Dissociation of Antibacterial and Hemolytic Activities of an Amphipathic Peptide Antibiotic

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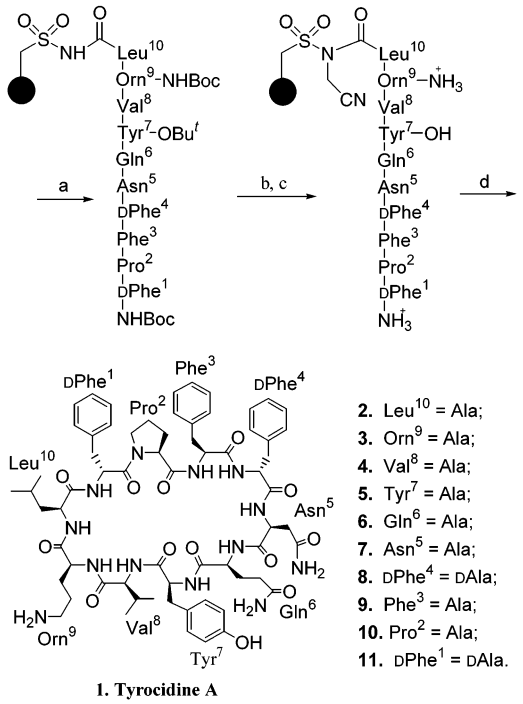
Abstract: Using an alanine-scanning method, we have found that the antibacterial and hemolytic activities of the amphipathic cyclic decapeptide antibiotic tyrocidine A depend on different structural components. Single substitution of glutamine-6 of the natural product with a cationic amino acid results in a therapeutic index enhancement of up to 140-fold. Successful dissociation of the two intimately associated properties should enable discovery of novel analogues with both high bacterial selectivity and antibacterial potency to counter microbial resistance.

Introduction. Microbial resistance to currently available antibiotics has become a serious public health threat.[1] Among efforts to contain the resistance, interest has been focused on the antibacterial α-helical peptides, including melittin[2] and pardaxin,[3] and amphipathic β-sheet peptide antibiotics such as gramicidin S and tyrocidine A.[4] These natural products act on microbial cell membranes where their accumulation results in disruption of the barrier functions.[5] Development of resistance to such molecules is unlikely because it requires significant alteration of the lipid composition of the microbial membranes. Indeed, no resistance to such antibiotics has been reported.[6] However, it is well-known that the high antibacterial potency of these amphipathic peptides is often intimately associated with high hemolytic activity, preventing their direct use in combating the microbial resistance.

To develop drug leads against resistant microbes on the basis of these natural peptide scaffolds, the structure−activity relationship was exploited to increase the therapeutic index, namely, minimizing the high hemolytic activity while maintaining the original high antibacterial activity. For example, D-amino acids have been systematically introduced to the α-helical[7] and β-sheet[8] peptides to disrupt the amphipathic structures for enhancement of selectivity against bacteria. In addition, chemoenzymatic replacement of a hydrophobic resident of tyrocidine A with a cationic one results in significant increase in microbial specificity.[9] To further unravel the structure−activity relationship for the small peptide antibiotic tyrocidine A, we took a systemic approach to identify the structural determinants of its antibacterial and hemolytic properties that led to dissociation of the two closely associated properties.

Synthesis. Tyrocidine A and its analogues were synthesized by a safety-catch method developed earlier by us as shown in Scheme 1,[10] based on the propensity of the biosynthetic precursor of tyrocidine A to form a conformation highly favorable for head-to-tail cyclization.[11] Briefly, the linear precursors of the cyclic peptides were synthesized on 4-sufamylbutyryl AM resin using standard Fmoc/DIC/HOBt chemistry. The carboxy terminus of the linear peptide was activated by cyanomethylation with ICH_2CN. After deprotection of the Boc and *t*-Bu groups, the activated linear precursors were cyclized under basic conditions to afford specific head-to-tail cyclic products, without interference of the reactive side chain $-NH_2$ and $-OH$ groups. The products were purified by HPLC and characterized by ^1H NMR and FAB-MS before determination of the biological activities.

Scheme 1. Synthesis of Tyrocidine A and Its Alanine-Substituted Analogues[a]



1. Tyrocidine A

[a] Reagents and conditions: (a) standard Fmoc solid-phase peptide synthesis; (b) ICH_2CN, NMP, DIPEA, 24 h; (c) CF_3COOH/phenol/*i*-Pr_3SiH/H_2O = 88:5:5:2, 1 h; (d) 20% DIPEA/THF.

Results and Discussion. To identify the structural determinants for the antibacterial and hemolytic activities, constituent amino acid residuals were systematically substituted with alanine in an alanine-scanning experiment. The alanine analogues were subjected to determination of the minimum inhibition concentration (MIC) against *Bacillus subtilis* and minimum hemolysis concentration (MHC) of human erythrocytes using published methods with slight modifications.[12,13] The results are summarized in Table 1. The relative therapeutic indices (relative MHC/MIC) of the analogues to the wild-type tyrocidine A are plotted in Figure 1.

Table 1 and Figure 1 indicate that most side chains of the constituent amino acid residuals affect the antibacterial and hemolytic activities to approximately the same extent. Alanine substitution of D-Phe-1, Tyr-7, Val-8, or Orn-9 resulted in analogues with therapeutic

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Table 1. Antibacterial and Hemolytic Activities of Tyrocidine A and Its Alanine-Substituted Analogues

compd	substituted residual	MHC[a] (μg/mL)	MIC[b] (μg/mL)	MHC/MIC
1	none	8.2 ± 2.0	8.3 ± 1.2	1.0 ± 0.4
2	Leu[10]	50 ± 5	25 ± 3	2.0 ± 0.5
3	Orn[9]	13 ± 2	13 ± 1	1.0 ± 0.3
4	Val[8]	16 ± 3	15 ± 2	1.1 ± 0.3
5	Tyr[7]	14 ± 2	14 ± 1	1.0 ± 0.2
6	Gln[6]	28 ± 2	1.5 ± 0.5	19 ± 6
7	Asn[5]	39 ± 3	15 ± 1	2.6 ± 0.4
8	D-Phe[4]	47 ± 4	10 ± 1	4.7 ± 0.8
9	Phe[3]	47 ± 4	19 ± 2	2.5 ± 0.4
10	Pro[2]	53 ± 5	18 ± 1	2.9 ± 0.5
11	D-Phe[1]	20 ± 3	15 ± 1	1.3 ± 0.3

[a] Minimum hemolysis concentration determined with fresh human erythrocytes. [b] Minimum inhibition concentration determined with *Bacillus subtilis* (ATCC 82).

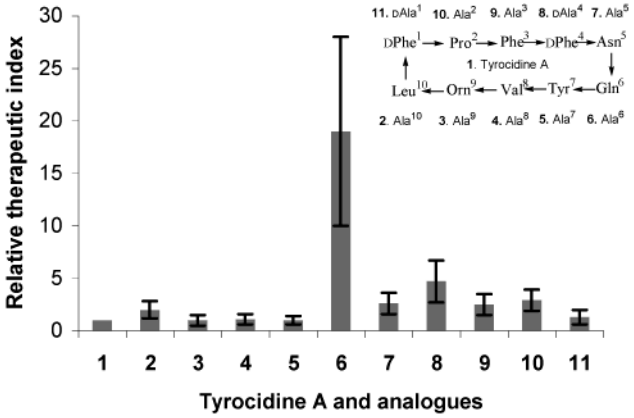
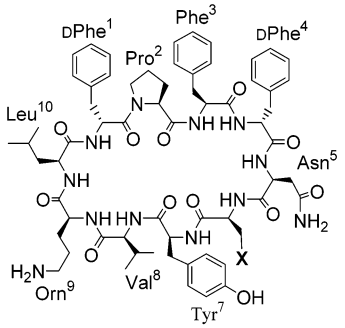


Figure 1. Determination of effects of amino acid side chains on the therapeutic index of tyrocidine A by alanine scanning.

indices essentially equal to that of the wild type natural product, with modest loss of antibacterial potency. This indicates that modification of these residuals is unlikely to improve the antibacterial selectivity of the resulting cyclic peptides. On the other hand, substitution of residuals Pro-2, Phe-3, Asn-5, and Leu-10 by alanine brings about modest improvement of the MHC/MIC ratio (2- to 3-fold). However, the improvement of the selectivity against bacteria is accompanied by a decrease in the antibacterial activity by 2- to 3-fold, indicating that the modification provides modest differentiation of the two biological properties of the cyclic peptides with sacrifice of the antibiotic potency. These observations show that the biological activities of the cyclic peptides are indeed intimately associated and mostly dependent on the structure, consistent with previous observations.[4,8,12]

Of particular interest are the results of the alanine substitution at Gln-6 and D-Phe-4, which show significant differentiation of the antibacterial and hemolytic activities with an MHC/MIC of 19 and 4.7, respectively. Most importantly, the significant increase of the therapeutic index is achieved without affecting or increasing the antibacterial activity, showing a complete segregation of the two intimately correlated biological properties. Increase of the therapeutic index for the alanine substitution at D-Phe-4 is consistent with the results of a previous investigation in which an even higher MHC/MIC ratio was achieved when the hydrophobic side chain was changed to a cationic one such as D-Arg, D-Lys, or D-Orn.[9] On the other hand, an even more



12. X = Leu **19.** X = Lys
13. X = Val **20.** X = Arg
14. X = Ile **21.** X = Ser
15. X = Phe **22.** X = Asp
16. X = Trp **23.** X = Glu
17. X = Tyr **24.** X = Asn
18. X = Orn

Figure 2. Tyrocidine A analogues displaying various side chain functionalities at the position of Gln-6.

Table 2. Antibacterial and Hemolytic Activities of Tyrocidine A Analogues with Various Side Chains at the Position of Gln-6

compd	amino acid at position-6	MHC[a] (μg/mL)	MIC[b] (μg/mL)	MHC/MIC
12	Leu	20 ± 2	0.3 ± 0.1	67 ± 12
13	Val	35 ± 2	2.9 ± 0.5	12 ± 3
14	Ile	45 ± 4	1.8 ± 0.3	25 ± 5
15	Phe	18 ± 2	0.6 ± 0.1	30 ± 8
16	Trp	125 ± 8	3.8 ± 0.3	33 ± 4
17	Tyr	120 ± 6	5.0 ± 0.5	24 ± 4
18	Orn	25 ± 3	0.2 ± 0.1	125 ± 35
19	Lys	99 ± 5	1.1 ± 0.2	90 ± 22
20	Arg	42 ± 3	0.3 ± 0.1	140 ± 43
21	Ser	16 ± 2	0.3 ± 0.1	53 ± 18
22	Asp	32 ± 2	5.3 ± 0.7	6.0 ± 1.2
23	Glu	30 ± 2	7.5 ± 0.5	4.0 ± 0.5
24	Asn	20 ± 2	0.4 ± 0.2	50 ± 20

pronounced effect of alanine substitution at the Gln-6 side chain position as shown in Table 1 and Figure 1 has not been reported before. Substitution of the polar side chain amide on this residual by a small methyl group led to substantial weakening of the hemolytic activity with concurrent significant enhancement of antibiotic potency of the resulting cyclic peptide. Both effects of such a structural modification are beneficial to identification of novel antibiotic candidates without cytotoxic activity.

To further exploit the potential of the sensitivity of the biological activities to the residual Gln-6, various side chain functionalities were individually displayed at this position to examine their effects on the activities of the resulting cyclic peptide products. Synthesis of these tyrocidine A analogues (Figure 2) was accomplished with the method illustrated in Scheme 1. After HPLC purification and characterization of the cyclic peptide products, they were subjected to the same antibacterial and hemolytic activity determination.

As shown in Table 2 and Figure 3, significant further increase in therapeutic index is achieved when the cationic amino acid residual Orn, Lys, or Arg is introduced into position 6, in comparison to the alanine-substituted analogue at the same position. The enhancement is mainly due to the increase of antibiotic potency. In contrast, incorporation of the anionic side chain of Asp or Glu results in a decrease in the MHC/MIC ratio. When the alanine is replaced by other small aliphatic amino acids, further enhancement of the therapeutic index was only observed for the leucine- and serine-substituted analogues. The branched hydrophobic aliphatic side chain of valine or isoleucine does not increase the bacterial selectivity of the resulting analogues nor do the aromatic side chain functionalities of phenyla-

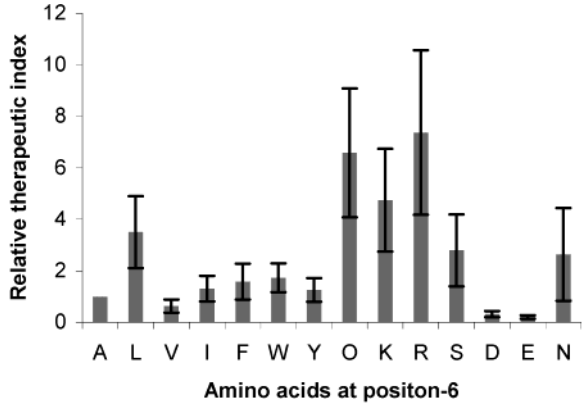


Figure 3. Effect of side chain functionalities at the position of Gln-6 on the therapeutic index.

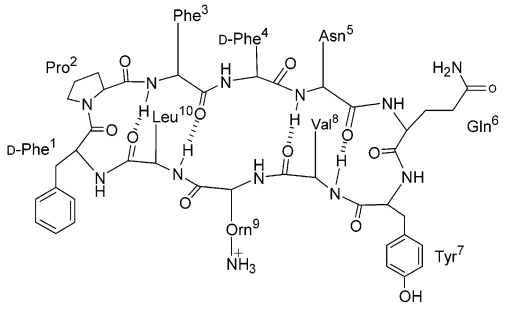


Figure 4. Antiparallel β-pleated sheet structure of tyrocidine A.

laine, tryptophan, and tyrosine. These results indicate that the antibacterial potency and the bacterial selectivity can be significantly increased when a cationic or unbranched small aliphatic amino acid is incorporated into the position of Gln-6 of the wild-type tyrocidine A template. Notably, analogues with high therapeutic indices also exhibit significant increase in activity against drug-resistant microbes.[14]

It is interesting to note that the hemolytic and antibiotic properties of the cyclic peptide natural product depend on different structural components. The potentially useful effects of modifications at D-Phe-4 and Gln-6 come from the unique structure and mode of action of the cyclic peptide scaffold molecule. Tyrocidine A is an amphipathic peptide antibiotic with one hydrophobic side consisting of Phe-2, Asn-5, Val-8, and Leu-10 and a hydrophilic cationic Orn-9 on the other side of the rigid antiparallel β-pleated sheet structure (Figure 4).[15] Previous studies on α-helical and β-sheet peptide antibiotics showed that the amphipathicity often affects the antibiotic potency and the hemolytic activity in parallel.[4b,8,12] Being on the same side as the hydrophobic patch of tyrocidine A, the aromatic side chain of D-Phe-4 contributes to the amphipathicity of the antibiotic. Increase of antibiotic potency and concurrent decrease of the hemolytic activity of the cyclic peptide by substituting this residual with alanine indicate that the aromatic side chain is detrimental to the interaction with mammalian cell membrane while facilitating its binding with the bacterial membrane, showing a differentiating amphipathicity effect on the two biological activities of the cyclic analogue. A similar differentiating effect of the polar side chain of Gln-6 may also underlie the dissociation of antibiotic and hemolytic activities caused by its substitution with an alanine or leucine side chain. However, the contribution of the Gln-6 side chain to the amphipathicity of the cyclic peptide is less certain because it is not obvious whether it belongs to the hydrophobic or hydrophilic side of the scaffold molecule. Substitution at both D-Phe-4 and Gln-6 is less likely to exert its effect on the biological activities through conformational change because the rigid antiparallel β-pleated sheet structure of the cyclic peptides is maintained by four strong interstrand hydrogen bonds that are not susceptible to side chain variations.

A significant increase in the therapeutic index was reported when a cationic side chain functionality was introduced into the position of D-Phe-4. This was ascribed to the differential ionic interactions between the analogues and charged headgroups of lipid components of the membranes.[9] Similar differential ionic interaction should also be responsible for the increase in therapeutic index when positively charged side chains are introduced into the position of Gln-6 in the current study because the additional positive charge will greatly strengthen binding affinity of the resulting analogue for the prokaryotic membrane that contains predominantly negatively charged phospholipids, whereas its effect on the analogue's affinity for eukaryotic membrane will be much less significant because of the zwitterionic nature of the phospholipids in mammalian cells. However, effects of the additional positive charge at the positions of D-Phe-4 and Gln-6 are different. Substitution of the former residual with a basic amino acid results in a significant increase of MHC without much effect on MIC,[9] whereas the increase in the therapeutic index caused by the same substitution at the latter position is mostly due to the enhancement of the antibacterial potency (decrease in MIC) with a concurrent moderate increase in MHC. Interestingly, substitution with small aliphatic amino acids at Gln-6 also results mainly in a decrease of MIC. These different effects indicate that analogues with high antibacterial specificity and potency are accessible by simultaneous structural modification at both positions.

Conclusion. We have discovered a novel position on the scaffold of natural cyclic decapeptide antibiotic tyrocidine A at Gln-6 whose modification will significantly lower the unwanted hemolytic activity and simultaneously enhance the desired antibacterial activity, in addition to our confirmation of similar effects of structural variation at another position of D-Phe-4 in a previous report. Combination of the favorable structural variations at both positions should enable identification of analogues of the natural product that are capable of combating microbial resistance without provoking new resistance.

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Supporting Information Available: Experimental procedures and HPLC, MS, and ^{1}H NMR data for **1**−**24**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) For example, MICs in μg/mL for **20** and tyrocidine A (**1**; value given in parentheses) for various microorganisms: *E. coli*, >50 (>50); *Pseudomonas aeruginosa*, >50 (>50); a clinical strain of methicillin-resistant *Staphylococcus aureus*, 5.0 ± 0.8 (17 ± 3); vancomycin-resistant *Enterococcus facecium* ATCC 70221, 1.0 ± 0.3 (15 ± 3); *Candida albicans*, 16 ± 3 (17 ± 3).

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