High-Throughput Synthesis and Screening of Cyclic Peptide Antibiotics

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Received March 12, 2007

Cyclic peptides are a rich source of biologically active compounds and are produced in nature by plants, bacteria, fungi, and lower sea animals. A high-throughput methodology has been developed for the combinatorial synthesis, screening, and identification of cyclic peptide natural product analogues with improved biological activities or useful new activities. The methodology was applied to generate a library of 1716 tyrocidine A analogues, which were screened for antibacterial activity in 96-well plates. The identity of the active peptides was determined by partial Edman degradation/mass spectrometry. This has resulted in the discovery of a series of tyrocidine analogues that have significantly improved therapeutic indices compared to the natural product. The availability of tyrocidine analogues with varying antibacterial activities has provided important insights into the structure−function relationship of tyrocidine A, which should help reveal its mechanism of action.

Introduction

Cyclic peptides and depsipeptides are a class of privileged molecular structures. In comparison to linear peptides, cyclic peptides are more stable against proteolytic degradation due to their lack of free N- or C-terminus and reduced conformational freedom. The entropic advantages associated with the increased rigidity also make cyclic peptides tighter-binding and potentially more specific ligands of macromolecular receptors. In addition, cyclic peptides are more bioavailable (e.g., greater membrane permeability) than linear peptides due to the absence of N- and C-terminal charges and their ability to form intramolecular hydrogen bonds as they traverse the lipid bilayer.[1] Not coincidentally, cyclic peptides and depsipeptides are widely distributed in nature and possess a broad range of biological activities.[2−4] Several cyclic peptides such as cyclosporin A (an immunosuppressant),[5] caspofungin (an antifungal agent),[6] and daptomycin (an antibiotic)[7] are clinically used therapeutic agents. Given their potential as drugs, drug leads, and molecular tools in biomedical research, there has been much interest in the generation of cyclic peptide natural product analogues, either chemically[8−13] or enzymatically,[14−16] to further improve their native activities or to impart new activities. In the previous studies, cyclic peptide analogues were generally prepared through sequential or parallel synthesis, which practically limited the size of libraries to 10^2-10^3 variants. In principle, much larger cyclic peptide libraries (up to 10^7 variants) should be readily accessible via combinatorial synthesis by the split-and-pool method.[17,18] However, combinatorial synthesis necessitates post-screening sequence determination of "hit" peptides; until very recently,[13] sequence determination of cyclic peptides had been a very challenging problem.

Tyrocidine A (Scheme 1, compound **1**) is a cyclic decapeptide antibiotic produced by *Bacillus brevis*. It adopts a β-pleated sheet conformation under physiological conditions.[19] It is believed that tyrocidine A and other cationic peptides kill bacteria by disrupting their cell membranes.[20,21] As such, it is difficult for bacteria to develop resistance to this type of antibiotic because it would require significant alteration of the membrane structure. However, clinic application of tyrocidine A is limited by its low selectivity toward microorganism; it also disrupts mammalian cell membranes, as indicated by its high hemolytic activity. In attempts to improve the therapeutic index of tyrocidine A, other investigators have prepared a fairly large number of tyrocidine analogues by parallel synthesis. For example, Walsh and co-workers chemoenzymatically synthesized a 192-member library of tyrocidine analogues by replacing D-Phe at positions 1 and 4 with both natural and unnatural amino acids.[14] From this small library, they identified one compound that retained almost wild-type antibacterial activity but had significantly reduced hemolytic activity. Glycosylation of peptide side chains had a similar effect.[15] Guo and colleagues employed parallel chemical synthesis to prepare small libraries of tyrocidine analogues on the solid phase (up to 192 variants).[8,9] A safety-catch linker was used to effect simultaneous peptide cleavage from resin and cyclization. They found that a significant increase in therapeutic index was achieved by modifying residues at positions 3, 4, and 6. These earlier studies have demonstrated that it is possible to significantly improve the therapeutic index of tyrocidine A through structural modification. However, due to the limitation of parallel synthesis, their libraries only covered a very small fraction of the possible sequence space. The question remains whether it is possible to generate analogues with still better therapeutic properties. To address this question, we have recently developed a methodology for combinatorial synthesis and high-throughput screening of cyclic peptides and demonstrated its validity by the identification of a biologically active tyrocidine analogue from a 400-member library.[13] In this work, we have applied this method to generate a library containing 1716 tyrocidine A analogues and discovered several analogues of improved therapeutic indices.

Chemistry

Design of Tyrocidine Analogue Library. Previous studies have shown that substitution at position 1 (D-Phe) has relatively minor effect on tyrocidine activity.[8,14] In contrast, mutations at positions 4 (D-Phe) or 6 (Gln) resulted in large changes in both antibacterial and hemolytic activities.[9,14] Asn at position 5 was selected as the anchoring residue for attachment of the peptide to solid support. Positions 7 and 8 appeared to be more tolerant

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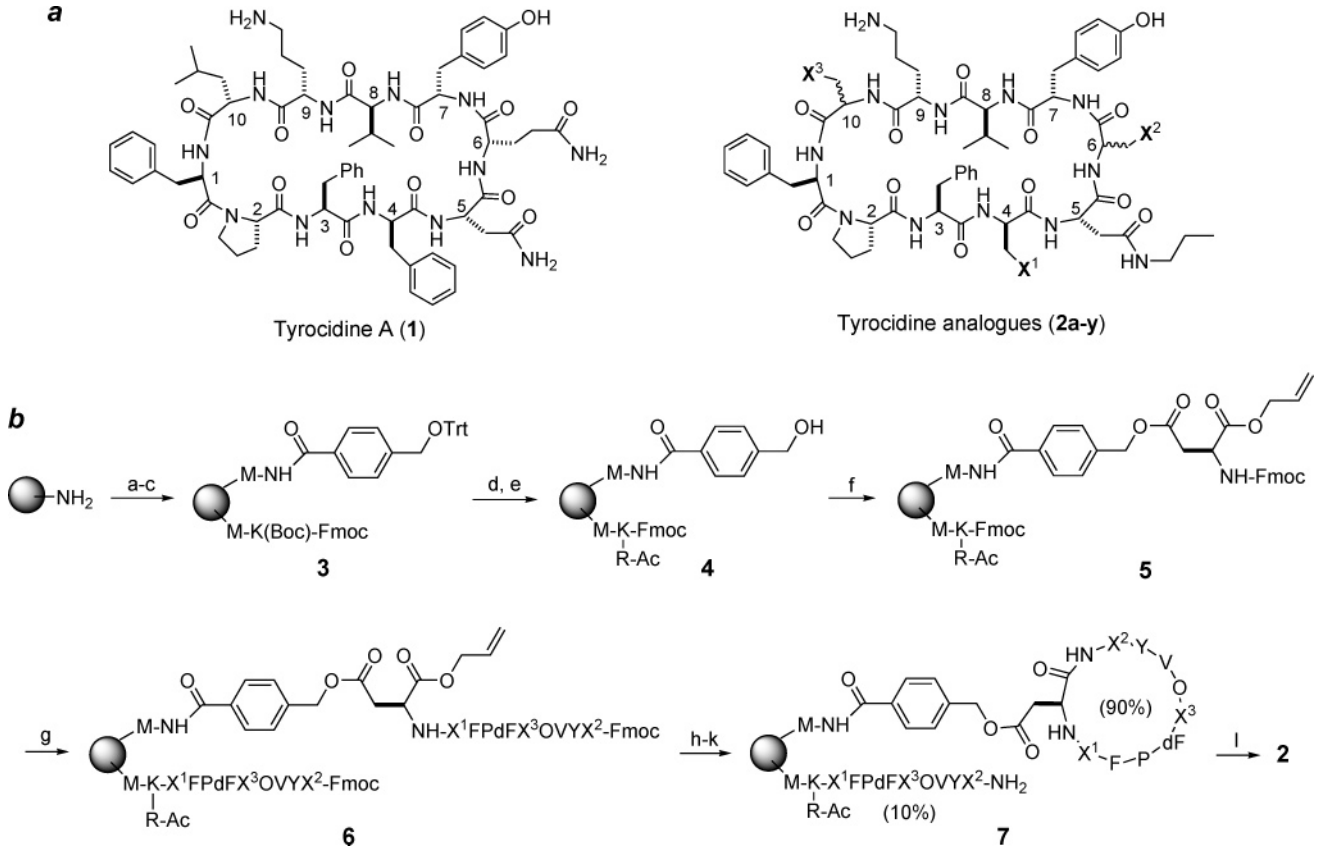
10.1021/jm070282e CCC: $37.00
Published on Web 06/05/2007

Scheme 1. (a) Structures of Tyrocidine A and Its Analogues; (b) Solid-phase Synthesis of the Tyrocidine Analogue Library[a]



[a] X^1-X^3 indicates the randomized amino acids. Reagents and conditions: (a) Fmoc-Met-OH/HBTU; (b) 20% piperidine; (c) 9:1 (mol/mol) Trt-HMBA/Fmoc-Lys(Boc)-OH, HBTU; (d) TFA; (e) N^{α}-Ac-Arg(Pmc)-OH/HBTU; (f) Fmoc-Asp-OAll/DIPC/DMAP; (g) peptide synthesis by Fmoc/HBTU (or HATU) chemistry; (h) $Pd(PPh_3)_4$; (i) 20% piperidine; (j) PyBOP/HOBt; (k) reagent K; (l) $CH_3CH_2CH_2NH_2$. Abbreviations: dF, D-Phe; O, ornithine.

to mutations,[9,13] while ornithine (Orn[a]) at position 9 was found to be important for antibacterial activity, likely because it provides the positive charge to the peptide.[13] Position 10 has not yet been targeted for extensive structure−activity relationship studies.[8] We thus decided to randomize positions 4, 6, and 10 of tyrocidine A (Scheme 1; X^1, X^2, and X^3 in compound **2**, respectively). Three amino acids, including D-Phe (wild-type), D-Arg, and D-Lys, were incorporated at position 4 because a previous study has shown that a positively charged residue at this position reduces the hemolytic activity of tyrocidine.[14] Gln at position 6 was replaced by 22 proteinogenic, D-, and other unnatural amino acids [D-Ala, Arg, D-Asn, Asp, L-4-fluorophenylalanine (Fpa), Gln, D-Glu, Gly, His, Ile, D-Leu, Lys, norleucine (Nle), Orn, D-Phe, L-phenylglycine (Phg), Pro, Ser, Thr, Trp, Tyr, and D-Val]. At position 10, Leu was replaced by 26 amino acids including four N^{α}-methylated amino acids [D-Ala, Arg, D-Asn, Asp, Fpa, Gln, D-Glu, Gly, His, Ile, D-Leu, Lys, L-N^{α}-methylalanine (Mal), L-N^{α}-methylleucine (Mle), L-N^{α}-methylphenylalanine (Mpa), Nle, Orn, D-Phe, Phg, Pro, sarcosine (Sar), Ser, Thr, Trp, Tyr, and D-Val]. The nonproteinogenic amino acids (D- and N^{α}-methylated amino acids, Fpa, Nle, Orn, and Phg), many of which are frequently found in naturally occurring nonribosome-synthesized peptides,[2−4] were included to increase the structural diversity of the peptide library and the stability of the resulting peptides against proteolytic degradation. The N^{α}-methylated amino acids were excluded from position 6 due to anticipated difficulty in peptide cyclization involving secondary amines. The theoretical diversity of the library is $3 \times 22 \times 26$ or 1716.

Library Synthesis and Evaluation. The tyrocidine analogue library was synthesized on TentaGel macrobeads (280−320 μm, 0.27 mmol/g, and 3.5 nmol/bead). To facilitate sequence determination of hit peptides, we adopted a "one-bead two-compound" strategy.[13,22] In this case, each resin bead was rendered to contain two peptide molecules; approximately 90% of the peptide molecules were cyclic and attached to the TentaGel resin by a labile ester linkage, whereas the other 10% of the molecules were the corresponding linear peptide, linked to the resin via a stable amide bond (Scheme 1b). Library synthesis started with the addition of a methionine to the resin, which allows later peptide release by CNBr prior to MS analysis. Next, the resin was reacted with a 9:1 (mol/mol) mixture of *O*-trityl hydroxymethylbenzoic acid (HMBA) and Fmoc-Lys-(Boc)-OH to afford resin **3**. Treatment with trifluoroacetic acid (TFA) removed the trityl group as well as the Boc group on the lysine side chain. The lysine side chain was selectively acylated with N^{α}-acetyl-Arg(Pmc)-OH by using HBTU as the coupling agent. This C-terminal arginine provides a fixed positive charge to the linear peptide, facilitating later MS analysis. Synthesis of the tyrocidine analogues started with L-Asn at position 5, because its side chain provides a convenient

[a] Abbreviations: Fpa, L-4-fluorophenylalanine; Fmoc-OSU, *N*-(9-fluorenylmethoxy-carbonyloxy)succinimide; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HMBA, hydroxymethylbenzoic acid; HOBt, 1-hydroxybenzotriazole; Mal, L-N^{α}-methylalanine; MHC, minimal hemolysis concentration; MIC, minimal inhibitory concentration; Mle, L-N^{α}-methylleucine; Mpa, L-N^{α}-methylphenylalanine; Nle, norleucine; NMM, *N*-methylmorpholine; Orn, ornithine; PED/MS, partial Edman degradation/mass spectrometry; Phg, L-phenylglycine; PITC, phenyl isothiocyanate; PrAsn, aspartic acid propylamide; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; Sar, sarcosine; TFA, trifluoroacetic acid.

anchoring point for attachment to the solid support. Thus, the free hydroxyl group of resin **4** was acylated with the side chain carboxyl group of N^{α}-Fmoc-Asp-*O*-allyl to give ester **5**. Subsequent peptide chain elongation employed standard Fmoc/HBTU chemistry, and the random residues were introduced by the split-and-pool synthesis[17,18] to afford linear peptide **6**. Next, the allyl group on Asp-5 was removed by $Pd(PPh_3)_4$ followed by Fmoc group removal with piperidine. Subsequent treatment with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) resulted in the cyclization of the peptides that were linked to the resin by the benzyl ester linkage (~90%), whereas the amide-linked peptides remained linear (~10%). The resin-bound peptides (**7**) were deprotected by treatment with TFA.

The quality of the cyclic peptide library was assessed by a number of methods. First, 20 beads immediately before and after the cyclization reaction were randomly picked from the library and subjected to quantitative ninhydrin tests. The results indicated that 90.2% of the resin-bound peptides were cyclized (theoretical yield 90%). Next, 30 beads were randomly selected from the deprotected library (resin **7**), and the peptides were released by CNBr and analyzed by MALDI-TOF MS. The cyclic monomer gave two peaks at *m*/*z* M + 1 (where M is the molecular mass of the cyclic peptide monomer) and *m*/*z* M + 218 (cyclic peptide + HMBA−homoserine lactone linker; Figure S1 in Supporting Information). Any uncyclized peptide should give peaks at *m*/*z* M + 19 and *m*/*z* M + 236, whereas the cyclic dimer should produce peaks at *m*/*z* 2M + 1, *m*/*z* 2M + 218 (cyclic dimer + one linker), and *m*/*z* 2M + 435 (cyclic dimer + two linkers). Sixteen beads showed uncyclized peptides, but their peak intensities were 0.9−5.3% compared to those of the corresponding cyclic monomers (Table S1 in Supporting Information). Eight beads had visible dimer signals, at intensities 0.2−23% of the corresponding cyclic monomers (average 1.9%). Assuming that the uncyclized peptide, cyclic monomer, and cyclic dimer forms all have similar ionization efficiency in the MS, the yields of cyclic monomers were calculated to be 77−100% (average 97%; Table S1). We have previously shown that linear and cyclic monomers of the same sequence have more or less similar ionization effieciencies.[13] Third, 10 selected peptides with antibacterial activity were resynthesized on a larger scale, and the products were analyzed by HPLC and MS (vide infra). The results showed that the desired cyclic monomer was usually the predominant species. The major impurity was usually a monomeric epimer in which the stereochemistry at Asn-5 was inverted (D-Asn). Previous studies have also shown that on-bead peptide cyclization is quite efficient.[8,9,13]

Results

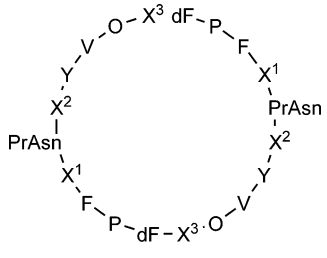
Approximately 2400 beads were randomly selected from the tyrocidine analogue library and placed in 96-well microtiter plates (one bead/well). The cyclic peptide on each bead was cleaved from the solid support by aminolysis of the ester linkage with neat propylamine. The linear encoding peptide, which was linked to the resin via an amide linkage, was unaffected (Scheme 1b). The excess propylamine was evaporated, and the resulting peptides (**2**) were redissolved in 40 μL of DMSO/water and neutralized by the addition of 20 μL of a sodium phosphate buffer. Assuming a 50% yield for cyclic peptide synthesis, the resulting stock solution should contain ~30 μM cyclic peptide. Antibacterial assays were carried out by the addition of 20 μL of the peptide stock solution to 80 μL of *Bacillus subtilis* culture (final peptide concentration of ~6 μM). Out of the 2400 samples screened, 22 (~1%) showed visible growth inhibition of *B. subtilis*. The 22 beads were retrieved from the microtiter plates, and the identity of the cyclic peptides was determined by sequencing the remaining linear encoding peptides by partial Edman degradation/mass spectrometry (PED/MS).[13,23] This resulted in 21 unambiguous sequences (compounds **2a**−**y** in Table 1; for their MALDI-TOF mass spectra, see Figure S2 in Supporting Information). Inspection of the selected sequences show that basic amino acids are clearly important for antibacterial activity, as 19 out of the 21 peptides contained at least one Arg or Lys at positions 4 and 6. When the residue at position 4 was D-Arg or D-Lys, a hydrophobic residue was typically found at position 10, while a more diverse set of amino acids were tolerated at position 6. When D-Phe was at position 4, an L-Lys was frequently found at position 6 and a hydrophilic amino acid was usually found at position 10. Interestingly, none of the peptides contained any positively charged residue at position 10 and, with the exception of peptide **2u** (which contains a Sar at position 10), the selected peptides did not contain N^{α}-methylated amino acids. These results offer several insights into the structure−activity relationship of tyrocidine A. First, the actual position of the positively charged residue(s) in the cyclic peptide is important for antibacterial activity. Second, there is strong sequence covariance among different positions, presumably because an active antibacterial agent must maintain an optimal balance in hydrophobicity and overall number of positive charges.[20,21] Finally, the absence of N^{α}-methylated amino acids suggests the importance of the backbone amides in maintaining the β-sheet conformation of tyrocidine through the formation of intramolecular hydrogen bonds.[19] Methylation of the backbone amide would disrupt the intramolecular hydrogen bonds.

Ten of the selected peptides (bead No. 2, 4, 9, 12, 13, 14, 15, 16, 17, and 20) were individually synthesized on a larger scale. The peptides were cleaved from the resin by treatment with propylamine, resulting in cyclic peptides containing an

Table 1. Sequences of Selected Tyrocidine Analogues with Antibacterial Activity

		peptide sequence			
bead No.	cmpd no.	position 4 (X^1)	position 6 (X^2)	position 10 (X^3)	position 5
1	**2a**	D-Lys	L-Fpa	L-His	L
2[a]	**2b**	D-Lys	L-Fpa	L-Pro	L
	8a	D-Lys	L-Fpa	L-Pro	L or D
3	**2c**	D-Lys	L-Fpa	L-Ile	L
4[a]	**2d**	D-Lys	L-Lys	L-Tyr	D
	2e	D-Lys	L-Lys	L-Tyr	L
5	**2f**	D-Lys	D-Leu	D-Phe	L
6	**2g**	D-Lys	D-Leu	D-Glu	L
7	**2h**	D-Lys	D-Leu	L-Nle	L
8	**2i**	D-Lys	L-Thr	L-Asp	L
9[a]	**2j**	D-Arg	D-Leu	D-Asn	L
10	**2k**	D-Arg	L-Arg	L-Phg	L
11	**2l**	D-Arg	L-Thr	L-Nle	L
12[a]	**2m**	D-Arg	D-Ala	L-Pro	L
	8b	D-Arg	D-Ala	L-Pro	L or D
13[a]	**2n**	D-Arg	Gly	L-Fpa	D
	2o	D-Arg	Gly	L-Fpa	L
14[a]	**2p**	D-Arg	L-Arg	L-Fpa	L
	2q	D-Arg	L-Arg	L-Fpa	D
15[a]	**2r**	D-Arg	L-Ser	L-Fpa	D
	2s	D-Arg	L-Ser	L-Fpa	L
16[a]	**2t**	D-Arg	L-Lys	D-Leu	L
17[a]	**2u**	D-Phe	D-Phe	Sar	L
18	**2v**	D-Phe	L-Lys	L-Gln	L
19	**2w**	D-Phe	L-Lys	L-Asp	L
20[a]	**2x**	D-Phe	L-Lys	Gly	L
21	**2y**	D-Phe	D-Leu	L-Ser	L

[a] Peptides on these beads were selected for resynthesis and antimicrobial analysis.



	X_1 =	X_2 =	X_3 =
8a	D-Lys	L-Fpa	L-Pro
8b	D-Arg	D-Ala	L-Pro

Figure 1. Structures of cyclic dimers **8a** and **8b**.

aspartic acid propylamide (PrAsn) at position 5 (Scheme 1). All 10 peptides (in their crude forms) inhibited bacteria growth (see Table S2 in Supporting Information). HPLC analyses indicated that the desired cyclic monomer was usually the predominant species (see Figure S3 in Supporting Information). A major impurity was usually an isomer that had the same molecular mass as the desired product but a different retention time on HPLC. We assigned this isomer as the cyclic epimer containing a D-PrAsn at position 5, which was formed during peptide cyclization. Cyclic peptide dimers (Figure 1, compound **8**) were another source of impurities in some samples. The crude peptides were purified by preparative reversed-phase HPLC (except for peptide **2u**, which had poor aqueous solubility and lower antibacterial activity). Due to the presence of epimers and dimers, a total of 14 different compounds were obtained (Tables 1 and 2). For example, bead No. 2 gave a cyclic monomer (**2b**) and a dimer (**8a**), while bead No. 4 gave a pair of monomeric epimers (**2d** and **2e**). The purity of the resulting peptides was assessed by analytical HPLC and found to be at least 95% (Figure 4S in Supporting Information). The authenticity of the peptides was confirmed by MALDI-TOF analysis. To determine which epimer from bead 4 (**2d** or **2e**) had L-PrAsn at position 5, we resynthesized the peptide and carried out the final cyclization reaction in the presence of $CuCl_2$, which was known to suppress epimerization during peptide fragment condensation.[24] We observed an increase in the amount of major product **2e** and a decrease of **2d** (data not shown). We thus concluded that **2e** was the "desired" epimer (which contained L-PrAsn at position 5). We tentatively assigned the corresponding major epimers **2b**, **2j**, **2m**, **2o**, **2p**, **2s**, **2t**, **2u**, and **2x** as the L-PrAsn-containing peptides (Table 2 and Figure S3).

The pure cyclic monomers derived from seven of the beads (compounds **2d**, **2e**, **2n**, **2o**, **2p**, **2q**, **2r**, **2s**, **2t**, and **2x**) showed potent antibacterial activity against *B. subtilis* cells, with minimal inhibitory concentration (MIC) values in the range of 1−8 μg/mL (Table 2). For the two epimers derived from bead No. 4, the D-PrAsn-containing isomer (**2d**) was more potent than the L-PrAsn isomer (**2e**; MIC values of 1−2 vs 4 μg/mL, respectively). Surprisingly, while the crude peptides derived from beads No. 2 and 12 were active against *B. subtilis* cells, after purification, the monomeric cyclic peptides (**2b** and **2m**) were completely inactive (Table 2). We, therefore, purified the minor components, the cyclic dimers **8a** and **8b**, and found that they were responsible for the observed antibacterial activities (MIC ~0.5 and 4 μg/mL against *B. subtilis*, respectively). Similarly, purified cyclic monomer **2j** (from bead No. 9) did not have any antibacterial activity. Unfortunately, our attempt to isolate the dimer of peptide **2j** failed due to the poor separation efficiency.

The cyclic peptides were next tested against *Escherichia coli* and Gram-positive pathogens *Enterococcus faecalis* and *Staphylococcus epidermidis*. Their hemolytic activities, defined as minimal hemolytic concentrations (MHCs), were also determined. Most of the cyclic monomers were active against all four bacterial species, with MIC values of 1−32 μg/mL (Table 2). Compared to wild-type tyrocidine A, which has an MHC/MIC ratio of 2, all of the analogues had substantially improved therapeutic indices (MHC/MIC in the range of 4−64). Compound **2n** had the best overall properties; it was essentially equipotent as tyrocidine A against all four bacteria but had an MHC/MIC ratio of 8−16. Cyclic dimer **8b** did not show any inhibition against *E. coli*, *E. faecalis*, or *S. epidermidis* (Table 2). It had an MHC value of 512 μg/mL and an MHC/MIC ratio of 1024. Its specific inhibition of *B. subtilis* and the high MHC suggest that this peptide may function via a mechanism other than disruption of cell membrane.

Discussion

Through our previous[13] and current work, we have developed a general methodology for high-throughput synthesis and screening of cyclic peptide libraries. Combinatorial synthesis can easily generate libraries of 10^7 different compounds. PED/MS sequencing provides a rapid, inexpensive method for post-screening hit identification.[23] Our method is compatible with both on-bead and in-solution screening methods. When on-bead screening is carried out, the entire library may be screened against a macromolecular target in minutes to hours.[13] For solution-phase assays, however, our method is currently limited by how fast one can separate the library beads into 96-well plates. In this work, individual beads were manually arrayed into 96-well plates by using a micropipet, which was a laborious and time-consuming process. We noted that bead arraying devices have recently been developed;[25] by coupling the bead arrayers with simple liquid-handling robotics, one should be able to screen up to 10^4−10^5 beads/compounds in a week. It is worth pointing out that our method is not limited to antibacterial assays. The method should be readily applicable to screening cyclic peptide libraries against other organisms, tissues, or molecular targets (e.g., enzymes and protein receptors).

Application of the methodology to tyrocidine A has resulted in a number of compounds with significantly improved therapeutic indices, including one compound that has equal antibacterial potency as the natural product but is 4−8 times less toxic to human erythrocytes. These compounds may provide useful leads for further development into clinically useful antibiotics. The availability of a large number of tyrocidine analogues of varying antibacterial potencies will help paint a clearer picture on the structure−activity relationship of cationic peptide antibiotics, which should in turn provide clues about their mechanism of action. Our results also demonstrate that generation and screening natural product analogues is a fruitful approach for discovery of compounds of improved or new biological activities.

Experimental Section

Materials and General Methods. Fmoc-protected D- and L-amino acids and *N*-(9-fluorenylmethoxy-carbonyloxy)succinimide (Fmoc-OSU) were purchased from Advanced ChemTech (Louisville, KY), Peptides International (Louisville, KY), or NovaBiochem (La Jolla, CA). N^{α}-Fmoc-L-aspartic acid α-allyl ester (Fmoc-Asp-OAll) and PyBOP were purchased from NovaBiochem. *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole hydrate (HOBt) were from Peptides International. Propylamine was purchased form ACRS. Chloranil was from Fluka. All solvents and other chemical reagents were obtained from Aldrich, Fisher Scientific (Pittsburgh, PA), or VWR

Table 2. Antimicrobial Activities and Therapeutic Indices of Selected Peptides

	MIC (μg/mL)					
cmpd	*B. subtilis*	*E. coli*	*E. faecalis*	*S. epidermidis*	MHC (μg/mL)	MHC/MIC[a]
1 (tyrocidine)	1	8	1	1	2	2
2b	NA[b]	NA	NA	NA		
2d	1−2	16	16	4	64	32−64
2e	4	32	16	4	128	32
2m	NA	NA	NA	NA		
2n	1−2	4	2	1	16	8−16
2o	4	32	4	4	32	8
2p	1−2	8	8	4	8	4−8
2q	2	8	8	4	16	8
2r	2	8	8	2	16	8
2s	2	16	8	2	16	8
2t	8	32	16	8	128	16
2x	8	NA	32	16	64	8
8a	4	32	8	32	128	32
8b	0.5	NA	NA	NA	514	1024

[a] MIC of *B. subtilis* was used in the calculation. [b] NA, no inhibition observed at 32 μg/mL.

(West Chester, PA) and were used without further purification unless noted otherwise. Phenyl isothiocyanate (PITC) was purchased in 1 mL sealed ampoules from Sigma-Aldrich, and a freshly opened ampule was used in each experiment. TentaGel Macrobead-NH_2 resin (280−320 μm, 0.27 mmol/g, and 3.5 nmol/bead) was obtained from Peptide International. NovaSynTGA resin (90 μm, 0.23 mmol/g) was from NovaBiochem. α-Cyano-4-hydroxy-cinnamic acid was purchased from Sigma and recrystallized prior to use.

Synthesis of Tyrocidine Analogue Library. The peptide library was synthesized on 1.0 g of TentaGel Macrobead-NH_2 resin (280−320 μm, 0.27 mmol/g). All of the manipulations were performed at room temperature unless otherwise noted. The C-terminal methionine was coupled onto the resin with 4 equiv of Fmoc-Met-OH using HBTU/HOBt/NMM. After removal of the Fmoc group with 20% piperidine in DMF, the resin was coupled with 1.05 equiv of a 9:1 (mol/mol) mixture of $TrtOCH_2C_6H_4COOH$ and Fmoc-Lys-(Boc)-OH at room temperature for 4 h. The trityl and Boc groups were removed with TFA/TES/ethanedithiol/CH_2Cl_2 (90:2.5:2.5:5) for 30 min. The beads were exhaustively washed with CH_2Cl_2 and DMF and then reacted with N^{α}-acetyl-Arg(Pmc)-OH (0.2 equiv) and HBTU (0.2 equiv). The resin was dried under vacuum overnight, swollen in anhydrous CH_2Cl_2 for 20 min, and treated twice with Fmoc-Asp-OAll (4 equiv), diisopropylcarbodiimide (5 equiv), and 4-dimethylaminopyridine (0.1 equiv) in CH_2Cl_2/DMF (9:1; 3 h each time). The resin was then treated with Ac_2O (3 equiv)/4-dimethylaminopyridine (0.1 equiv) in CH_2Cl_2 for 45 min to cap any remaining hydroxyl group. After removal of the N-terminal Fmoc group, peptide chain elongation was carried out using standard Fmoc/HBTU chemistry. For the addition of the random positions, the resin was split into the desired number of aliquots and each aliquot was coupled twice with 4 equiv of a different Fmoc-amino acid (2 h each time). To differentiate mass-degenerate amino acids during MS sequencing, 5% (mol/mol) CD_3CO_2D was added to the coupling reactions of D-Leu, L-Lys, and l-Orn, whereas 5% CH_3-CD_2CO_2D was added to the coupling of Nle. After the addition of residue X^3 at position 10, the resin from the 26 reaction vessels was combined into two different pools based on the identity of the X^3 residue. The beads that had N^{α}-methylated amino acids as the X^3 residue were combined into one pool and coupled to L-Orn by using HATU/NMM as the coupling agents. All other beads were combined as a second pool and L-Orn was added using HBTU/HOBt/NMM. After the addition of the X^2 residue at position 6, the C-terminal allyl group was removed by overnight treatment with $Pd(PPh_3)_4$ (1 equiv), triphenylphosphine (3 equiv), formic acid (10 equiv), and diethylamine (10 equiv) in anhydrous THF. The beads were washed sequentially with 0.5% diisopropylethylamine in DMF (10 min) and 0.5% sodium dimethyldithiocarbamate hydrate in DMF (10 min). The N-terminal Fmoc group was then removed with 20% piperidine, and the beads were washed with DMF, 1 M HOBt in DMF (3 × 10 min), and DMF. For peptide cyclization, a solution of PyBOP/HOBt/NMM (5, 5, and 10 equiv, respectively) in DMF was mixed with the resin, and the mixture was incubated on a carousel shaker for 3 h. To determine the cyclization efficiency, 20 beads were randomly picked for the ninhydrin test, with the same amount of beads before cyclization as control. Ninhydrin assay was performed by the addition of 300 μL of 76% phenol in ethanol (w/v), 300 μL of 20 μM KCN in pyridine, and 300 μL of 5% ninhydrin in ethanol (w/v) to 20 beads followed by heating at 120 °C for 5 min. The absorbance at 580 nm was measured on a Perkin-Elmer Lambda 25 UV/visible spectrometer, and the background was subtracted from the absorbance of the mixture without resin. For beads that contained proline as the X^2 residue, the cyclization reaction was performed separately and a chloranil test[26] was performed to monitor the completion of reaction. Beads were randomly picked and transferred into an microcentrifuge tube and 50 μL of 2% (w/w) chloranil in DMF and 50 μL of 2% (v/v) acetaldehyde in DMF were added. The color of the beads was examined under a microscope. After cyclization was complete, the resin was washed with DMF (3×) and CH_2Cl_2 (5×), and side chain deprotection was carried out with a modified reagent K (7.5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol, and 1% anisole in TFA). The resin was washed with CH_2Cl_2, dried under vacuum, and stored at 4 °C.

Library Screening. Resin beads were manually transferred into glass-coated 96-well microtiter plates using a micropipet (1 bead/well). The cyclic peptide was detached from each bead by overnight treatment with 50 μL of propylamine at room temperature. After overnight standing in a fume hood, most of the propylamine evaporated. The residual amine was removed by repeatedly dissolving the peptides in each well in 50 μL of CH_2Cl_2 and evaporation (three times). The peptide amides were then dissolved in 40 μL of 30% DMSO in water and the solution was neutralized by the addition of 20 μL of a 1 M sodium phosphate buffer (pH 7.0). The resulting stock solution (60 μL) should contain ∼30 μM cyclic peptide, assuming a loading capacity of 3.5 nmol/bead and an overall peptide synthesis yield of 50%. Antibacterial assays were performed using the standard microtiter plate assay method.[27] For each sample, 20 μL of the stock solution was added to a 1000-fold diluted overnight culture and the resulting culture (total volume of 100 μL) was incubated for 7 h at 37 °C and examined visually for bacterial growth. MIC was defined as the lowest peptide concentration that caused no visible cell growth.

Peptide Sequencing by PED/MS. The positive beads selected from the library were pooled and subjected to partial Edman degradation in a single reaction vessel, as described previously.[23] Mass analysis was performed on a Bruker III MALDI-TOF instrument in an automated manner at Campus Chemical Instrument Center of The Ohio State University. The data obtained were analyzed by either Moverz software (Proteometrics LLC, Winnipeg,

Canada) or Bruker Daltonics flexAnalysis 2.4 (Bruker Daltonic GmbH, Germany).

Large-Scale Synthesis of Tyrocidine Analogues. Each cyclic peptide selected for further analysis was synthesized on 100 mg of NovaSynTGA resin (0.23 mmol/g) in a manner similar to that described for the library construction. The cyclic peptide was released from the resin by incubating with propylamine (1.5 mL) for 6 h at room temperature. After the propylamine was removed under vacuum, side chain deprotection was effected with a cocktail containing 79:7.5:5:5:2.5:1 (v/v) TFA/phenol/H_2O/thioanisole/ethanedithiol/anisole. The cyclic peptides were precipitated in diethyl ether and dried under vacuum. The crude peptides were purified by reversed-phase HPLC on a C_{18} column (Varian Dynamax 250 × 10.0 mm), which was eluted with a linear gradient of acetonitrile in 0.05% TFA in water. The identity of each peptide was confirmed by MALDI-TOF MS analyses.

Determination of Minimal Hemolysis Concentration (MHC). A published method[28] was adapted for MHC determination. The cyclic peptide stock solutions were serially diluted (2-fold) in H_2O and the diluted solutions were added into a 96-well plate. Fresh human red blood cells were diluted 100-fold in PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4), and the diluted red blood sample was transferred into each well and mixed with the peptide solution (final volume of 50 μL). The plate was incubated overnight at room temperature and visually inspected for cell lysis. MHC was defined as the lowest concentration required to lyse half of the red blood cells.

Acknowledgment. We thank Dr. Susheela Tridandapani for providing the human red blood cells and Ms. Lisa M. Mutchler for assistance in library screening. This work was supported by a grant from the National Institutes of Health (GM062820).

Supporting Information Available: MALDI-TOF MS, HPLC, and bioactivity data of target peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM070282E