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On-resin cyclization and antimicrobial activity of Laterocidin and its analogues

Chuanguang Qin *, Chunlan Xu, Ruijie Zhang, Weining Niu, Xiaoya Shang

Faculty of Life Science, Northwestern Polytechnical University, Xi'an 710072, PR China

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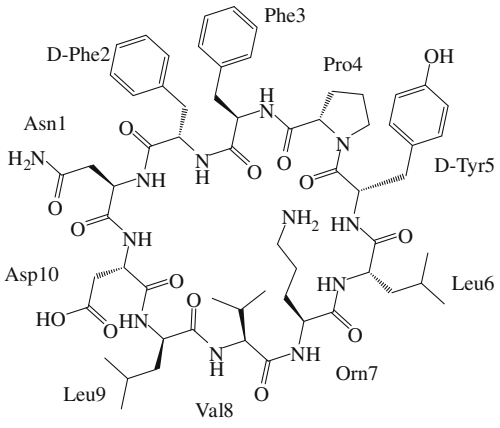
ABSTRACT

Total synthesis of cyclodecapeptide antibiotics from *Bacillus laterosporus*, laterocidin and its analogues, was accomplished for the first time by solid-phase peptide synthesis followed by traceless on-resin cyclization of the linear precursors with protection of α-carboxyl group on the Asp residue by Dmab as a temporary blocking group for on-resin head-to-tail cyclization, in which the carboxyl group of side chain of Asp was linked to Rink resin. Single alanine substitution or Asn substitution (Asp^{10}→Asn^{10}) demonstrated improvements in antibacterial activity. Of note, D-Phe^2 and Pro^4 play an important role in on-resin head-to-tail cyclization and exerting antibacterial activity of Laterocidin.

The emergence of highly resistant microbe strains is increasingly limiting the effectiveness of current therapeutic drugs such as penicillins, vancomycin, cephalosporins, quinolones, tetracyclines and macrolides. Even now, resistance to these highly potent drugs is being commonly seen in clinic.[1] 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.[2]

On the other hand, 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.[3] In light of this method, we have selected Laterocidin, a natural product isolated from the culture broth of *Bacillus laterosporus*, as a platform for this 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.[4] Natural cyclopeptide antibiotics such as gramicidins, polymyxins, Valinomycin and daptomycin have been used extensively for topical therapy with excellent results. Furthermore, cyclic peptide derivatives such as cyclosporine, streptogramins, glycopeptides and lipopeptides have been instrumental in controlling severe bacterial infections.

Recently, Tyrocidine A and Gramicidin S have received a great deal of attention due to their highly desirable mechanism of action. Both the compounds are known to form a β-type secondary structure leading to an amphipathic molecule.[5] Studies have found that bacteriocidal properties are influenced by several properties: rings size/rigidity, hydrophobicity and amphipathicity.[6] A collection of similar studies have been conducted for cyclopeptide Tyrocidine A and Gramicidin S. Formerly, we have demonstrated that by using an alanine substitution screen, minor changes in the peptide composition of Tyrocidine A can lead to improvements in antibacterial potency.[7] Similarly, we can envisage that by making point substitutions that alter the amphipathicity of the Laterocidin 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 that exhibit moderate changes in antibacterial properties.[8] The previous



Laterocidin

Figure 1. Structure of Laterocidin.

* Corresponding author. Tel.: +86 029 88491840; fax: +86 029 88460332.
E-mail addresses: qinchg@nwpu.edu.cn, qinchg2002@yahoo.com (C. Qin).

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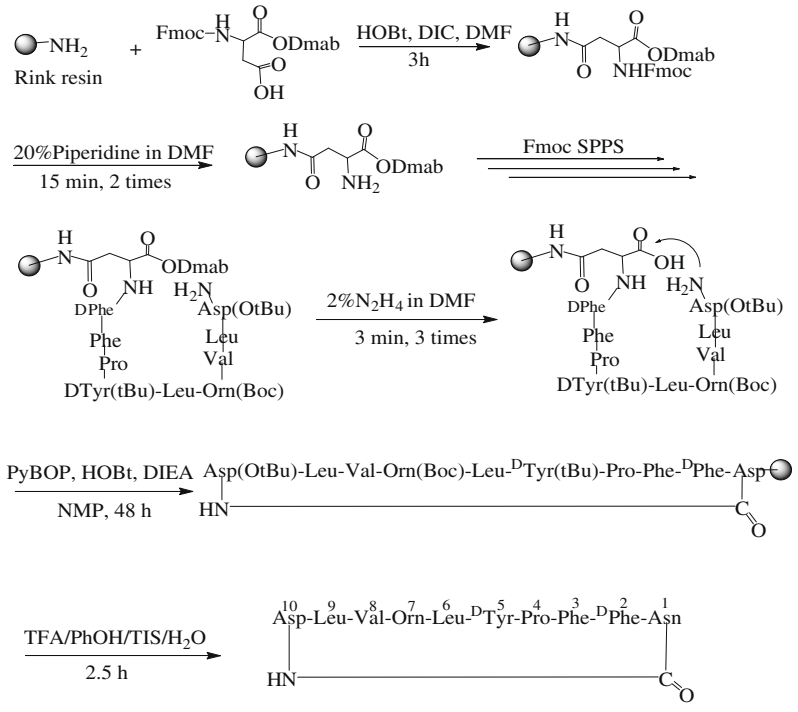
success of these groups in modulating antibacterial properties by using simple alanine screens and point amino acid substitutions is highly encouraging and sets the stage for our work.

With the favourable attributes of the cyclopeptides recognized, we have set out to improve the collective understanding of their structure–activity relationship using Laterocidin as our point of reference. On the basis of similar sequence and structure, we anticipated that the newly discovered Laterocidin adopts the same unique antibiotic mechanism and may have bacterial specificity different from other members in the family of amphipathic peptide antibiotics. Thus, we embarked on their total synthesis to verify the structures, to study their biological activities, and to develop a convenient method for analogue synthesis in an attempt to improve their selectivity against bacteria.

Different methods have been developed for the synthesis of cyclic peptides.[9] Traditionally, C-terminal activation by *N*-hydroxysuccinimide ester (ONSu) and azide of the linear peptide was used in the synthesis of tyrocidines and gramicidins.[10] More recently, cyclization of peptides anchored on resin through the side chain functionalities has been widely employed to afford the cyclic peptides.[11] Moreover, 'safety-catch' methods have been developed for the synthesis of head-to-tail cyclic products.[12] However, these methods involve tedious side chain protection–deprotection necessitated in the ring closure and are often hampered by the poor cyclizing tendency of the linear peptide precursors. Furthermore, thioesterases are successfully applied in the synthesis of tyrocidine A and analogues, which is suggested to be a general method for the generation of molecular diversity for enhancement of the therapeutic index of the natural products.[13] Recently, we found that the biosynthetic precursors of tyrocidine A[14] and gramicidin S[15] adopt a pre-organized conformation, which is highly favourable for specific head-to-tail cyclization. This led to a simpler synthetic method for the natural occurring cyclic decapeptides such as tyrocidines, streptocidins, gramicidin S and loloatins, using acylsulfonamide safety-catch linker,[16] without the need to protect the reactive side chain functionalities in the cyclization product release step.

Laterocidin differs from tyrocidines and gramicidin S. Thus, the convenient safety-catch linker method for Tyrocidine A can not be directly employed to synthesize the novel cyclic decapeptide antibiotics. In this study, we would like to report the novel solid-phase synthesis of Laterocidin via linking of the carboxyl group of side chain of Aspartate to Rink resin with the protection of α-carboxyl group of Aspartate by Dmab. In the experiment, we investigated the effect of side chains of the self-cyclizing tendency of the scaffold molecule. The constituent amino acids of the linear precursor were sequentially substituted by alanine in a process called 'alanine-scanning'. D-Alanine was used to replace the D-Phenylalanine in the parent molecule, since configuration of individual amino acid had been shown to be important to the conformation of the linear precursors.[14] The substituted linear precursors were synthesized and cyclized in parallel using IRORT's AccuTag100 Combinatorial Chemistry System,[17] according to the method shown in Scheme 1. Starting from 50 mg Rink resin (0.5 mmol/g,



Laterocidin = cyclo (-DLVOL^{d}YPF^{d}FN-)

1 = cyclo (-NLVOL^{d}YPF^{d}FN-)

2 = cyclo (-NLVOL^{d}YPF^{d}FA-)

3 = cyclo (-NLVOL^{d}YPF^{d}AN-)

4 = cyclo (-NLVOL^{d}YPA^{d}FN-)

5 = cyclo (-NLVOL^{d}YAF^{d}FN-)

6 = cyclo (-NLVOL^{d}APF^{d}FN-)

7 = cyclo (-NLVOA^{d}YPF^{d}FN-)

8 = cyclo (-NLVAL^{d}YPF^{d}FN-)

9 = cyclo (-NLAOL^{d}YPF^{d}FN-)

10 = cyclo (-NAVOL^{d}YPF^{d}FN-)

11 = cyclo (-ALVOL^{d}YPF^{d}FN-)

Scheme 1. On-resin cyclization strategy for solid-phase synthesis of Laterocidin and its analogues (1–11).

100–200 mesh, 1% DVB) for each compound, the alanine-substituted products were obtained in good yields and characterized after simplified purification, as summarized in Table 1. After solid-phase synthesis, the linear precursor was cyclized on resin. Subsequently, the cyclic product was simultaneously deprotected and cut down from the resin with cocktail reagent. Finally, the target cyclopeptides were precipitated with cool ether and separated with a centrifuge. After drying under vacuum, Laterocidin and its analogues were obtained and characterized. The overall yield of Laterocidin is 43.8%. In comparison with this, the yields of compound **2** ($Asn^1 \rightarrow Ala^1$ and $Asp^{10} \rightarrow Asn^{10}$) and compound **12** ($Asp^{10} \rightarrow Asn^{10}$), which were 46.5% and 43.1%, respectively, were higher than those of other compounds. However, the yield of compound **10** ($Leu^9 \rightarrow Ala^9$ and $Asp^{10} \rightarrow Asn^{10}$), which was 17.6%, was the lowest in comparison with those of other compounds. These results indicated that Alanine residue of N- or C-terminal, and different configuration of N- and C-terminal benefited to cyclization. Moreover, the hydrophobic residue such as alkyl group (Leu) on N-terminal is adverse to cyclization. Turn tendency amino acid (Pro) in the middle of linear peptide or trans-peptide bond which is good for forming β-turn is better for cyclization.

MALDI-TOF Mass Spectrum of the cyclization product showed only one molecular ion peak at 1224.5 (M+2 as isotope peak of $[M+H]^+$), consistent with the calculated mass of 1222.6 for Laterocidin, indicating the absence of hydrolytic products or other truncated peptide products. RP-HPLC analysis of the final products without chromatographic purification showed that they are of high purity (half >90%). Relatively overall low yield (17–25%) of the products was probably due to the overestimation of the resin loading value determined after attachment of the first amino acid (Fmoc-Asp-ODmab). No free amine ground was found after the cyclization reaction by Kaiser's test. The data of the synthetic Laterocidin and analogues 1–11 indicated that they are the correct head-to-tail cyclization products. These results show that the synthetic scheme indeed affords the correct head-to-tail cyclic products without interference from the reactive side chain $-NH_2$ and

Table 1
The cyclodecapeptide sequences of Laterocidin and its analogues

Entry	Cyclodecapeptide sequence	Calculated M^+	Found[a] $[M+H]^+$	Ret. time[b] (min)	Purity[c] (%)	Yield[d] (%)
Laterocidin	Cyclo(-DLVOL^{d}YPF^{d}FN-)	1222.64	1224.5	27.0	70.0	43.8
1	Cyclo(-NLVOL^{d}YPF^{d}FN-)	1221.65	1222.6	29.3	90.7	20.9
2	Cyclo(-NLVOL^{d}YPF^{d}FA-)	1178.65	1179.8	30.1	82.9	46.5
3	Cyclo(-NLVOL^{d}YPF^{d}AN-)	1145.62	1146.7	27.4	72.3	35.1
4	Cyclo(-NLVOL^{d}YPA^{d}FN-)	1145.62	1146.7	25.4	71.1	40.4
5	Cyclo(-NLVOL^{d}YAF^{d}FN-)	1195.64	1196.7	26.0	87.5	21.7
6	Cyclo(-NLVOL^{d}APF^{d}FN-)	1129.63	1130.6	29.7	93.2	24.6
7	Cyclo(-NLVOA^{d}YPF^{d}FN-)	1179.61	1180.6	25.2	83.1	24.5
8	Cyclo(-NLVAL^{d}YPF^{d}FN-)	1178.61	1180.6	30.4	91.2	40.3
9	Cyclo(-NLAOL^{d}YPF^{d}FN-)	1193.62	1194.7	27.3	91.0	24.9
10	Cyclo(-NAVOL^{d}YPF^{d}FN-)	1179.61	1180.7	26.2	91.6	17.6
11	Cyclo(-ALVOL^{d}YPF^{d}FN-)	1178.65	1180.6	26.9	95.0	43.1

[a] The found values M+2 of entries Lacterocidin, **8** and **11** are the isotope of $[M+H]^+$ as the main peak in MS.
[b] Retention time, RP-HPLC analyses were performed with a Kromasil RP-C18 column (No. NC-2546-06251151, 5 μm, 250 × 4.6 mm i.d.) on Waters 2696 separation module system equipped with a 996 photodiode array detector. Flow conditions were: 1.0 mL min^{-1} flow rate, a linear gradient of 80–20% A in 25 min, 20–0%A in another 10 min, washed with 100% B for 10 min and then calibrated at 80% A for 15 min. Solution A was 0.1% TFA in double-deionized H_2O and solution B was 0.1% TFA in acetonitrile.
[c] Purity was determined by HPLC analysis of the unpurified cyclization products after being precipitated and washed with cool ether.
[d] Overall yields were calculated from the loading value of the resin after first amino acid attachment.

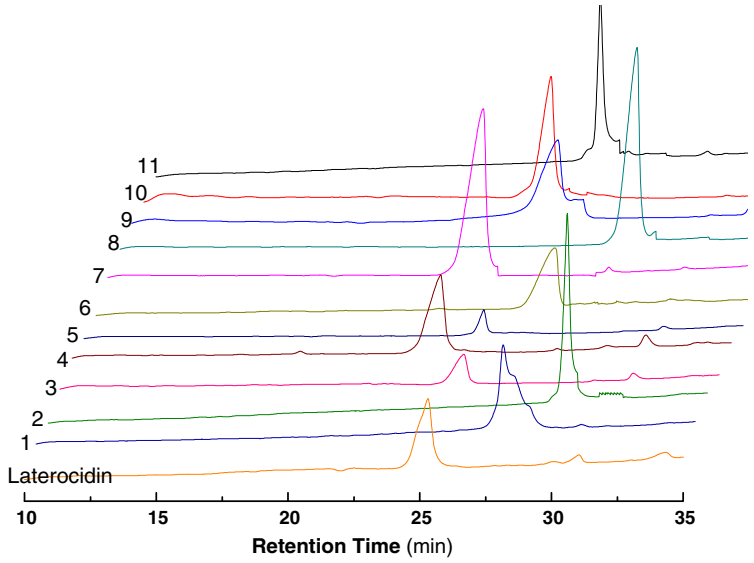


Figure 2. HPLC chromatograms of laterocidin and its synthetic analogues (1–11) purified through a reversed-phase semi-preparative column. Eluted products were monitored at 220 nm, collected and freeze-dried. Purification was performed with a reversed-phase semi-preparative Kromasil C18 300 Å column (No. NC-3010-07182, 5 μm, 300 × 10 mm id) on Waters 2696 separation module system equipped with a 996 photodiode array detector. Flow conditions were: 2.0 mL min^{-1} flow rate, a linear gradient of 80–20% A in 25 min, 20–0% A in another 10 min, washed with 100% B for 10 min and then calibrated at 80% A for 15 min. Solution A was 0.1% TFA in double-deionized H_2O and solution B was 0.1% TFA in acetonitrile.

Table 2
MIC of the cyclodecapeptide Laterocidin and its analogues (μg/mL)[a]

Cyclic decapeptide	Organism (strain)[b]						
	B. subtilis	*S. aureus*	L-MRSA	*E. coli*	*P. aeruginosa*	ESBLS *E. coli*	L-*E. coli*
Laterocidin	16	32	128	>256	>256	>256	>256
1	1	2	4	64	>256	256	64
2	4	8	16	128	>256	256	64
3	16	64	16	64	>256	128	64
4	4	32	8	64	>256	128	64
5	32	32	16	32	>256	>256	128
6	2	2	4	256	128	32	16
7	2	8	8	256	256	32	16
8	4	32	128	>256	>256	>256	>256
9	2	4	4	256	>256	32	16
10	2	2	4	>256	>256	64	32
11	2	4	8	256	>256	>256	256

[A] Minimal inhibition concentration of synthetic cyclopeptides after purification by semi-preparative RP-HPLC and freeze-dry (the purity more than 98%, see Fig. 2), required to completely inhibit bacterial growth.
[b] Gram-positive bacterium: *B. subtilis, Bacillus subtilis*; *S. aureus*, *Staphylococcus aureus*; L-MRSA, Clinical Methicilllin Resistance *Staphylococcus aureus*; Gram-negative bacterium: *E. coli, Escherichia coli*; *P. aeruginosa*, *Pseudononas aeruginosa*; ESBLs *E. coli*, Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli*; L-*E. coli*: Clinical Medicinal Resistance *Escherichia coli*.

–OH groups in the cyclization step, as expected. The side chains of the Laterocidin scaffold have minimal effect on the strong tendency of the linear precursors to self-cyclize, and they are replaceable for the generation of molecular diversity to enhance the natural product's activity or evolve a new biological function.

In the experiment, we further examined the antibiotic activity of the synthetic products purified by semi-preparative RP-HPLC and freeze-dry (purity more than 98%, HPLC chromatogram see Fig. 2) using a modified broth dilution method.[6a,18] As shown in Table 2, minimum inhibition concentration (MIC) of these products showed that they are indeed potent antibiotics against gram-positive bacterium and gram-negative bacterium, with potency comparable to that of Laterocidin. In further analysis of the antimicrobial activities of Laterocidin and its analogues, it was found that they are modestly potent towards gram-positive bacterium. Moreover, mutual replacement of Phenylalanine and Tyrosine had little effect on the antimicrobial activities of Laterocidin. However, configuration of amino acids had been shown to be important to the antimicrobial activities of the cyclodecapeptide, Laterocidin. In the experiment, we found that D-alanine-substitution for D-Phe^2 and Alanine-substitution for proline-4 have significant effects on the antimicrobial activities of Laterocidin amide with higher MIC. However, Alanine-substitution for other amino acids did not bring about the differentiation of antimicrobial activities. Asp^{10} substituted by Asn^{10} improved the antimicrobial activity of parent Laterocidin, which may be related to the increase of the positive charge in the molecule. The results indicated that D-Phe^2 and Pro^4 play an important role in the antimicrobial activity of cyclodecapeptide Laterocidin.

In summary, the synthetic method of Laterocidin and its analogues has been successfully established for the first time via linking of the carboxyl group of side chain of Aspartate to Rink resin with the protection of α-carboxyl group of Aspartate by Dmab. In comparison with the reported method, this synthetic approach is simple and efficient with high purification and yields for cyclopeptide product. Results of antimicrobial activity test showed that D-amino acid residues, D-Phe^2 and Pro^4 play an important role for the antimicrobial activities of Laterocidin amide. This work may lead to analogous chemical strategies for the efficient generation of their analogues for structural optimization or discovery of new biological functions.

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Supplementary data

Supplementary data (experimental procedure, Mass Spectrum of Laterocidin and its analogues) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.11.007.

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