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Novel antimicrobial peptides that exhibit activity against select agents and other drug resistant bacteria

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ABSTRACT

One of the greatest challenges facing modern medicine is the evolution of drug resistant strains of bacteria. In addition to traditional methods of exposure to traditional bacterial organisms there is a growing concerned of the use of bacteria as bio-terrorism agents. To counter the evolution of drug resistant and potential bio-terrorism bacterial agents new antibiotic drugs must be developed. One potential source of new therapeutic agents that act via a novel mechanism of action are natural and synthetic antimicrobial peptides (AMPs). In our laboratories we have developed a series of AMPs incorporating the un-natural amino acids Tic-Oic to impart organism selectivity and potency while increasing metabolic stability. Herein the in vitro activity of these peptides, including ten new compounds, against eight potential bio-terrorism bacterial agents and three other bacterial strains is presented and discussed. These peptides exhibit a wide range of organism potency and selectivity. Calcein fluorescence leakage and circular dichroism studies were conducted to confirm that these peptides interact with zwitterionic and anionic liposomes.

1. Introduction

One of the research focus areas in our laboratories is the development of novel antimicrobial peptides as potential antibiotic therapeutic agents.[1] This is due to the dramatic and continued evolution of drug resistant strains of bacteria, which has created an international crisis in health care.[2,3] This crisis has resulted in an intensive research effort to develop a new class of compounds that exhibit novel mechanisms of antibacterial activity.[2,4–6] As a class, natural and synthetic antimicrobial peptides exhibit a very high potential as new therapeutic agents because of their novel mechanisms of antibiotic activity, coupled with the difficulty of bacteria to develop resistance to them.[5,7–9] The most promising applications of AMPs are currently focused on their clinical development as topical antibiotics.[10]

Antimicrobial peptides (AMP) have evolved in almost every class of living organisms as a host defense mechanism against invading micro-organisms.[11–13] These compounds are also considered to be key players in innate immunity.[14,15] AMP's are generally small peptides consisting of 5–50 amino acid residues and are highly positively charged (+3 to +9)[16] amphipathic molecules with well defined hydrophobic and hydrophilic regions.[9,17] AMP's found in nature exhibit a wide range of size, structure and amino acid sequence with amphiphilicity and positive charge as the only common factor between them.[18] As of January 2009, more than 1330[19] natural and synthetic antimicrobial peptides have been characterized and have shown, a wide range of biological activity.[20]

Originally all AMPs were believed to exhibit their antibiotic activity via membrane disruption, however there is growing evidence that some AMPs exhibit their antibiotic activity by interacting with a multitude of intracellular targets.[10] The exact mechanism of cytotoxicity of these peptides is currently hotly debated in the literature[21] with AMP's divided into two mechanistic classes, membrane-disruptors and non-membrane-disruptors.[9,10,21,22] Five different mechanisms of membrane disruption have been proposed at one time or another to explain the antibiotic activity of these peptides.[13,23–25] Independent of the exact mechanism of membrane disruption a specific 'threshold crossing' concentration of the AMP is

Abbreviations: Ahx, 6-aminohexanoic acid; AMP, antimicrobial peptide; βAla, beta alanine; Dpr, diaminopropionic acid; Fpa, 4-fluorophenylalanine; Gaba, gama aminobutyric acid; ITC, isothermal titration calorimetry; LUV, large unilamellar vesicles; Oic, octahydroindolecarboxylic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt); Tic, tetrahydroisoquinolinecarboxylic acid.

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required for antibiotic activity.[18,26–28] The 'threshold crossing' concentration is very different than the solution concentration of the peptide, it is the concentration required on the surface to disrupt the membrane.[18,26–28] Melo et al. report, 'the local peptide concentration in a model bacterial membrane is approximately 10,000 times higher than its concentration in the aqueous phase'.[18] The observed MIC values for a particular AMP is the measurable macroscopic threshold indicating the onset of antibiotic activity, thus the relationship between a low lipid to peptide ratio and the MIC values clearly indicate that a high membrane coverage by the peptide may be a requirement for antibiotic activity.[18]

The selectivity of AMP's for prokaryotic versus eukaryotic cells is believed to be derived from the difference in the chemical compositions of their respective membranes.[12] Hancock and co-worker[21] 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.[29] Other differences also exist including membrane composition (sterols, lipopolysaccharide, peptidoglycan etc.)[13] structure, transmembrane potential and polarizability. These differences are in part responsible for the observed selectivity of some AMPs for bacterial versus mammalian cells.[13,30] Melo et al. have characterized the physical properties of bacterial membranes that are conducive for AMP interactions.[18]

In addition to the differences between eukaryotic and prokaryotic cells, 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.[12,31] 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.[13,21]

In our laboratory we have focused on developing antimicrobial peptides that contain un-natural amino acids to control conformation and physicochemical properties[1] and to take advantage of the inherent metabolic stability of un-natural amino acids.[9,32] Our original skeletal design of an un-natural AMP incorporated placement of multiple L-Tic-L-Oic dipeptide units into the polypeptide backbone to induce an ordered structure onto the peptide.[1] Kyle et al. reported, using NMR and molecular modeling methods, that the dipeptide consisting of the un-natural amino acids (D)-Tetrahydroisoquinolinecarboxylic acid (Tic) and (L)-Octahydroindolecarboxylic acid (Oic) when placed in positions $i+1$ and $i+2$ of a four amino acid sequence induced a β-turn.[33] The AMPs developed in our laboratory the Tic-Oic units are connected via one or two amino acid spacers 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.[1,34]

Since the chemical compositions of the membranes of different bacterial strains vary greatly[12,31,35] the resulting physicochemical surface properties presented by the membrane to the external environment will therefore be different as well as specific for each type of bacterial strain.[34] It has been clearly demonstrated in the literature that the physicochemical properties of charge, hydrophobicity and conformation, among others, are known to play a major role in defining antimicrobial activity and organism selectivity.[9,12,13,21] Our research hypothesis evolved from the above assertion, which in its simplest form states; the 3D-physicochemical surface properties of target cell membrane interact with the 3D-physicochemical surface properties of the approaching AMP in a very specific way (via bioactive conformation) thus defining the resulting organism selectivity and potency.[34] A 3D-QSAR analysis has shown that within this series of peptides, specific and different physicochemical properties are responsible for anti-bacterial activity and selectivity against two different bacteria. Based on the 3D-QSAR there are six physicochemical properties that are responsible for the majority of the antibiotic activity against *Staphylococcus aureus*, while five different physicochemical properties are responsible for the majority of the antibiotic activity against *Mycobacterium ranae*.[34] These results support the hypothesis that for any particular AMP, organism selectivity and potency are controlled by the chemical composition of the target cell membrane and the resulting physicochemical surface properties presented to the attacking AMP.[34]

Many of the peptides included in this study have exhibited broad spectrum in vitro activity against the following bacterial strains: (1) *Salmonella typhimurium* (ATCC 13311) Gram-negative,[36,37] (2) *S. aureus*—Methicillin/Gentamicin/Tetracycline Resistant (ATCC 33592) Gram-positive,[38] (3) *Bacillus subtillis* (ATCC 43223) Gram-positive,[36,37] (4) *M. ranae* (ATCC 110)[36,37] at the high nM to low μM concentration.[1] Herein we report the in vitro activity of 22 compounds (including 10 new analogs) against a series of select bacterial biological warfare agents and drug resistant bacteria.

2. Results and discussion

The basic skeleton of the AMPs based on three Tic-Oic dipeptide units is given in Figure 1. Spacer #1 defines the distance between the two Tic-Oic dipeptide units. This Spacer is involved in defining the flexibility of any induced turn or helical structure. Spacer #2 defines the distance between the polypeptide backbone atoms and the positively charged side chain nitrogen. Spacer #2 is also involved in determining the overall surface charge density as well as the distance between the peptide backbone and the membrane surface. Spacer #3 defines the distance between the last Tic residue and the C-terminal Lys residues. Spacer #3 provides additional conformational flexibility for binding to the cell membrane surface. The basic skeleton of the AMPs based on six Tic-Oic dipeptide units is given as a cartoon in Figure 2. Spacer A defines the distance between the polypeptide backbone atoms and the positively charged side chain nitrogens. Spacer B, a Gly residue defines, the distance between the two Tic-Oic dipeptide units and is located on the N-terminal side of the charged residue in the primary sequence of the peptide.

The results for the in vitro antibacterial activity (MIC in μM concentration) against 11 bacterial strains are given in Table 1. The amino acid sequences for each of the 22 AMPs tested are given in Table 2.

2.1. Membrane interaction studies

The primary methods used to investigate an AMPs mechanism of antibacterial action is observing how they interact with model membranes of bacterial cells.[9,10] We have previously investigated using circular dichroism (CD), isothermal calorimetry (ITC) and calcein leakage assays the interactions that occur between compound **23** and model membranes.[39] POPC liposomes were used as a simple model for eukaryotic membranes[40–42] and 4:1 POPC/POPG liposomes were used as a simple model for prokaryotic membranes.[42,43] CD, ITC, and calcein leakage data clearly indicate that compound **23** interacts via very different mechanisms with the two different liposome membranes.[39] Compound **23** exhibits weaker binding and induces less calcein leakage in POPC liposomes than POPC/POPG (4:1 mole ratio) liposomes. The predominant binding mechanism to POPC appears to be limited to surface inter-

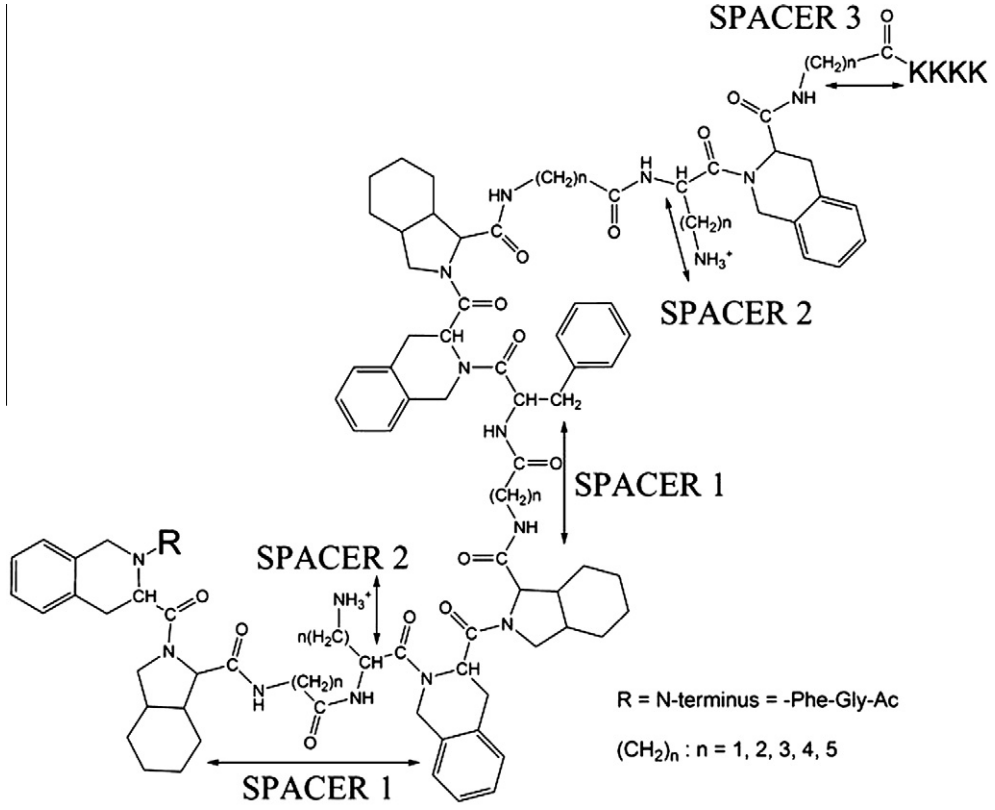

Figure 1. Skeleton of the analogs containing three Tic-Oic dipeptide units defining SPACER #1, SPACER #2 and SPACER #3.

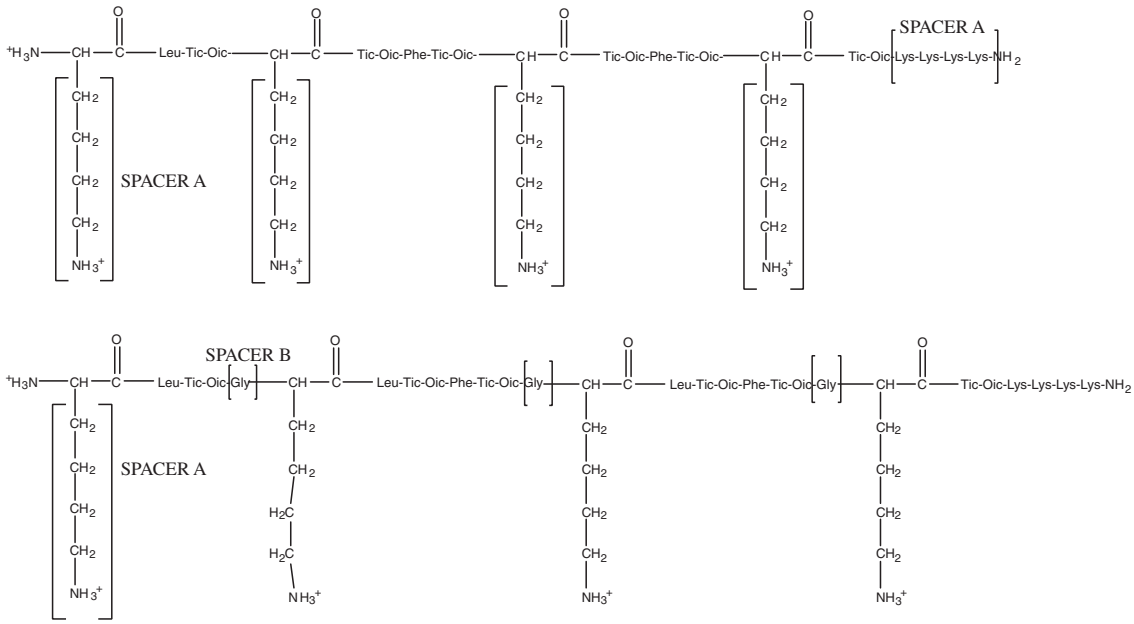

Figure 2. Cartoon description of the analogs containing six Tic-Oic dipeptide units defining SPACER A and SPACER B.

actions while the mechanism of binding to 4:1 POPC/POPG most likely involves some type of pore formation.[39] ITC data supports two different binding mechanisms of compound **23** to the two different liposomes.[39] Also CD studies indicated that compound **23** adopts different conformations on binding to the two different liposomes.[39] This investigation strongly supports our hypothesis that the analogs containing three Tic-Oic dipeptide units most likely exhibit their antibacterial activity by membrane disruption.

2.1.1. Calcein leakage studies

Calcein fluorescence leakage experiments were conducted to evaluate the potential for several of these compounds (four compounds containing three Tic-Oic dipeptide units and three compounds containing six Tic-Oic dipeptide units) to induce leakage and/or pore formation of POPC and 4:1 POPC/POPG liposomes. Peptide induced calcein leakage monitored through fluorescence is a well documented technique for probing AMP activity.[44,45] Calcein

Table 1
In vitro activity MIC activity (μM) against specific bacterial strains[a]

Compd #	*Acinetobacter baumanii* ATCC 19606	*Acinetobacter baumanii* WRAIR	*Staphylococcus aureus* ATCC 33591 (MRSA)	*Yersinia pestis* CO92	*Brucella melitensis* 16 M	*Brucella abortus* 2308	*Brucella suis* ATCC 23445	*Bacillus anthracis* AMES	*Francisella tularensis* SCHU-S4	*Burkholderia mallei* ATCC 23444	*Burkholderia pseudomallei* BURK003 (1026b)
22	40.5	162	162	162	162	500	162	40.5	81	162	162
23	3.2	3.2	205	205	205	205	205	12.8	100	205	205
29	3.1	3.1	196	196	196	196	196	12.5	196	196	196
35	3.2	3.2	208	3.2	26.00	26.00	26.00	0.2	0.01	208	208
36	1.5	6.2	200	200	200	200	200	25	100	200	200
37	5.90	2.90	187	23.40	93	12	47	1.50	23.4	187	187
38	6.30	6.30	25.30	101	202	101	13.00	0.75	202	202	202
40	5.80	2.90	46.60	46.60	46.60	>186	93	0.71	186	186	186
41	6.50	6.50	52.40	26.20	104	209	13.00	0.81	104.80	209	209
42	3.10	6.20	101	202	202	202	202	12.60	202	202	202
43	6.60	6.60	53.00	26.50	106	106	106	0.82	106	212	212
45	3.60	3.60	114	228	228	228	228	14.80	228	228	228
46	3.40	3.40	109	219	219	219	219	6.80	219	219	219
50	6.20	6.20	199	199	199	199	199	12.40	199	199	199
52	3.00	6.10	196	48.90	196	196	196	48.90	196	196	196
53	3.40	3.40	220	220	220	220	220	6.80	220	220	220
56	5.98	24.00	96	192	192	192	192	5.98	192	192	192
61	12.80	12.80	102	13.10	51	51	25.60	0.40	51	204	204
62	3.20	3.20	207	207	207	207	207	26.00	104	207	207
64	6.70	3.40	54	27.00	54	27.00	>216	27.00	0.02	0.02	0.02
70	9.30	150	150	150	150	150	150	150	150	150	150
71	9.77	156	156	156	156	156	156	156	156	156	156
74	4.60	144	17.90	144	144	144	144	8.97	144	144	144
Dox	0.56	0.56	17.60	0.56	0.56	0.56	0.56	0.27	0.09	0.09	0.09
Amp	179	1432.00	93.00	0.80	11.00	11.00	0.80	0.40	0.09	0.09	0.09

Dox = doxycycline, Amp = ampicillin.
[a] MIC drug concentrations based on serial dilutions from a maximum concentration of 500 μg/mL. Mic values were then converted to μM concentrations.

is a water soluble relatively large molecule and its release from a liposome is assumed to involve the formation of some type of pore in the liposome, or disruption of the surface of the liposome.[46,47]

Induced calcein leakage was measured at peptide concentrations of 4, 8, 12, 16, and 20 μM against POPC and 4:1 POPC/POPG liposomes. While the induced calcein leakage of the analogs containing three Tic-Oic dipeptide units was somewhat concentration dependant, leakage induced by the analogs containing six Tic-Oic dipeptide units were not concentration dependant. In fact the lowest concentration of peptide (4 μM) induced almost 100% leakage.

Table 2
Amino acid sequences for the AMP tested for antibacterial activity

Compd #	Amino acid sequence
	Analogs containing 3-Tic-Oic dipeptide units
23	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKKK-$CONH_2$
29	Ac-Gaba-F-Tic-Oic-Gaba-K-Tic-Oic-Gaba-F-Tic-Oic-Gaba-K-Tic-KKKK-$CONH_2$
35	Ac-GF-F-Oic-GK-F-Oic-GF-F-Oic-GK-F-KKKK-$CONH_2$
36	Ac-βAla-F-Tic-Oic-βAla-K-Tic-Oic-βAla-F-Tic-Oic-βAla-K-Tic-KKKK-$CONH_2$
37	Ac-Ahx-F-Tic-Oic-Ahx-K-Tic-Oic-Ahx-F-Tic-Oic-Ahx-K-Tic-KKKK-$CONH_2$
38	Ac-F-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-KKKKKK-$CONH_2$
40	Ac-Gaba-F-Tic-Oic-Gaba-K-Tic-Oic-Gaba-F-Tic-Oic-Gaba-K-Tic-KKKKK-$CONH_2$
41	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-Orn-Orn-Orn-Orn-$CONH_2$
42	Ac-G-Fpa-Tic-Oic-GK-Tic-Oic-G-Fpa-Tic-Oic-GK-Tic-KKKK-$CONH_2$
43	Ac-GF-Tic-Oic-G-Orn-Tic-Oic-GF-Tic-Oic-G-Orn-Tic-Orn-Orn-Orn-Orn-$CONH_2$
45	Ac-GF-Tic-Oic-G-Dpr-Tic-Oic-GF-Tic-Oic-G-Dpr-Tic-Dpr-DprDpr-Dpr-$CONH_2$
46	Ac-βAla-Fpa-Tic-Oic-βAla-Dpr-Tic-Oic-βAla-Fpa-Tic-Oic−βAla-Dpr-Tic-Dpr-Dpr-Dpr-Dpr-$CONH_2$
50[a]	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-βA-KKKK-$CONH_2$
52[a]	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-Ahx-KKKK-$CONH_2$
53[a]	Ac-GF-Tic-Oic-G-Dab-Tic-Oic-GF-Tic-Oic-G-Dab-Tic-Dab-Dab-Dab-Dab-$CONH_2$
56[a]	Ac-GF-Tic-Oic-GR-Tic-Oic-GF-Tic-Oic-GR-Tic-RRRR-$CONH_2$
61[a]	Ac-KKKK-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-$CONH_2$
62[a]	Ac-GF-Oic-Oic-GK-Oic-Oic-GF-Oic-Oic-GK-Tic-KKKK-$CONH_2$
64[a]	Ac-GF-Tic-Oic-GK-Tic-Oic-GF-Tic-Oic-GK-Tic-KKK-$CONH_2$
	Analogs containing 6-Tic-Oic dipeptide units
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[a]	H_2N-KL-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-F-Tic-Oic-K-Tic-Oic-KKKK-$CONH_2$
71[a]	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-$CONH_2$
74[a]	H_2N-KL-Tic-Oic-GK-Tic-Oic-F-Tic-Oic-GK-Tic-Oic-F-Tic-Oic-GK-Tic-Oic-KKKK-$CONH_2$

Dpr, 2,3-diaminopropionic acid; Dab, 2,4-diaminobutanoic acid; ßAla, beta alanine; Gaba, gama aminobutyric acid; 6-Ahx, 6-aminohexanoic acid; Fpa, 4-fluorophenylalanine; Tic, tetrahydroisoquinoline-3-carboxylic acid; Oic, octahydroindole-3-carboxylic acid.
[a] Indicate 11 new compounds not previously reported in the literature.

As an example the effect of compound **74** on inducing calcein leakage from both POPC and 4:1 POPC/POPG liposomes is given in Figure 3. A comparison of the ability of the four compounds containing three Tic-Oic dipeptide units and three compounds containing six Tic-Oic dipeptide units at a peptide concentration of 12 μM to induce leakage and/or pore formation of POPC and 4:1 POPC/POPG liposomes is shown in Figure 4. As can be seen in Figure 4, the shorter analogs containing three Tic-Oic dipeptide units, compounds **23** and **36** (Spacer #1 is Gly and β-Ala respectfully) induce the least amount of calcein leakage from both POPC and 4:1 POPC/POPG liposomes. Increasing the overall length of the peptide backbone in compounds **29** and **37** (where Spacer #1 is a Gaba residue and a Ahx residue, respectively) increases the amount of calcein leakage from both POPC and 4:1 POPC/POPG liposomes. Increasing the length of the peptide backbone with the incorporation of six Tic-Oic units induces complete leakage of calcein. The Calcein fluorescence leakage data clearly indicates that both, the analogs containing three Tic-Oic dipeptide units and the analogs containing six Tic-Oic dipeptide units interact with both neutral and zwitterionic liposomes and induce calcein leakage from the liposomes. The exact mechanism of action is not fully known at this time.

2.1.2. Circular dichroism studies

The application of CD spectroscopy to monitor conformational changes in peptides and proteins is well documented.[48–50] The technique is very sensitive and the observed spectrum represents the linear combination of all conformers present in solution.[48,51] The CD spectra of compounds **22**, **70**, **71**, and **74** are shown in Figure 5, POPC is shown at the top and 4:1 POPC/POPG liposomes is shown at the bottom of the figure. The CD spectra of compounds **22**, **70**, and **71** in buffer (shown in both parts of Fig. 5) are different from the CD spectra of these three compounds in the presence of POPC and 4:1 POPC/POPG liposomes. The observation of changes in the intensity or shape of the CD spectrum of a peptide in the presence of a liposome indicates that the peptide is adopting different conformations on interacting with that particular liposome as compared to another environment such as a buffer.[48,51] Only minor changes in the CD spectra of compound **74** in liposomes compared to buffer are observed, and in both cases these changes are observed below 200 nM. This observation suggests a minor conformational change on binding to the liposomes. However, CD data clearly indicates that these four analogs interact in some manner with liposomes which is consistent with the calcein leakage results. Due to high percentage of un-natural amino acids incorporated into the peptide under investigation here, no quantitative estimation of secondary structural features are possible.

2.2. In vitro antibacterial activity

2.2.1. Six Tic-Oic dipeptide unit analogs

Compounds **22**, **70**, **71**, and **74** are very active in vitro against *Acinetobacter baumanii* (MIC values in the range of 40.5–4.6 μM, compared to MIC values of 0.56 μM for doxycycline, and 179 μM for ampicillin) however they are not active against drug resistant strains of *A. baumanii* (MIC greater than 150 μM) Ampicillin is also inactive against this same strain with a MIC value of 1432.00 μM. Since all of these peptides are believed, based on calcein leakage and CD data, to act via the mechanism of membrane disruption, this suggests that the membrane composition of the drug resistant *A. baumanii* is somehow different from the ATCC 19606 strain. Compound **22** which contains two positively charged amino acids (one Arg and one Lys) at the C-terminus is much less active against normal *A. baumanii* (40.5 μM) as compared to compound **70** (9.30 μM) which is identical except for the placement of four Lys residues at the C-terminus. The incorporation of the two additional Lys residues results in a fourfold increase in activity. This indicates that positive charge density at the C-terminus is of critical importance in the interactions of these peptides with the membranes of normal *A. baumanii*. We have previously reported a similar increase in activity against *M. ranae* with increasing positive charge at the C-terminus of the peptide.[1,34] Compounds **70** and **71** exhibited activity of 9.30 and 9.77 μM, respectively, against normal *A. baumanii*.

The overall in vitro activity of compounds **22**, **70**, **71**, and **74** is very interesting. As seen in Table 1, these compounds are relatively inactive against the 11 bacterial strains tested except for normal *A. baumanii*. The two exceptions are compounds **22** and **74**. Compound **22** exhibits moderate in vitro activity against the AMES strain of *Bacillus anthracis* (40.5 μM) and against *Francisella tularensis* SCHU-S4 (81 μM). Compound **74** exhibits good activity against the AMES strain of *B. anthracis* (8.97 μM) and it exhibits the highest activity of all the compounds screened against *S. aureus*—(MRSA) (17.90 μM). In fact on a molar basis compound **74** is equally potent as doxycycline, (MIC of 17. 60 μM) while all other analogs in this series (**22**, **70**, **71**, **74**) are inactive against *S. aureus*—(MRSA). Compound **74** is the only one of the analogs that contains six Tic-Oic units that also contains a Gly residue for Spacer B. This data suggests the following for activity of these compounds against *S. aureus*—(MRSA). The increased molecular flexibility obtained by incorporation of three Gly residues for Spacer B must allow compound **74** to adopt a stable conformation on binding to the surface of *S. aureus*—(MRSA) that favors membrane disruption that is not accessible to the other analogs.

2.2.2. Three Tic-Oic dipeptide unit analogs

The remaining analogs in this study all contain three Tic-Oic dipeptide units except for compound **35** which contains the Phe-Oic dipeptide unit instead. (Phe is essentially the open chain analog of the bicyclic amino acid Tic, with very similar hydrophobicity but greater molecular flexibility as compared to Tic) Compound **35** exhibited excellent in vitro activity against both *B. anthracis* AMES (MIC 0.20 μM, compared to MIC values of 0.27 μM for doxycycline, and 0.40 μM for ampicillin) and *F. tularensis* SCHU-S4 (0.01 μM compared to MIC values of 0.09 μM for doxycycline, and 0.09 μM for ampicillin) and good activity against *Yersinia pestis* CO92, (3.20 μM) *Brucella melitensis* 16 M (26 μM), *Brucella abortus* 2308 (26 μM), *Brucella suis* 23444 (26 μM) and both strains of *A. baumanii* (ATCC 19606 and WRAIR isolate) (3.20 μM).

The remaining compounds exhibited diverse activity against all of the bacterial strains. The activity against specific bacterial strains is discussed below.

2.2.2.1. *A. baumanii.* The evolution of drug resistant strains of *A. baumanii* illustrates the growing crisis associated with drug resistant Gram-negative bacteria and the critical need for the development of novel therapies to address this world-wide epidemic.[10,52] All of the compounds listed in Table 1 exhibited very good activity against both strains of *A. baumanii*. These compounds exhibited activity against *A. baumanii* ATCC 19606, with MIC values in the range of 1.5–40.5 μM, which was better than that reported for ampicillin (179 μM). These compounds exhibited activity against the clinical drug resistant isolate *A. baumanii* **WRAIR** with MIC values in the range of 2.9–24 μM. These compounds are much more active than ampicillin against this strain (MIC = 1432 μM).

Other researchers[53] have reported antimicrobial peptide activity against *A. baumanii* including Batoni et al.,[54] who reported that analogs of the peptide human beta defensin-3 exhibited MIC values of 0.78–4 μg/mL against *A. baumanii* and Andra et al.,[55] reported that analogs of the peptide NK23c exhibited a range of MIC values of 2 to >256 μM against *A. baumanii* ATCC 19606. The best MIC values exhibited by these compounds are very similar to the best MIC values exhibited by our compounds.

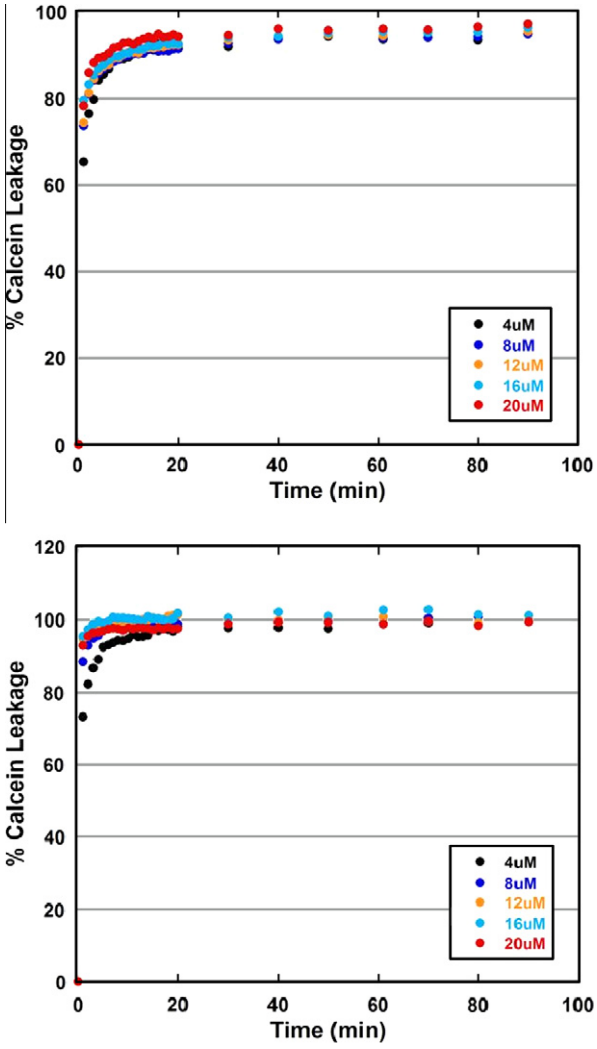

Figure 3. The time dependence of the calcein leakage fluorescence induced by compound **74** (contains six Tic-Oic dipeptide units) in liposomes. (Top) POPC liposomes (Bottom) 4:1 POPC/POPG liposomes. For all studies the liposomes concentration was 36 μM. A measurement was taken every minute for the first 20 min of the experiment and every 10 min after until the emission intensity showed no further significant increase (approximately 90 min). One hundred percent leakage was determined with the addition of 50 μL of 10% Triton X. The apparent percent leakage was calculated using the following equation: % leakage = $[1 - (F_0/F_T)] \times 100\%$, where F_0 and F_T are the initial fluorescence before introduction of peptide and after the addition of Triton X, respectively.[87,101–104] The study was repeated with a peptide concentration of 4, 8, 12, 16 and 20 μM, clearly induction of calcein leakage is not a peptide concentration dependant event.

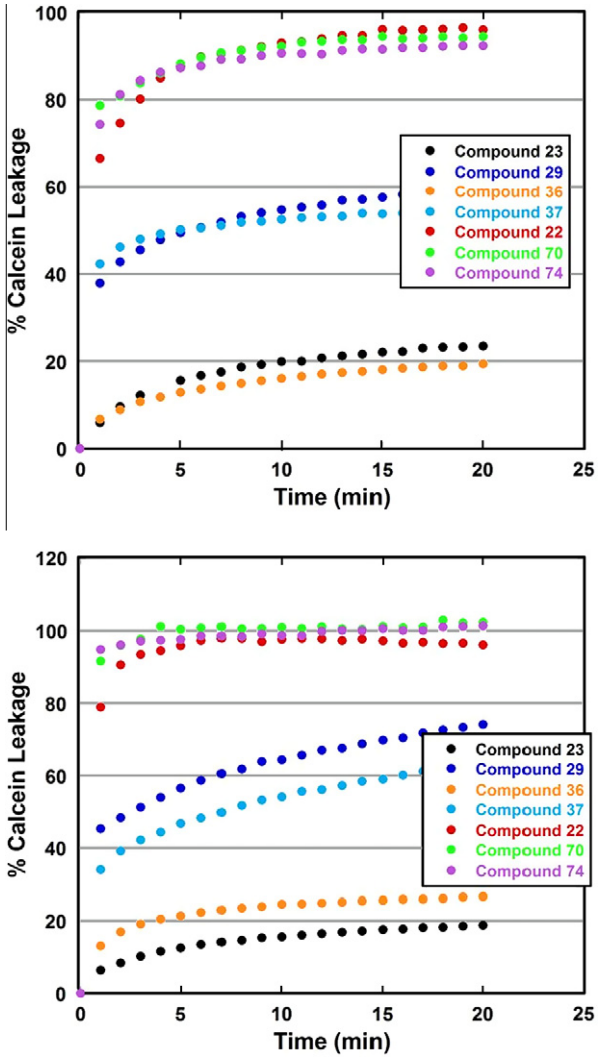

Figure 4. The time dependence of the calcein leakage fluorescence induced by a representative set of compounds. Compounds, **23**, **29**, **36** and **37** (containing three Tic-Oic dipeptide units and compounds **22**, **70** and **74** (contains six Tic-Oic dipeptide units) all at a peptide concentration of 12 μM (Top) POPC liposomes (Bottom) 4:1 POPC/POPG liposomes. For all studies the liposomes concentration was 36 μM. A measurement was taken every minute for the first 20 min of the experiment and every 10 min there after until the emission intensity showed no further significant increase (approximately 90 min). One hundred percent leakage was determined with the addition of 50 μL of 10% Triton X. The apparent percent leakage was calculated using the following equation: % leakage = $[1 - (F_0/F_T)] \times 100\%$, where F_0 and F_T are the initial fluorescence before introduction of peptide and after the addition of Triton X, respectively.[87,101–104] As can be seen the shorter analogs containing three Tic-Oic dipeptide units, compounds **23** and **36** (Spacer #1 is Gly and β-Ala respectfully) induce the least amount of calcein leakage form both POPC and 4:1 POPC/POPG liposomes. Increasing the over all length of the peptide backbone in compounds **29** and **37** (Spacer #1 is a Gaba residue and a Ahx residue respectfully) increase the amount of calcein leakage from both POPC and 4:1 POPC/POPG liposomes. Increasing the length of the peptide backbone with the incorporation of six Tic-Oic units induces complete leakage of calcein. From this data it must be concluded that the analogs containing six Tic-Oic dipeptide units interact very strongly with both liposomes.

2.2.2.2. *S. aureus*—MRSA. Spacer #1 appears to play no role in the activity against *S. aureus*—(MRSA). Compound **23** which has Spacer #1 as a Gly residue, compound **36** with Spacer #1 being a β-Ala residue, compound **29** with Spacer #1 being a Gaba residue, and compound **37** with Spacer #1 being the much larger 6-amino-hexanoic acid, all exhibited very weak activity (MIC values of 205, 200, 196, and 187 μM, respectively) against *S. aureus*—(MRSA). This observation is consistent with what was observed for these four compounds against *S. aureus* ME/GM/TC where the in vitro activity (MIC values 3, 10, 100, 10, respectively) was observed to be independent of Spacer #1 length.[1]

As stated previously, compound **23**, which contains four Lys residues at the C-terminus, exhibited very weak activity (MIC value 205 μM) against *S. aureus*—(MRSA) while compound **64** which contains only three Lys residues at the C-terminus, exhibited much better activity (MIC values of 54 μM) against *S. aureus*—(MRSA). This observation of the number of positively charged residues at the C-terminus effecting organism potency and selectivity was previously documented by the 3D-QSARs generated for members of this class of compound against *S. aureus* and *M. ranae*.[34] It is interesting to point out that transposing the four Lys residues from the C-terminus to the N-terminus in compound **61** (keeping the rest of the amino acid sequence the same) resulted in an analog with intermediate activity (MIC values of 108 μM) against *S. aureus*—

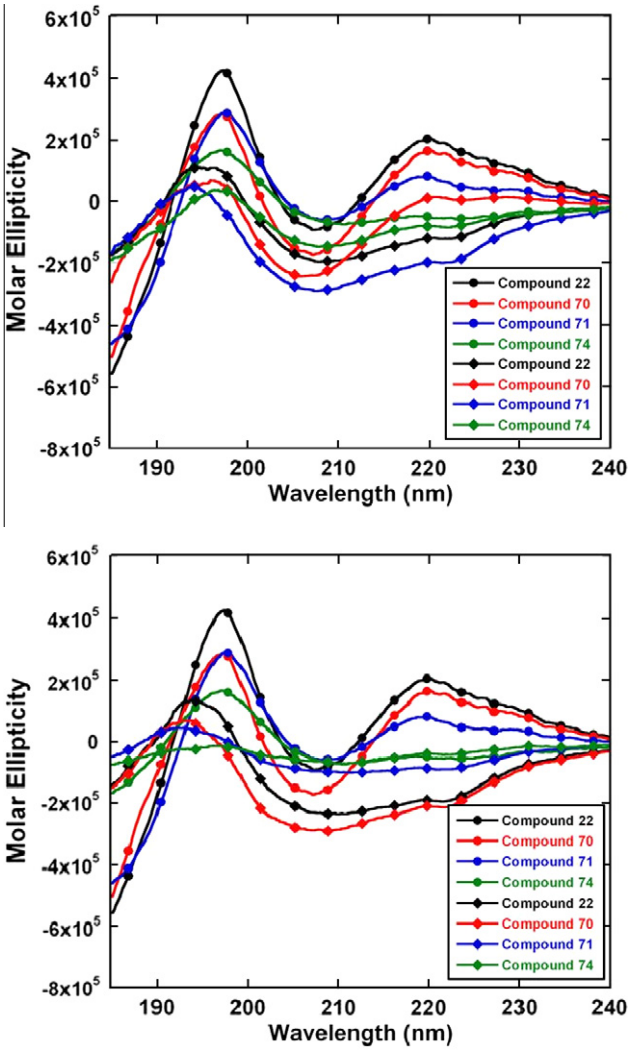

Figure 5. Far-UV Circular Dichroism spectra of compounds **22**, **70**, **71** and **74** (1 mg/mL) in the presence of (Top) Indicated with diamonds 10 mM 4:1 POPC LUVs dissolved in 40 mM phosphate buffer (pH 6.83), indicated with circles—buffer only. (Bottom) Indicated with diamonds 10 mM 4:1 POPC/POPG LUVs dissolved in 40 mM phosphate buffer (pH 6.83), indicated with circles—buffer only. All CD spectra were obtained by acquiring eight 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, 1 nm bandwidth, data pitch 0.2 nm, response time 0.25 s and 5 mdeg sensitivity at room temperature (~25 °C).

(MRSA). Converting the four C-terminal Lys residues to Orn residues in compound **41** results in moderate in vitro activity (MIC value of 52.40 μM) against *S. aureus*—(MRSA). This modification reduces the number of carbon atoms in the side chain of the charged residue by one. Thus, probably reducing the conformational freedom of the binding process, as well as, bringing the positive charge density closer to the peptide backbone compared to the Lys analogs. Substitution of four Arg residues for the four Lys C-terminal residues in compound **56** resulted in a modest increase of in vitro activity against *S. aureus*—(MRSA) (MIC value of 96 μM). The side chain length for Arg and Lys differ by one carbon atom (Lys-4 carbons, Arg-3 carbons). However, the guanidino group of Arg has the positive charge delocalized over two nitrogen atoms, thus the charge density at the end of the side chain is dispersed. The delocalized charge of the guanidino group will interact differently with the cell membrane than the 'harder' or localized charge of the amine analogs of Lys. Replacement of all the Lys residues (the two within the sequence and the four C-terminal residues) with Orn (compound **43**) or Dpr (compound **45**) resulted in analogs somewhat more active (MIC values 53 and 114 μM, respectively), against *S. aureus*—(MRSA) as compared to compound **23**. This suggests that the optimum length for the positively charged side chain is three carbons (Orn residue) and lengthening the chain (Lys) or shortening the chain (Dpr-2 carbon atoms) decrease activity. It is interesting to note that changes in the hydrophobicity of the aromatic residues by replacing the two Phe residues with a 4-Fluoro-Phe residue resulted in a modest increase in the in vitro activity against *S. aureus*—(MRSA) (MIC value of 101 μM) compared to compound **23** with a MIC value of 205 μM.

Removing Spacer #1 from the sequence and placement of six Lys residues at the C-terminus in compound **38** produces a very active analog (25.30 μM) against *S. aureus*—(MRSA). In compound **29** a Gaba residue is used for Spacer #1 coupled with the incorporation of one additional Lys residue, to give a total of five Lys residues at the C-terminus, resulted in compound **40** which increases the in vitro activity against *S. aureus*—(MRSA) compared to compound **29** by a factor of 4 (MIC values of 46.60 and 196 μM, respectively). This data suggests that there exists a complex interaction between the overall molecular flexibility of the polypeptide sequence and the positive charge density at the C-terminus which defines the in vitro activity against *S. aureus*—(MRSA).

Other researchers[53] have reported antimicrobial peptide activity against methicillin-resistant *S. aureus* including Steffen et al.,[56] who reported that peptides DCD-1L SSL 23, SSL 25, and SSL 29 exhibited a MIC values of 1.7, >89, 19.9, and >69 μM, respectively; Bikker et al.[57] reported that analogs of the peptide drosocin exhibited a MIC value of >100 μM; De Smet and Contreras[58] reported that peptide HNP-1 and LL-37 exhibited MIC value of 21.2 μg/mL and 3.4 μg/mL, respectively and Zelezetsky et al.[59] reported that peptide protegrin 1 exhibited a MIC value of 1.5 μM. Clearly some of our peptides exhibit similar activity against *S. aureus*—(MRSA) as those sited here.

2.2.2.3. *Y. pestis*. The compounds tested exhibited a diversity of activity against *Y. pestis* CO92[60] with the Phe-Oic analog compound **35** being the most active (MIC value of 3.20 μM). Again compound **23** with four Lys residues at the C-terminus was only weakly active (MIC value of 205 μM) while compound **64** with only three Lys at the C-terminus was much more active with a MIC value of 27 μM.. Transposing the four Lys residues from the C-terminus to the N-terminus in compound **61** (keeping the rest of the amino acid sequence the same) resulted in an analog with increased in vitro activity (MIC value of 13.1 μM). Replacement of the four C-terminal Lys residues of compound **23** with four Orn residues in compound **41** or all Lys residues with Orn in compound **43** resulted in two equally potent analogs against *Y. pestis* (MIC values of 26.2 and 26.50 μM). It is interesting to note that compound **23** where Spacer #1 is a Gly residue, and compound **36** where Spacer #1 is a β-Ala residue, and compound **29** where Spacer #1 is a Gaba residue exhibited weak activity against *Y. pestis* (MIC values of 205, 200 and 196 μM, respectively) while compound **37** were Spacer #1 is 6-aminohexanonic acid exhibited relative potent in vitro activity against *Y. pestis* (MIC value of 23.40 μM). This is most likely due to the increased flexibility of the peptide backbone allowing much greater conformational change on binding to the cell membrane. Other researchers[53] have reported antimicrobial peptide activity against *Y. pestis* including Bikker et al.,[57] who reported that analogs of the peptide drosocin exhibited a MIC value of <100 μM, while Andra et al.[55] reported that analogs of the peptide NK23c exhibited a MIC value in the range of 16 to >256 μM. Clearly our peptides exhibit similar MIC values to reported in the literature against *Y. pestis*.

2.2.2.4. *B. anthracis*. All of the analogs containing three Tic-Oic units exhibited good to excellent in vitro activity with MIC val-

ues in the range of 48.90–0.40 μM against *B. anthracis*. Compound **23** where Spacer #1 is a Gly residue and compound **36** where Spacer #1 is a β-Ala residue, and compound **29** where Spacer #1 is a Gaba residue exhibited very similar activity against *B. anthracis* (MIC values of 12.8, 25, and 12.5 μM, respectively) while compound **37** where Spacer #1 is 6-aminohexanonic acid exhibited very potent in vitro activity against *B. anthracis* (MIC value of 1.5 μM). Removing Spacer #1 and adding two additional Lys residues to the C-terminus to yield compound **38** resulted in a very active analog with a MIC value of 0.7 μM. The importance of positive charge density on activity against *B. anthracis* is illustrated by the activity of compound **40** compared to compound **29**. The addition of one additional Lys residue at the C-terminus yielding a total of five Lys residues at the C-terminus results in MIC value of 0.71 μM compared to the previous MIC value of 12.5 μM. Replacing the four C-terminal Lys residues in compound **23** with Orn residues (while maintaining the two internal Lys residues) resulted in compound **41** with a MIC value of 0.81 μM. Replacing all Lys residues with Orn residues resulted in compound **43** (MIC value of 0.82 μM) which was equally potent as compound **41**. Replacement of all the Lys residues with Dpr residues in compound **45** resulted in a reduction in the in vitro activity of *B. anthracis* (MIC value of 14.80 μM) compared to that of compound **43**. Replacing all of the Lys residues in compound **23** with Dap residues in compound **53** resulted in a MIC value of 6.80 μM, an increase in activity compared to compound **23**, but a reduction of activity as compared to compound **43**. Placement of a β-Ala residue and an Ahx residue as Spacer #3 in compound **23** yielding in compounds **50** and **52** resulted in MIC values of 12.40 and 48.90 μM, respectively. Replacement of the four C-terminal Lys residues of compound **23** with four Arg residues yield compound **56** which exhibited a MIC value of 5.98 μM which is more potent than the parent compound **23** (MIC value of 12.8 μM). Reducing the number of Lys residues at the C-terminus from four in compound **23** to three in compound **64** reduces activity by approximately 50% to a MIC value of 27.0 μM. This is opposite of the trend observed for MRSA where reducing the number of C-terminal Lys resulted in an increase in activity compared to compound **23**. Transposing the four C-terminal Lys residues of compound **23** to the N-terminus of compound **61** increases the activity against *B. anthracis* by a factor of 32 (MIC value 0.4 μM) compared to compound **23**. This trend is again different that what was observed for the other Gram-positive strain MRSA where the increase in activity of compound **61** versus compound **23** was only twofold. Clearly the placement and the density of positive charge plays a major role in defining the in vitro potency of these compounds against *B. anthracis*. Other researchers[53] have reported antimicrobial peptide activity against *B. anthracis* including Loose et al.,[61] who reported that the peptides D28 and D51 exhibited a MIC value of 16 μg/mL while other analogs in this series exhibited MIC values in the range of 64 to >256 μg/mL; Bikker et al.[57] reported that analogs of the peptide drosocin exhibited a MIC value of >100 μM; Wang et al.[62] report that peptides protegrin 1 and HNP-1 exhibited a MIC values of 0.89 and 0.85 μg/mL, respectively. The peptides synthesized in our laboratory exhibit similar or improved activity against *B. anthracis* compared to other AMPs. The importance of the Tic-Oic dipeptide unit to the in vitro activity against *B. anthracis* is confirmed by compound **62** where the three Tic-Oic units are replaced with three Oic-Oic dipeptide units. The resulting activity is reduced to a MIC value of 26 μM.

2.2.2.5. Other strains. Very few of the analogs exhibited good activity against *Burkholderia mallei*, *Burkholderia pseudomallei* or *F. tularensis*. Only compound **64** exhibited excellent in vitro activity (0.02 μM) against all three organisms and compound **35** exhibited excellent in vitro activity (0.01 μM) against *F. tularensis*. At this time we cannot explain the observed isolated activity of these two compounds. Only a very few of the compounds tested showed reasonable in vitro activity against the three *Brucella* strains, *B. melitensis* 16 M, *B. abortus* 2308 and *B. suis* ATCC 23445. Compounds **23**, **36**, and **29** exhibited very weak in vitro activity (>180 μM) against the three strains. However compound **37**, where Spacer #1 is an Ahx residue, exhibited in vitro MIC values of 93, 12, and 47 μM, respectively. The observation that compound **37** is the most active of this series of four compounds was also observed for *B. anthracis* and *Y. pestis*. This is very interesting since two of the bacterial strains are Gram-negative and one is Gram-positive. In compound **43** where all of the Lys residues of compound **23** are replaced with Orn residues, the in vitro activity against all three strains is increased (MIC values of 106 μM) compared to compound **23** (MIC value 205 μM). Reducing the number Lys residues at the C-terminus from four to three in compound **64** resulted in a dramatic increase in activity (MIC values of 27, 54, and >216 μM, respectively) compared to compound **23** (MIC value of 205 μM against all three strains). Again this increase in activity compared to compound **23** was observed with MRSA and *Y. pestis*. Transposing the four C-terminal Lys residues of compound **23** to the N-terminus of compound **61** increases the activity against all three *Brucella* strains (MIC values of 51, 51, and 25.60 μM, respectively) compared to compound **23**. This increase in activity compared to compound **23** was also observed against *Y. pestis*, *B. anthracis* and MRSA. Other researchers[53] have reported antimicrobial peptide activity against *B. abortus* including Andra et al.,[55] who reported that analogs of the peptide NK23c exhibited a MIC value of 2 to >256 μM.

3. Materials and methods

Minimum Inhibitory Concentration (MIC) were determined for the following organisms; Plague (*Y. pestis*),[60,63] *Brucella*, (*B. melitensis*, *suis*, or *abortus*),[64,65] Anthrax (*B. anthracis*),[63,66,67] Glanders (*B. mallei*),[68–70] Melioidosis (*B. pseudomallei*),[71,72] *S. aureus*—MRSA[73,74] and Tularemia (*F. tularensis*)[63,75] *A. baumanii* and a drug resistant clinical isolate strain of *A. 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]

3.1. Preparation of bacteria samples

A small amount of the organism was streaked onto the appropriate agar plates and the plates Incubated at either 30 °C or 37 °C (depending on organism to be tested—Plague and Anthrax were incubated at 30 °C, *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 suspend 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 600 nm. The suspension was adjusted to obtain a volume of 5 mL containing a 1.0 OD solution. One 50 mL disposable Erlenmeyer flask containing 20 mL of broth with 1 mL of the organism dilution was then inoculated for 24–48 h (dependent on growth characteristics of the organism in question) in a shaking incubator rotating at 200 rpm.[76,78] After 24–48 h, the suspension was thoroughly mixed, the tubes centrifuged at high speed (2000×*g*) for 15 min, the supernatant removed, and the pelleted organisms resuspended to yield a total volume of 45 mL. This

was repeated for a total of three 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 1×10^5 cfu/mL with sufficient volume for the number of plates being used for the MIC determination.[76,78]

3.2. Minimum inhibitory concentration

A solution of Mueller Hinton Broth + IsoVitaleX (Bekton Dikinson, Inc.) was prepared containing 1% 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.[76,78] Using a robotic sample processor (Precision XS, Biotek Instruments, Inc.), 15 μL of HIBCD broth was transferred into all wells of a 384 well plate and the plate was then inoculated using 15 μL 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 600 nm and repeated at 24 h 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.[76,78]

3.3. Peptide synthesis

Peptide synthesis was performed manually using tBOC chemistry[79–83] (Synthetic Proteomics, Carlsbad, CA) or in an automated instrument using FMOC chemistry[81–84] (Peptide Core Facility in the School of Medicine of the University of North Carolina at Chapel Hill, Chapel Hill, North Carolina). Selection of synthetic method to synthesize the peptides was based on the techniques used by the specific facility.

Automated synthesis[85] was performed using a Biosearch model 9500 peptide synthesizer at approximately a 0.4–0.8 millimole scale giving rise to between 700 mg and 1.0 g of crude material. Amino acids were purchased from Novabiochem (EMD Chemicals, Gibbstown, NJ) as was the Rink amide (MBHA) resin. Solvents (dimethylformamide and methylene chloride (DMF and MECL)) were purchased from Fischer Scientific (Fair Lawn, NJ) and were low water synthesis/sequencing quality. Piperidine (99+%) was purchased from Sigma (St. Louis, MO) and diluted with DMF to make a 20% solution. Acetic anhydride was also purchased from Sigma. All other amino acids were purchased from Chem-Impex (Wood Dale, IL). Synthesis chemistry utilized diisopropylcarbodiimide (Sigma, St. Louis, MO) and 1-hydroxyl-7-azabenzo-triazole (HOAt, GL Biochem, Shanghai, China) as reagents for chain elongation. FMOC synthesis consisted of addition of a twofold excess of amino acid/HOAt from the amino acid reservoir of the synthesizer and simultaneous addition of DIPCDI/methylene chloride from its reservoir. Constant nitrogen mixing occurred for 12 min, the reaction vessel was drained and a second addition of amino acid/HOAt and DIPCDI was reacted for an additional 12 min. The resin was washed with a solution of 50% DMF/MECL, the reaction vessel drained, and an aliquot of 20% piperidine added and mixed with nitrogen for 5 min. After draining, a second aliquot of 20% piperidine was added and allowed to react for an additional 5 min. After draining and solvent washing, the cycle was repeated with the next amino acid.

Peptide cleavage from resin was performed for 90 min at room temperature with agitation utilizing a cleavage cocktail consisting of 95% trifluoroacetic acid (Halocarbon Industries, River Edge, NJ) with 2.25% water, 2.25% triisopropylsilane, and 0.5% 1,2 ethane dithiol (both from Sigma, St. Louis, MO). Cleaved peptide was filtered to remove resin and the filtrate precipitated in an ice bath with anhydrous ethyl ether (Mallinckrodt, Phillipsburg, NJ). The precipitate was spun in the cold, the supernatant decanted and discarded, and the precipitate resuspended in ether, centrifuged again, that supernatant also discarded, and the washed precipitate dried in vacuum and stored at 4 °C.

Some peptides were synthesized manually in fritted syringes (Supelco, Bellefonte, PA) using tBOC chemistry with either *N*-dimethylamino-1*H*-1,2,3-triazolo-4.5-b-pyridin-1-ylmethylene-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) or *N*-1*H*-benzotriazol-1-yl-dimethylaminomethylene-*N*-methylmethaniminium hexafluorophosphate *N*-oxide (HBTU) as chain elongation reagent in the presence of *N*,*N*-diisopropylethylamine (all purchased from Chem-Impex, Wood Dale, IL). Chain elongation was effected by addition of twofold excess of amino acid/HBTU (or HATU) to MBHA resin and addition of a twofold excess solution of DIPEA with nitrogen mixing for 12 min. A small aliquot of washed resin was assayed using the Kaiser test—if positive, the addition of activated amino acid/DIPEA was repeated. When the Kaiser test was negative, the washed resin was treated with a solution of 10% acetic anhydride in DMF for 5 min and extensively washed with DMF. The BOC group[86] was removed by treatment of the resin with neat TFA for 10 seconds, the TFA was evacuated, and another aliquot of neat TFA was allowed to react with the resin for 2 min. The resin was neutralized and the next cycle begun.

Cleavage of tBOC synthesized peptide from resin was performed using anhydrous hydrogen fluoride in an Immuno-Dynamics (San Diego, CA) HF cleavage apparatus. Briefly, the peptide/resin complex was extensively washed and dried from the final ether wash in vacuo. For each gram of peptide/resin, 1 mL of anisole was added to the Teflon reaction vessel as scavenger and approximately 10 mL of HF was condensed into the reaction vessel and allowed to react for 1 h at −5 °C. The HF was removed by flowing nitrogen gas, ether added, the ether/peptide/resin mixture transferred to a sintered frit filter, washed with ether, and dried in the filter. Peptide was solubilized and extracted with 50% acetonitrile/water and lyophilized.

3.4. Purification of peptides

Crude products of synthesis were purified by reverse phase HPLC (Waters model 600E, Milford, MA) at a flow rate of 2 mL/min using Amberchrom CG 1000S resin (TOSOHAAS, Montgomeryville, PA) in a 1 cm × 25 cm steel column (Alltech, Deerfield, IL). Loading/starting buffer was 0.05% trifluoroacetic acid in water and the gradient was developed with increasing 95% acetonitrile/5% 0.05% trifluoroacetic acid. Elution was monitored at 229 nm. Leading and trailing fractions of insufficient purity as judged by MALDI-TOF mass spectrometry and analytical HPLC were further purified by reverse phase HPLC using Amberchrom XT20 resin in a steel 0.46 × 30 cm column utilizing the same solvents used for the semi-preparative separation.

Individual collected fractions (1 min) were analyzed by either an Applied Biosystems (Foster City, CA) model 4700 or 4800 TOF-TOF MALDI mass spectrometer with 10 mg/mL alpha cyano 4-hydroxycinnamic acid (Sigma/Aldrich, St. Louis, MO.) matrix.

Analytic scale HPLC evaluation of fractions was performed using a Waters system and a steel column (0.2 × 15 cm) containing Amberchrom XT20 resin with the 0.05% TFA/acetonitrile gradient and monitoring at 229 nm. Selected, purified fractions were lyophilized and immediately weighed and stored at −20 °C.

3.5. Preparation of liposomes

A predetermined amount of dried POPC or POPC/POPG (4:1 mol to mol) was weighed, depending on the experiment, and sus-

pended in buffer (40 mM sodium phosphate, pH 6.8) and spun for 30 min giving a total lipid concentration of 35 mM. LUVs for circular dichroism and ITC were prepared by extrusion using a Mini-Extruder (Avanti Polar Lipid Inc).[87,88] The solution was passed through a 100 nm pore size polycarbonate membrane 21 times. Previously we have reported the preparation of liposomes using this procedure resulted in a homogeneous population of liposomes with >95% of the particles falling into the particle size range of 70–100 nm.[89] We have also previously shown by ^{31}P NMR that these liposomes are unilamellar.[89] The starting concentration of 35 mM of the liposomes was based on the dry lipid weight. The extrusion process yields approximately 125 μL out of starting volume of 150 μL liposome.[43,87,90–96] All liposomes were made fresh for each experiment and used within 48 h.

3.5.1. Preparation of encapsulated calcein liposomes

A defined amount of dried POPC or POPC/POPG (4:1 mol to mol) to give a starting concentration of approximately 35 mM was weighed and suspended in a calcein-containing buffer (70 mM calcein, 10 mM Bis–Tris, 150 mM NaCl, 1 mM EDTA, pH 7.2). The pH was corrected using 1 mM NaOH and the final lipid concentration was calculated based on dilution. The pH was determined using a model Orion 720A+ pH meter (Thermo Electron Corporation) using three point calibrations. The resulting solution was vortexed for 1 min (five times). For the calcein leakage assays a calcein-containing lipid suspension was extruded using a Mini-Extruder (Avanti Polar Lipid Inc).[87,88] The solution was passed through a 100 nm pore size polycarbonate membrane 21 times.[89] The self-quenching efficiency (Q) for each lipid suspension was, at minimum, 80% and 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 Triton X, respectively.[97–100]

3.6. Calcein leakage assays

Peptide induced calcein leakage was investigated using an ISS PC1 photon counting spectrofluormeter (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 7.2) was added to the cell containing liposomes (36 μM lipid concentration). A measurement was taken every minute for the first 20 min of the experiment and every 10 min after until the emission intensity showed no further significant increase (approximately 90 min). One hundred percent leakage was determined with the addition of 50 μL of 10% Triton X. The apparent percent leakage was calculated using the following equation: % leakage = $[1 - (F_0/F_T)] \times 100\%$, where F_0 and F_T are the initial fluorescence before introduction of peptide and after the addition of Triton X, respectively.[87,101–104]

3.7. Circular dichroism

Peptides (1 mg/mL) were dissolved in 40 mM phosphate buffer (pH 6.8). Binding studies were conducted using freshly prepared liposome samples at the specific concentration in 40 mM phosphate buffer (pH 6.8) at room temperature. All CD spectra were obtained by acquiring eight 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, 1 nm bandwidth, data pitch 0.2 nm, response time 0.25 s and 5 mdeg sensitivity at room temperature (~25 °C). Minor contributions of light scattering due to the LUVs were eliminated by subtracting the lipid spectra of the corresponding peptide-free suspensions. Spectra were processed using Jasco spectra analysis software. They were smoothed by the means-movement method with a convolution width ranging from 10–15 then converted to molar ellipticity using the spectra analysis program. All data below 185 nm was omitted because it produced a HT value over 600 V giving a low signal-to-noise ratio.[51,87,94,101,104,105]

4. Conclusion

The members of this family of antimicrobial peptides exhibited a diversity of in vitro activity against 11 strains of bacteria. The observed deviations in activity can be attributed to the variation of the physicochemical properties of these peptides and how they interact with the different chemical compositions of the membranes of each strain of bacteria. Small modifications of molecular length, flexibility, and charge have a dramatic impact on organism selectivity and potency. The analogs containing six Tic-Oic dipeptide units, compounds **22**, **70**, **71**, and **74**, exhibit a varying degree of selectivity against *A. baumanii* but were inactive against a drug resistant clinical isolate strain of *A. baumanii* (Walter Reed Army Institute of Research). All of the analogs containing three Tic-Oic dipeptide units were active against both strains of *A. baumanii* as well as the AMES strain of *B. anthracis*. Very few of the three Tic-Oic dipeptide unit containing analogs exhibited good activity against *B. mallei*, *B. pseudomallei*, and *F. tularensis*. Only compound **64** exhibited excellent in vitro activity (0.02 μM) against all three organisms and compound **35** exhibited excellent in vitro activity (0.01 μM) against *F. tularensis*.

For the analogs containing three Tic-Oic dipeptide units the placement of positive charge and the resulting charge density plays a major role in determining in vitro activity particularly against Gram-positive bacteria (*B. anthracis* and *S. aureus*—MRSA) The placement of positive charge and the resulting charge density does not seem to effect significantly the in vitro activity against either Gram-negative strain of *A. baumanii*. *Y. pestis*, another Gram-negative bacteria also exhibited susceptibility to placement of positive charge and the resulting charge density. The other Gram-negative bacteria (*Burkholderia*, *Francisella*, and *Brucella*) seem to be insensitive to the placement of positive charge. The effect on in vitro activity of the length of Spacer #1 seems to vary between different bacteria strains in an unpredictable manner. For those bacteria strains sensitive to placement of positive charge and the resulting charge density, the length of Spacer #2 affect on the observed in vitro activity varies from organism to organism.

The design approached followed has resulted in the development of a series antimicrobial peptides that exhibit potency and selectivity for different strains of bacteria based on the physicochemical interactions that occur between the peptides and the varying chemical composition of the cell membranes of the different bacterial strains. From what has been learned from this investigation we will be able to design more potent and selective analogs for specific bacteria strains.

Note: Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publications. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.

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Supplementary data

Supplementary data (HPLC and HR mass spec data for each compound is attached) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.065.

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