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Solid-phase synthesis and antibiotic activities of cyclodecapeptides on the scaffold of naturally occurring Laterocidin

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

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

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ABSTRACT

The development of new antibacterial therapeutic agents capable of halting microbial resistance is a chief pursuit in clinical medicine. Laterocidin and its analogues were synthesized for the first time by solid-phase synthesis method via linking of the carboxyl group on side chain of Aspartate to Rink resin with the protection of side chain α-carboxyl group of Aspartate by Dmab as a temporary α-COOH protecting group for the on-resin cyclization. Different configuration of N- and C-terminal was benefit to peptide cyclization. Laterocidin analogue **3** ($Asp^1 \rightarrow Asn^1$, $Phe^4 \rightarrow Tyr^4$ and $D\text{-}Tyr^6 \rightarrow D\text{-}Phe^6$) demonstrated potent and broad antimicrobial properties, especially exhibited activity against clinical Methicillin-resistant *Staphylococcus aureus* (L-MRSA) and the gram-negative extended-spectrum β-lactamases-producing *Escherichia coli* (ESBLs *E. coli*) and L-*E.coli*. This finding has important significance to exploit new antibiotic medicine.

The development of new antibacterial therapeutic agents capable of halting microbial resistance is a chief pursuit in clinical medicine. 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.[1–3] Natural peptide antibiotics such as the gramicidins and polymyxins have been used extensively for topical therapy with excellent results. Practical use of available linear antimicrobial peptides has not been completely satisfactory due to the conformational flexibility of their structure which is often associated to low target selectivity, poor bioavailability, and low stability towards protease degradation. However, cyclic peptides exhibit better metabolic stability, selectivity and bioavailability.[4,5] For instance, potent cyclic antimicrobial peptides have been successfully developed and cyclization of cytolytic amphipathic α-helical peptides has been shown to increase the selectivity for bacteria by substantially reducing the hemolytic activity.[4,6,7] More commonly, entirely new generations of drugs within a class have been produced by making structural changes to existing scaffolds, perhaps the most notable being the penicillins, cephalosporins, quinolones, tetracyclines, and macrolides.[8–10] Furthermore, cyclic peptide derivatives such as the streptogramins, glycopeptides, and lipopeptides have been fundamental in controlling severe bacterial infections.

Therefore, to generate molecular diversity base on those natural product cyclic peptides aiding to search for improved or new biological function has aroused much interest. Currently, some researchers utilized the modular biosynthesis of the natural product peptide to achieve this goal. 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.[11] In contrast, available chemical methods for cyclic peptide synthesis have been perceived not to be suitable for this engineering purpose, largely because of the poor cyclizing tendency of the precursors and the tedious side chain protection–deprotection necessitated in the ring closure.[12] However, it is well known that chemical synthesis of these natural peptide products, particularly in the ring closure, can be greatly facilitated by the conformational preference of their linear precursors,[13] rendering the hope that a convenient strategy can be developed to generate molecular diversity on the basis of natural peptide products. Moreover, up to now, synthesis of peptide with amino acid residue with carboxyl group mostly utilized allyl group protection, which needs to be deprotected with method of palladium reagent. This method was expensive with great toxicity and could not be deprotected completely. Moreover, acetic acid acetylation of N-terminal was adverse to cyclize.[14]

With the favorable attributes of the cyclopeptides recognized, we have set out to improve the collective understanding of their structure–activity relationship (SAR) using Laterocidin (Fig. 1) as our point of reference. 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 on side chain of Aspartate to Rink resin with

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

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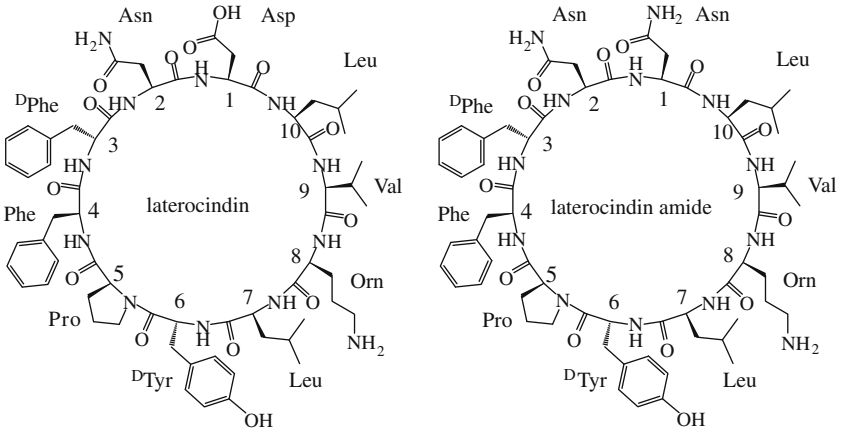
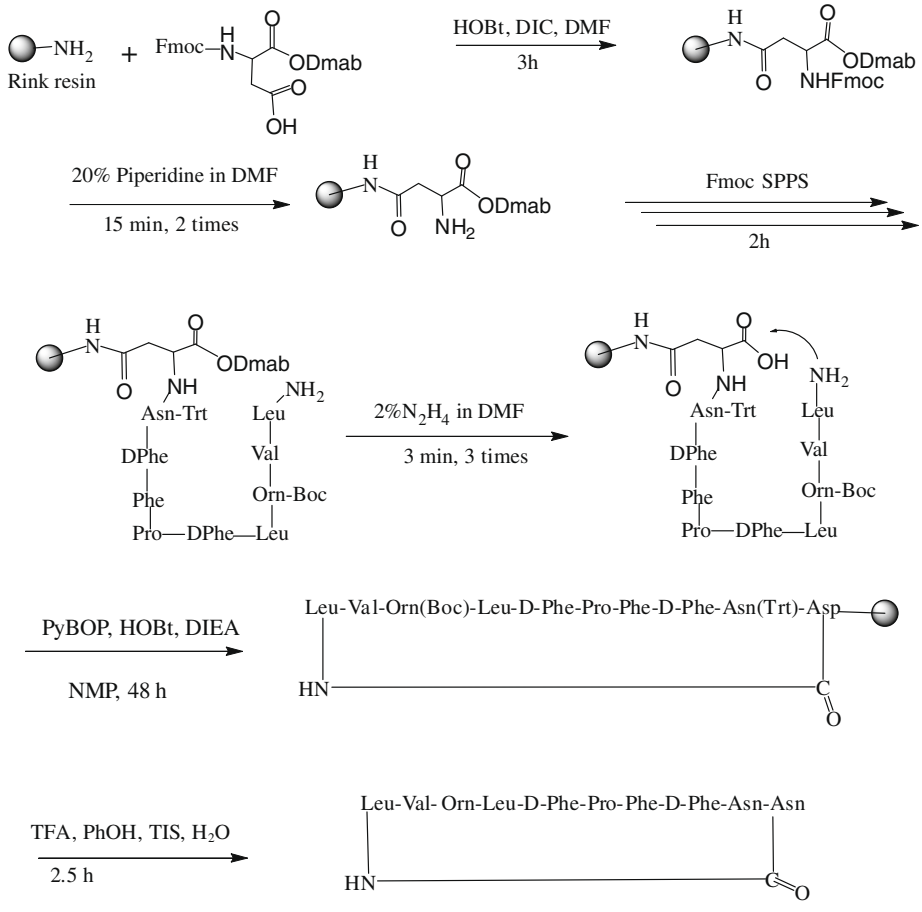


Figure 1. Structure of Laterocidin and Laterocidin amide.



Laterocidin: cyclo[-LVOL(^{D}Y)PF(^{D}F)ND-]

1: cyclo[-LVOL(^{D}Y)PF(^{D}F)NN-]

2: cyclo[-LVOL(^{D}F)PF(^{D}F)NN-]

3: cyclo[-LVOL(^{D}F)PY(^{D}F)NN-]

4: cyclo[-LVOL(^{D}F)PYFNN-]

5: cyclo[-LVOLFPY(^{D}F)NN-]

6: cyclo[-LVOLFPYFNN-]

Scheme 1. Solid-phase synthesis scheme of Laterocidin and its analogues (**1–6**).

the protection of side chain α-carboxyl group of Aspartate by Dmab in the cyclization. Configuration of individual amino acid had been shown to be important to the conformation of the linear precursors.[15] Therefore, we investigated the impacts of type and conformation of amino acid residue on the yield of peptide cyclization. The parent compound, Laterocidin, as well as six other closely

Table 1
The cyclodecapeptide sequences of Laterocidin and its analogues

Entry	Sequence	Calculated Mw	Found[a] Mw	Ret. time[b] (min)	Yield[c] (%)	Purity[d] (%)
Laterocidin	*cyclo*[-LVOL(^{D}Y)PF(^{D}F)ND-]	1222.64	1224.5	27.0	43.8	70.0
1	*cyclo*[-LVOL(^{D}Y)PF(^{D}F)NN-]	1221.65	1222.6	29.3	20.9	90.7
2	*cyclo*[-LVOL(^{D}F)PF(^{D}F)NN-]	1205.66	1206.57	31.3	46.5	50.4
3	*cyclo*[-LVOL(^{D}F)PY(^{D}F)NN-]	1221.65	1222.8	27.1	42.7	91.7
4	*cyclo*[-LVOL(^{D}F)PYFNN-]	1221.65	1223.6	21.8	33.0	68.2
5	*cyclo*[-LVOLFPY(^{D}F)NN-]	1221.65	1223.7	21.6	34.8	76.6
6	*cyclo*[-LVOLFPYFNN-]	1221.65	1222.5	21.2	23.7	58.8

[a] The found values M+2 of entry Lacterocidin, **4** and **5** 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 id) on Waters 2696 separation module system equipped with a 996 photodiode array detector. Flow conditions were: 0.5 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 from HPLC analysis of the unpurified cyclization products after being precipitated and washed with cool ether.
[d] Overall yields calculated from the loading value of the resin after first amino acid attachment.

related analogues **1**–**6** was synthesized. The substituted linear precursors were synthesized and cyclized in parallel using IRORT's AccuTag100 Combinatorial Chemistry System,[16] according to the method shown in Scheme 1. Starting from 50 mg Rink resin (0.5 mmol/g, 100–200 mesh, 1% DVB) for each compound, the synthesis products were obtained in good yields and characterized without 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 centrifuge. After dry under vacuum, Laterocidin and its analogues were obtained and characterized. The overall yield of Laterocidin is 43.8%. According to the yield, compound **2** ($Asp^1 \rightarrow Asn^1$ and $D\text{-}Tyr^6 \rightarrow D\text{-}Phe^6$) with yield of 42.7% was higher than other compounds except for Laterocidin. However, compound **1** ($Asp^1 \rightarrow Asn^1$) and compound **6** ($Asp^1 \rightarrow Asn^1$, $D\text{-}Phe^3 \rightarrow Phe^3$, $Phe^4 \rightarrow Tyr^4$ and $D\text{-}Tyr^6 \rightarrow Phe^6$) with yield of 20.9% and 23.7% respectively were lower than others. Relatively overall low yield (17–25%) of the products were probably due to the overestimation of the resin loading value determined after attachment of the first amino acid (Fmoc-Asp-ODmab). These results indicated that different type and conformation of amino acid residue in the linear precursors have great effect on the yield of cyclic peptides.

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 an absence of hydrolytic products including the linear precursor of Laterocidin or other truncated peptide products. HPLC analysis (Table 1) of compound **1** ($Asp^1 \rightarrow Asn^1$) and compound **3** ($Asp^1 \rightarrow Asn^1$, $Phe^4 \rightarrow Tyr^4$ and $D\text{-}Tyr^6 \rightarrow D\text{-}Phe^6$) without chromatographic purification showed that they are of high purity (>90%). No free amine ground was found after the cyclization reaction by Kaiser's test. The data of the synthetic Laterocidin and analogues **1**–**6** indicated that they are the correct head-to-tail cyclization products. These results showed that the synthetic scheme indeed affords the correct head-to-tail cyclic products without interference from the reactive side chain-NH_2 and –OH groups in the cyclization step, as expected.

The in vitro antibacterial activities of Laterocidin and its analogues **1**–**6** purified by semi-preparative RP-HPLC and freeze-dry (purity more than 98%, HPLC chromatogram see Fig. 2) were measured using a modified broth dilution method.[17,18] As shown in Table 2, results were expressed as minimum inhibition concentration (MIC). The parent compound, Laterocidin, exhibited moderate antibacterial activity against gram-positive bacterial. Compounds **1** and **2** showed a marked improvement in antibacterial potency, with an MIC improving from 8- to 16-fold over the parent Laterocidin. Compound **3** demonstrated the most potent and broadest antimicrobial properties of all the compounds tested, with MICs increasing from 32- to 128-fold over the parent Laterocidin. Of note, Compound **3** also exhibited activity against L-MRSA and the

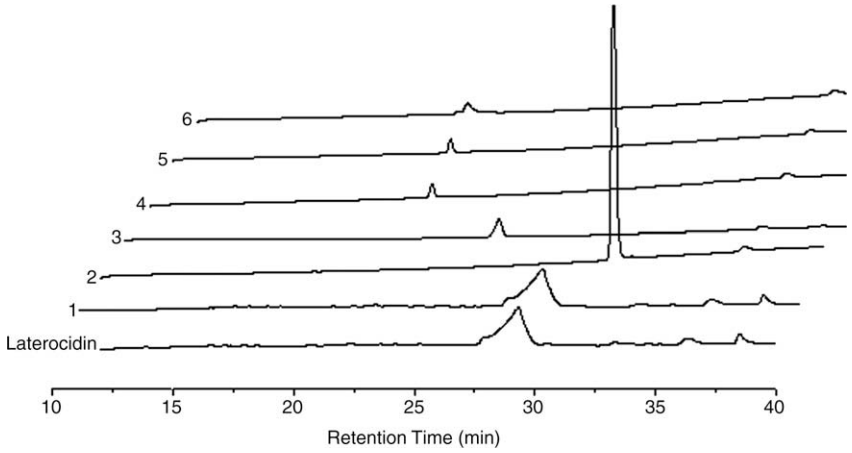


Figure 2. RP-HPLC chromatograms of Laterocidin and its analogues (**1**–**6**) purified through a reversed-phase semi-preparative column. Eluted products were monitored at 220 nm, collected and freeze-dried. Purification was performed with a reversed-phase semi-preparative Kromasil C18 300 Å column (No.: 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, 2–0% 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	Microorganism[b] (strain)						
	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	2	2	4	>256	>256	128	128
3	0.5	1	1	>256	>256	64	16
4	32	32	16	>256	>256	>256	>256
5	32	128	256	>256	>256	>256	>256
6	64	64	128	>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-resistant *S. aureus*; Gram negative bacterium: *E.coli, Escherichia coli*; *P. aeruginosa*, *Pseudomonas aeruginosa*; ESBLs *E. coli*, Extended spectrum beta-lactamase producing *Escherichia coli*; L-*E.coli*: Clinical drug-resistant *E. coli.*

gram-negative ESBLS *E. coli* and L-*E.coli*. Further analysis of the antimicrobial activities of Laterocidin and its analogues found that they are modestly potent toward gram-positive bacterium. However, compounds **4**–**6** demonstrated similar antibacterial activities to Latrocidin. The results indicated that configuration D-amino acids on the positions **3** and **6**, D-Phe^3 especially play an important role in antibacterial activity of cyclodecapeptide Laterocidin. Asp^1 substituted by Asn^1 improved antimicrobial activity of parent Laterocidin, which may be related to the increase of the positive charge in the molecule. Previous researches have found that bacteriocidal properties are influenced by several properties: rings size/rigidity, hydrophobicity, and amphipathcity.[19–22] Qin et al. have demonstrated that by making point substitutions, minor changes in the peptide composition of Tyrocidine A can yield improvements in antibacterial potency.[18,23]

In summary, we accomplished a solid-phase total synthesis of Laterocidin and its analogues. The synthetic method was via linking of the carboxyl group of side chain of Aspartate to Rink resin by using Dmab group as a temporary α-COOH protecting group during solid-phase synthesis with Fmoc chemistry. In comparison to the reported method, this synthetic approach is simple and efficient with high purification and yields for cyclopeptide product. D-amino acid residues, D-Phe^3 especially play an important role for the antimicrobial activities of Laterocidin. This work may lead to analogous chemical strategies for efficient generation of their analogues for structural optimization or discovery of new biological functions. Laterocidin analogue (Asp^1→Asn^1, Phe^4→Tyr^4 and D-Tyr^6→D-Phe^6) demonstrated potent and broad antimicrobial properties, especially exhibited activity against clinical Methicillin Resistance *Staphylococcus aureus* (L-MRSA) and L-*E.coli*. This finding has important significance to exploit new antibiotic medicine.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.11.009.

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