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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 6667–6677
Bioorganic & Medicinal Chemistry

Development of Tyrocidine A analogues with improved antibacterial activity

Michael A. Marques,[a] Diane M. Citron[b] and Clay C. Wang[a,*]

[a]*Departments of Pharmacology and Chemistry, University of Southern California, 1985 Zonal Avenue, Los Angeles, CA 90089, USA*
[b]*Microbial Research Laboratory, Los Angeles County, University of Southern California Medical Center, 1801 East Marengo Street 2G-24, Los Angeles, CA 90033, USA*

Received 17 January 2007; revised 5 July 2007; accepted 7 August 2007
Available online 11 August 2007

Abstract—The development of new antibacterial therapeutic agents capable of halting microbial resistance is a chief pursuit in clinical medicine. Classes of antibiotics that target and destroy bacterial membranes are attractive due to the decreased likelihood that bacteria will be able to generate resistance to this mechanism. The amphipathic cyclic decapeptide, Tyrocidine A, is a model for this class of antibiotics. Tyrocidine A is composed of a hydrophobic and a hydrophilic face, allowing for insertion into bacterial membranes, creating porous channels and destroying membrane integrity. We have used a combination of molecular modeling and solid phase synthesis to prepare Tyrocidine A and analogues **1**–**8**. The minimum inhibitory concentrations (MICs) of these compounds were determined for a host of gram positive species and *E. coli* as a representative gram negative bacterium. Analogues **2** and **5** demonstrated moderate 2- to 8-fold increases in antibacterial activity over the parent Tyrocidine A for a variety of pathogenic microbes (best MICs for *E. coli* 32 μg/mL and 2 μg/mL for most gram positives). Examination of the structure– activity relationship between the analogues demonstrated a preference for increased amphipathicity but did not show a clear preference for increasing hydrophilicity versus hydrophobicity in improving antibacterial activity. Of note, movement of positively charged lysine residues or neutral pentafluorophenyl residues to different positions within the cyclopeptide ring system demonstrated improvements in antibacterial activity.
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1. Introduction

The market share for antibiotics is greater than $25 billion per year. Unfortunately, the emergence of highly resistant microbe strains is increasingly limiting the effectiveness of our current arsenal of therapeutics, making drugs such as vancomycin, daptomycin, the streptogramins, and linezolid, which were previously reserved for highly resistant bacterial strains, our last and only line of defense. Even now, resistance to these highly potent drugs is being seen clinically.[1–5] Making matters worse, there is less motivation on the part of pharmaceutical companies to spend billions on research and development of a novel antibiotic when bacterial resistance is likely to occur rapidly.[6–8]

Antimicrobial agents fall into one of several classes: β-lactams, β-lactamase inhibitors, aminoglycosides, tetracyclines, rifamycins, macrolides, lincosamides, glycopeptides, streptogramins, sulfonamides, oxazolidinones, quinolones, gramicidins and others.[7] These compounds target a variety of bacterial systems including DNA replication, transcription, folic acid metabolism, protein synthesis, and cell wall synthesis/integrity. Each class has its own specificities or coverage for a variety of bacteria. The means by which bacteria acquire resistance to such an expansive array of agents is highly variable and complex. It has been proposed that antimicrobial agents that target individual enzymes are the most likely to induce resistance, whereas therapeutics that target several structures irreversibly generate resistance at a slower rate.[9] The clearest mechanisms of genetic resistance include: inactivation of the drug, modification of the target active site, modified permeability of the cell wall, upregulation of the target enzyme, or the complete bypass of inhibited steps.[10,11]

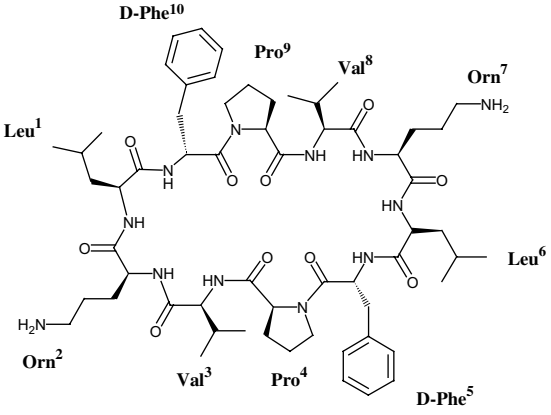
With so many different paths to resistance, the goal of generating successful new therapeutics to combat infec-

Keywords: Tyrocidine; Gramicidin; Cyclopeptide; Antibiotics; Amphipathic peptides; Sulfamylbutyryl resin; Solid phase synthesis.
* Corresponding author. Tel.: +1 323 442 1670; fax: +1 323 442 1365; e-mail: clayw@usc.edu

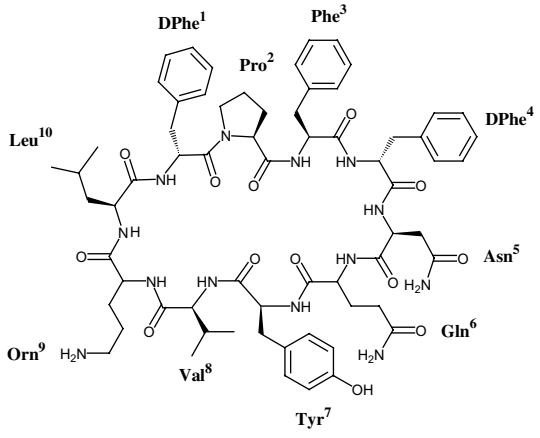
0968-0896/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmc.2007.08.007

tion is also challenging. There are several common methodologies for accomplishing this task.[12,13] With the advancements in genomic sequencing technologies, there has been a hope that novel targets that are less prone to cross resistance can be developed.[14,15] Unfortunately, deciphering bacterial genomes has not yet led to a wealth of new targets or therapeutics. Further, as mentioned above, even new classes of antimicrobials such as the oxazolidinones have met with resistant bacterial strains.[4,16–18]

More commonly, entirely new generations of drugs within a class have been produced by making structural changes to existing scaffolds, perhaps the most notable being the penicillins, cephalosporins, quinolones, tetracyclines, and macrolides.[6,18–22] In light of this method, we have selected Tyrocidine A, a natural product related to Gramicidin S, as a platform for study (Fig. 1). Polypeptide antimicrobials are not new to the field of microbiology. Nature has long used linear and cyclic polypeptides as natural defenses against competing or pathogenic bacteria.[23–25] Natural peptide antibiotics such as the gramicidins and polymyxins have been used extensively for topical therapy with excellent results. Furthermore, cyclic peptide derivatives such as the streptogramins (quinupristin/dafloprístin), glycopeptides (vancomycin, teicoplanin), and lipopeptides (daptomycin) have been fundamental in controlling severe bacterial infections.



Gramicidin S



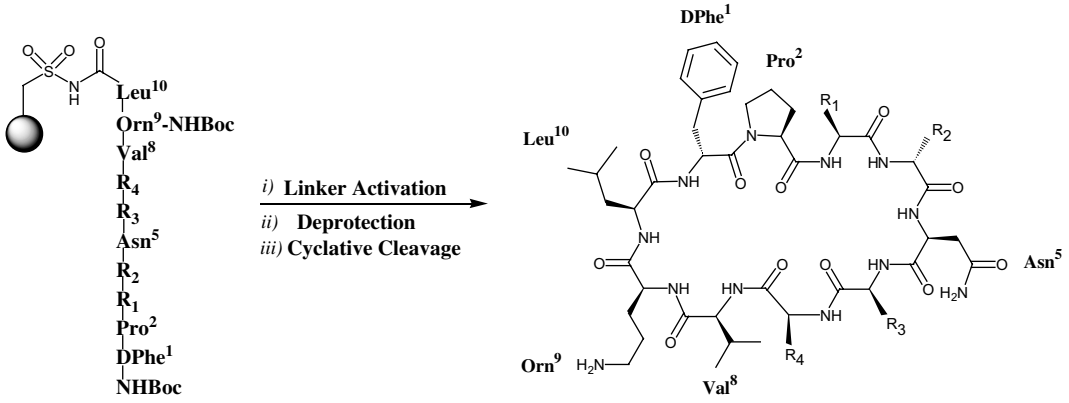
Tyrocidine A

Figure 1. Structures of amphipathic cyclopeptides Gramicidin S and Tyrocidine A.

Recently, Gramicidin S and Tyrocidine A have received a great deal of attention due to their highly desirable mechanism of action. Both compounds are decapeptide natural products isolated from *Bacillus subtilis*.[26,27] Structurally, the compounds differ only slightly with respect to their primary sequence. Both compounds are known to form a β-type secondary structure leading to an amphipathic molecule.[28–33] More specifically, one face of the molecule is hydrophobic, while the other is hydrophilic. The β-type structure, and associated amphipathicity, imparts Gramicidin S and Tyrocidine A with their specific antibacterial mechanism. These cyclic amphipathic peptides are known to associate with bacterial membranes, causing them to destabilize, lose structural integrity and ultimately fragment, resulting in bacterial cell death.[34–38] It seems that the hydrophilic residues of the cyclopeptide associate with the negatively charged phosphate groups of the bacterial lipids, while the hydrophobic groups form a nonselective porous channel.

The amphipathic cyclopeptides demonstrate a mixed selectivity for bacterial membranes, mildly preferring to associate with the highly negative phosphates found on bacterial walls, as opposed to the zwitterionic phospholipids found in eukaryotes.[25] Furthermore, the cholesterol content of eukaryotic membranes helps to stabilize and protect against binding of these cyclopeptides.[39] It is noteworthy that bacteria have not yet demonstrated appreciable resistance to this class of antibiotics, making them attractive for further study.[23,25] Due to the fact that the target of these cyclopeptides is the bacterial membrane, pathogens would have to alter their membrane composition and organization to achieve resistance, a much more complicated and costly process than simply the mutation of an enzymatic binding site.

With the favorable attributes of the cyclopeptides recognized, we have set out to improve the collective understanding of their structure– activity relationship (SAR) using Tyrocidine A as our point of reference. The question arises, how does altering the overall hydrophobicity, hydrophilicity, and amphipathicity of the cyclopeptide beta sheet affect the drugs' ability to inhibit bacterial growth. Using molecular modeling, we rationalized generating a small set of Tyrocidine A analogues with specific point amino acid substitutions that would affect either the polar or non-polar face of the amphipathic cyclopeptide. A solid-phase synthetic methodology was used to prepare the parent compound, Tyrocidine A, as well as eight other closely related analogues **1**–**8** (Figs. 2 and 3). These novel analogues were then tested against a host of clinically important gram positive and select gram negative bacterial strains to further correlate structure to function.



Tyrocidine A: R_1 = Phe, R_2 = DPhe, R_3 = Gln, R_4 = Tyr
1: R_1 = Phe, R_2 = DLys, R_3 = Gln, R_4 = Tyr
2: R_1 = Phe, R_2 = DLys, R_3 = Lys, R_4 = Tyr
3: R_1 = PFPhe, R_2 = DPhe, R_3 = Gln, R_4 = Tyr
4: R_1 = PFPhe, R_2 = DPhe, R_3 = Gln, R_4 = PFPhe
5: R_1 = Phe, R_2 = DPhe, R_3 = Lys, R_4 = Tyr
6: R_1 = Phe, R_2 = DPhe, R_3 = Gln, R_4 = PFPhe
7: R_1 = PFPhe, R_2 = DPhe, R_3 = Lys, R_4 = Tyr
8: R_1 = PFPhe, R_2 = DLys, R_3 = Lys, R_4 = Tyr

Figure 2. Solid phase synthesis of Tyrocidine A and analogues **1**–**8**. (i) Linker activation with 25 equiv IACN, 11 equiv DIEA, in NMP; (ii) deprotection with TFA/Phenol/TIPS/H_2O (88:5:5:2), rt 2 h; (iii) cyclative cleavage with 20% DIEA in THF, rt 24 h. **R1** = Phe or Pentafluorophenylalanine (PFPhe), **R2** = DLys or DPhe, **R3** = Gln or Lys, **R4** = Tyr or PFPhe.

2. Results

The retention time of each compound was measured by reverse phased LCMS, with retention time correlating with overall predicted hydrophobicity/hydrophilicity. Molecular modeling clearly depicted both the hydrophobic and hydrophilic faces of the Tyrocidine A scaffold (Fig. 4). Hydrophobicity/hydrophilicity was modulated by the placement of positive charges or the incorporation of pentafluorophenyl residues. The order of increasing hydrophobicity for Tyrocidine A and analogues **1**–**8** was as follows: **2** > **8** > **1** > **7** > **5** > **6** > Tyrocidine A > **3** > **4** (Table 1).

The parent compound, Tyrocidine A, exhibited only moderate antibacterial activity ranging from greater than 128 μg/mL for *E. coli* to 16 μg/mL for the remaining gram positive bacteria (Table 2). Analogue **1** showed a similar profile as Tyrocidine A. Compounds **2** and **3** showed a marked improvement in antibacterial potency, with an MIC improving from 2- to 8-fold over the parent Tyrocidine A. Of note, compound **2** was the only compound exhibiting activity against the gram negative *E. coli*. Compound **4** displayed the least antibacterial activity with an MIC range from 32 μg/mL to greater than 256 μg/mL. Compound **5** demonstrated the most potent and broadest antibacterial properties of all the compounds tested, with MICs increasing from 2- to 8-fold over the parent Tyrocidine A. Compounds **6**–**7** demonstrated similar antibacterial profiles with moderate improvement in activity against gram positive pathogens compared to Tyrocidine A. Compound **8** demonstrated improved activity against both gram positive pathogens, and the selected gram negative pathogen *E. coli*.

Daptomycin exhibited broad activity and high potency ranging from greater than 128 μg/mL for *E. coli* to 2–0.5 μg/mL for the gram positive bacteria. As previously reported, daptomycin is a membrane disrupting cyclic lipopeptide with broad therapeutic activity, however, its in vitro potency has a dependency on calcium concentration.[40,41] To determine if Tyrocidine A and the novel analogues shared the same dependency on calcium concentration, we screened activity at the standard calcium concentration of 25 mg/L, as well as the reported maximal value for daptomycin of 50 mg/L. Broadly, increasing the calcium concentrations from 25 to 50 mg/L for Tyrocidine A and analogues moderately decreased antibacterial activity. In contrast, daptomycin showed a marked increase in potency with increasing calcium concentration as previously reported.[40]

3. Discussion

Several groups have been studying the structure–activity relationships of streptogramin cyclopeptides Gramicidin S[34,35,42–48] and Tryocidine A (Fig. 1).[49–53] Initial work with Gramicidin S analogues has yielded a handful of useful facts concerning the structure–activity relationships of these cyclopeptides. Studies have found that bacteriocidal and hemolytic properties are influenced by several properties: rings size/rigidity, hydrophobicity, and amphipathicity.[44–47,54] A collection of similar studies have been conducted for the cyclopeptide Tyrocidine A. Guo et al. have demonstrated that by using an alanine substitution screen, minor changes in the peptide composition of Tyrocidine A can yield improvements in antibacterial potency.[52] More specifically, by making point substitutions that alter the amphipathicity of the Tyrocidine analogue the antibacterial activity may be systematically improved. Other recent studies by Kohli et al. have demonstrated the utility of using nonribosomal polypeptide synthetase (NRPS) technology for generating a privileged library of Tyrocidine A analogues

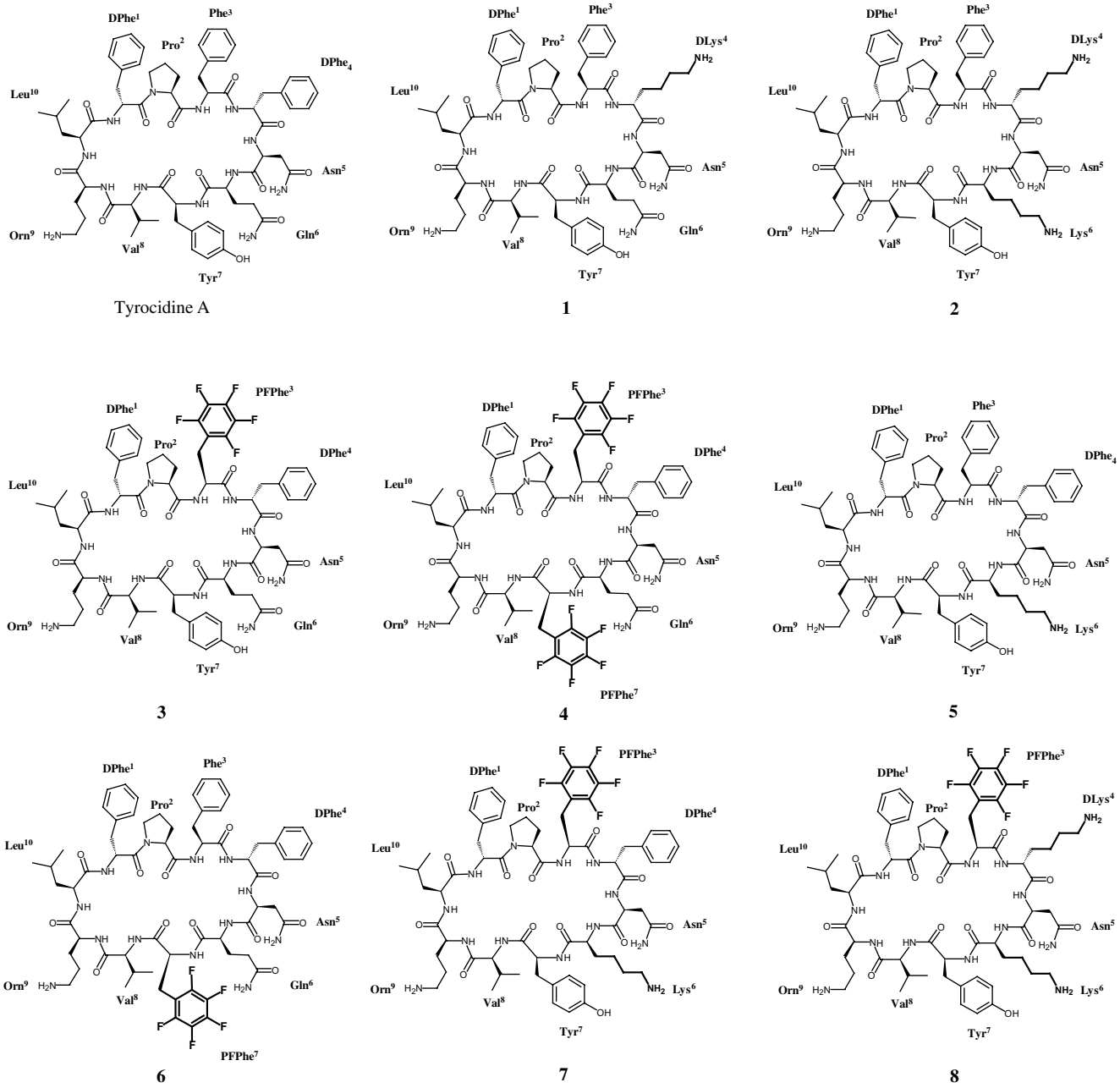


Figure 3. Structure of Tyrocidine A and analogues **1**–**8**. Residue substitutions on the hydrophilic and hydrophobic faces shown in bold above.

that exhibit moderate changes in antibacterial properties.[50] The previous success of these groups at modulating antibacterial properties by using simple alanine screens and point amino-acid substitutions is highly encouraging and sets the stage for our work.

While the use of NRPS enzymes is an elegant approach, the ability to complete a total synthesis on resin with a single purification step is a clearly attractive option, which we chose to utilize. A further advantage to the complete solid phase synthesis approach was being able to introduce point mutation without concern for affecting enzymatic activity. This methodology makes use of a commercially available sulfamylbutyryl safety catch resin. A linear peptide may be loaded onto the resin by standard solid phase techniques, the protecting groups removed, and the resin subjected to cyclative cleavage by treatment with diisopropylethylamine in THF (Fig. 2). A recent study by Tariq et al. showed that both biosynthetic and chemical mediated cyclizations were successful for generating streptogramin–tyrocidine derivatives, but that the chemical cyclative cleavage was superior for generating quantities necessary for bioassay.[55] Using similar solid phase synthesis techniques, we were able to prepare the natural product Tyrocidine A and eight closely related analogues **1**–**8**. Of note, yields were substantially improved by using a combination of HOAt in place of HOBt, NMP in place of DMF, and heating the coupling reactions at 38 °C.[51,52] In contrast to biosynthetic methods, the synergistic benefits of easily modulating the chemical structure without regard for affecting enzyme efficiency, coupled with simplified

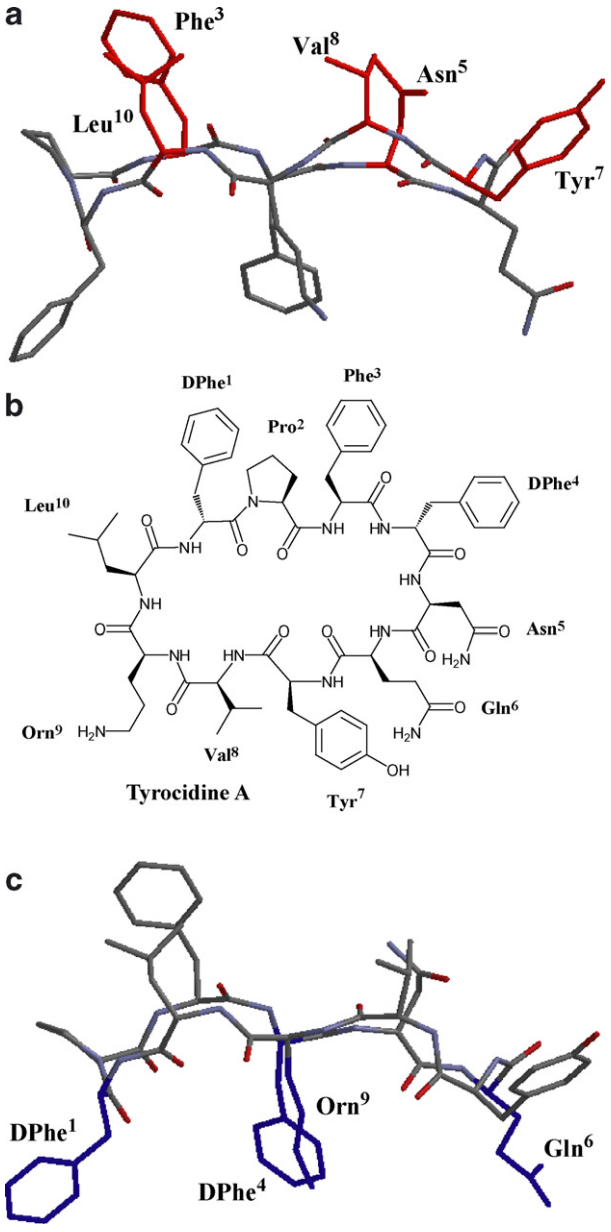


Figure 4. Hydrophobic and hydrophilic faces of Tyrocidine A. (a) Residues on hydrophobic face: Phe[3], Asn[5], Tyr[7], Val[8], Leu[10]. (b) Chemical structure of Tyrocidine A. (c) Residues on hydrophilic face: DPhe[1], DPhe[4], Gln[6], Orn[9].

loading and purification steps that provide milligram quantities of product for assay, validate the protocol.

Previously, a substantial body of work by Kondejewski et al. on the the Gramicidin S scaffold examined how specific changes in hydrophobicity, hydrophilicity, amphipathicity, affinity for negative phospholipid substrate, and cycle size all have intricate interlocking relationships.[44–47] Such analysis remains to be accomplished for the Tyrocidine A scaffold. Thus far, the majority of works done to understand the intricate interplay between structure and function of Tyrocidine A have involved alanine screens or a small positional library without much discussion of how these point substitutions may affect the overall geometric or electronic properties

Table 1. LCMS retention times for Tyrocidine A and analogues **1–8**

Compound	Charge[a]	R_t (min)[b]
Tyrocidine A	+1	18.8
(1)	+2	11.1
(2)	+3	9.7
(3)	+1	19.4
(4)	+1	27.2[c]
(5)	+2	15.3
(6)	+1	18.7
(7)	+2	12.4
(8)	+3	9.8

[a] Charge based on number of primary amines.
[b] Retention time by reversed phase LCMS.
[c] Retention time correlates to hydrophilicity: increasing hydrophilicity leads to shorter retention time. Addition of pentafluorophenyl (PFPhe) residues decreases hydrophilicity.

of the cyclopeptide in question.[50,52] Discussion is predominantly limited to antibacterial and hemolytic assay results. Granted, there has yet to be definitive structural evidence of how these antibiotics associate with the cellular membrane, and molecular modeling must be treated with mild skepticism due to the variable degree of possible conformations these cyclopeptides may take. However, the structure of the cyclopeptide backbone for Gramicidin S and Tyrocidine A is generally agreed upon, and the assignment of individual residues within the structure to either the hydrophobic or hydrophilic face by modeling seems reasonable.[56–59]

The overall beta sheet conformation of these cyclopeptides is stabilized by intramolecular hydrogen bonding between the carbonyl oxygen and the distal amides on the opposing side of the cyclopeptide.[60–62] It is likely that this structural organization is what allows these linear peptides to self-cyclize using a pure solid phase chemistry approach, as well as proving to be highly efficient substrates for NRPS enzymes.[49,51,60] Further, this beta sheet conformation plays a key role in the cyclopeptides' ability to disrupt bacterial membranes.[34–38] The covalent linkage of the cyclopeptide locks in the beta sheet conformation of Tyrocidine A allowing for topographic organization and presentation of given substituents to either the hydrophobic or hydrophilic face of the molecule. We can take advantage of the locked conformation provided by Tyrocidine by choosing to manipulate the substituents presented on either the hydrophobic or hydrophilic face of the cyclopeptide.

Figure 4 shows the putative hydrophobic and hydrophilic faces of Tyrocidine A. We used this model to select positions Phe[3] and Tyr[7] on the hydrophobic face, along with positions D-Phe[4] and Gln[6] on the hydrophilic face for amino acid substitution. Compound **1** with a substitution of a polar charged D-Lys[4] in place of a hydrophobic D-Phe[4] on the hydrophilic face of the cyclopeptide showed decreased antibacterial activity in comparison to Tyrocidine A. This result is mildly surprising in light of the fact that adding an additional positive charge positioned on the hydrophilic face of the amphipathic cyclopeptide would arguably increase the analogues' affinity for the negatively charged bacterial membrane. Of note, further increasing the charge of

Table 2. Minimum inhibitory concentrations for Tyrocidine A analogues (μg/mL)[a]

Organism[b]	Strain[c]	TyrA	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	Daptomycin (+Ca[d])	
MRSA	ATCC 33592	32	64	16	16	>256	4	16	8	8	1	0.25
MSSA	ATCC 29213	32	32	16	8	>256	4	16	16	8	1	0.25
E. faecalis	ATCC 29212	16	64	32	4	32	2	2	4	16	1	1
E. coli[e]	ATCC25922	>128	128	32	>256	>256	128	>256	>256	32	>128	>128
MRSE	MRL19010	16	32	4	8	256	2	2	4	4	1	0.5
VRE	MRL 17001	16	32	8	8	32	2	2	4	4	2	1
B. subtilis	MRL 18734	16	16	2	4	128	2	8	8	2	1	0.25
B. cereus	MRL 18731	16	64	16	4	256	2	8	8	4	0.5	0.25

[a] Inhibitory concentration required to completely inhibit bacterial growth.
[b] All gram positive bacteria with the exception of *E. coli*; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; *E. faecalis*, *Enterococcus faecalis*; *E. coli*, *Escherichia coli*; MRSE, methicillin-resistant *Staphylococcus epidermidis*; VRE, vancomycin-resistant *Enterococcus*; *B. subtilis*, *Bacillus subtilis* and *B. cereus*, *Bacillus cereus*.
[c] MRL clinical isolates from USC-LAC Hospital.
[d] Calcium supplemented to 50 mg/L (normal 25 mg/L). Tyrocidine analogues did not show the same calcium dependence.
[e] *E. coli* is a gram negative bacterium.

compound **1** by adding another lysine residue ($Gln^6 \rightarrow Lys^6$ compound **2**) markedly improves the antibacterial properties, providing as much as an 8-fold improvement over the parent Tyrocidine A (Table 2). The increased charges of compounds **2** and **8** provide them with an improved activity toward the gram negative *E. coli*, indicating that future efforts may be focused on tuning these compounds to target both gram negative and gram positive pathogens.

Compound **5**, similar to compound **1**, has a single lysine addition, however, the substitution is at a different position in the cyclopeptide. Interestingly, unlike compound **1**, which showed poor antimicrobial activity when a single lysine substitution was made ($D\text{-}Phe^4 \rightarrow D\text{-}Lys^4$), there is a substantial increase in antimicrobial activity when the lysine is positioned at a different point in the cyclopeptide ($Gln^6 \rightarrow Lys^6$) as in compound **5**. Such a comparison supports our reasoning that simply increasing charge does not directly lead to an increase in antimicrobial properties, and that it is in fact a more complicated interplay of structure vs. activity exists.

Substitution of the Phe^3 and Tyr^7 residues on the hydrophobic face of Tyrocidine A with pentafluorophenyl (PFPhe) derivatives provided mixed results. The PFPhe residue is particularly interesting due to its similar surface area but dramatically modified electronic and hydrophobic nature when compared to Phe. Analogue **3**, with a single PFPhe substitution ($Phe^3 \rightarrow PFPhe^3$), showed increased gram positive antibacterial activity similar to that of compound **2** but failed to show gram negative activity (Table 2). Movement of the PFPhe substitution across the cyclopeptide to a different position, as is demonstrated for compound **6** ($Tyr^7 \rightarrow PFPhe^7$), maintains a similar antibacterial potency overall, but shows moderate improvements in certain gram positive strains. Interestingly, addition of a second PFPhe substitution, **4** ($Phe^3 \rightarrow PFPhe^3$) ($Tyr^7 \rightarrow PFPhe^7$), virtually abolished antibacterial activity across the spectrum of strains tested.

Combining lysine and pentafluorophenyl substitutions within the same molecule proved successful in increasing the overall antibacterial activity of compounds **7** and **8** when compared to the parent Tyrocidine A. Similar to compound **2**, compound **8** demonstrated a modest increase in activity against the gram negative pathogen *E. coli*, further supporting that these compounds may eventually be tuned to target gram negative pathogens.

With respect to delineating the importance of hydrophilicity versus hydrophobicity in improving antibacterial activity, this study has shown that there is not a clear preference for a single parameter in determining activity. Of note, simply adding positive charge to the hydrophilic face of Tyrocidine A does not result in improved antibacterial activity, demonstrated by **1**. Further, most analogues showed significantly increased antibacterial properties over the parent Tyrocidine A, while having substantially different electronic profiles. For example, while compounds **2** and **8** had the greatest positive charge and hydrophilicity, compounds **3** and **6** had a markedly increased hydrophobic nature and only a single positive charge on the hydrophilic face (Table 1). Such a similarity between the activities of these two groups, respectively, seems to indicate that increasing amphipathicity, either by increasing hydrophobicity or hydrophilicity on an individual face, results in a moderate increase in antibacterial activity. This is in contrast to previous studies with Gramicidin S analogues assayed against gram negative bacteria, which demonstrated a negative correlation with increasing amphipathicity.[46] Compound **4** demonstrates that there may be a limit to how much we can increase the overall amphipathicity of Tyrocidine A before activity is lost.

The substantially improved antibacterial profile of compound **5** versus **1** seems to indicate that the actual ring position of charged residues, and not just the placement of the charge itself on the hydrophilic face of the compound, may be of importance. The notion that residue position and not simply facial substitution may be a factor in moderating activity is supported, albeit to a lesser extent, by the improvement of antibacterial activity of compound **6** when compared to **3** for strains of MRSE, VRE, and *E. faecalis*.

It is noteworthy that Tyrocidine A and analogues, while sharing the same mechanism of action as daptomycin, do not share the same dependence on calcium.[40] Fur-

ther, the addition of calcium actually decreases the measured in vitro activity for Tyrocidine A and its analogues. Whether this difference in calcium dependence would have an implication for differences in molecular mechanism seems unclear.

4. Conclusion

With antibacterial resistance increasing at an alarming rate, the development of new antibacterial therapeutic agents capable of staving off microbial resistance is a chief concern in clinical medicine.[7,18,63] One class of antibiotics, those that target and destroy bacterial membranes, is attractive due to the decreased likelihood that bacteria will be able to generate resistance to this mechanism.[23–25] The amphipathic cyclic decapeptide, Tyrocidine A, is a model system for this class of antibiotics.[26,27]

We have been able to use molecular modeling and solid phase synthetic techniques to design and efficiently synthesize Tyrocidine A and multiple novel analogues with improved antibacterial activity. Further, we have shown that the relationship between increasing hydrophilicity, by the substitution of positive charge on the polar face of the amphipathic structure, does not necessarily provide an increase in antibacterial activity, and that charge/residue placement may be of increasing importance. Overall, we have demonstrated that an increase in amphipathicity within a finite range improves antibacterial activity. A substantial increase in the hydrophobic nature of Tyrocidine A by substitution with multiple pentafluorophenyl residues demonstrates a marked decrease in antibacterial activity, indicating a narrower window for electrochemical modification than originally envisioned. Finally, we demonstrated Tyrocidine A analogues do not have the same in vitro reliance on calcium concentration as demonstrated by our comparator daptomycin.

Moving forward there are a variety of other questions that we would like to address. For example, we chose to use lysine as our means of introducing positive charge but would the antimicrobial activities be moderated differently by delivery of positive charge through a different functionality or linker length? We were able to demonstrate an increase in gram negative antibacterial activity by substituting the analogues with multiple positive charges and the ability to tune these compounds for both gram negative and gram positive pathogens with some sort of selectivity is certainly of interest. Testing these compounds against a larger panel of clinically important gram negative pathogens is in order.

5. Materials and methods

5.1. General

Methanol, diethyl ether, acetonitrile, and dichloromethane (all HPLC grade) were purchased from VWR, San Diego, CA, and used without further purification. Anhydrous tetrahydrofuran (THF), dimethylformamide (DMF), 1-methyl-2-pyrrolidinone (NMP), 1,3-diisopropylcarbodiimide (DIC), piperidine, diisopropylethylamine (DIEA), 1-methylimidazole (NMI), activated aluminum oxide, iodoacetonitrile, *d*6-dimethylsulfoxide, and Sigmacoat were purchased from Aldrich St. Louis, MO. 4-Sulfamylbutyryl AM resin (1.1 mmol/g) (01-64-0152), Fmoc and Boc amino acids, and all other solid phase coupling reagents were purchased from Novabiochem, San Diego, CA. Fluorinated amino acid derivatives were purchased from PepTech Corp, Burlington, MA.

Analytical and preparatory HPLC was acquired using a Waters 600 controller equipped with a 2487 dual wavelength detector and RCM 8×10 semi-prep C18 column. Mass spectra were acquired using a Thermo/Finnigan LCMS equipped with an Altech 10 mm $\times$ 2.1 mm C18 column (43871), autosampler, and triple wavelength detector. UV spectra were acquired using a Shimadzu UV-2401 spectrophotometer. NMR spectra were acquired using a Varian 400 Hz instrument.

5.2. Molecular modeling

A molecular model of Tyrocidine A was constructed with a semi-empirical energy minimization (PM3) using the Spartan Essential Software Package (2000). Modeling allowed visualization of amino acid side chains reported to be on either the hydrophobic or hydrophilic face of the amphipathic cyclopeptide (Fig. 4).[50,52,57,59] Positions Phe^3 and Tyr^7 on the hydrophobic face, along with positions $DPhe^4$ and Gln^6 on the hydrophilic face, were selected for amino acid substitution. To ensure that an observed change in antibacterial activity could be attributed to an individual structural modification, only a single progressive amino acid substitution was made between Tyrocidine A and each respective analogue (Fig. 3).

5.3. Solid phase synthesis (resin loading)

Tyrocidine A and subsequent analogues were synthesized using manual solid phase synthesis Fmoc and Boc protocols. A glass resin reaction vessel was pretreated with Sigmacoat before use to prevent resin from sticking to the glass surface during shaking. Loading of the sulfamylbutyryl AM resin was accomplished as follows. Sulfamylbutyryl AM resin (1 g, 1.1 mmol), DCM (4 mL), and DMF (1 mL) were added to the glass reaction vessel and shaken at room temperature for 20 min. The resin was then drained. A mixture of Fmoc-Leu-OH (1.94 g, 5.5 mmol), 1-methylimidazole (452 mg, 436 μL, 5.5 mmol), 1,3-diisopropylcarbodiimide (DIC) (694 mg, 851 μL, 5.5 mmol), DCM (4 mL), and DMF (1 mL) were added to the resin and the mixture was shaken for 24 h at room temperature. After 24 h, the resin was washed and the loading procedure was repeated a second time as described above. Resin loading was near quantitative as determined by UV assay.[64] Following resin loading, the resin was washed with DMF, DCM, MeOH, and Et_2O, dried under vacuum, and stored at 4 °C for later use.

5.4. Manual solid phase synthesis

To a solid phase reaction vessel charged with 0.2 g of resin pre-loaded with Fmoc-Leu was added DMF (2 mL) and allowed to swell at room temperature for 20 min. The resin was then drained and washed with 20% piperdine in DMF (continuous flow for 30 s), followed by the addition of more 20% piperdine in DMF (4 mL) and shaking at room temperature for 30 min to accomplish Fmoc deprotection. Following deprotection, the resin was washed thoroughly with DMF in preparation for the coupling step. In a separate 20 mL glass vial, a mixture of Fmoc-Orn(Boc)-OH (500 mg, 1.1 mmol), HOBt (149 mg, 1.1 mmol), DIC (139 mg, 171 μL, 1.1 mmol), and DMF (2 mL) was agitated until homogeneous and allowed to stand for 10 min. This mixture was then added to the solid phase reaction vessel containing the deprotected H_2N-Leu-R resin and shaken for 3–4 h at room temperature. Following coupling, the resin was washed thoroughly with DMF in preparation for the next round of deprotection and coupling. If the resin was to be coupled at a later time, it was washed with a series of DMF, DCM, MeOH, and Et_2O, dried under vacuum, and stored for later use. The coupling of all subsequent amino acids to synthesize resin bound linear derivatives of Tyrocidine A and **1**–**4** was accomplished as described above.

5.5. Modified coupling conditions

It is noteworthy that changing the above conditions by replacing HOBt with HOAt, utilizing NMP in place of DMF, and heating the reactions to 38 °C provided compounds **5**–**8** in significantly greater quantities.

5.6. Safety catch linker activation

Following attachment of the terminal amino acid, the resin was washed with NMP in preparation for resin activation. To a 10 mL syringe equipped with a 2 μm filter tip were added activated basic alumina (200 mg), NMP (3.5 mL), DIEA (180 μL), and iodoacetonitrile (280 μL). The mixture was then filtered into the reaction vessel containing the unactivated resin. The vessel was then wrapped in aluminum foil to exclude light and shaken at room temperature for 24 h. Following 24 h the mixture was filtered and the resin washed with DMF and DCM in preparation for orthogonal Boc and *O*-*t*Bu deblocking.

5.7. Orthogonal deprotection

A mixture of TFA/Phenol/TIPS/H_2O (88:5:5:1) was freshly prepared and stored at 4 °C for subsequent use. To the activated resin prepared as described above was added 3 mL of the TFA/Phenol/TIPS/H_2O deblocking mixture. The mixture was shaken at room temperature for 2 h. Following deprotection, the resin was washed with DCM and THF in preparation for on resin self-cyclization of the linear peptide.

5.8. Resin self-cyclization and cleavage

The resin was washed with THF as described above. Following washing, a 20% solution of DIEA in THF (2.5 mL) was added and the mixture was shaken at room temperature for 24 h. After 24 h the resin was filtered and washed with THF (2 × 2 mL) and all filtrates were combined in a round bottom flask. The solvent was evaporated to provide a viscous solution or solid, depending on the compound. In all cases a small amount of methanol (200–400 μL) was added to the round bottom to dissolve the solid or dilute the solution. The mixture was then transferred to a 1.5 mL Eppendorf tube. To the Eppendorf tube was added cold Et_2O to provide a white precipitate. The Eppendorf tube was centrifuged at 14,000 rpm at 4 °C for 5 min to provide a solid white pellet. The Eppendorf tube was decanted, the pellet taken up in 200 μL of methanol and subjected to a second round precipitation. The pellet was then taken up in 500 μL of acetonitrile, flashed frozen with liquid nitrogen, and subjected to lyophilization to provide the final cyclized products (Tyrocidine A and analogues **1**–**8**) as fine white solids. It is noteworthy that with different structural variants, such as the fluorinated derivatives that show some solubility in the MeOH/Et_2O precipitation mixture, the use of 0.1% TFA in place of Et_2O is more effective.

5.9. Purification and characterization

In most cases, precipitated products were shown to be sufficiently pure by HPLC and H NMR, and reported yields are based on recovery following precipitation and lyophilization. Compounds that were not pure by precipitation were subjected to reverse phase preparatory HPLC purification. The HPLC purification protocol is as follows: no more than 15 mg of crude precipitate was dissolved in MeOH (1 mL) and loaded onto the HPLC equipped with a 5 mL injection loop and Waters 8 × 10 semi-prep C18 colum. The solvent system was a 0.1% TFA (solvent A) and acetonitrile (solvent B) mixture. The method consisted of a ramp from 0 to 10% B over 5 min, followed by 10–80% B over 50 min, followed by 80–100% B over 5 min, with a flow rate of 3 mL/min. The column was then washed at 100% B for 5 min, followed by re-equilibration to 100% A for the next run. HPLC spectra were recorded at 220 nm. HPLC fractions were analyzed by LCMS. The LCMS was equipped with an Altech 10 mm × 2.1 mm C18 column and was run using a solvent mixture of 1% phosphoric acid (solvent A) and acetonitrile (solvent B). Briefly, 25 microliters of a dilute sample was autoinjected to the LCMS and the standard method consisted of the following: the column was equilibrated at 10% B running at 125 μL/min followed by a ramp from 10 to 15% B over 5 min, followed by 15 to 80% B over 30 min, followed by 80–100% B over 5 min. The column was then washed for 2 min at 100% B, followed by re-equilibration. Tyrocidine A and analogue **1**–**8** retention times were recorded and used as an indicator of their hydrophobicity. Pooling of the appropriate fractions followed by lyophilization provided pure cyclized compounds.

5.10. Tyrocidine A

Isolated as a fine white powder (7.4 mg 2.7% Yield) after HPLC purification of the appropriate fractions. ^{1}H NMR (400 MHz, d_6-DMSO, 25 °C); 9.27 (s, 1H), 9.16 (s, 1H), 9.02 (m, 2H), 8.91 (d, 1H, J = 8.8 Hz), 8.70 (d, 1H, J = 4.4 Hz), 8.43 (d, 1H, J = 10 Hz), 8.01 (s, 1H), 7.91 (d, 1H, J = 7.6 Hz), 7.45 (s, 1H), 7.39 (d, 2H, J = 8.4 Hz), 7.23–7.01 (m, 16H), 6.92 (d, 2H, J = 8.4 Hz), 6.61 (d, 2H, J = 8.4 Hz), 5.57 (m, 1H), 5.26 (m, 1H), 4.55–4.42 (m, 4H), 4.35–4.25 (m, 2H), 4.06 (d, 1H, J = 8.4), 3.80 (m, 1H), 3.09–2.70 (m, 8H), 2.19 (m, 2H), 1.99 (m, 4H), 1.62 (m, 4H), 1.43 (m, 2H), 1.21 (m, 2H), 1.00–0.85 (m, 12H), 0.36 (br, 2H); ES-MS m/z 1270.88 (M+H 1270.66 calcd $C_{66}H_{88}N_{13}O_{13}$). Retention time LCMS = 18.8 min.

Compound **1**: Isolated as a fine white powder (7.2 mg 3.5% Yield) after HPLC purification of the appropriate fractions. ^{1}H NMR (400 MHz, d_6-DMSO, 25 °C); 9.26 (s, 1H), 9.17 (s, 1H), 8.99 (d, 1H, J = 9.2 Hz), 8.86 (s, 1H), 8.73 (d, 1H, J = 9.2 Hz), 8.66 (s, 1H), 8.31 (d, 1H, J = 10 Hz), 8.23 (m, 1H), 7.93 (s, 1H), 7.38–7.12 (m, 11H), 6.91 (d, 2H, J = 8.4 Hz), 6.61 (d, 2H, J = 8.4 Hz), 5.10 (m, 1H), 4.74 (m, 1H), 4.58 (m, 1H), 4.50 (m, 1H), 4.45 (m, 1H), 4.37 (m, 1H), 4.27 (m, 1H), 4.14 (m, 1H), 3.76 (m, 1H), 3.0–2.6 (m, 6H), 1.64 (m, 3H), 1.47 (m, 3H), 1.34 (m, 2H), 1.23 (m, 2H), 0.92–0.78 (m, 12), 0.40 (br, 2H); ES-MS m/z 1251.69 (M+H 1251.69 calcd $C_{63}H_{91}N_{14}O_{13}$). Retention time LCMS 11.1 min.

Compound **2**: Isolated as a fine white powder after precipitation purification (15 mg 5.4% Yield) ^{1}H NMR (400 MHz, d_6-DMSO, 25 °C); 9.27 (s, 1H), 9.00 (d, 1H, J = 9.2 Hz), 8.86 (s, 1H), 8.75 (d, 1H, J = 9.2 Hz), 8.24 (d, 2H, J = 9.2), 7.99 (d, 1H, J = 4 Hz), 7.93 (s, 1H), 7.36–7.12 (m, 14H), 6.92 (d, 2H, J = 8.8 Hz), 6.63 (d, 2H, J = 8.4), 5.10, (s, 1H), 4.73 (m, 1H), 4.57 (m, 1H), 4.43 (m, 1H), 4.37 (m, 1H), 4.27 (m, 1H), 4.14 (d, 1H, J = 8.4 Hz), 4.09 (s, 1H), 3.70 (m, 1H), 2.98–2.54 (m, 6H), 1.64 (s, 3H), 1.48 (m, 6H), 1.34 (m, 5H), 1.24 (m, 2H), 1.18–1.0 (m, 3H), 0.94–0.70 (m, 12H), 0.38 (br, 2H); ES-MS m/z 1251.88 (M+H 1251.73 calcd $C_{64}H_{95}N_{14}O_{12}$). Retention time LCMS 9.7 min.

Compound **3**: Isolated as a fine white powder after precipitation purification (21 mg 7.0% Yield) ^{1}H NMR (400 MHz, d_6-DMSO, 25 °C); 9.27 (d, 1H, J = 9.6 Hz), 9.18 (s, 1H), 9.10, (s, 1H), 9.03 (d, 1H, J = 6 Hz), 8.96 (d, 1H, J = 9.2 Hz), 8.69 (s, 1H), 8.40 (d, 1H, J = 9.6 Hz), 8.03 (s, 1H), 7.87 (d, 1H, J = 8.4 Hz), 7.44 (m, 6H), 7.24–7.09 (m, 12H), 6.94 (d, 2H, J = 8.4 Hz), 6.63 (d, 2H, J = 8.8 Hz), 5.50 (m, 1H), 5.11 (m, 1H), 4.53 (m, 3H), 4.31 (m, 1H), 4.22 (m, 1H), 4.17 (m, 1H), 3.81 (m, 1H), 3.01–2.67 (m, 12H), 2.00 (m, 2H), 1.66 (m, 7H), 1.25 (m, 9H), 0.92 (m, 12), 0.81 (br, 2H); ES-MS m/z 1361.03 (M+H 1360.62 calcd $C_{66}H_{83}F_5N_{13}O_{13}$). Retention time LCMS 19.4 min.

Compound **4**: Isolated as a fine white powder (8.4 mg 2.7% Yield) after HPLC purification of the appropriate fractions. ^{1}H NMR (400 MHz, d_6-DMSO, 25 °C); 9.28 (d, 1H, J = 9.6 Hz), 9.11 (s, 1H), 9.07 (d, 1H, J = 7.2 Hz), 9.00 (d, 1H, J = 9.2 Hz), 8.67 (s, 1H), 8.63 (d, 1H, J = 10 Hz), 8.04 (s, 1H), 7.84 (d, 1H, J = 8.4 Hz), 7.64 (d, 1H, J = 8 Hz), 7.41 (d, 1H, J = 10 Hz), 7.35 (d, 1H, J = 12.4 Hz), 7.25–7.09 (m, 11H), 6.88 (s, 1H), 5.49 (m, 1H), 5.13 (m, 1H), 4.78 (m, 1H), 4.52 (m, 3H), 4.21 (m, 1H), 4.17 (m, 1H), 3.84 (m, 1H), 3.23–2.68 (m, 8H), 2.03 (m, 2H), 1.91 (m, 4H), 1.5–1.23 (m, 6H), 0.93–0.76 (m, 12H), 0.47 (br, 2H); ES-MS m/z 1434.63 (M+H 1434.57 calcd $C_{66}H_{78}F_{10}N_{13}O_{12}$). Retention time LCMS 19.4 min.

Compound **5**: Isolated as a fine white powder after precipitation purification (45.2 mg 16.1% Yield). ^{1}H NMR (400 MHz, d_6-DMSO, 25 °C); 9.07 (d, 1H, J = 7.2 Hz), 8.95 (d, 1H, J = 8.8 Hz), 8.81 (d, 1H, J = 8.4 Hz), 8.34 (d, 1H, J = 9.6 Hz), 8.00 (m, 4H), 7.36 (m, 2H), 7.26–7.04 (m, 20H), 6.93 (d, 2H, J = 8.4 Hz), 6.63 (d, 2H, J = 8.4 Hz), 5.62 (m, 1H), 5.21 (m, 1H), 4.54–4.47 (m, 4H), 4.31–4.25 (m, 2H), 4.05 (d, 1H, J = 8.0 Hz), 3.73 (s, 1H), 3.34–2.53 (m, 10H), 2.44–2.39 (m, 3H), 2.33 (m, 1H), 2.19–2.00 (m, 4H), 1.60–1.17 (m, 20H), 0.93–0.89 (m, 12H), 0.32 (br, 2H); ES-MS m/z 1270.70 (M+H 1270.70 calcd $C_{67}H_{92}N_{13}O_{12}$). Retention time LCMS 15.25 min.

Compound **6**: Isolated as a fine white powder after precipitation purification (59 mg 20% Yield). ^{1}H NMR (400 MHz, d_6-DMSO, 25 °C); 9.28 (s, 1H), 9.06 (m, 2H), 8.96 (d, 1H, J = 10 Hz), 8.65 (m, 1H) 8.03 (s, 1H), 7.88 (d, 1H, J = 8.4 Hz), 7.60 (d, 1H, J = 8 Hz), 7.32–7.03 (m, 15H), 6.87 (s, 1H), 5.57 (m, 1H), 5.29 (m, 1H), 4.56–4.42 (m, 4H), 4.28 (m, 2H), 4.05 (d, 1H, J = 8.0 Hz), 3.84 (m, 1H), 3.15–2.65 (m, 8H), 2.43–1.98 (m, 6H), 1.68 (m, 4H), 1.61–1.21 (m, 7H), 0.93–0.88 (m, 12H), 0.34 (br, 2H); ES-MS m/z 1344.77 (M+H 1344.62 calcd $C_{66}H_{83}F_5N_{13}O_{12}$). Retention time LCMS 18.65 min.

Compound **7**: Isolated as a fine white powder after precipitation purification (40.4 mg 13.5% Yield). ^{1}H NMR (400 MHz, d_6-DMSO, 25 °C); 9.19 (s, 1H), 9.00 (m, 2H), 8.87 (d, 1H, J = 8 Hz), 8.28 (d, 1H, J = 9.2) 7.96 (m, 1H), 7.86 (m, 1H), 7.41 (m, 2H), 7.22–7.07 (m, 10H), 6.92 (d, 2H, J = 8.8 Hz), 6.62 (d, 2H, J = 8.8 Hz) 5.45 (m, 1H), 5.05 (m, 1H), 4.73 (m, 1H), 4.47 (m, 2H), 4.27–4.21 (m, 4H), 4.13 (d, 1H, J = 8.0 Hz), 3.73 (m, 1H), 3.10–2.64 (m, 8H), 1.70–1.21 (m, 12H), 0.94–0.81 (m, 12H); ES-MS m/z 1360.81 (M+H 1360.65 calcd $C_{67}H_{87}F_5N_{13}O_{12}$). Retention time LCMS 12.39 min.

Compound **8**: Isolated as a fine white powder after precipitation purification (26.1 mg 8.8% Yield). ^{1}H NMR (400 MHz, d_6-DMSO, 25 °C); 8.91 (s, 1H), 8.81 (s, 1H), 8.19 (m, 2H), 7.97–7.84 (m, 4H), 7.43–7.24 (m, 6H), 6.94 (d, 2H, J = 7.6 Hz), 6.63 (d, 2H, J = 8 Hz) 4.92 (m, 1H), 4.80 (m, 1H), 4.46 (m, 2H), 4.34–4.20 (m, 4H), 4.02 (m, 1H), 3.88 (m, 1H), 3.10–2.55 (m, 6H), 1.50–1.11 (m, 24H), 0.88–0.83 (m, 12H); ES-MS m/z 1341.78 (M+H 1341.68 calcd $C_{64}H_{90}F_5N_{14}O_{12}$). Retention time LCMS 9.80 min.

5.11. Bacterial inhibition assays

The in vitro activities of Tyrocidine-A, **1**–**8**, and daptomycin were measured using a microdilution method (CLSI) according to standard procedures.[65] The compounds were dissolved in cation adjusted Mueller-Hinton broth (CAMHB) or a small amount of dimethylsulfoxide (dmso), followed by broth. Serial twofold dilutions were prepared in CAMHB in micro titer trays. Daptomycin and vancomycin were also tested as controls.

5.11.1. Bacterial strains. The test organisms were *Staphylococcus aureus* ATCC 33592 (oxacillin-resistant), *Staphylococcus aureus* ATCC 29213 (oxacillin-susceptible), *Staphylococcus epidermidis* (clinical isolate MRL 19010), *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* (clinical isolate MRL 17001, vancomycin-resistant), *Bacillus subtilis* (clinical isolate MRL 18734), *Bacillus cereus* (clinical isolate MRL 18731), and *Escherichia coli* ATCC 25922.

5.11.2. Method. Overnight cultures of the strains were suspended in saline to equal the 0.5 McFarland turbidity standard. They were further diluted 1:100 in CAMHB and added to the wells containing the diluted compounds. Drug-free wells were quantitatively subcultured to determine the actual colony counts in the test. The trays were incubated overnight at 36 °C. After growth had occurred, they were examined using an inverted mirror apparatus. The MIC was the concentration that completely inhibited growth of the organisms.

Acknowledgments

This work was funded by the National Institutes of Health through the NIH Roadmap for Medical Research, Grant (1R01GM075857-01). Information on this RFA (RM-05-013) can be found at (http://grants.nih.gov/grants/guide/notice-files/NOT-RM-05-013. html, links to nine initiatives are found here http://nihroadmap.nih.gov). We also thank the Baxter Foundation for fellowship to M.A.M., and A.P. and K.W. for help with instrumentation and fruitful conversation.

References and notes

1. Chang, S.; Sievert, D. M.; Hageman, J. C.; Boulton, M. L.; Tenover, F. C.; Downes, F. P.; Shah, S.; Rudrik, J. T.; Pupp, G. R.; Brown, W. J.; Cardo, D.; Fridkin, S. K. *N. Engl. J. Med.* **2003**, *348*, 1342–1347.
2. Jones, R. N.; Farrell, D. J.; Morrissey, I. *Antimicrob. Agents. Chemother.* **2003**, *47*, 2696–2698.
3. Sievert, D. M.; Boulton, M. L.; Stoltman, G.; Johnson, D.; Stobierski, M. G.; Downes, F. P.; Somsel, P. A.; Rudrik, J. T.; Brown, W.; Hafeez, W.; Lundstrom, T.; Flanagan, E.; Johnson, R.; Mitchell, J. *JAMA* **2002**, *288*, 824–825.
4. Tsiodras, S.; Gold, H. S.; Sakoulas, G.; Eliopoulos, G. M.; Wennersten, C.; Venkataraman, L.; Moellering, R. C.; Ferraro, M. J. *Lancet* **2001**, *358*, 207–208.
5. Cui, L. Z.; Tominaga, E.; Neoh, H. M.; Hiramatsu, K. *Antimicrob. Agents. Chemother.* **2006**, *50*, 1079–1082.
6. Bax, R. P. *Clin. Infect. Dis.* **1997**, *24*, S151–S153.
7. Coates, A.; Hu, Y. M.; Bax, R.; Page, C. *Nat. Rev. Drug Dis.* **2002**, *1*, 895–910.
8. Cohen, M. L. *Science* **1992**, *257*, 1050–1055.
9. Spratt, B. G. *Science* **1994**, *264*, 388–393.
10. Sefton, A. M. *Drugs* **2002**, *62*, 557–566.
11. Walsh, C. *Nature* **2000**, *406*, 775–781.
12. Silver, L.; Bostian, K. *Eur. J. Clin. Microbiol. Infect. Dis.* **1990**, *9*, 455–461.
13. Walsh, C. *Nat. Rev. Microbiol.* **2003**, *1*, 65–70.
14. Rosamund, J.; Allsop, A. *Science* **2000**, *287*, 1973–1976.
15. Tettelin, F. *Drug Dis. Today* **2001**, 887–892.
16. Gonzales, R. D.; Schreckenberger, P. C.; Graham, M. B.; Kelkar, S.; DenBesten, K.; Quinn, J. P. *Lancet* **2001**, *357*, 1179.
17. Pai, M. P.; Rodvold, K. A.; Schreckenberger, P. C.; Gonzales, R. D.; Petrolatti, J. M.; Quinn, J. P. *Clin. Infect. Dis.* **2002**, *35*, 1269–1272.
18. Bax, R. P.; Anderson, R.; Crew, J.; Fletcher, P.; Johnson, T.; Kaplan, E.; Knaus, B.; Kristinsson, K.; Malek, M.; Strandberg, L. *Nat. Med.* **1998**, *4*, 545–546.
19. Souli, M.; Kontopidou, F. V.; Koratzanis, E.; Antoniadou, A.; Giannitsioti, E.; Evangelopoulou, P.; Kannavaki, S.; Giamarellou, H. *Antimicrob. Agents Chemother.* **2006**, *50*, 3166–3169.
20. Rose, W. E.; Rybak, M. J. *Pharmacotherapy* **2006**, *26*, 1099–1110.
21. Fraise, A. P. *J. Infect.* **2006**, *53*, 293–300.
22. Doan, T.-L.; Fung, H. B.; Mehta, D.; Riska, P. F. *Clin. Therapeutics* **2006**, *28*, 1079–1106.
23. Hancock, R. E. W.; Chapple, D. S. *Antimicrob. Agents. Chemother.* **1999**, *43*, 1317–1323.
24. Zasloff, M. *N. Engl. J. Med.* **2002**, *347*, 1199–1200.
25. Zasloff, M. *Nature* **2002**, *415*, 389–395.
26. Hotchkiss, R. D.; Dubos, R. J. *J. Biol. Chem.* **1940**, *136*, 803–804.
27. Hotchkiss, R. D.; Dubos, R. J. *J. Biol. Chem.* **1941**, *141*, 155–162.
28. Jelokhani-Niaraki, M.; Prenner, E. J.; Kay, C. M.; McElhaney, R. N.; Hodges, R. S. *J. Pept. Res.* **2002**, *60*, 23–36.
29. Jelokhani-Niaraki, M.; Prenner, E. J.; Kondejewski, L. H.; Kay, C. M.; McElhaney, R. N.; Hodges, R. S. *Biophys. J.* **2000**, *78*, 286A.
30. Jelokhani-Niaraki, M.; Prenner, E. J.; Kondejewski, L. H.; Kay, C. M.; McElhaney, R. N.; Hodges, R. S. *J. Pept. Res.* **2001**, *58*, 293–306.
31. Jones, C. R.; Sikakana, C. T.; Hehir, S.; Kuo, M. C.; Gibbons, W. A. *Biophys. J.* **1978**, *24*, 815–832.
32. Jones, C. R.; Sikakana, C. T.; Kuo, M. C.; Gibbons, W. A. *J. Am. Chem. Soc.* **1978**, *100*, 5960–5961.
33. Kato, T.; Izumiya, N. *Biochimica. Et Biophysica. Acta* **1977**, *493*, 235–238.
34. Lewis, R.; Prenner, E. J.; Kiricsi, M.; Jelokhani-Niaraki, M.; Hodges, R. S.; McElhaney, R. N. *Biophys. J.* **2005**, *88*, 575A.
35. Prenner, E. J.; Jelokhani-Niaraki, M.; Kondejewski, L. H.; Hodges, R. S.; McElhaney, R. N. *Biophys. J.* **2000**, *78*, 321A.
36. Salgado, J.; Grage, S. L.; Kondejewski, L. H.; Hodges, R. S.; McElhaney, R. N.; Ulrich, A. S. *J. Biomol. NMR* **2001**, *21*, 191–208.
37. Shai, Y. *Biochimica. Et Biophysica. Acta-Biomembr.* **1999**, *1462*, 55–70.
38. Shai, Y. *Biopolymers* **2002**, *66*, 236–248.
39. Prenner, E. J.; Lewis, R.; Jelokhani-Niaraki, M.; Hodges, R. S.; McElhaney, R. N. *Biochimica. Et Biophysica. Acta-Biomembr.* **2001**, *1510*, 83–92.

40. Fuchs, P. C.; Barry, A. L.; Brown, S. D. *Diagn. Microbiol. Infect. Dis.* **2000**, *38*, 51–58.
41. Ball, L. J.; Goult, C. M.; Donarski, J. A.; Micklefield, J.; Ramesh, V. *Org. Biomol. Chem.* **2004**, *2*, 1872–1878.
42. Ando, S.; Nishikawa, H.; Takiguchi, H.; Lee, S.; Sugihara, G. *Biochimica. Et Biophysica. Acta Biomembr.* **1993**, *1147*, 42–49.
43. Jelokhani-Niaraki, M.; Kondejewski, L. H.; Farmer, S. W.; Hancock, R. E. W.; Kay, C. M.; Hodges, R. S. *Biochem. J.* **2000**, *349*, 747–755.
44. Kondejewski, L. H.; Farmer, S. W.; Wishart, D. S.; Hancock, R. E. W.; Hodges, R. S. *Int. J. Pept. Protein. Res.* **1996**, *47*, 460–466.
45. Kondejewski, L. H.; Farmer, S. W.; Wishart, D. S.; Kay, C. M.; Hancock, R. E. W.; Hodges, R. S. *J. Biol. Chem.* **1996**, *271*, 25261–25268.
46. Kondejewski, L. H.; Jelokhani-Niaraki, M.; Farmer, S. W.; Lix, B.; Kay, C. M.; Sykes, B. D.; Hancock, R. E. W.; Hodges, R. S. *J. Biol. Chem.* **1999**, *274*, 13181–13192.
47. Kondejewski, L. H.; Lee, D. L.; Jelokhani-Niaraki, M.; Farmer, S. W.; Hancock, R. E. W.; Hodges, R. S. *J. Biol. Chem.* **2002**, *277*, 67–74.
48. McInnes, C.; Kondejewski, L. H.; Hodges, R. S.; Sykes, B. D. *J. Biol. Chem.* **2000**, *275*, 14287–14294.
49. Bu, X. Z.; Wu, X. M.; Xie, G. Y.; Guo, Z. H. *Org. Lett.* **2002**, *4*, 2893–2895.
50. Kohli, R. M.; Walsh, C. T.; Burkart, M. D. *Nature* **2002**, *418*, 658–661.
51. Qin, C. G.; Bu, X. Z.; Wu, X. M.; Guo, Z. H. *J. Comb. Chem.* **2003**, *5*, 353–355.
52. Qin, C. G.; Zhong, X. F.; Bu, X. Z.; Ng, N. L. J.; Guo, Z. H. *J. Med. Chem.* **2003**, *46*, 4830–4833.
53. Ruttenbe, Ma.; Mach, B. *Biochemistry* **1966**, *5*, 2864.
54. Katayama, T.; Nakao, K.; Akamatsu, M.; Ueno, T.; Fujita, T. *J. Pharm. Sci.* **1994**, *83*, 1357–1362.
55. Mukhtar, T. A.; Koteva, K. P.; Wright, G. D. *Chem. Biol.* **2005**, *12*, 229–235.
56. Kuo, M. C.; Drakenberg, T.; Gibbons, W. A. *J. Am. Chem. Soc.* **1980**, *102*, 520–524.
57. Kuo, M. C.; Gibbons, W. A. *Biochemistry* **1979**, *18*, 5855–5867.
58. Kuo, M. C.; Gibbons, W. A. *J. Biol. Chem.* **1979**, *254*, 6278–6287.
59. Kuo, M. C.; Gibbons, W. A. *Biophys. J.* **1980**, *32*, 807–836.
60. Trauger, J. W.; Kohli, R. M.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7092–7098.
61. Kohli, R. M.; Trauger, J. W.; Schwarzer, D.; Marahiel, M. A.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7099–7108.
62. Trauger, J. W.; Kohli, R. M.; Mootz, H. D.; Marahiel, M. A.; Walsh, C. T. *Nature* **2000**, *407*, 215–218.
63. Bax, R.; Mullan, N.; Verhoef, J. *Int. J. Antimicrob. Agents* **2000**, *16*, 51–59.
64. Kay, C.; Lorthioir, O. E.; Parr, N. J.; Congreve, M.; McKeown, S. C.; Scicinski, J. J.; Ley, S. V. *Biotech. Bioeng.* **2000**, *71*, 110–118.
65. CLSI, Performance Standards for Antimicrobial Susceptibility Testing; Sixteenth Informational Supplement. In *CLSI Document M100-S16*, 7th ed.; Clinical and Laboratory Standards Institute: Wayne, Pennsylvania, 2006; Vol. 26, pp M100–S16.