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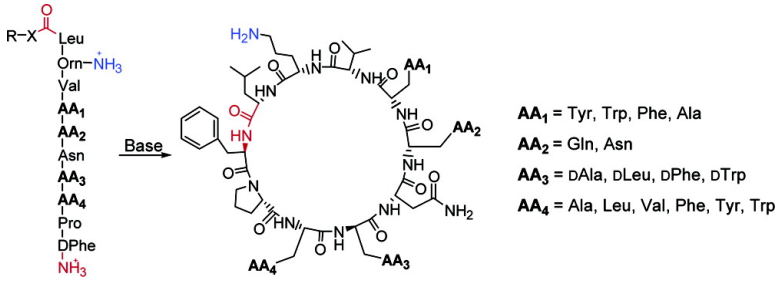

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Optimization of Antibacterial Cyclic Decapeptides

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A previously developed method for cyclic peptide synthesis was demonstrated to be able to provide convenient access to large combinatorial libraries of analogues, and this methodology was applied to the optimization of natural product cyclic decapeptides. Using this method, a 192-member library was designed and successfully constructed on the basis of the natural products tyrocidines, streptocidins, and loloatins to increase the therapeutic indices of these antibiotics. Library screening identified nine analogues whose therapeutic indices were increased by up to 90-fold in comparison to the natural products. Three of these analogues showed significant increase in antibacterial potency and concurrent drastic decrease in hemolytic activity. Since the natural products target the bacterial cell wall, the newly discovered analogues are promising leads for drug development against drug-resistant bacteria.

Introduction

Emergence of resistance to currently available antibiotics by pathogenic microbes is posing an increasing threat to public health.[1] There is a growing interest in the discovery of new antibiotics to counter this microbial resistance. One potential source of novel antibiotics is peptide natural products that kill microbes quickly by physical disruption of their cell membranes.[2,3] Peptide antibiotics are unlikely to cause rapid emergence of resistance since they target the cell membranes nonspecifically and development of resistance requires significant alteration of the lipid composition.[2a] These peptide natural products include the structurally diverse antimicrobial α-helical cationic peptides found in all organisms as an important part of their innate defense against infections and the β-pleated amphipathic cyclic peptide antibiotics predominantly from bacterial sources.[4] The cyclic β-pleated peptide natural products can be structurally classified as a family of antimicrobial cyclic decapeptides composed of gramicidin S (**1**),[5] tyrocidines (**2**),[6] loloatins (**3**),[7] and the recently isolated streptocidins (**4**),[8] as shown in Figure 1. In comparison to the antimicrobial cationic peptides, the small size and structural simplicity of these β-pleated peptides make them attractive targets for drug development.[9] This class of peptide natural products are broad-spectrum antibiotics with high potency; however, they also disrupt mammalian cell membranes, as denoted by their high hemolytic activity.[10] To increase their ability in differentiating bacteria from mammalian cells, these peptide products must be structurally optimized so that their hemolytic activity is minimized and their antibacterial potency maximized at the same time.

The main obstacle to the optimization of the β-pleated peptide antibiotics is the poor availability of analogues for functional screening. Traditional synthetic methods are poor at generating large numbers of analogues, whereas poor cyclization tendency hampers the development of an efficient general method for construction of the cyclic structures.[11] Recently, we found that the biosynthetic precursors of tyrocidine A[12] and gramicidin S[13] adopt a preorganized conformation which is highly favorable for specific head-to-tail cyclization, leading to a simple synthetic scheme for tyrocidine A synthesis using an acylsulfonamide safety-catch linker, which is activated prior to deprotection and cyclization-mediated product release (Scheme 1).[14] The readily adaptability of this conformation-dependent method to combinatorial synthesis should enable easy access to tyrocidine A analogues for optimization of this peptide antibiotic.

In our previous work, substitution of single amino acid residues with alanine showed the side chains of the constituent amino acids on the tyrocidine A scaffold do not affect the strong propensity of the linear peptide precursors to self-cyclize.[14] Although the independence of the self-cyclizing propensity from the side-chain functionalities of the synthetic method stems from the backbone antiparallel β-pleated sheet structure involving four strong backbone hydrogen bonds,[14] it still remained to be proved whether substitution of multiple residues on the tyrocidine A scaffold would be allowed. In addition, it was of interest whether the constituent amino acid residues of the tyrocidine A scaffold were amenable to substitution by residues other than alanine. Herein, these issues are examined.

Results and Discussion

Scope of the Tyrocidine A Analogue Library. Multiple substitution of the constituent amino acids in the parent

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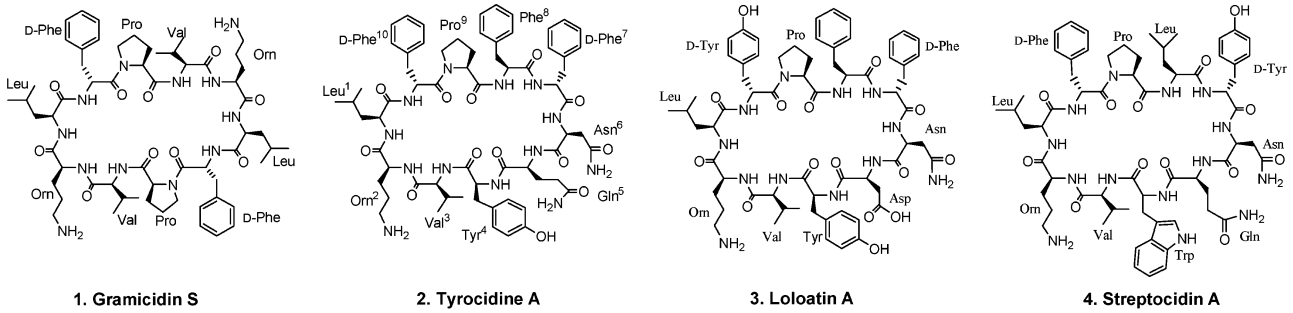
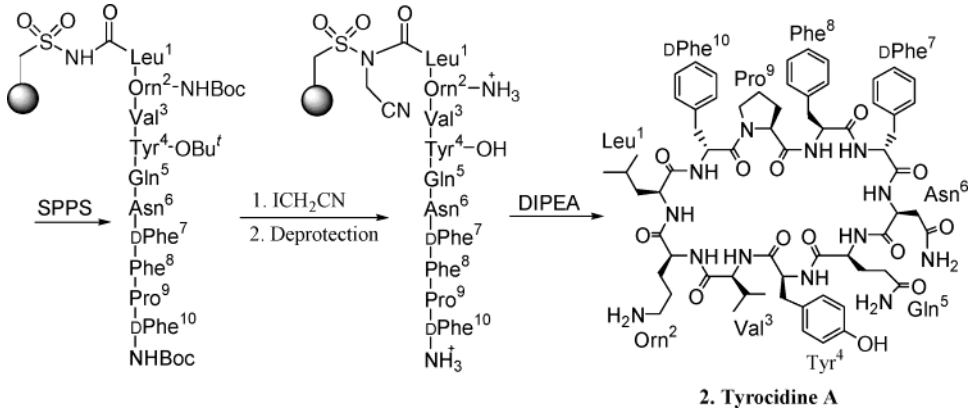
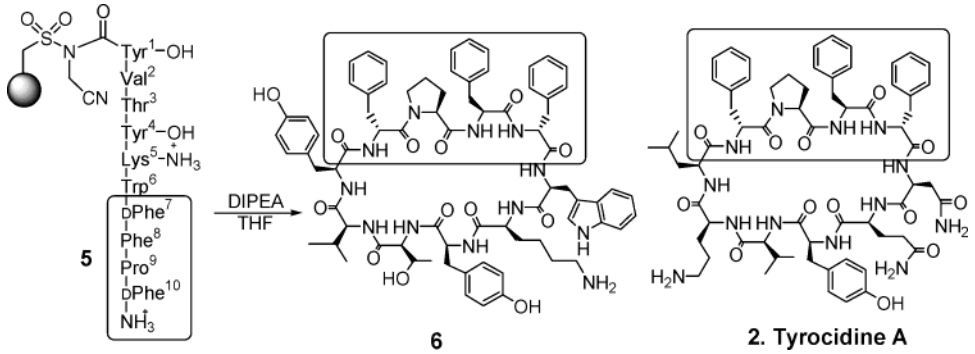


Figure 1. Representative structures of β-pleated cyclic decapeptide antibiotics.

Scheme 1. Illustration of the Conformation-Dependent Self-Cyclization of Linear Peptide Precursors on the Basis of the Tyrocidine A Template



Scheme 2. Self-Cyclizing Synthesis of the Substitution Product (**6**) of Six Consecutive Amino Acids on the Tyrocidine A Scaffold

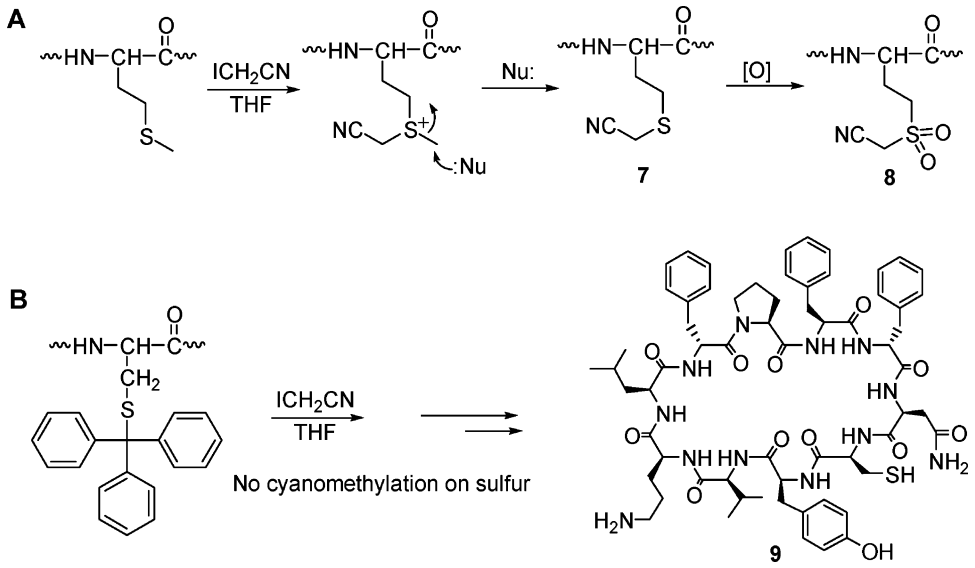


The unchanged structural moiety is circled.

compound using amino acids other than alanine were carried out. The method was first applied to synthesis of the streptocidins that differ from tyrocidine A at Tyr-4 (changed to Tyr/DTrp/Trp), DPhe-7 (changed to DTrp/DPhe), and Phe-8 (changed to Leu).[16] The analogous cyclic peptide natural products were successfully obtained in high purity, similar to the results obtained for the tyrocidine A and its alanine-substituted analogues.[14] In another experiment, a hexapeptide fragment was randomly selected to replace six consecutive residues (Leu-1 to Asn-6, Scheme 2) at the carboxy terminus of the linear precursor of tyrocidine A. The solid-phase synthesis of the substituted precursor (**5**) and its cyclization after cyanomethylation activation were performed following the published procedure[14] to obtain the head-to-tail cyclization product (**6**) in high purity (>95%).

The possible restrictions on the amino acid side chain functionalities that could be used to replace the residues on the tyrocidine A template were examined. Since a highly reactive electrophile, ICH_2CN, was used for activation of the carboxy terminal sulfonamide in the synthetic scheme, nucleophilic side chains, particularly that of methionine and cysteine, might not survive the synthetic process. Indeed, the methylthio side chain of methionine was found to be substituted with a cyanomethylthio group (product **7**) that underwent oxidation upon exposure to air to form product **8** after the cyanomethylation activation and cyclization. The reaction mechanism is shown in Scheme 3 (A). However, the cysteine side chain partly survived the cyanomethylation activation process because of protection of the thioyl group from reacting with iodoacetonitrile, by the bulky trityl protecting group employed in the Fmoc solid-phase peptide synthesis. When a cysteine residue was incorporated into position 5 of the scaffold molecule, the correct cyclic product (**9**, Scheme 3B) was obtained, as shown by mass spectro-

Scheme 3. Fate of the Nucleophilic Thiol Ether Functionality in the Conformation-Dependent Synthesis of Cyclic Peptides



scopy (calcd mass, 1244.6; found $[M + H]^+$, 1245.9). Nevertheless, the product (**9**) was very low in yield (<2%) and escaped HPLC detection and further characterization. Therefore, both methionine and cysteine could not be incorporated into the cyclic template.

Another concern in the use of the self-cyclization reaction was the possible interference of the side chain amino groups in the head-to-tail cyclization of the linear precursors. Our previous studies adequately proved that δ-NH_2 of Orn-2 of the tyrocidine A template does not interfere in the final cyclization reaction;[12,14] however, it is expected that side chain NH_2 would attack the activated sulfonamide if it belongs to the C-terminal residue, on the basis of a previous observation of a side chain-to-tail cyclization of a similar linear peptide precursor.[17] Although not systematically investigated, the amine side chain can be introduced to other positions on the scaffold. For example, in the synthesis of compound (**6**), a lysine residue was successfully incorporated into position 5; thus, the self-cyclizing propensity of the tyrocidine-based linear peptide precursors is not influenced by the side-chain functionalities.

Despite the problems associated with the active side-chain amine and thioyl groups mentioned above, the exchangeability of multiple residues in the scaffold still ensures access to numerous analogues through the synthetic method.

Library Design and Synthesis. To increase the ability of the natural products to differentiate bacteria from mammalian cells without affecting their high antibiotic activity, a focus library was synthesized on the basis of currently available structure−activity relationship information. Much work has been done on the relationship of structure vs function for the antibiotic potency of tyrocidines.[11] It has been reported that significant changes of the side chain of most of the constituent residues of the tyrocidine template results in the loss of antibiotic potency or an inability to form the antiparallel β-sheeted structure necessary for the biological property;[11] however, the structural dependence of the unwanted hemolytic activity of tyrocidine A is less clear. The only known example is the introduction of a cationic residue in the DPhe-7 position which results in a significant increase in minimum hemolysis concentration (MHC).[18] Instead of relying on side-chain swapping of single amino acid residues, the antibacterial and hemolytic activities could be differentiated by combinations of the simultaneous changes in the scaffold.

To determine the amino acid residues that could be altered to improve the function of the cyclic structure, it was noticed that there exist a variety of congeners and analogous peptide natural products for the scaffold tyrocidine A. Alignment of the sequences of these cyclic peptides and tyrocidine A (Figure 2) showed that small structural alterations occurred at the positions of Tyr-4, Gln-5, DPhe-7, and Phe-8 of the cyclic scaffold, whereas other amino acid residues were highly conserved. On the basis of this comparison, a library of 192 members was designed, as shown in Figure 2. Since all the listed cyclic peptide natural products are potent antibiotics against Gram-positive bacteria, it was expected that most members of the designed library would also possess high antibiotic potency. Most natural products in Figure 2 were also included in the attempted library to serve as internal controls for the resulting library.

The 192-member library was synthesized with the conformation-dependent self-cyclization method (Scheme 1) following the reported procedures, using the Irori AccuTag-100 Combinatorial Chemistry System.[19] After synthesis, 24 random library members were subjected to product identification and purity analysis. The results are summarized in Table 1. Reversed-phase HPLC analysis identified a major peak between t_R 24 and 33 min under the employed conditions for each unpurified product whose purity was >80%. Mass spectroscopy (FAB) showed that the unpurified products gave rise to a single molecular ion consistent with the calculated mass of the expected head-to-tail cyclic product. Finally, the major product for each library member was purified by HPLC and examined by ^{1}H NMR to reveal its structure by comparison with that of the template. In all cases, the isolated compound was confirmed to be the correct head-to-tail cyclization product of the corresponding linear peptide precusor, because the proton signals of the unchanged amino acid residues of the scaffold were found to be either

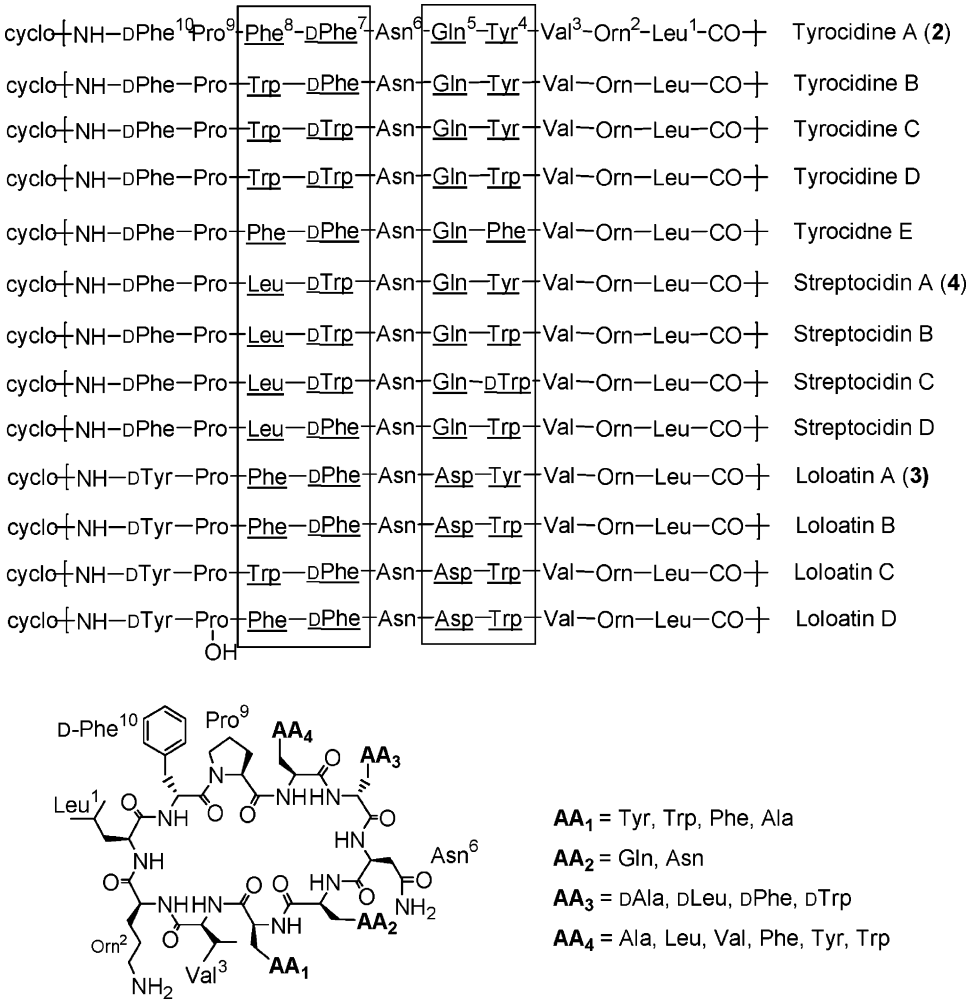


Figure 2. Sequence alignment of the decapeptide natural product antibiotics and design of an analogue library for their functional optimization.

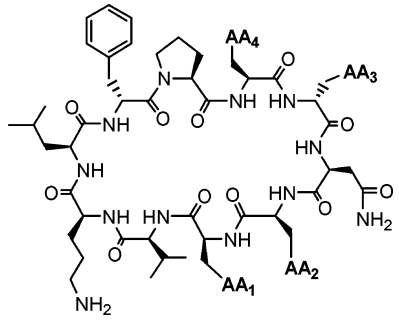
identical or slightly altered in comparison to that of the wild-type tyrocidine A.[16a] These results show the synthesis of the attempted library was successful and that the self-cyclization method is indeed a suitable general method for construction of cyclic peptide libraries based on the tyrocidine A scaffold.

Screening Results. The library in a 96-well microtiter plate was dissolved in DMSO after solvent evaporation and subjected to activity screening without purification and determination of the compound concentration. The minimum inhibition concentration (MIC) was determined by a microdilution method in liquid culture[20] using *Bacillus subtilis* (ATCC82) as the model Gram-positive organism. Minimum hemolysis concentration (MHC) of the library members was determined by a published method with slight modifications using human erythrocytes.[21] Since both MIC and MHC have the same concentration dependence that was expressed in dilution factors, the therapeutic index (MHC/MIC) of the library members was independent of the compound concentration and used as the evaluation measure for the ability of the library members in differentiating bacteria from mammalian cells. The screening results are shown in Figure 3.

From the results in Figure 3, the MHC/MIC ratios for the natural peptide products were very close to unity, consistent with previous findings that these wild-type peptide antibiotics do not discriminate between bacteria and mammalian cells. Consistent results for these internal controls showed that the simple workup procedure in the library synthesis was compatible with the screening assays and that any trace possible impurities were not toxic and did not interfere in the biological activity measurement. Most other library members did not show an improved therapeutic index. This was expected because the structural variation at the four selected amino acid residues are small and the template molecule has a very low index. Interestingly, a significantly increased therapeutic index was found for certain combinations of the subtle variations at the altered positions. Identities of the positive library members are shown in Table 2 with a MHC/MIC ratio of >10. No regularity is apparent for these positive structures.

To confirm the hit compounds from the above screening experiments, they were individually synthesized using the same self-cyclization method along with a negative control (compound **43**), purified by HPLC, characterized by spectroscopic techniques, and subjected to MIC and MHC determination again. The results are summarized in Table 2. The activity data show that the therapeutic indices of these compounds are, indeed, much increased in comparison to that of the natural cyclic peptide products, which is consistent with the primary screening results using the unpurified products. The small discrepancies of the index values before and after the resynthesis may come from the impurities contained in the samples of the unpurified library.

Table 1. Characterization of the 24 Randomly Selected Library Members



	AA_1	AA_2	AA_3	AA_4	t_R (min)[a]	calcd mass	[M + H][b]	yield (%)[c]	purity (%)[d]
10	Ala	Asn	D-Trp	Leu	26.9	1168.6	1169.8	19	87
11	Phe	Asn	D-Trp	Tyr	27.3	1294.7	1295.8	15	86
12	Trp	Gln	D-Phe	Leu	30.8	1258.7	1260.8[e]	21	90
13	Phe	Gln	D-Ala	Leu	28.0	1143.6	1144.7	17	84
14	Ala	Gln	D-Ala	Val	23.7	1053.6	1054.6	16	86
15	Phe	Gln	D-Leu	Tyr	28.5	1235.7	1236.6	17	86
16	Trp	Asn	D-Leu	Phe	32.3	1244.7	1245.9	17	92
17	Ala	Gln	D-Trp	Val	27.3	1260.7	1261.8	18	91
18	Ala	Gln	D-Trp	Ala	24.1	1140.6	1141.7	18	84
19	Tyr	Asn	D-Ala	Leu	26.7	1145.6	1146.7	18	86
20	Trp	Gln	D-Ala	Leu	28.5	1182.7	1183.7	18	91
21	Tyr	Gln	D-Leu	Ala	25.8	1159.6	1160.7	16	88
22	Trp	Asn	D-Trp	Leu	29.8	1283.7	1285.8[e]	13	84
23	Trp	Asn	D-Ala	Val	27.0	1154.6	1156.6[e]	18	81
24	Ala	Asn	D-Leu	Trp	28.4	1168.6	1169.7	14	87
25	Ala	Asn	D-Phe	Tyr	25.4	1179.6	1180.7	15	94
26	Ala	Gln	D-Ala	Trp	26.0	1140.6	1141.8	16	95
27	Phe	Gln	D-Trp	Val	28.5	1244.7	1245.9	16	81
28	Tyr	Asn	D-Ala	Trp	27.3	1218.6	1219.8	15	90
29	Ala	Asn	D-Phe	Ala	24.7	1087.6	1088.6	17	94
30	Trp	Gln	D-Trp	Ala	26.7	1255.7	1256.7	17	88
31	Ala	Asn	D-Phe	Val	27.0	1115.6	1116.7	20	82
32	Ala	Asn	D-Phe	Trp	27.9	1202.6	1203.7	15	89
33	Ala	Asn	D-Leu	Phe	29.2	1129.6	1130.6	10	80

[a] Retention time of the product peak on the HPLC chromatograms. [b] Molecular ion from FAB-MS. [c] Overall yield based on the loading value of the resin after first amino acid coupling. [d] Calculated as percentage of the product peak area over the total peak area between 10 and 35 min. [e] Samples in DMSO-d_6 solution when mass spectrum was taken.

From Table 2, it can be concluded that there are not apparent patterns for the amino acid residues at positions 4 and 5 among the compounds with an increased therapeutic index. In contrast, when the two hydrophobic aromatic side chains of DPhe-7 and Phe-8 of the tyrocidine A scaffold were replaced by two smaller aliphatic side chains (compound **34**) or one aliphatic side chain and one aromatic side chain (compound **35**−**42**), a significant increase in the therapeutic index was achieved. These effects of the structural variations most likely stem from the unique structure and the mode of action for the template tyrocidine A molecule. Like its well-studied relative gramicidin S,[22] tyrocidine A is a cyclic decapeptide antibiotic adopting a preformed β-pleated sheet conformation under physiological conditions.[16] Its membrane-disrupting activity is dependent on its amphipathic structure, in which side chains of Leu-1, Val-3, Asn-6, and Phe-8 constitute one hydrophobic "side", and the cationic Orn-2 side chain forms a hydrophilic patch on the other side of the rigid antiparallel β-pleated sheet. The residues at positions 4 and 5 form a type II′ β-turn and do not belong to either side of the antiparallel sheet structure. The small structural perturbations at both positions are, thus, believed to have little effect on the amphipathicity that has been shown to affect both the antimicrobial potency and specificity in other amphipathic antimicrobial model peptides.[23] On the contrary, both phenyl side chains at positions 7 and 8 are on the hydrophobic side of the antiparallel β-pleated sheet structure and contribute significantly to the amphipathicity of this molecule. When small hydrophobic aliphatic side chains are introduced into either or both positions, the hydrophobicity of the molecule is reduced, and the overall amphipathicity of the cyclic structure is subtly modulated to increase the therapeutic index, which is consistent with similar function enhancement through amphiphaticity modulation achieved by systematic configuration alteration in the component amino acids of other antimicrobial peptides.[24] Interestingly, compounds such as **34** in Table 2 show significant increase of both the therapeutic index and the antimicrobial potency, making them very promising leads for further optimization. These effects of structural variation at both positions 7 and 8 of the tyrocidine A scaffold on the antibacterial activity and cellular selectivity show that the natural peptide products can, indeed, be optimized to counter the microbial resistance that has become an increasing threat to public health.

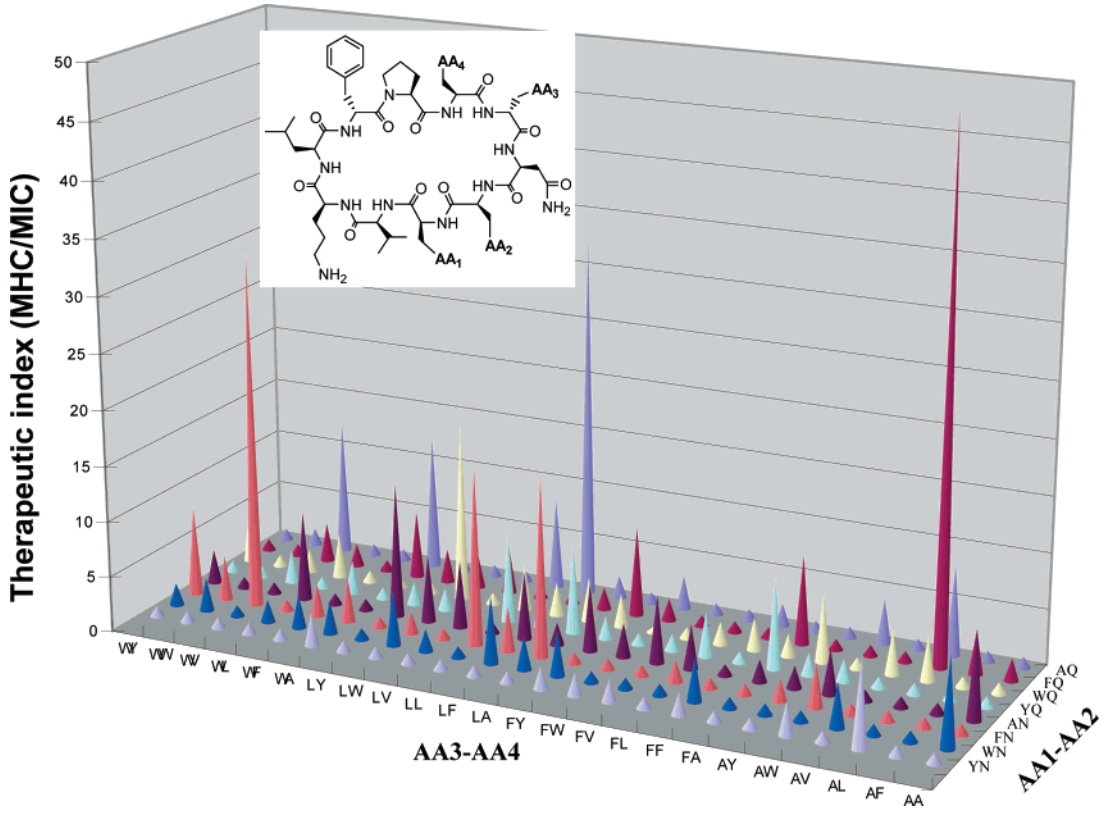
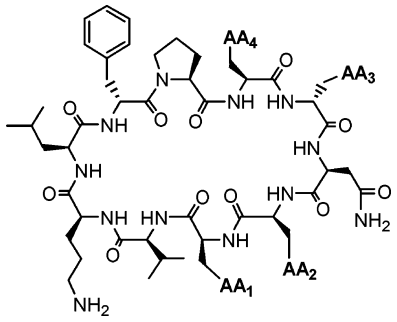


Figure 3. Screening results for the 192-member tyrocidine A analogue library. The *x* and *y* axes list the amino acid residues at the AA_3/AA_4 and AA_1/AA_2 positions, respectively.

Table 2. Characterization and Biological Activities of Positive Hit Compounds after Resynthesis



	AA_1	AA_2	AA_3	AA_4	t_R (min)	calcd mass	[M + H]	MIC (μg/mL)	MHC (μg/mL)	MHC/MIC
2	Tyr	Gln	D-Phe	Phe				8.3	8.5	1
34	Phe	Gln	D-Ala	Leu	28.0	1143.6	1144.7	0.8	70	90
35	Phe	Asn	D-Trp	Val	28.5	1230.7	1232.0	8.0	160	20
36	Ala	Gln	D-Leu	Phe	26.5	1143.6	1144.8	1.5	36	24
37	Phe	Asn	D-Leu	Ala	27.5	1129.6	1130.8	16	150	9
38	Trp	Gln	D-Leu	Trp	30.6	1297.7	1299.0	5.0	40	8
39	Phe	Asn	D-Leu	Leu	30.1	1171.7	1172.9	6.0	60	10
40	Ala	Gln	D-Trp	Val	26.1	1168.6	1169.9	3.0	45	15
41	Ala	Asn	D-Leu	Tyr	25.8	1145.6	1146.7	10	100	10
42	Trp	Asn	D-Leu	Phe	32.3	1144.7	1145.9	1.4	13	9
43	Tyr	Gln	D-Trp	Leu	27.3	1260.7	1262.0	2.5	10	4

Conclusion

In this report, we further explored a combinatorial cyclic peptide library accessible by a conformation-dependent self-cyclization method based on the decapeptide antibiotic tyrocidine A.[14] Multiple substitutions are allowed on the scaffold molecule in addition to the known exchangeability of each individual amino acid with alanine;[14] however, methionine and cysteine were found to be incompatible with the synthetic scheme because of low product yields. In addition, amino acids with a side-chain amino group, such as lysine or ornithine, shall not be used to replace the C-terminal residual of the linear peptide precursors because of the side chain-to-end cyclization observed for a similar linear peptide.[17] The exchangeability of multiple residues in the scaffold allows access to numerous analogues through the synthetic method.

A 192-member combinatorial library was designed and successfully constructed using the conformation-dependent self-cyclization method. Library screening identified nine analogues that were increased in their therapeutic indices by 8−90-fold in comparison to that of the wild-type natural products without compromising the original high antibiotic potency. More interestingly, three of the identified analogues show significant increases in both antibacterial activity and therapeutic index. These newly discovered analogues are promising leads for further drug development.

Experimental Section

General. All reactions were carried out under protection of nitrogen atmosphere. All Fmoc and Boc-protected amino acids were from GL Biochem (Shanghai) Ltd. 4-Sufamylbutyryl AM resin was from Novabiochem. Peptide grade *N*,*N*-dimethylformamide (DMF) was from Fisher Scientific and dried with 4-Å molecular sieves before use. Tetrahydrofuran (THF) was refluxed over sodium metal and freshly distilled before use. Chloroform ($CHCl_3$) was refluxed over CaH_2 and freshly distilled before use. Benzotriazole-1-yl-oxy-trispyriolidinophosphonium (PyBOP), diisopropylethylamine (DIEA), 1-methyl-2-pyrrolidinone (NMP), *N*,*N*′-diisopropylcarbodiimine (DIPCPI), 1-hydroxybenzotriazole (HOBt), iodoacetonitrile (ICH_2CN), and all other chemicals were from Acros and used directly.

NMR spectra were recorded on a Varian INOVA 500 and calibrated with the residual undeuterated solvent or TMS. Mass spectra were recorded on a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer using fast atom bombardment (FAB) ionization mode. HPLC analysis and purification was carried out using a Waters 600E system with a model 2487 dual λ absorbance detector.

Synthesis of Cyclic Peptides and an Analogue Library of Tyrocidine A. Solid-phase synthesis of the linear peptide precursors and their cyclization into tyrocidine A and analogues followed a published procedure with slight modification.[14] Typically, attachment of the first amino acid was performed in a 25-mL round-bottle flask with $CHCl_3$ (5 mL), DIEA (1.67 mmol, 160 μL), Fmoc-Leu-OH (192.5 mg), and 4-sulfamylbutyryl AM resin (150 mg, 0.18 mmol). The suspension was stirred for 20 min under N_2 protection and subsequently cooled to −20 °C. PyBOP (0.54 mmol, 283.1 mg) was then added, and the reaction mixture was stirred for 10 h at −20 °C. After the resin was washed with $CHCl_3$ (5 × 5 mL), the coupling reaction was repeated once. Loading of the first amino acid was determined by reacting 1 mg of dried resin with 0.5 mL of 20% piperidine in DMF for 10 min at room temperature and then measuring the absorbance at 290 nm (A_{290}), using the equation

$$\text{loading (mmol/g)} = [(A_{290} - \text{blank}) \times 0.5\ \text{mL}]/(5253\ \text{M}^{-1}\ \text{cm}^{-1} \times 0.001\ \text{g resin} \times 1\ \text{cm})$$

The loading value was in the range of ∼0.7 to 0.8 mmol/g.

Peptide synthesis was subsequently carried out through repetitive cycling between Fmoc deprotection and coupling of an appropriate Fmoc-amino acid residue according to the sequence of the target molecule. Deprotection of Fmoc was carried out by addition of 2 mL of 20% piperidine in DMF solution to the resin, followed by agitation for 30 min at room temperature under the protection of bubbling N_2. After washes with DMF (5 × 5 mL), a freshly prepared mixture of the Fmoc-protected amino acid (5 × 0.12 mmol), HOBt (38 mg, 5 × 0.12 mmol), and DIPCDI (40.4 mg, 5 × 0.12 mmol) was added, and the reaction mixture was kept under N_2 bubbling for 2h. The coupling reaction was repeated if a Kaiser test of the resin was positive after DMF washing (5 × 5 mL). Boc-DPhe-OH was used in coupling of the last amino acid.

The sulfonamide linker was activated by immersing the resin in a mixture of NMP (2 mL), DIEA (90 μL), and iodoacetonitrile (137.6 μL, 0.36 mmol) with slow N_2 bubbling for 24 h at room temperature in a 25-mL round-bottle flask wrapped in aluminum foil. After sequential washing of DMF (5 × 5 mL) and CH_2Cl_2 (3 × 5 mL), Boc and *t*-Bu protecting groups on the linear peptide were removed by addition of 3 mL of reagent B (CF_3COOH/phenol/*i*-Pr_3SiH/H_2O = 88:5:5:2) and stirring for 2 h at room temperature. The resulting resin was finally washed with THF (5 × 5 mL) and immersed in 2.5 mL of 20%(v/v) DIEA in THF for 6 h at room temperature. After the cleavage reaction, the resin was filtered and washed with THF (3 × 2 mL). The filtrates were combined, precipitated with cold ether (4 °C), and dissolved in 200 μL of DMSO for analysis or HPLC purification.

The 192-member library was synthesized by adapting the aforementioned solid-phase synthetic method to the Irori's AccuTag-100 Combinatorial Chemistry System according to the manufacturer's instructions. Reactions were carried out in MicroKan reactors with 30 mg of 4-sulfamylbutyryl AM resin (0.8 mmol/g) for each compound. The amounts of the reagents and the solvent were scaled up proportionally. The cleavage products were directly evaporated off the solvent without ether precipitation to obtain crude cyclic peptides that were each dissolved in 200 μL of DMSO for activity assays and HPLC analysis or purification.

Typically, initial yield and purity of a peptide product or library member before HPLC purification were 10−20% and >80%, respectively. After HPLC purification, the yield was 5−10%. A summary of characterization data for compounds **10**−**43** is provided in the Supporting Information.

HPLC Analysis and Purification of Cyclic Products. HPLC analysis and purification were performed with a Waters 600E system with a reversed-phase semipreparative XTerra RP_{18} column, 7 μm, 7.8 × 300 mm. Separation conditions were 2.0 mL min^{-1} flow rate, a linear gradient of 80−20% A over 25 min, 20−0%A over 10 min. Solution A was 0.1% TFA in double-deionized H_2O, and solution B was 0.1% TFA in acetonitrile. The cyclic peptide products were obtained as white solids after solvent evaporation.

Determination of Minimum Inhibition Concentration (MIC). A published method[20] was used with slight modification. Briefly, an overnight culture of *B. subtilis* (ATCC 82) was diluted to a density of approximately ∼7×10^5 colony-forming units/mL and transferred into a 96-well plate with 300 μL of culture in each well. The peptides in 2-fold serial dilution were added to the wells, and the culture without any additives was used as a negative control. The cultures

were incubated at 30 °C for 20 h in an orbital shaker (200 rpm). Cell growth was measured by optical density at 600 nm in a microtiter plate reader, and the minimal inhibition concentration (MIC) was determined from a plot of the cell growth vs peptide concentration.

Determination of Minimum Hemolysis Concentration (MHC). A published procedure[21] was adapted for MHC determination. Erythrocytes were separated as a precipitate from 20 mL of anticoagulant-treated fresh human blood by fractionation in an equal volume of Ficoll-Paque PLUS (Amersham Bioscience) by centrifugation at 2500*g* for 20 min at 20 °C. The cells were washed three times with 40 mL of PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) by resuspension and centrifugation at 2500*g* for 15 min. The red blood cells were resuspended to 5×10^7 cells/mL in PBS buffer and added to a 96-well plate (100 μL/well) containing 100 μL of peptide solution in the same buffer in 2-fold serial dilutions. The microplates were incubated at 37 °C for 4 h, and the turbidity of the cell cultures was then measured at A_{690} with a microtiter plate reader (TECAN) for determination of minimum hemolysis concentrations. Triton X-100 and the buffer PBS (pH 7.4) were used as the positive and negative controls, respectively.

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Supporting Information Available. Two-dimensional ^{1}H NMR spectra and assignments for tyrocidine A; summary of characterization data for compounds **10**−**43**; HPLC chromatograms compounds **6**, **10**−**43**; FAB-MS spectra of **6**, **9**, **10**−**43**; and ^{1}H NMR spectra of compounds **10**−**43**. This material is available free of charge via the Internet at http://pubs.acs.org.

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