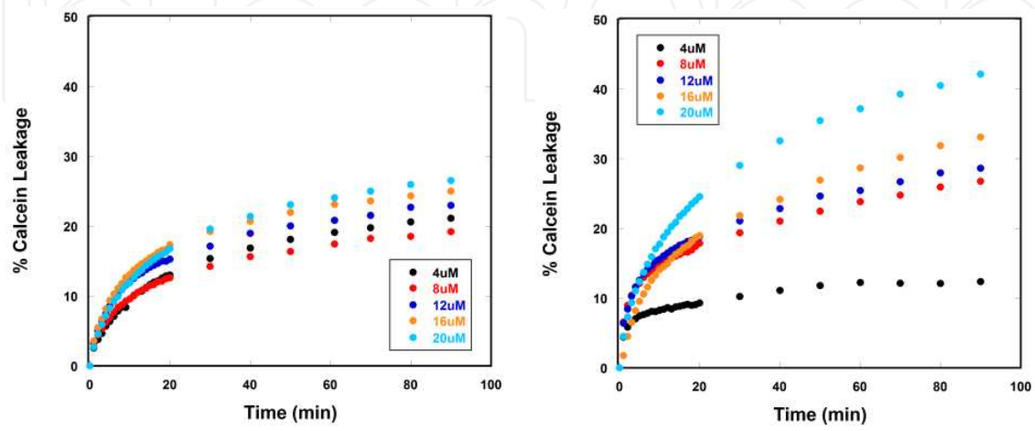

Fig. 3. The calcein leakage induced by compound **23** as a function of peptide concentration from LEFT) POPC LUVs and RIGHT) 4:1 POPC/POPG LUVs

The corresponding experiment using compound **74** were conducted. Compound 74 at various concentrations (4 – 20 μM) was introduced to solutions of either POPC or 4:1 POPC/POPG LUVs and their induced fluorescence, due to leakage of calcein was monitored over a 90 minute time period. It can be seen in Fig. 4, that compound **74** interacts with POPC LUVs, and 4:1 POPC/POPG LUVs in a totally concentration independent manner. In fact almost 100% calcien leakage was observed at all concentrations. This suggests that the analogs containing six Tic-Oic dipeptide units bind to the surface of the LUV and cause the LUV to lysis or break down in some fashion. After causing this disruption of the surface of the LUVs these peptides most likely then separate from the "disrupted" LUV and bind to another LUV and repeat the process.

3.2 Circular dichroism studies

Circular dichroism (CD) spectroscopy is very sensitive and its use to monitor conformational changes in peptides and proteins is well documented (Glattli, Daura, Seebach, & van Gunsteren, 2002), (Ladokhin, Selsted, & White, 1999) (Ladokhin, Vidal, & White, 2010). Traditionally, SUVs have been employed almost exclusively to investigate the binding of peptides and proteins with lipids in CD studies in order to minimize the contribution of light scattering on the spectra (Ladokhin et al., 2010) (C. H. Huang, 1969).

In the analysis of CD data, it must be noted that the spectrum of a peptide represents the linear combination of a number of different conformers (Glattli et al., 2002)-(Berova, Nakanishi, & Woody, 2000). This is particularly true when liposomes are used as there are several different peptide-liposome interactions possible depending on the lipid to peptide ratio.

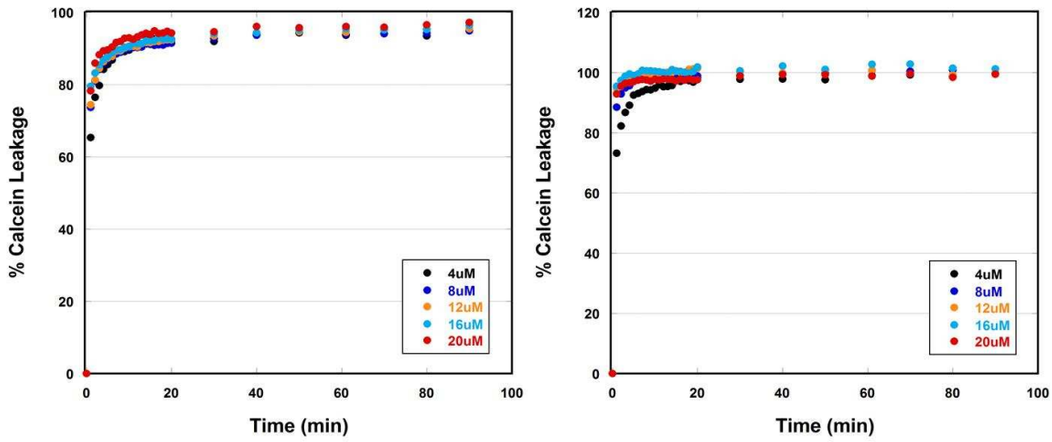

Fig. 4. The calcein leakage induced by compound **74** as a function of peptide concentration from LEFT) POPC LUVs and RIGHT) 4:1 POPC/POPG LUVs

Changes in the intensity or shape of the CD spectrum of a peptide in the presence of a liposome indicate that the peptide is adopting different conformations on interacting with that particular liposome as compared to another environment such as a buffer. (Berova et al., 2000; Glattli et al., 2002). Due to the high percentage of unnatural amino acids incorporated into the peptide under investigation here, no quantitative estimation of secondary structural features are possible. Therefore, analysis of CD spectra will be limited, at best, to qualitative comparisons highlighting differences in the spectra of these compounds with references to possible secondary structural features.

3.2.1 CD spectra in buffer

The CD spectra of 100 µM solutions of compounds **22**, **70, 71, 72, 73, 74, 75, 76, 77, 78** and **79** in 40 mM phosphate buffer, pH = 6.8 are given in Fig. 5. As can be seen in Fig. 5, these peptide adopt a variety of different conformations varying from random coil to possible β-sheet like. However, overall these peptides are unordered in buffer only.

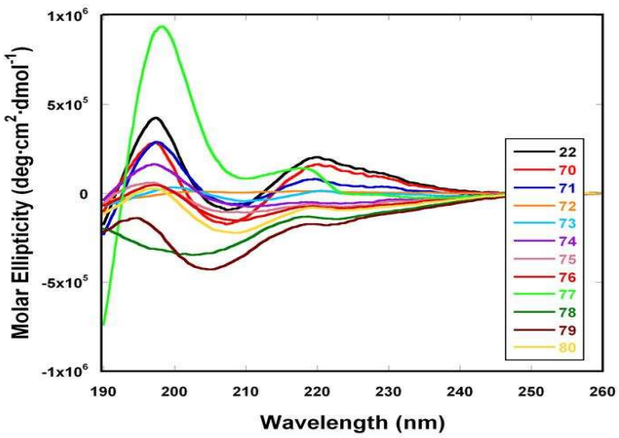

Fig. 5. Far-UV Circular Dichroism spectra of 100 µM solutions of compounds **22**, **70, 71, 72, 73, 74, 75, 76, 77, 78** and **79** in 40 mM phosphate buffer, pH = 6.8.

3.2.2 CD spectra in the presence of POPC SUVs

The CD spectra of compounds **70, 71, 72, 73, 74, 75, 76, 77, 78** and **79** in the presence of 1.75 mM POPC SUVs are shown in Fig. 6. This figure illustrates the diversity of conformations adopted by these compounds on binding to POPC SUVs. The CD spectra of these compounds in the presence of POPC SUVs are different in most cases from the corresponding spectra in buffer. This indicates that these compounds are interacting with the POPC SUVs via some mechanism. This is consistent with the very strong interactions observed with POPC LUVs in the induced calcein leakage studies. From this figure it is difficult to characterize the conformational difference associated with the variations in the five Spacers. To accomplish analysis, these CD spectra will be divided into smaller sub sets of spectra.

The first sub set of CD spectra to be discussed will be those of compounds **70**, **74**, **75**, and **76** which contain Spacers A or C, or both. The CD spectra of these four compounds, given in Fig. 7, are different indicating that these compounds adopt different conformations on binding to POPC SUVs. This observation is critical in explaining possible organism selectivity. Since this POPC SUVs are a model for mammalian cells, this data suggests that these compounds would adopt different conformations on binding to red blood cells. From a therapeutic perspective this information may allow for the development of analogs with reduced toxicity. The CD spectrum of compound **75** exhibits a maxima at approximately 195 nm and double minima at approximately 220 and 205 nm which would suggest the presence of α-helical conformers (Ladokhin et al., 1999) (Fuchs et al., 2006). While the CD spectra of compounds **74** and **76** exhibit double minima at approximately 220 and 205 nm without the maxima at 195 nm suggesting these compound adopt a mixture of β-turn and β-sheet like conformers on binding to POPC SUVs (Ladokhin et al., 1999) (Fuchs et al., 2006). The CD spectrum of compound **70** exhibits a maxima at approximately 195 nm, however no negative minima is observed. It is very difficult to qualitatively characterize the possible secondary structures adopted by compound **70**.

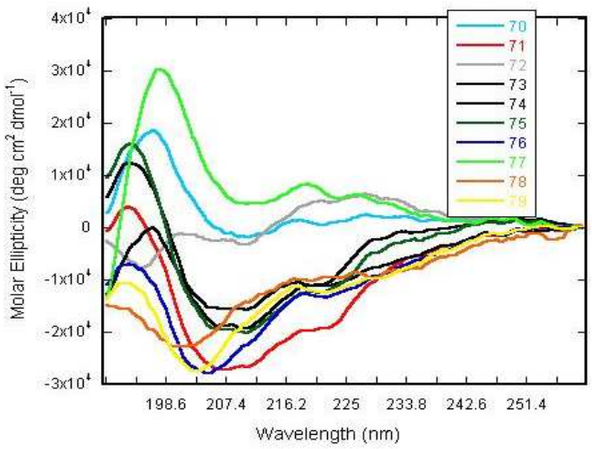

Fig. 6. Far-UV Circular Dichroism spectra of 100 μM solutions of compounds **70, 71, 72, 73, 74, 75, 76, 77, 78** and **79** in the presence of 1.75 mM POPC SUV in 40 mM phosphate buffer, pH = 6.8.

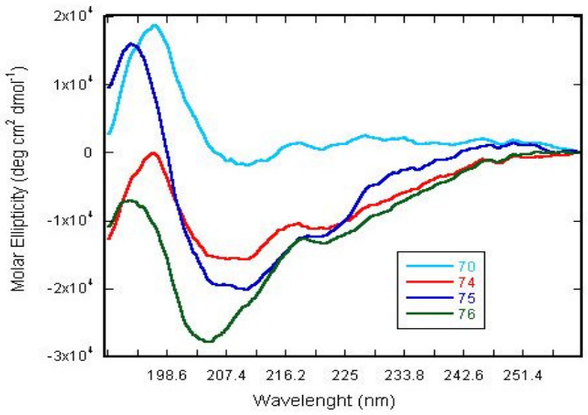

Fig. 7. Far-UV Circular Dichroism spectra of 100 µM solutions of compounds **70, 74, 75** and **76** in the presence of 1.75 mM POPC SUV in 40 mM phosphate buffer, pH = 6.8

The next sub set of CD spectra to be discussed in the presence of 1.75 mM POPC SUVs are of compounds **77**, **78** and **79 (**Fig. 8) which contain Spacers B and D. The CD spectrum of compound **77** is similar in shape and intensity to the CD spectrum of compound **70,** and therefore it is very difficult to qualitatively characterize the possible secondary structures adopted by compound **77**. While compounds **78** and **79** exhibited similar CD spectra to each other, both with minim at approximately 202 to 205 nm, it is very difficult to qualitatively characterize the possible secondary structures adopted by compounds **78** and **79,** however, this suggests these compound adopt a mixture of β-turn and β-sheet like conformers on binding to POPC SUVs (Ladokhin et al., 1999) (Fuchs et al., 2006) . What is clear from these three CD spectra is Spacer D (compound **77**) doesn't play a major role in stabilizing a particular secondary structure on binding to POPC SUVs. Spacer B (contained in compounds **78** and **79**) seems to play a more significant role in stabilizing a β-turn/sheet conformation on binding to POPC SUVs.

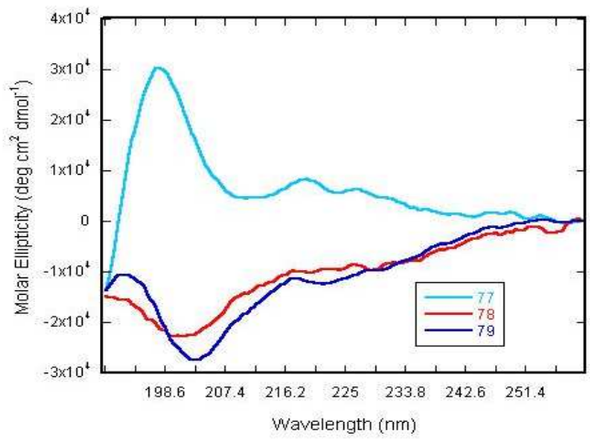

Fig. 8. Far-UV Circular Dichroism spectra of 100 µM solutions of compounds **77, 78** and **79** in the presence of 1.75 mM POPC SUV in 40 mM phosphate buffer, pH = 6.8.

The last sub set of CD spectra to be discussed in the presence of 1.75 mM POPC SUVs are those of compounds **71**, **72**, **73**, and **74** (Fig. 9) which contain Spacer E. Compounds **71**, and **74** exhibit double minima at approximately 220 and 205 nm without the maxima at 195 nm suggesting these compound adopt a mixture of β-turn and β-sheet like conformers on binding to POPC SUVs (Ladokhin et al., 1999) (Fuchs et al., 2006). While the CD spectrum of compound **72** exhibits characteristics of a random coil conformation. The CD spectrum of compound **73** exhibited a maxima at approximately 195 nm and a double minima at approximately 220 and 205 nm which would suggest the presence of α-helical conformers (Ladokhin et al., 1999) (Fuchs et al., 2006). This data clearly indicates that the length of Spacer E and the resulting location of the positive charge density relative to the peptide's backbone plays a major role in determining the conformations adopted by these peptides on binding to POPC SUVs.

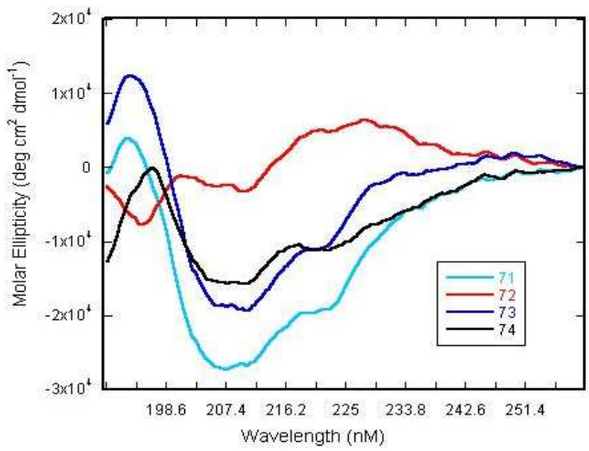

Fig. 9. Far-UV Circular Dichroism spectra of 100 μM solutions of compounds **71, 72, 73,** and **74** in the presence of 1.75 mM POPC SUV in 40 mM phosphate buffer, pH = 6.8.

3.2.3 CD spectra in the presence of 4:1 POPC/POPG SUVs

The CD spectra of compounds **22**, **70, 71, 72, 73, 74, 75, 76, 77, 78** and **79** in the presence of 1.75 mM 4:1 POPC/POPG SUVs are shown in Fig. 10. This figure illustrates the diversity of conformations adopted by these compounds binding to 4:1 POPC/POPG SUVs. The CD spectra of these compounds in the presence of 4:1 POPC/POPG SUVs are different in most cases from the corresponding spectra in buffer. This indicates that these compounds are interacting with the 4:1 POPC/POPG SUVs. From this figure it is difficult to characterize the conformational difference associated with the variations in the five Spacers. To accomplish this analysis, these CD spectra will be divided into smaller sub sets of spectra. As will become evident from the analysis of the sub sets of spectra, many of these compounds adopt different conformation on binding to POPC and 4:1 POPC/POPG SUVs.

The first sub set of spectra to be discussed will be focused on Spacers A and C, consisting of compounds **70**, **74**, **75**, and **76**. The CD spectra (Fig. 11) of these four compounds are different indicating that these compounds adopt different conformations on binding to 4:1 POPC/POPG SUVs. The differences in the CD spectra for these compounds binding to 4:1

POPC/POPG SUVs is critical in that it helps to explain the observed organism selectivity between the different bacteria strains. The CD spectrum of compound **75** exhibits a maxima at approximately 195 nm and double minima at approximately 220 and 205 nm which would suggest the presence of α-helical conformers just as it did in the presence of POPC SUVs (Ladokhin et al., 1999) (Fuchs et al., 2006).

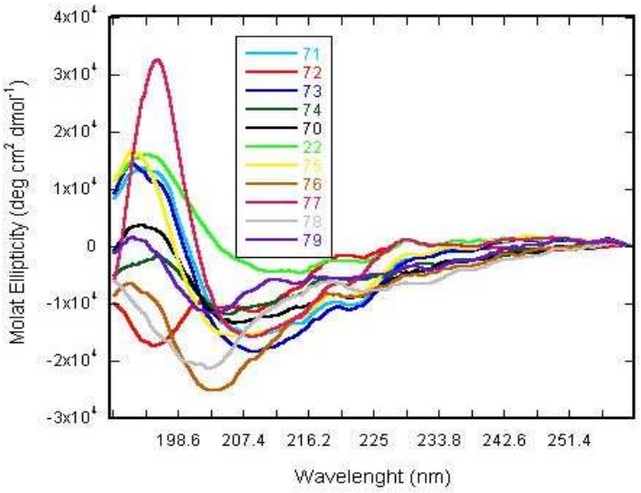

Fig. 10. Far-UV Circular Dichroism spectra of 100 μM solutions of compounds **22**, **70, 71, 72, 73, 74, 75, 76, 77, 78** and **79** in the presence of 1.75 mM 4:1 POPC/POPG SUV in 40 mM phosphate buffer, pH = 6.8.

The CD spectra of compounds **76** exhibited a double minima at approximately 220 and 205 nm without the maxima at 195 nm suggesting these compound adopt a mixture of β-turn and β-sheet like conformers on binding to 4:1 POPC/POPG SUVs just as it did with POPC SUVs (Ladokhin et al., 1999) (Fuchs et al., 2006). The CD spectrum of compound **70** exhibits a weak maxima at approximately 195 nm and a weak double minima at approximately 220 and 205 nm is also observed which would suggest the presence of α-helical conformers just as it did in the presence of POPC SUVs (Ladokhin et al., 1999) (Fuchs et al., 2006). This CD spectrum is different from the one observed in the presence of POPC SUVs. The CD spectrum of compound **74** was very interesting as it exhibits only a single minima at approximately 205 nm. Also the overall spectra intensity is less than that observed in the presence of POPC SUVs.

The second set of CD spectra to be discussed are of compounds **77**, **78** and **79** (Fig. 12**)** (Spacers B and D) in the presence of 4:1 POPC/POPG SUVs. The CD spectrum of compound **77,** unlike in the presence of POPC SUVs, is very different in shape and intensity from the CD spectrum of compound **70**. Compound **77** in the presence of 4:1 POPC/POPG SUVs exhibits a strong and a weak minima at approximately 207 nm. Again compounds **78** and **79** exhibited similar CD spectra to each other both with minima at approximately 202 to 205 nm. These spectra are similar to those observed in the presence of POPC SUVs. These CD spectra are different in shape, thus implying a different conformation of binding to the SUVs, from the CD spectra of the active analogs, which from a therapeutic respective is a critical observation.

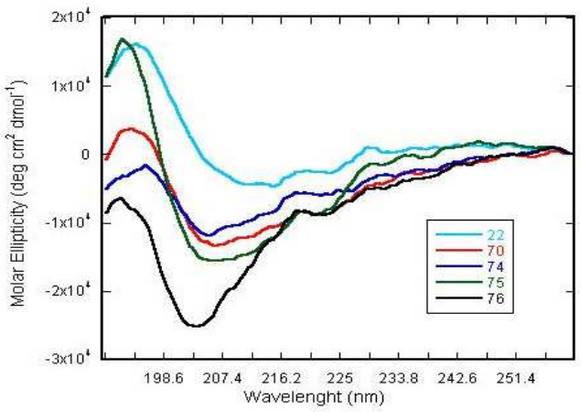

Fig. 11. Far-UV Circular Dichroism spectra of 100 μM solutions of compounds **22**, **70, 74, 75,** and **76** in the presence of 1.75 mM 4:1 POPC/POPG SUV in 40 mM phosphate buffer, pH = 6.8.

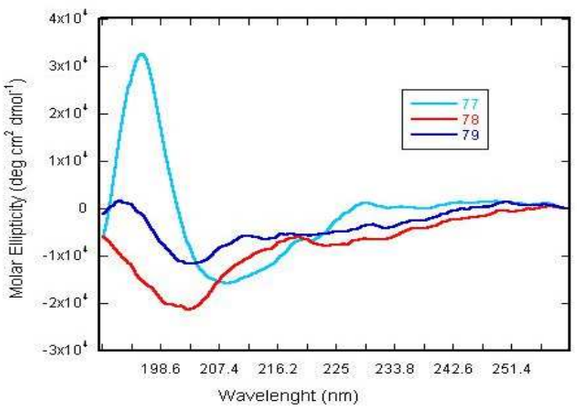

Fig. 12. Far-UV Circular Dichroism spectra of 100 μM solutions of compounds **77, 78** and **79** in the presence of 1.75 mM 4:1 POPC/POPG SUV in 40 mM phosphate buffer, pH = 6.8.

The fact that the CD spectra of the inactive compounds **77**, **78** and **79** in the presence of 4:1 POPC/POPG SUVs are very different from the CD spectra of the active compounds supports our hypothesis that the incorporation of multiple Tic-Oic dipeptide units that are connected via an amino acid spacer with defined properties of charge and hydrophobicity will result in peptides with well-defined physiochemical properties while maintaining sufficient conformational flexibility to allow the peptide to adopt different conformations on interacting with membranes of different chemical compositions, and thus exhibit organism selectivity.

The last sub set of CD spectra to be discussed in the presence of 1.75 mM 4:1 POPC/POPG SUVs are those of compounds **71**, **72**, **73**, and **74** (Fig. 13) which contain Spacer E. The CD spectra of compounds **71**, and **73** exhibited a maxima at approximately 195 nm and a double minima at approximately 220 and 205 nm which would suggest the presence of α-helical conformers (Ladokhin et al., 1999) (Fuchs et al., 2006). In the presence of POPC SUVs the CD

spectrum of compound **71** exhibit double minima at approximately 220 and 205 nm without the maxima at 195 nm suggesting that this compound adopts a mixture of β-turn and β-sheet like (Ladokhin et al., 1999) (Fuchs et al., 2006). While the CD spectrum of compound **72** exhibits a minima at approximately 195 nm. This spectrum was very different from the CD spectrum observed in the presence of POPC SUVs. The CD spectrum of compound **74** exhibited a double minima at approximately 220 and 205 nm which would suggest the presence of β–turn or β- sheet conformers (Ladokhin et al., 1999) (Fuchs et al., 2006). Again the observed difference in the CD spectra in the presence of 4:1 POPC/POPG SUVs are consistent with the observed differences in bacteria strain selectivity and potency.

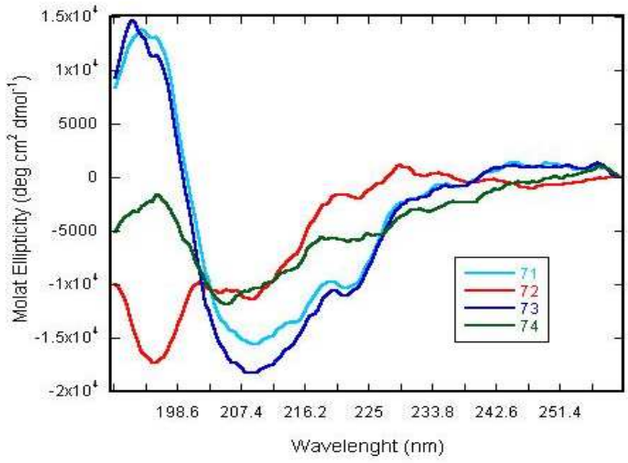

Fig. 13. Far-UV Circular Dichroism spectra of 100 μM solutions of compounds **71, 72, 73,** and **74** in the presence of 1.75 mM 4:1 POPC/POPG SUV in 40 mM phosphate buffer, pH = 6.8.

3.3 Isothermal titration calorimetry studies

The isothermal titration calorimetry (ITC) binding enthalpies for peptides with SUVs, generally, are more exothermic than those in the presence of LUVs. Therefore, SUVs are not ideal membrane models for thermodynamic measurements. (Beschiaschvili & Seelig, 1992; Seelig & Ganz, 1991; Wieprecht, Apostolov, & Seelig, 2000). LUVs formed by extrusion methods are equilibrium structures and are more appropriate to use as a membrane model in thermodynamic studies (Enoch & Strittmatter, 1979). ITC studies were conducted on the most active analogs in hopes of gaining insight into the possible explanations for the observed organism selectivity.

3.3.1 ITC studies of POPC LUVs

The application of isothermal titration calorimetry (ITC) to the study of peptide-membrane interactions is well documented in the literature (Hunter et al., 2005) (Wieprecht, Beyermann, & Seelig, 2002) (Meier & Seelig, 2007). The binding interactions between these AMPs and 35 mM POPC LUVs was investigated using ITC. In an ideal case, ITC would allow for the complete analysis of the thermodynamic parameters (free energy, enthalpy, entropy, binding constants and heat capacity changes) associated with the binding interactions between peptides and LUVs (Wieprecht et al., 2002) (Thomas, Surolia, &

Surolia, 2001). The interaction of peptides with lipid vesicles are driven by three forces: the hydrophobic effect, the coil-helix transition and non-classical hydrophobic effect (Wieprecht, Apostolov, Beyermann, & Seelig, 2000) (Wieprecht, Beyermann, & Seelig, 1999). Further, Wieprecht and co-workers (Wieprecht, Apostolov, Beyermann, et al., 2000) proposed that the binding of a peptide to a LUV is dependent on the global structural physicochemical properties including the overall charge, hydrophobicity, and amphipathicity of the AMP.

As seen in Fig. 14 the titration of 35 mM POPC LUVs into compounds **22**, **70**, **71**, **72**, **74**, **75,** resulted in different thermograms, suggesting that the process that contributes to the binding interactions vary in magnitude with each peptide. All six peptides begin their thermograms with an endothermic phase (Wieprecht, Apostolov, Beyermann, et al., 2000). Previous studies reported in the literature (Abraham, Lewis, Hodges, & McElhaney, 2005) of the interaction between peptides and lipids using ITC have noted that an endothermic phase can be attributed to a combination of several events. These events may include electrostatic interactions between the peptide and the membrane surface, disruption of polar head groups accompanied by reorganization of lipids on the surface of the membrane, disruption of the solvation spheres on both the peptide and the membrane surfaces, and other less understood phenomena (Abraham et al., 2005).

Compounds **22**, **74** and **75** all produced single endothermic phases. With these compounds, titration with POPC resulted in the continuous decrease of heat as the experiment progressed until only the heat of dilution was observed. This behavior is similar to that previously observed for compound 23. While compounds **70**, **71** and **72** all produced two phased thermograms. These thermograms begin with an endothermic phase of varying intensity and then transition into a second exothermic phase of varying intensity.

This behavior is rare, but not unknown. Jelokhani-Niaraki et. al. reported a similar observation for the interaction of aromatic amino acid analogues of gramicidin S with phospholipid membranes (Jelokhani-Niaraki, Hodges, Meissner, Hassenstein, & Wheaton, 2008). Andrushchenko and co-workers reported similar thermograms for the binding of the tryptophan-rich cathelicidin antimicrobial peptides tritrpo4 and tritrpo6 with 7:3 POPE/POPG LUVs (Andrushchenko et al., 2008). This type of two phased thermogram was not observed for the titration of the corresponding three Tic-Oic didpeptide containing analogs into POPC LUVs, only single phased endothermic thermograms were observed.

Several factors may contribute to the observed enthalpies of binding to LUVs such as attractive and repulsive electrostatic interactions, conformational changes of the peptide, changes in Van der Waals contacts due to the dehydration of both the peptide and membrane surfaces and disruption of polar head groups on the surface of the LUV (Seelig, 1997) (Andrushchenko et al., 2008) (Seelig, 2004) (Abraham et al., 2005).The endothermic heat event occurring at low lipid to peptide ratios has been interpreted as the summation of several simultaneous processes. These processes include, but are not limited to, pore formation (Wieprecht, Apostolov, Beyermann, et al., 2000), peptide aggregation (Andrushchenko et al., 2008), as well as changes in the phase properties of the lipids at different points during the titration (Epand, Segrest, & Anantharamaiah, 1990) (Seelig, 2004). The exothermic heat event at high lipid-to-peptide ratios has been attributed to the binding of the peptide to lipid surfaces (Seelig, 1997).

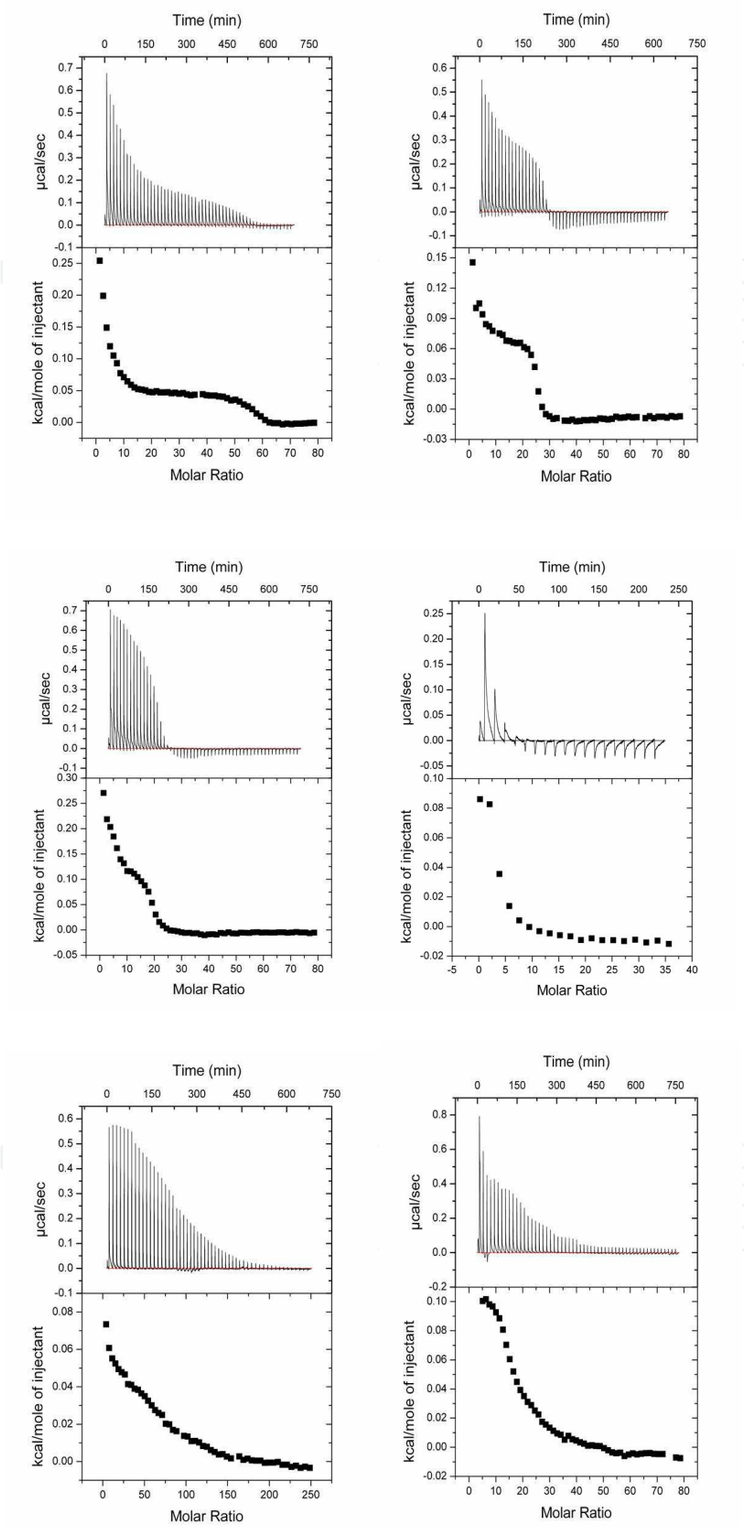

Fig. 14. The ITC thermograms of POPC LUVs titrated into compounds **22**, **70**, **71**, **72**, **74**, and **75**.

3.3.2 ITC studies of 4:1 POPC/POPG LUVs

Titration of 35 mM 4:1 POPC/POPG LUVs into compounds **22**, **70**, **71**, **74** and **75** all resulted in two phased thermograms beginning (Fig. 15) with an endothermic phase of varying intensity transitioning into an exothermic phase of varying intensity. This behavior is similar to that previously observed for compound **23** (Russell et al., 2010). This behavior is very similar to the thermograms observed for the three Tic-Oic dipeptide containing analogs. Not only does the magnitude of the endothermic and exothermic components vary, also the transition point between the endothermic and exothermic phase vary at different lipid to peptide molar ratios. These ratios are given in Table 6. Replacement of the Lys residues in compound **74** with Orn residues in compound **71** reduce the lipid to peptide ratio for the transition from the endothermic to the exothermic phase from 30 to 12. This reduction in the lipid to peptide ratio for the transition from the endothermic to the exothermic phase may possibly help explain the reduction in the overall antibacterial activity of compound **71** compared to compound **74**. Also compound **22** exhibits the most intense exothermic phase of the five compounds investigated, at a lipid to peptide ratio for the transition from the endothermic to the exothermic phase of 18. This observation suggests that a combination of factors such as the lipid to peptide ratio for the transition from the endothermic to the exothermic phase, and the magnitude of the exothermic and endothermic phase all play major roles in defining antibacterial activity, with no single factor correlating directly with the observed antibacterial activity.

Compound	Lipid to Peptide Ratio for transition
22	18
70	18
71	12
74	30
75	10

Table 6. Lipid to Peptide molar ratios for the transition from the endothermic to the exothermic phase of the thermogram for the titration of 4:1 POPC/POPG LUVs into the following peptides

As previously stated there is a growing preponderance of evidence in the literature, indicating that the selectivity and potency of a specific AMP for bacterial cells is determined in a large measure by the chemical composition of the target cell's membrane (Powers & Hancock, 2003; Yeaman & Yount, 2003). Thus we and other researchers have postulated that the bacteria cell membrane's physicochemical surface interactions with the physicochemical surface of the AMP defines organism selectivity.(Dennison et al., 2005; Giangaspero et al., 2001; Glukhov, Stark, Burrows, & Deber, 2005; Powers & Hancock, 2003) In addition, it is our hypothesis that the physicochemical properties of the target cell's membrane interact with the physicochemical properties of the approaching AMP defining its selectivity and potency against that particular cell. During this process conformational changes are induced onto the AMP that will maximize the attractive interactions between the two to facilitate AMP-membrane binding. The observation of different thermograms for these compounds on interacting with POPC and 4:1 POPC/POPG LUVs supports our hypothesis.

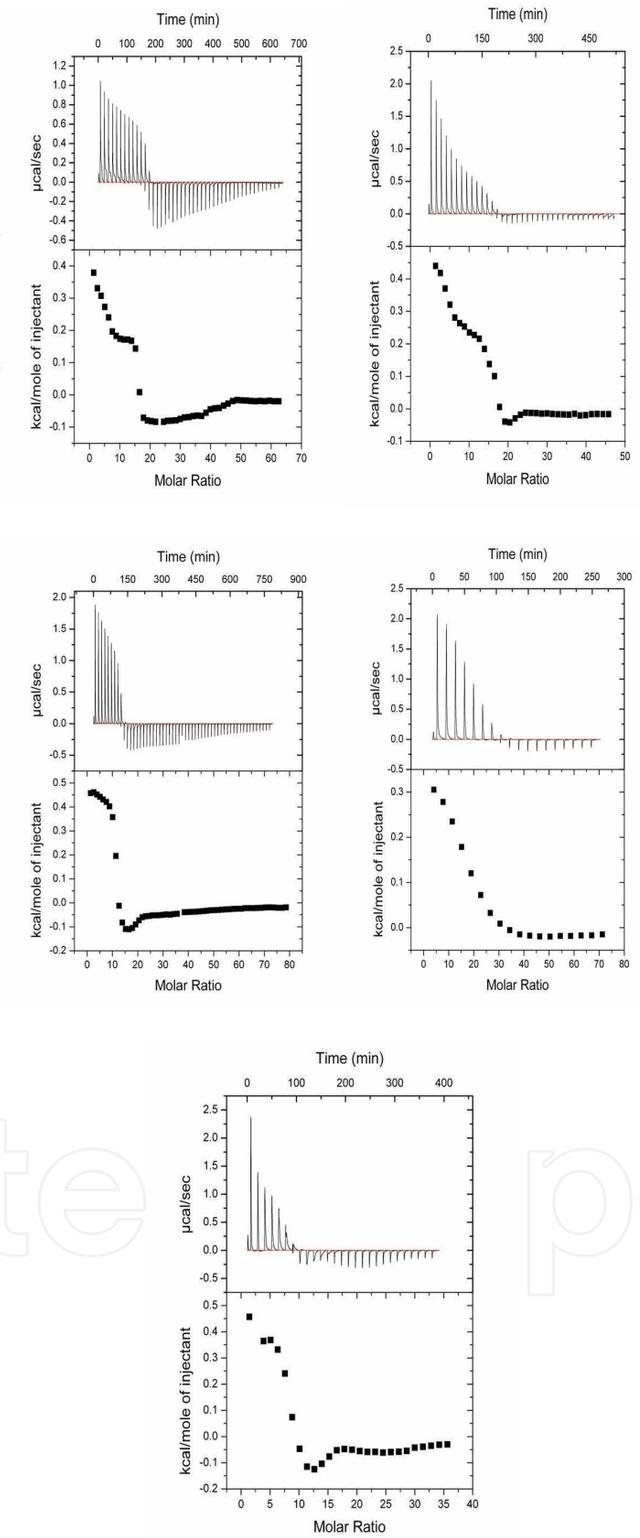

Fig. 15. ITC thermogram of the titration of 4:1 POPC/POPG into compounds **22**, **70**, **71**, **74**, **75**.

4. Material and methods

Sodium dodecyl sulfate (SDS) and Bis-Tris buffer were purchased from Sigma-Aldrich. Monobasic and dibasic sodium phosphate, EDTA and NaCl were purchased from Fischer Scientific. POPC (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine), POPG (1-Palmitoyl-2-Oleoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (Sodium Salt)) and Dodecylphosphocholine (DPC) were purchased from Avanti Polar Lipids. High purity calcein was purchased from Invitrogen. All chemicals were used without further purification.

4.1 Peptide synthesis

Peptide synthesis was performed either manually using tBOC chemistry or with an automated peptide synthesizer using FMOC chemistry (Grant, 2002) as previously reported (Hicks et al., 2007) (Venugopal et al., 2010) (Russell et al., 2010). All peptides were purified by Reverse Phase HPLC using an Agilent 1100 Series Preparative Instrument and a Vydac C18 Reverse Phase Preparative HPLC Column as previously reported (Venugopal et al., 2010) (Russell et al., 2010). All purified peptides were analyzed again by HPLC and Mass-Spec. Mass Spectral analyses were carried out using a Finnigan LTQ ESI-MS instrument running Xcalibur 1.4SR-1 or a Kratos PC Axima CFR Plus instrument (MALDI) running Kompact V2.4.1. ESI-MS showed multiply charged ions and the accurate mass was calculated. MALDI analyses were performed in reflectron mode (Venugopal et al., 2010) (Russell et al., 2010).

4.2 Preparation of POPC and 4:1 POPC/POPG SUVs

The appropriate amount of dried POPC or 4:1 POPC/POPG (mol to mol) lipid was weighed out to yield a final lipid concentration of 35 mM. The lipid was dissolved in chloroform and vortexed for 3 minutes. The sample was dried under a stream of nitrogen gas for four hours and under high vacuum overnight. The lipid was then hydrated with 2 mL of buffer (40 mM sodium phosphate, pH = 6.8) and vortexed extensively. SUVs were prepared by sonification of the milky lipid suspension using a titanium tip ultrasonicator (Qsonica Sonicators model Q55) for approximately 40 minutes in an ice bath until the solution became transparent. The titanium debris was removed by centrifugation at 14,000 rev/min for 10 minutes using an Eppendorf table top centrifuge (Wieprecht, Apostolov, & Seelig, 2000). It has been reported that the mean diameter for SUVs formed by sonication is approximately 30 nm (C. H. Huang, 1969; Ladokhin et al., 2010). Final concentration used for CD was 1.75 mM or as otherwise stated in the text.

4.3 Preparation of POPC and 4:1 POPC/POPG LUVs

A defined amount of dried POPC or 4:1 POPC/POPG (mol to mol) was weighed, suspended in buffer (40 mM sodium phosphate, pH = 6.8) and spun for 30 minutes, resulting in a total lipid stock solution concentration of 35 mM. Other concentrations of LUVs used are noted in the text and resulted from the dilution of the stock solution. Large unilamellar vesicles (LUVs) were prepared by extrusion using a Mini-Extruder (Avanti Polar Lipid Inc) (Wei, 2006) (Hunter et al., 2005). The solution was extruded through a 100 nm pore size polycarbonate membrane 21 times. After extrusion the LUVs were allowed to "rest" for at least two hours before use to allow for equilibration to occur. The final lipid

concentration was calculated based on the weight of the dried lipid (Wieprecht, Apostolov, & Seelig, 2000) (Wieprecht, Apostolov, Beyermann, et al., 2000) (Russell et al., 2010) (Wieprecht et al., 2002) (Wieprecht & Seelig, 2002) (Wen, 2007). Kennedy and co-workers previously reported the preparation of LUVs using this procedure resulting in a homogeneous population of LUVs with >95% of the particles falling into the particle size range of 70-100 nm (Kennedy et al., 2002). We have also previously shown by ^{31}P NMR that these LUVs are unilamellar (Kennedy et al., 2002).

4.4 LUVs for calcein release experiments

A defined amount of dried POPC or 4:1 POPC/POPG LUVs (mol to mol) was weighed and suspended in a calcein-containing buffer (70mM calcein, 10mM Bis-Tris, 150mM NaCl, 1mM EDTA, pH=7.1, the pH was corrected using 3mM NaOH and the final calcein concentration was calculated based on dilution). The resulting solution was vortexed for one minute (5 times). The calcein-encapsulated LUVs were extruded using the same technique as described above. Following extrusion the unencapsulated calcein was removed by gel filtration on a Sephadex G50 column (eluent: buffer containing 10 mM Bis-Tris, 150 mM NaCl, 1 mM EDTA, pH =7.1) The fraction containing calcein-encapsulated liposomes was collected and retained for fluorescence studies (Wieprecht, Apostolov, & Seelig, 2000) (Wieprecht, Apostolov, Beyermann, et al., 2000) (Russell et al., 2010) (Wieprecht et al., 2002) (Wieprecht & Seelig, 2002) (Wen, 2007) (Wieprecht et al., 1996) (Dathe, 1996) (Tamba & Yamazaki, 2005). Prior to use each batch of calcein encapsulated LUVs was subjected to a self-quenching efficiency test. The self-quenching efficiency (Q) for each lipid suspension was set at a minimum value of 80% before it could be used in these investigations. The Q value was calculated using the following equation: $Q = (1 - (F_0/F_T)) \times 100\%$ where F_0 and F_T are the background fluorescence of the lipid suspension and the total fluorescence after the addition of a solution of 10% Triton X, respectively (Jing, Hunter, Hagel, & Vogel, 2003) (Benachir & Lafleur, 1995) (Tachi T, Epand RF, Epand RM, & K., 2002) (Mason, Marquette, & Bechinger, 2007).

4.5 Circular dichroism studies

Peptide stock solutions were dissolved in 40mM phosphate buffer (pH = 6.8). Binding studies were conducted using SUV preparations consisting of 1.75 mM POPC or 4:1 POPC/POPG in 40 mM phosphate buffer (pH = 6.8) with peptide concentrations of 100 µM. All CD spectra were obtained by acquiring 8 scans on a Jasco J-815 CD Spectrometer using a 0.1 mm cylindrical quartz cell (Starna Cells, Atascadero, CA) from 260 to 178 nm at 20 nm/min, with a 1 nm bandwidth, a data pitch of 0.2 nm, a response time of 2.0 sec and a sensitivity of 5 mdeg at room temperature (~25°C). Contributions due SUVs were eliminated by subtracting the lipid spectra of the corresponding peptide-free solutions. All analysis of CD spectra was conducted after smoothing (with a means-movement function) and conversion to molar ellipticity using the JASCO Spectra Analysis program (Russell et al., 2010) (Wei, 2006) (Bringezu et al., 2007). CD spectra that exhibited HT values greater than 400 were not used due to excessive light scattering and / or absorption.

4.6 Isothermal titration calorimetry studies

Data was acquired using a Microcal VP-ITC calorimeter (Microcal, Northampton, MA). All experiments were run in 40 mM sodium phosphate buffer, pH = 6.8, at 25°C. All solutions

were degassed for approximately 10 minutes under vacuum before loading the reaction cell and syringe. For full titration experiments, 15 µL aliquots of 35 mM lipid solutions in buffer were titrated into peptide (100 – 200 µM). For binding single injection experiments, 15 µL of dilute samples of peptide (100-200 µM) were titrated into excess lipid (15-20 mM). A stirring speed of 220 rpm and injection duration of 30 sec were chosen to ensure sufficient mixing while keeping the baseline noise to a minimum. To ensure complete equilibration between injections, a delay of 700 sec between injections was used. The background heat of dilution was obtained by titrating LUVs into the reaction cell containing only buffer and was subtracted prior to analysis. Data was analyzed with Origin® software (version 7.0). ITC data collection was obtained in duplicate in an effort to ensure reproducibility (Wieprecht, Apostolov, & Seelig, 2000) (Wieprecht, Apostolov, Beyermann, et al., 2000) (Russell et al., 2010) (Wei, 2006) (Wieprecht et al., 2002) (Wieprecht & Seelig, 2002) (Wen, 2007).

4.7 Calcein leakage assays

Peptide induced calcein leakage studies were conducted using an ISS PC1 photon counting spectrofluorometer (ILC Technology) at an excitation wavelength of 494 nm and an emission wavelength of 518 nm. An aliquot of peptide (4 – 20 µM) in buffer (10 mM Bis-Tris, 150 mM NaCl, 1 mM EDTA, pH = 7.1) was added to the cell containing calcein-encapsulated liposomes (36.6 µM lipid concentration). Measurements were taken every minute for the first 20 minutes of the experiment and every 10 minutes after until no further changes in the emission intensity occurred (approximately 90 minutes). To determine the maximum fluorescence intensity that corresponded to hundred percent leakage, an aliquot of 10 µL of a 10% Triton X solution was added to the sample at the end of each experiment. The apparent percent leakage was calculated using the following equation: % leakage = $[(F_I-F_0)/F_T)]$ x 100 %, where F_0 and F_T are the initial fluorescence before introduction of peptide and after the addition of Triton X, respectively (Dathe, 1996; Russell et al., 2010) (Wieprecht, Apostolov, Beyermann, et al., 2000) (Tamba & Yamazaki, 2005) (Wieprecht et al., 1996) (Wei, 2006).

4.8 Preparation of bacteria samples

A small amount of the organism was streaked onto the appropriate agar plates and the plates incubated at either 30° or 37° C (depending on organism to be tested – Plague and Anthrax were incubated at 30°, Brucella, Francisella, and Burkholderia were incubated at 37°C) overnight. At the end of the incubation period the organisms were harvested using a sterile loop and suspended into a 15 mL centrifuge tube containing 5 mL Mueller Hinton Broth + IsoVitaleX (Bekton Dikinson, Inc.) and thoroughly mixed. A standardized suspension of 1.0 OD_{600} was prepared from a suspension using sterile saline as the diluent and the absorbance was read against a tube containing saline only as a blank using either a spectrophotometer (Spectronix 20, Bausch & Lomb, Inc.) or by a microplate reader (Spectramax Plus384, Molecular Devices, Inc.) at a wavelength of 600nm. The suspension was adjusted to obtain a volume of 5 mL containing a 1.0 OD solution. One 50 mL disposable Erlenmeyer flask containing 20mL of broth with 1 mL of the organism dilution was then inoculated for 24-48 hours (dependent on growth characteristics of the organism in question) in a shaking incubator rotating at 200 rpm (Standards, 2000) (Standard, 2002) (Venugopal et al., 2010) . After 24-48 hours, the suspension was thoroughly mixed, the tubes centrifuged at high speed (2,000 x g) for 15 minutes, the supernatant removed, and the

pelleted organisms resuspended to yield a total volume of 45 mL. This was repeated for a total of 3 washes. After the final wash the pelleted organisms were resuspended in 15 mL of broth and a 0.1 OD_{600} standardized suspension was then prepared by making dilutions in sterile cuvettes using broth as the diluent and the absorbance read blanked against a tube containing broth. The suspensions were then diluted to a final concentration of $1x10^5$ cfu/ml with sufficient volume for the number of plates being used for the MIC determination (Standards, 2000) (Standard, 2002; Venugopal et al., 2010) .

4.9 Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) was determined for the following organisms; Plague (Yersinia pestis), (Pohanka & Skladal, 2009; Revazishvili et al., 2008) Brucella, (Brucella melitensis, suis, or abortus),[64, 65] Anthrax (Bacillus anthracis), (Hicks et al., 2005; Koehler, 2009; Pohanka & Skladal, 2009) Glanders (Burkholderia mallei),(Harley, Dance, Drasar, & Tovey, 1998; Lehavi, Aizenstien, Katz, & Hourvitz, 2002; Manzeniuk et al., 1999) Melioidosis (Burkholderia pseudomallei), [71, 72] Staphylococcus aureus –MRSA [73, 74] and Tularemia (Francisella tularensis) (Pohanka & Skladal, 2009; Santic, Al-Khodor, & Abu Kwaik, 2009) Acinetobacter baumanii and a drug resistant clinical isolate strain of Acinetobacter baumanii (Walter Reed Army Institute of Research) using the following protocol. All protocols used were approved by the appropriate review committee at the Walter Reed Army Institute of Research and are summarized below. Modifications of the NCCLS methods were employed for these analyses. [76, 77] A solution of Mueller Hinton Broth + IsoVitaleX (Bekton Dikinson, Inc.) was prepared containing1% Dimethylsulfoxide (DMSO Sigma Scientific) and referred to herein as HIBCD broth. Frozen antibiotic solutions were thawed and diluted to a final concentration of 500 µg/mL in HIBCD broth (Standards, 2000) (Standard, 2002). Using a robotic sample processor (Precision XS, Biotek Instruments, Inc.), 15µL of HIBCD broth was transferred into all wells of a 384 well plate and the plate was then inoculated using 15µL of the organism suspension into all 384 wells using one organism per plate. The plates were incubated overnight and the optical density was read using the microplate reader (Spectromax Plus384) at a wavelength of 600nm and repeated at 24 hour intervals until the control wells reached an optical density of 1.0OD. The MIC values correspond to the highest compound dilution with no measurable growth as determined from OD readings compared to both negative and control measurements (Standards, 2000) (Standard, 2002) (Venugopal et al., 2010).

5. Conclusion

Our laboratory has previously reported the development of a series of novel antimicrobial peptides (AMPs) that incorporate unnatural amino acids into their primary sequence to impart specific three-dimensional physicochemical properties onto the peptide (Hicks et al., 2007,) (Bhonsle et al., 2007) (Venugopal et al., 2010) (Russell et al., 2010). Those AMPs exhibited low µM to nM in-vitro MIC antibacterial activity against several strains of Gram positive, Gram negative and mycobacterium. Many also exhibited very low hemolytic activity and low acute in vivo toxicity (125 mg/kg) in mice; these AMPs have also exhibited excellent metabolic stability in pooled human liver microsomes for up to 60 minutes (unpublished results). (Hicks et al., 2007)(Venugopal et al., 2010). The first generation AMPs focused on the incorporation of three Tic-Oic dipeptide units separated by an amino acid

spacer (Spacer #1) and either a charged residue (Spacer 2) or a hydrophobic residue to control the conformational physicochemical properties of the AMP. Unnatural amino acids were selected for incorporation into the primary sequence of the AMP to provide peptide chemists with a "toolbox" of new functionality. This "toolbox" would allow for the development of novel peptides with specific physicochemical properties that will interact with cell membranes in novel ways thus providing entry to the synthesis of organism specific AMPs. The incorporation of unnatural amino acids into the peptide sequence of an AMP offers several advantages over an AMP consisting exclusively of naturally occurring amino acids. 1) Unnatural amino acids inherently exhibit greater metabolic stability. Therefore, incorporation of unnatural amino acids into the primary sequence generally results in an increase in metabolic stability compared to peptides consisting of only natural amino acids (Toke, 2005, Hancock and Lehrer, 1998). 2) The use of the conformationally restrained amino acids (Tic an Oic) reduces the local flexibility of the peptide backbone and thus reduces the total conformational space that may be sampled by the peptide during lipid binding.

Here in the in vitro antibacterial activity and physical characterization of a new series of AMPs incorporating six Tic-Oic dipeptide units as well as four spacers, A, B, C and D on either side of the intervening hydrophobic and charged residues as well as a fifth spacer, E, which defines the distance between the polypeptide backbone and the positively charged side chain amine group is reported. This new series of AMPs is overall less active than the series containing only three Tic-Oic dipeptide units and two spacers. However, the new analogs exhibit far greater bacteria strain selectivity than the previously reported analogs. Induced calcein leakage studies, CD and ITC investigations indicate that the analogs containing six Tic-Oic dipeptide units and the additional spacers interact with POPC and 4:1 POPC/POPG LUVs and SUVs somewhat differently than the previous analogs. These differences could account for the observed differences in bacteria strain selectivity. Induced calcein leakage studies, CD and ITC studies also indicate that the five spacers, A, B, C, D, and E contribute differently to organism selectivity and potency. For example Spacer D completely eliminates any activity, while Spacer A and E seem to play more important roles in defining organism selectivity and potency.

What is clear from this study is that correct positioning of amino acids that influence the conformational and physicochemical properties that are favorable to interactions with a membrane with a specific chemical composition is critical to obtain organism selectivity and potency.

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