4-23-2013

Design, Synthesis and Evaluation of Antimicrobial Activity of N-terminal Modified Leucocin A Analogues

Krishna Chaitanya Bodapati
University of Alberta

Rania Soudy
University of Alberta

Hashem Etayash
University of Alberta

Michael Stiles
CanBiocin Inc.

Kamaljit Kaur
Chapman University, kkaur@chapman.edu

Recommended Citation

Bodapati K, Soudy R, Etayash H, Stiles M, Kaur K. Design, synthesis and evaluation of antimicrobial activity of N-terminal modified leucocin A analogues. *Bioorg. Med. Chem.* 2013;21(13):3715-3722. doi: 10.1016/j.bmc.2013.04.045

Design, Synthesis and Evaluation of Antimicrobial Activity of N-terminal Modified Leucocin A Analogues

Design, synthesis and evaluation of antimicrobial activity of N-terminal modified Leucocin A analogues

Krishna Chaitanya Bodapati[a], *Rania Soudy*[a], *Hashem Etayash*[a], *Michael Stiles*[b], *and Kamaljit Kaur*[a,*]

[a]Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada, T6G 2E1

[b]CanBiocin Inc., Edmonton, Alberta, Canada, T5J 4P6

kkaur@ualberta.ca

RECEIVED DATE (to be automatically inserted after your manuscript is accepted if required according to the journal that you are submitting your paper to)

Tel. 780-492-8917; Fax. 780-492-1217; email. kkaur@ualberta.ca

ABSTRACT

Class IIa bacteriocins are potent antimicrobial peptides produced by lactic acid bacteria to destroy competing microorganisms. The N-terminal domain of these peptides consists of a conserved YGNGV sequence and a disulphide bond. The YGNGV motif is essential for activity, whereas, the two cysteines involved in the disulphide bond can be replaced with hydrophobic residues. The C-terminal region has variable sequences, and folds into a conserved amphipathic α-helical structure. To elucidate the structure-activity relationship in the N-terminal domain of these peptides, three analogues (**1**-**3**) of a class IIa bacteriocin, Leucocin A (LeuA), were designed and synthesized by replacing the N-terminal β-sheet residues of the native peptide with shorter β-turn motifs. Such replacement abolished the antibacterial activity in the analogues, however, analogue **1** was able to competitively inhibit the activity of native LeuA. Native LeuA (37-mer) was synthesized using native chemical ligation method in high yield. Solution conformation study using circular dichroism spectroscopy and molecular dynamics simulations suggested that the C-terminal region of analogue **1** adopts helical folding as found in LeuA, while the N-terminal region did not fold into β-sheet conformation. These structure-activity studies highlight the role of proper folding and complete sequence in the activity of class IIa bacteriocins.

Keywords: Antimicrobial peptide; Class IIa bacteriocins; Leucocin A (LeuA); Solid phase peptide synthesis; Native chemical ligation; Antimicrobial activity; Solution conformation; Structure-activity relationships

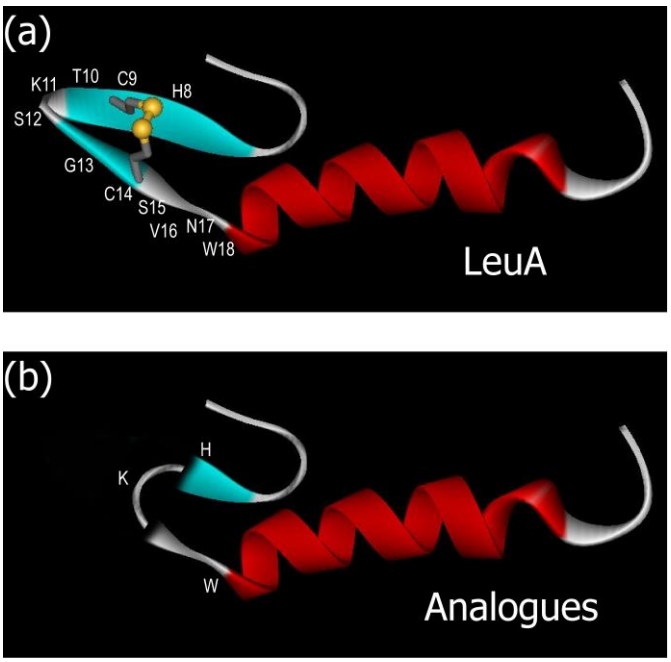
1. Introduction

Bacteriocins are potent antimicrobial peptides with a potential in a variety of applications such as food preservation, replacement of conventional antibiotics, and treatment of bacterial multiple-drug-resistance.[1-3] Bacteriocins derived from lactic acid bacteria are produced as a part of their self defense mechanism to destroy competing microorganisms.[4] Bacteriocins like class I nisin and class IIa pediocin PA-1 are currently used for food preservation and their potential in medical applications is being explored.[1] Class IIa bacteriocins are unmodified cationic peptides ranging from 37–48 residues that display activity against a variety of food pathogens, including *Listeria monocytogenes* in the nanomolar range.[2]

Bacteriocins display potent activity by one or more of the proposed mechanisms of actions. These peptides can (i) permeabilize the target cell membrane and reduce the proton motive force of bacterial cells, (ii) induce lysis of the cell by activating autolysins [1, 5, 6] or (iii) bind to the membrane bound receptor and cause pores in the cell wall.[7, 8] For class IIa bacteriocins, it is becoming clear that peptides act by interacting with a specific receptor, mannose phosphotransferase system (PTS) permease, on the target cell membrane and for this peptide-receptor interaction folded conformation of the peptide is a key element. Comparison of the amino acid sequence of class IIa bacteriocins shows that these peptides consist of a highly conserved hydrophilic and charged N-terminal region harboring the consensus YGNGV sequence and a disulfide bond. The C-terminal region is hydrophobic with a more variable sequence. In contrast, the three dimensional solution structures of several class IIa bacteriocins show that N-terminus region folds into different conformations, such as anti-parallel β-sheet or coiled structure, and the C-terminal region maintains a conserved amphipathic α-helical structure.[9-11] The C-terminal helical region imparts target specificity and likely interacts with the

target cell membrane bound receptor, whereas the positively charged N-terminal region interacts with the cell surface by electrostatic interaction.[12-14]

Leucocin A (LeuA) is a 37 residue class IIa bacteriocin from *Leuconostoc gelidium*.[15] A series of experiments conducted by Fleury *et al.*[16] to elucidate structure activity relationship (SAR) of LeuA show that N-terminal YGNGV sequence and C-terminal tryptophan are essential for activity. The N-terminal of LeuA (residues 2-16) consists of a three stranded anti-parallel β-sheet which is stabilized by the disulphide bond between residues 9 and 14, and C-terminal residues Trp18-Ala30 form an amphipathic α-helix. (**Figure 1a**).[9, 17] The conserved disulphide bond between two cysteines maintains the correct geometry of other residues in the sequence and this geometry is essential for its activity.[18] Studies conducted by Derksen *et al.*[18] confirmed that the disulphide bond only contributes to maintain correct geometry in the molecule but does not bind to the receptor on the bacterial cell membrane. The authors substituted the disulphide bond with carbocyclic rings without losing activity while maintaining flexibility and geometry in the synthetic LeuA. More recently it was shown that the replacement of the two Cys residues in the N-terminal region of LeuA with hydrophobic residues such as Phe, Nva (Norvaline), AllylGly yields a fully active analogue.[19] On the other hand, substitution of the Cys with Ser residues in LeuA made the peptide inactive. These results suggests that although the disulfide bond is conserved among class IIa bacteriocins, it can be replaced with residues which can induce similar geometry in the molecule without losing its antibacterial activity.



(c)

Peptide	Sequence[a]	Charge	No. of residues
LeuA	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	+2	37
1	KYYGNGVH--KP---VNWGEAFSAGVHRLANGGNGFW	+2	32
2	KYYGNGVH--KSG---NWGEAFSAGVHRLANGGNGFW	+2	32
3	KYYGNGVH--KPNG---WGEAFSAGVHRLANGGNGFW	+2	32

[a] For analogues **1-3**, residues in black are the same as in the native LeuA sequence, green represents mutations, and – stands for deleted residue.

Figure 1. (a) NMR solution structure of native LeuA (PDB code: 1CW6)[9] in 90% TFE. (b) Schematic of the designed LeuA analogues depicting the replacement of a portion of N-terminal β-sheet region from LeuA with a smaller sequence. (c) Amino acid sequences of native LeuA and peptide analogues studied herein.

We hypothesized that analogues of LeuA can be designed by replacing a portion of the N-terminal β-sheet region (Cys9-Ser15) with a smaller β-turn. To validate this and study the SAR of LeuA, three analogues (**1-3**) of LeuA were designed and synthesized by manipulating the N-terminal region of the native LeuA (**Figure 1**). LeuA was synthesized as a control

employing native chemical ligation (NCL) of two smaller fragments of LeuA. The antimicrobial activity of analogues and LeuA was evaluated against two indicator strains, *Carnobacterium divergens and Listeria monocytogenes*. In general the analogues did not show any activity whereas synthetic LeuA was highly active. Interestingly, analogue **1** was able to competitively inhibit the activity of LeuA. Solution conformation study using circular dichroism (CD) spectroscopy suggested that only analogue **1** most likely adopts similar helical folding as LeuA. Further, molecular dynamics (MD) simulations of **1** revealed that the C-terminal region folds into a well-defined α-helix, however, the N-terminal region does not form a β-sheet structure like the native LeuA emphasizing the role of proper folding in the activity of class IIa bacteriocins.

2. Results and discussion

2.1 Design of LeuA Analogues

As mentioned above, the N-terminal region of LeuA mainly interacts with electrostatic interaction with the target cell membrane. Therefore, the exact role of folded conformation in the N-terminal region of class IIa bacteriocins is not clear. We designed three analogues of LeuA by replacing the N-terminal β-strand residues ranging from Cys9-Asn17 with smaller β-turn sequences (**Figure 1**). The analogues were 32-residue long, five residues less compared to the native LeuA. Lys11 was kept in all the analogues to maintain the overall charge of +2. The β-strand residues Cys9-Thr10 and Gly13-Ser15 were deleted in analogue **1**, and Ser→Pro mutation was introduced to increase the conformational stability of the β-turn. Proline has a unique restricted ϕ angle that allows it to be entropically favourable at turn positions.[20] In analogue **2**, the β-turn sequence Lys-Ser-Gly (KSG) from the native LeuA was maintained

whereas, in analogue **3**, the β-turn was substituted with another β-turn motif, a conserved tripeptide sequence Pro-Asx-Gly.[21]

2.2 Synthesis of LeuA and Analogues

Peptide analogues **1**-**3** were synthesized on 2-chlorotrityl chloride resin using Fmoc solid-phase peptide synthesis.[22] Crude peptides were purified using semi-preparative reversed-phase HPLC prior to characterization by MALDI-TOF mass spectrometry. All peptide analogues were purified up to >95% purity as shown by analytical RP-HPLC and mass spectrometry (**Figure S1, SI**), and were obtained with an overall yield of 15-30%.

LeuA (37-mer) is a fairly hydrophobic peptide that gives rise to several truncated sequences during stepwise linear synthesis. We attempted to establish a NCL method[23-24] for LeuA which allows synthesis of two smaller fragments that are ligated to give the complete product. NCL method leads to high purity, higher yields, low racemisation, low aggregation/truncation of peptide, and allows synthesis of larger peptides. Therefore, it is a promising alternative for the synthesis of LeuA and similar peptides. In order to perform NCL reaction of two fragments, the first fragment should have C-terminal thioester and the other fragment should have N-terminal cysteine residue. In general, the fragments are chosen based on the presence of cysteine residue somewhere in the middle of the sequence. However this is not a prerequisite as other residues, such as Alanine, can also be chosen at the fragmentation point.[23] After NCL was first reported by Dawson et al,[24] a number of peptides, protein, and enzymes have been synthesized using this strategy.[25]

For the synthesis of LeuA using NCL, Cys14 was chosen as the potential ligation site giving rise to two fragments, 13-residue fragment 1 (1-13) and 24-residue fragment 2 (14-37)

(**Figure 2a**). The NCL reaction is initiated by a chemo selective nucleophilic attack of the thiol of Cys (N-terminal) of unprotected peptide fragment 2 on the C-terminal thioester moiety of

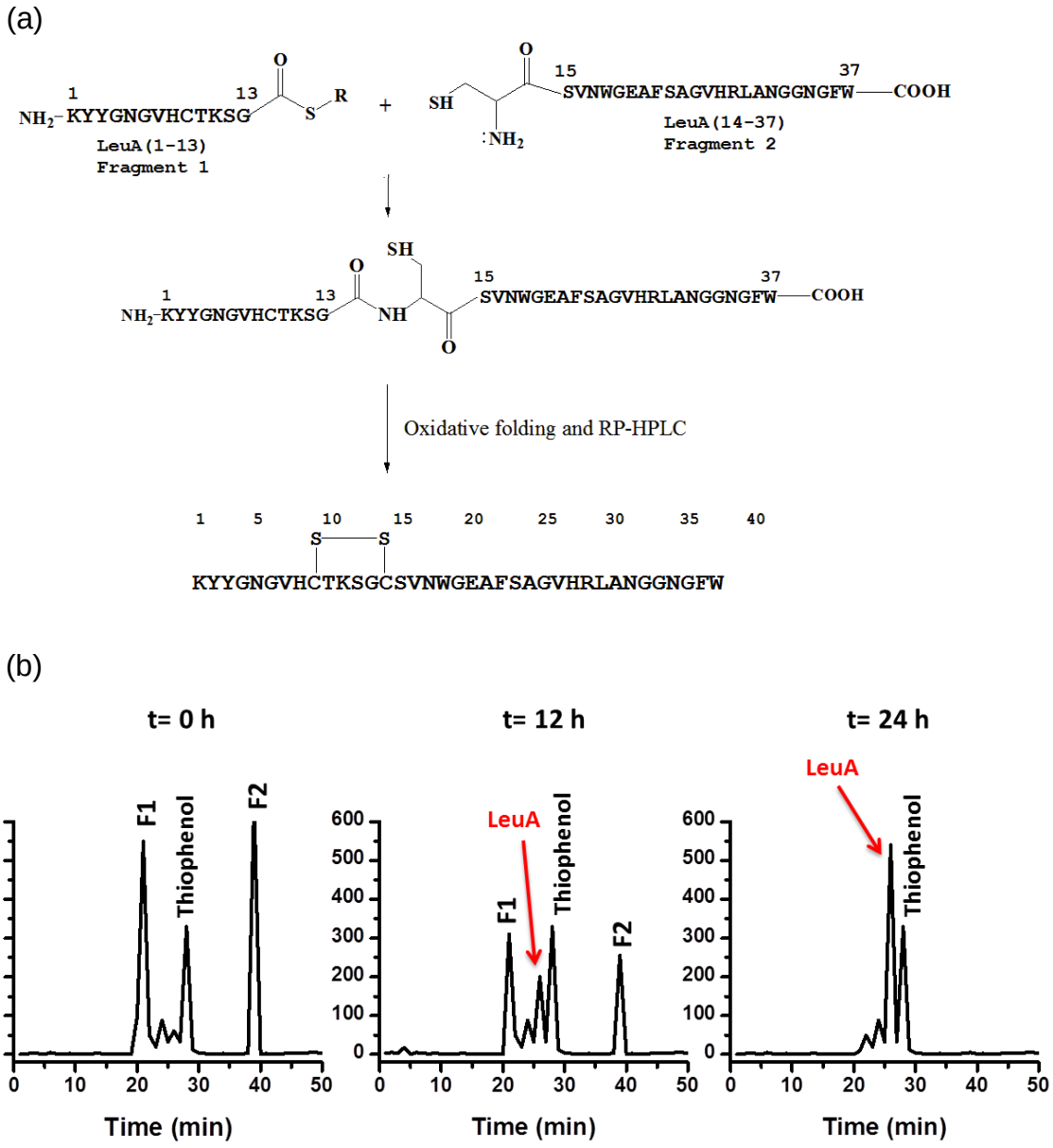


Figure 2. (a) Protocol for the synthesis of LeuA using NCL method. (b) Progress of the NCL reaction monitored using RP-HPLC at time 0, 12 and 24 h. The two fragments, LeuA-13AA (residues 1-13, F1) and LeuA-24AA (residues 14-37, F2) elute at 21 and 39 min, respectively, whereas, the ligated product LeuA (residues 1-37) elutes at 26 min. The NCL reaction was complete in 24 h.

unprotected peptide fragment 1. This reaction is followed by rapid spontaneous rearrangement, through an S-N acyl shift, giving rise to a ligated product.

The 13-residue fragment 1 (thioester) was synthesized using two different protocols as shown in **Figure S2**. First method utilizes sulfonamide safety-catch resin for the thioester synthesis[26, 27] whereas the second method employs thioesterfication of the peptide synthesized using 2-chlorotrityl resin.[28] For the synthesis using sulfonamide safety catch resin, first amino acid Fmoc-Gly (activation with PyBOP) was coupled manually to the 4-sulfamylbutyryl AM resin followed by assembly of the remaining amino acids on automated synthesizer. After complete peptide sequence, activation of the sulfonamide with iodoacetonitrile and displacement with the thiol nucleophile ethyl-3-mercaptopropionate produced the protected thioester peptide. LeuA 1-13 thioester (Fragment 1) was obtained after side chain deprotection using a mixture containing TFA, TIS and water. Typical yield of the HPLC purified fragment 1 synthesized using 4-sulfamylbutyryl AM resin was 21 mg (7% based on 0.2 mmol scale). Fragment 1 was characterized by RP-HPLC and MALDI-TOF mass spectrometry. For the second method, the peptide (1-13) was synthesized on 2-chlorotrityl resin. After cleavage from the resin with 1% TFA, peptide with C-terminal carboxylate was obtained which was converted into thioester by reaction with DIC, HOBt, DIPEA, and ethyl 3-mercatopropionate. Finally, the side chain protections and N-terminal Boc was removed as done for the first method. The second method gave higher yield (100 mg, 33% based on 0.2 mmol scale) of the pure thioester fragment 1.

Fragment 2, 24-residue (14-37), was synthesized a 0.2 mmole scale on 2-chloro trityl chloride resin following stepwise SPPS. After purification using RP-HPLC, fragment 2 was obtained with an overall yield of 270 mg (55% based on 0.2 mmol scale) and purity greater than 95%. Next, the two fragments, fragment 1 thioester (4 μmol) and fragment 2 (4 μmol), were

ligated in phosphate buffer (pH 7.6) containing 6 M GnHCl and thiophenol (4%)/ benzyl mecaptan (4%) as catalysts.[29] The reaction was monitored at different time intervals (0, 12 h, 24 h) by RP-HPLC. As shown in **Figure 2b**, the ligation was completed in 24 h, after which the resultant ligated product was purified using semi-preparative RP-HPLC. The presence of thiophenol with excess with 3-mercaptopropionic acid ethyl ester significantly enhanced the ligation reaction rate through nucleophilic catalysis.[30] The pure peptide was characterized by RP-HPLC and MALDI-TOF mass spectrometry (**Figure S3**). The yield of the chemical ligation reaction to give pure peptide in the reduced form was 95-98% (14 mg).

The overall yield of the purified reduced LeuA was 0.06 mmol (30%) on a 0.2 mmol scale synthesis. Oxidative folding or disulfide bond formation of the reduced peptide (14 mg) was carried out to give 9.5 mg of oxidized LeuA peptide with an overall yield of 21% based on 0.2 mmol scale synthesis.

We also attempted the stepwise SPPS of LeuA using Cl-trityl resin and the overall yield of LeuA obtained was 4.2%. In addition, others have attempted synthesis of Leu A using specialized resins such as polyamide/kieselguhr resin with an overall yield 16%.[16] Syntheses of several unnatural analogues of LeuA, such as *ent*-LeuA and carba-LeuA and diallyl-LeuA have been reported,[18, 31, 32] and the overall yield for the analogues has been low, such for *ent*-LeuA the yield was 6% using Wang resin as a solid support.[31] The NCL method certainly seems more promising for the synthesis of class IIa bacteriocins and analogues. Due to the hydrophobic nature of these peptides, coupling amino acids after 20-residues to the solid support during stepwise SPPS becomes very difficult. The elongated peptide tends to aggregate most likely due to the hydrophobic C-terminal domain leading to the appearance of increased number of

truncated peptides. To increase the yield and avoid loss of LeuA due to truncated peptides, chemical ligation seems to be a better method for the synthesis of LeuA and analogues.

2.3 Antimicrobial Activity

The activity spectra of the LeuA analogues **1**-**3** were evaluated against two indicator strains, *Carnobacterium divergens* and *Listeria monocytogenes* using liquid growth inhibition assay as described previously.[18] Synthetic LeuA (or LeuA) was used as a positive control. LeuA displayed potent activity against *C. divergens* and *L. monocytogenes* with MIC values of 39 and 37 nM, respectively (**Figure 3a**). These MIC values were similar to the wild type peptide reported previously.[18, 22] However, none of the analogues (**1**-**3**) displayed any activity up to 200 μM concentration. Next, the ability of analogues **1**-**3** to inhibit the antimicrobial activity of LeuA was explored. Analogues **2** and **3** did not inhibit the activity of native LeuA, whereas, the presence of analogue **1** led to significant inhibition of LeuA activity against *C. divergens* in a dose-dependent manner (**Figure 3b**). At high concentration of analogue **1** (100 μM), there was almost complete inhibition of LeuA activity (OD 0.55±0.08). The optical density for the control *C. divergens* sample, without LeuA and analogue **1**, was 0.61±0.09. These results suggest that the C-terminal helical region of analogue **1** may play an important role in mediating specific interaction with the target cell membrane receptor.

The N-terminal sequence in analogue **1** that lacks the β-sheet folding is likely responsible for inactivity of **1**. Chen et al. previously showed using N-terminal pediocin PA-1 fragments that electrostatic interaction direct peptide binding to the target membrane.[14] In our analogue design (**1**-**3**), the charge of the N-terminal region was maintained (Table 1). Despite that analogues did not show any activity suggesting that proper folding and/or complete sequence is essential for

(a)

Peptide	MIC[a] (nM)	
	C. divergens	*L. monocytogenes*
Wild type LeuA[48]	35	35
Synthetic LeuA	39 ± 0.2	37 ± 0.4
1	-	-
2	-	-
3	-	-

-, no activity detected up to 200 μM. Each assay against the two strains was repeated at least three times.

(b)

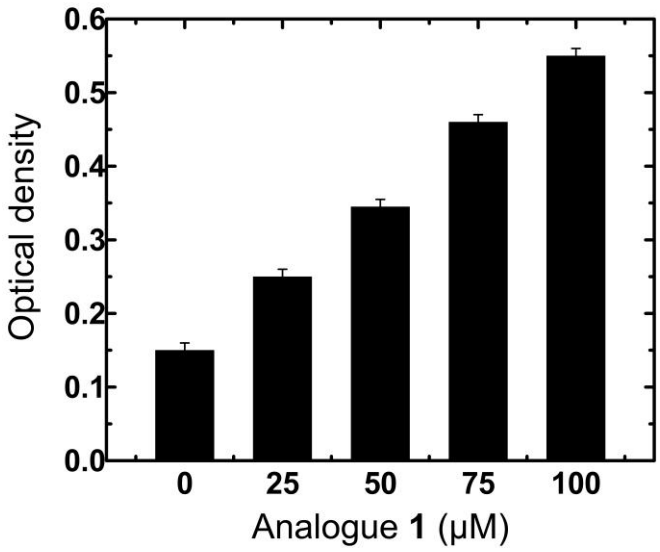


Figure 3. (a) Antibacterial activity of LeuA and analogues against *C. divergens* and *L. monocytogenes*. The MIC values represent peptide concentration that inhibited the growth by 50%. Data are represented as means ± standard errors of the means. The values are results of at least three independent measurements. (b) Dose dependent decrease in antimicrobial activity of LeuA in the presence of analogue **1** against *C.divergens*. Antimicrobial activity is expressed as optical density at 600 nm ± standard deviation.

peptide bactericidal activity. In addition, it is important to note that in class IIa bacteriocins like divercin V41, peptide fragment from the C-terminal domain (from residue 18 to 43) displays activity.[33] On the other hand, a change in the N-terminal domain in the native sequence while

keeping the remaining sequence intact led to complete loss of activity, as observed for analogue **1**. Likewise, fragment from C-terminal region of pediocin PA-1 (from residue 20 to 34) did not display activity but were able to specifically inhibit the bactericidal activity of 44-residue pediocin PA-1.[13] Finally, it has been shown that long fragments (15-mer) from the N-terminal of class IIa peptides such as pediocin PA-1, mesentericin Y105 and, enterocin CRL35, display some antimicrobial activity, however, smaller fragments from the N-terminal are inactive.[34]

2.4 Structure-Activity Relationship

In order to understand the structural basis of inactivity of analogues, we next studied their solution conformations using CD spectroscopy and MD simulations.

CD Spectroscopy. CD spectra of LeuA and analogues were obtained in 90% aqueous TFE (pH~2.5) to compare the secondary structure of the peptides. TFE was used as a solvent as class IIa bacteriocins form well-defined secondary structures in membrane mimicking solvents like TFE, detergent micelles or vesicles, but are essentially unstructured in water.[17, 22] Among the three analogues, only analogue **1** displayed significant helical structure as indicated by the appearance of a distinct negative band at 208 nm (Θ = -11.4 x 10^3), a negative shoulder near 220 nm, and a positive band at 195 nm (**Figure 4**). In comparison, analogues **2** and **3** were much less structured as observed from the CD spectra. The CD spectrum of **1** resembled that of LeuA suggesting similar helical folding in the two peptides.[22] Notably, the CD spectra of synthetic LeuA shows similar peaks as observed previously for wild type LeuA[22] with a negative band at 206 nm (θ = -10.5 x 10^3) and a negative shoulder near 220 nm (θ = -6.0 x 10^3) suggesting similar folding pattern.

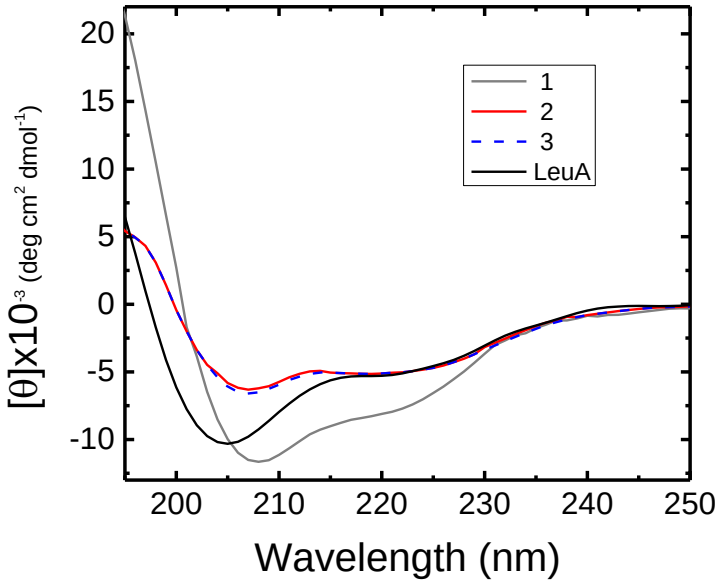


Figure 4. Circular Dichroism (CD) spectra of synthetic LeuA and analogues **1**, **2**, and **3** in 90% TFE (0.1% TFA final concentration, pH 2.5) at 200 μM concentration.

Homology Modeling and MD Simulations. The secondary structure of analogue **1** was further explored using MD simulations. First, a homology model of analogue **1** was constructed using the 3D structure of native LeuA (PDB code-1CW6 [9]).[22] Homology model was placed in the middle of a cubic box (**Figure 5a**). The box was filled with solvent (TFE) followed by MD simulations at two different temperatures, 298 K and 310 K. Snapshots were extracted after the simulations at each nanosecond and were analysed using VMD software.[35] Figure **5b** shows the overlay of several snapshots during simulation at 298 K. The peptide formed a stable structure with a coiled N-terminal region and C-terminal folded into an α-helical conformation. Clearly, the N-terminal region of **1** was very different from the native LeuA. Next, the simulation was conducted at an elevated temperature of 310 K. Peptide **1** showed the appearance of an N-terminal β-sheet (**Figure 5c**); however, this conformation was present only for short duration toward the end of the simulation (~ 9-10 ns). Overall, MD results suggest that the C-terminal

region of analogue **1** fold more like native LeuA, whereas, the N-terminal region displays marked differences from the native peptide.

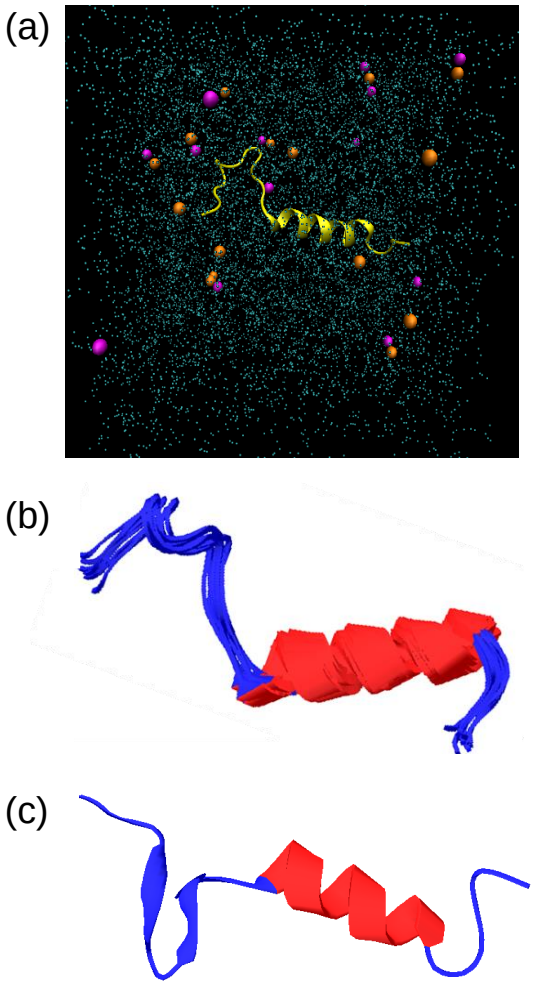


Figure 5. (a) Initial setup of a simulation box with box size X, Y and Z = 6 nm for MD simulation of analogue **1**. TFE is rendered in blue, analogue **1** in yellow ribbon, and Na^+ and Cl^- ions in magenta and blue, respectively. (b) Overlay of snapshots of **1** (9-10 ns) extracted from simulation at 298 K. The superimposed images show that the peptide conformation was stable with an N-terminal coil and C-terminal helix. (c) Snapshot of **1** extracted (~9 ns) from simulation at 313 K.

3. Conclusion

In conclusion, LeuA analogues (**1**-**3**) studied here support earlier investigations that the C-terminal region is required for specificity and dictates the antimicrobial profile, whereas, the N-terminal sequence is necessary for activity. Replacement of few N-terminal residues with conservative substitutions is allowed as shown previously,[19] however, the complete N-terminal domain is required for activity. Further, we found that NCL is a better approach for the solid phase synthesis of LeuA like peptides. NCL method gives high yield and is an efficient method for obtaining large amounts of LeuA. The NCL method described here can be utilized in future for the synthesis of other class IIa bacteriocins and library of analogues for performing comprehensive SAR studies. NCL method avoids unnecessary repeated synthesis of the unaltered sequences in a peptide during analogues syntheses.

4. Experimental Section

4.1 Synthesis of Peptide Analogues

Synthesis of peptide analogues **1**-**3** was carried out on 1.0% DVB cross-linked chlorotrityl resin (0.2 mmol, loading 1.05 mmol/g) following the standard Fmoc solid-phase peptide chemistry with acid labile side chain protections (t-Bu, Boc, Trt) as described previously.[36] Peptides were synthesized using MPS 357 automated peptide synthesizer robot (Advanced Chemtech Inc., USA). To assist the coupling of difficult residues (Asn, Ser, and for all residues after position 10), increased coupling times were employed. Test cleavage was performed at regular intervals (every 5 amino acids) by adding 50% TFA in DCM to a small amount of resin followed by shaking for 30 min at room temperature. The complete peptide sequences were released from support with concomitant removal of acid-labile side chain

protecting groups using the same procedure as used for the test cleavages. The filtrate from the cleavage reactions was combined with TFA washes (3x 2 min, 1 mL), and concentrated in vacuum. Cold diethyl ether (~15 mL) was added to precipitate the crude cleaved peptide. After trituration for 2 min, the peptides were collected upon centrifugation. The crude peptides were dissolved in 20% aqueous isopropanol and purified on HPLC using a semi-preparative VYDAC C8 RP-HPLC column (1 x 25 cm, 5 μm) with an isopropanol/water gradient in the presence of 0.05% TFA. The gradient used was 25-95% isopropanol/water in 45 minutes at a flow rate of 1.0 mL/min. Pure peptides obtained in 15-30% yield were characterized by analytical reversed-phase HPLC and MALDI-TOF mass spectrometry (**Figure S1, SI**). The purity of the peptides was verified to be ≥95% by reversed-phase chromatography. The pure lyophilized peptide analogues were stored at -20 ˚C under nitrogen.

4.2 Synthesis of LeuA

The synthesis of native LeuA was attempted using native chemical ligation of two fragments, 13-mer N-terminal fragment 1 and 24-mer C-terminal fragment 2.

4.2.1. Synthesis of Fragment 1 Thioester

In order to obtain higher yield of the thioester for fragment 1, two methods were attempted for the synthesis (**Figure S2).** The first method employed sulfonamide safety catch resin, whereas the second method used 2-chlorotrityl resin. The synthesis of fragment 1 thioester using Fmoc chemistry on sulfonamide safety catch resin (4-sulfamylbutyryl AM resin, 0.9 mmol/g, 0.2 mmol) followed procedure described by Ingenito *et al*.[26] The first amino acid glycine (1 mmol, 5 eq) was coupled manually to the resin using coupling agents PyBOP (1

mmol) and iPr_2EtN (0.75 mmol). The reaction mixture was agitated for 8 h at -20 ˚C followed by 8 hr at r.t. to get maximum loading of the first amino acid[27]. After deprotection of the first amino acid using freshly prepared 20% piperidine in DMF, rest of the amino acids were coupled using DIC/HOBt activation protocol on automated peptide synthesizer. During the entire synthesis, coupling reactions were monitored using ninhydrin test. After completion of synthesis, the full length peptide was released from resin with concomitant removal of acid-labile side chain protecting groups and formation of C-terminal thioester. The resin was first treated with DIPEA (2 mmol, 10 eq) and iodoacetonitrile (1.6 mmol, 8 eq) in NMP in dark for 24 h at room temperature. Following this, the resin was washed and ethyl-3-mercaptopropionate (50 eq) and sodium thiophenol (0.5 eq) in DMF were added to a final concentration of 1 M. The reaction mixture was agitated for 24 h at r.t. The resin was filtered and washed with DMF (3 x 2 mL). The combined filtrate and washings were collected in flask, vacuum concentrated and washed with cold anhydrous diethyl ether to remove traces of mercaptans. Finally, the amino terminal Boc group and the side chain protecting groups were removed from the peptide by addition of a cleavage cocktail (95:5:5 TFA/TIS/H_2O) and the mixture were stirred at r.t. for 2.5 h. The solution was evaporated in vacuum and the residual oil was treated with cold anhydrous ether (~15 mL). The resulting precipitate was collected by centrifugation and freeze dried. Precipitate was dissolved in 20% acetonitrile/water and purified by semi-preparative RP-HPLC to give pure fragment 1 thioester yield 7% (21 mg, 0.013 mmol). MALDI-TOF calcd for fragment 1, $[M+H]^+$ 1530.1; found 1530.3.

The second method employed 2-chlorotrityl resin (loading 1.6 mmol/g, 0.2 mmol) for the preparation of fragment 1 thioester. The peptide assembly was achieved following procedure described for the analogues except that the N-terminal lysine was Boc-protected. After peptide

assembly, peptide was cleaved from the resin using 1% TFA/DCM (3 x 4 mL, each for 3 min). The acidic supernatants were combined in reaction vessel containing DIPEA (6.65 mmol, 1160 μL) to yield a final peptide concentration of 4 mM. To this solution, ethyl 3-mercatopropionate (6 mmol, 30 eq), HOBt (4 mmol, 20 eq), DIC (4 mmol, 20 eq), and additional DIPEA (1.33 mmol, 230 μL) were added. The mixture was stirred overnight and concentrated. The protected peptide thioester was precipitated by multiple triturations with cold diethyl ether followed by centrifugation. Deprotection of side chains and N-terminal Boc was carried out as described above. The crude product was purified using semi-preperative RP-HPLC to give pure fragment 1 thioester with a 33% yield (100 mg, 0.065mmol). Calcd. $[M+H]^+$ 1530.1, found 1530.7.

4.2.2. Synthesis of Fragment 2

Synthesis of 24-residue fragment 2, LeuA (14-37), was carried out on 2-chlorotrityl chloride resin (0.2-mmol) as described for the analogues. After purification of the crude peptide using RP-HPLC, pure fragment 2 was obtained with 55% yield. MALDI-TOF calcd for fragment 2, $[M + H]^+$ 2536.1; found 2536.6.

4.2.3. Native Chemical Ligation

The chemical ligation of unprotected linear peptide fragments 1 and 2 was done following literature protocol.[29, 37] Fragment 1 (0.004 mmol, 6 mg) and fragment 2 (0.004 mmol, 10 mg) were dissolved in freshly degassed sodium phosphate buffer (0.2 M) containing guanidine HCL, (6 M, pH 7.5), and the ligation reaction was started by addition of a mixture of thiophenol (4%) and benzylmecaptan (4%). The solution was stirred at 25 °C for 24 hr under argon at r.t. and monitored by semi-preparative RP-HPLC and MALDI-TOF MS. The ligation

reaction was completed after 24 hr. The precipitate was collected, freeze dried, followed by purification with RP-HPLC method and characterization. MALDI-TOF calcd for reduced linear LeuA, $[M+H]^+$ 3930.7; found $[M+H]^+$ 3930.3; overall yield 30%. The pure peptide was subjected to oxidative folding (disulfide formation) by air oxidation in freshly degassed Tris buffer (50 mM, pH 8.4). Briefly, peptide (10.3 μmol, 41 mg) was dissolved in Tris buffer at a concentration of 1 mg/mL, and 20% DMSO was added to the buffer to enhance peptide solubility and oxidation. The solution was gently stirred in an open-air flask for 48 h, and the reaction was monitored using Ellman test[38] and mass spectrometry (**Figure S3**). Oxidized peptide was subjected to RP-HPLC to obtain pure cyclized LeuA in 70% yield. MALDI-TOF calcd $[M+H]^+$ 3928.7, found $[M+H]^+$ 3929.1. The overall yield of LeuA using NCL method was 21%.

Leu A was also prepared using Fmoc-SPPS on Cl-trityl resin where 37 amino acids were added linearly followed by peptide cleavage from resin and oxidation (disulfide formation). Using this method, the overall yield of cyclized LeuA after purification was 4.2%.

4.3 Antimicrobial Activity

Peptide stock solutions (25 μM) were prepared in methanol/water (1:3) and serial dilutions were made as required. Peptide concentrations of the stock solutions was determined by measuring UV absorbance at 280 nm following literature procedure.[39] Antimicrobial activity assay followed by determination of minimum inhibitory concentrations (MICs) were done using the liquid growth inhibition assay in microtiter plates as described previously.[36] The indicator strains used were *Carnobacterium divergens* UAL9 (grown in APT broth at 25 °C) and *Listeria monocytogenes* ATCC 15313 (TSYBE, 37 °C). The indicator strains were obtained from the

culture collection of CanBiocin Inc. (Edmonton, Alberta, Canada). MICs were defined as the peptide concentration that inhibited the growth of the indicator strain by 50%. For the competition experiments using the liquid growth inhibition assay, a range of concentrations of analogue **1** (0-100 μM) was incubated with indicator strain (*C. divergens*, diluted 1:100) in the presence of LeuA (slightly above its MIC, 50 nM). The final volume of each well of the microtiter plate was made 200 μL with the culture medium. The microtiter plate culture was incubated overnight at 25 $^{\circ}$C, after which the growth of the indicator strain was measured spectrophotometrically at 600 nm using microtitre plate reader (TECAN, Männedorf, Switzerland).

4.4 Characterization of Leu A analogues

4.4.1. CD Spectroscopy

CD spectra of LeuA analogues **1**-**3** and synthetic LeuA were obtained using an Olis CD spectrometer (Georgia, USA) in a thermally controlled quartz cell with 0.02 cm path length over 196-250 nm. Peptide samples with a final concentration of 200 μM were prepared in 2,2,2-trifluoroethanol (90% TFE) containing 0.1% aqueous TFA (pH~2.5). The baseline scan was performed by taking the buffer alone and subtracted from experimental readings. Data were collected every 0.05 nm and were average of 6 scans. The normalized CD data was expressed in units of molar ellipticity per residue (deg cm^2 $dmol^{-1}$) and plotted versus the wavelength.

4.4.2. Molecular Dynamic Simulations

MD simulation of peptide analogue **1** was conducted using GROMCAS as described previously.[22, 40] BLAST [41] and FASTA [42] sequence alignment tools were used to search for other similar sequences apart from LeuA. Analogue **1** displayed high sequence identity with LeuA and the solution NMR structure of LeuA in TFE (PDB code 1CW6)[9] was used to construct homology model of **1** using the magic fit tool in the VMD-software.[35] The homology model was chosen as the starting structure for MD simulations using GROMACS MD simulation package (GROMOS96 force field).[43, 44] TFE was used to solvate the simulation box and simulations were conducted in the NPT ensemble at 298 or 313 K using periodic boundary conditions. Weak coupling of the peptide to a solvent bath of constant temperature was maintained using the Berendsen thermostat [45] and pressure was controlled using the Berendsen algorithm at 1 bar with a coupling constant 4 ps, using a compressibility of liquid TFE of 1.22 x 10^{-4} and 1.34 x 10^{-4} kJ^{-1} mol nm^3 at 298 and 313 K, respectively.[46] The electrostatic and van der Waals interactions were truncated at a cutoff distance of 1.2 nm. The integration time step was 2 fs, and the coordinates and velocities were saved every 2 ps. The LINCS algorithm was used to restrain all bond lengths. A cubic box (6×6×6 nm^3) was constructed and used to run the simulation. Parameters for TFE solvent were prepared according to Fioroni et al.[46] Homology model of analogue **1** was placed in the centre of the box by replacing the TFE molecules equivalent to the peptide size. The N-terminal was positively charged (NH_3^+) and C-terminal was considered to be negatively charged (COO^-). In the sequence, residues Asp, Arg, Glu and Lys were charged and His residues were kept neutral. Sufficient counter ions (Na^+, Cl^-) were added to make the system electro neutral and to provide a final concentration of ~100 mM. Energy within the system was minimized before MD simulation using 200 steps of the steepest descent energy minimization method in order to relax any steric conflicts generated during the set-up. The equilibration of the analogue

1-TFE system was achieved by performing a 3 ns MD run with positional restrain on the peptide molecule. Following this, a full MD run of 10 ns was performed without any restraints at 298 K followed by at 310 K. Snapshots of the peptides were extracted from 1-10 ns. Simulations were analyzed using various GROMACS post processing routines.[43] Swiss-Pdb Viewer [47] and VMD[35] softwares were used to visualize, analyse and superimpose structures.

ACKNOWLEDGEMENT

We thank Wael Soliman for assistance with MD simulations, Sahar Ahmed for help in writing, and Liru Wang (CanBiocin Inc.) for assistance in performing antimicrobial assays. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC). The infrastructure support from the Canada Foundation for Innovation (CFI) is also acknowledged.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at . These data include synthetic scheme for fragment 1, RP-HPLC chromatograms and mass spectrometric characterization of LeuA and analogues **1**-**3** (Figures S1-S3) described in this article.

Abbreviations

AMP, antimicrobial peptide; CD, circular dichroism; DIC, 1,3-diisopropylcarbodiimide; Fmoc, 9-Fluorenylmethyloxycarbonyl; HOBt, N-hydroxybenzotriazole; RP-HPLC, reversed phase high performance liquid chromatography; LAB, lactic acid bacteria; LeuA, Leucocin A; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MD, molecular dynamics; MIC,

minimum inhibitory concentration; NMR, nuclear magnetic resonance; NCL, native chemical ligation; OD, optical density; PDB, protein data bank; SPPS, solid phase peptide synthesis; SAR, structure activity relationship; 3D, three-dimensional; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol

References

1. Cotter, P. D.; Hill, C.; Ross, R. P. *Nat. Rev. Microbiol.* **2005,** *3*, 777.
2. Lohans, C. T.; Vederas, J. C. *Int. J. Microbiol.* **2012,** *2012*, 386410.
3. Abriouel, H.; Franz, C. M. A. P.; Omar, N. B.; Gálvez, A. *FEMS Microbiol. Rev.* **2011,** *35*, 201.
4. Corr, S. C.; Li, Y.; Riedel, C. U.; O'Toole, P. W.; Hill, C.; Gahan, C. G. M. *Proc. Natl. Acad. Sci. U.S.A.* **2007,** *104*, 7617.
5. Héchard, Y.; Sahl, H.-G. *Biochimie* **2002,** *84*, 545.
6. Martinez-Cuesta, M. C.; Kok, J.; Herranz, E.; Pelaez, C.; Requena, T.; Buist, G. *Appl. Environ. Microbiol.* **2000,** *66*, 3174.
7. Kjos, M.; Nes, I. F.; Diep, D. B. *Microbiology* **2009,** *155*, 2949.
8. Diep, D. B.; Skaugen, M.; Salehian, Z.; Holo, H.; Nes, I. F. *Proc. Natl. Acad. Sci. U.S.A.* **2007,** *104*, 2384.
9. Wang, Y.; Henz, M. E.; Fregeau Gallagher, N. L.; Chai, S.; Gibbs, A. C.; Yan, L. Z.; Stiles, M. E.; Wishart, D. S.; Vederas, J. C. *Biochemistry* **1999,** *38*, 15438.
10. Uteng, M.; Hauge, H. H.; Markwick, P. R. L.; Fimland, G.; Mantzilas, D.; Nissen-Meyer, J.; Muhle-Goll, C. *Biochemistry* **2003,** *42*, 11417.
11. Haugen, H. S.; Fimland, G.; Nissen-Meyer, J.; Kristiansen, P. E. *Biochemistry* **2005,** *44*, 16149.
12. Johnsen, L.; Fimland, G.; Nissen-Meyer, J. *J. Biol. Chem.* **2005,** *280*, 9243.
13. Fimland, G.; Jack, R.; Jung, G.; Nes, I. F.; Nissen-Meyer, J. *Appl. Environ. Microbiol.* **1998,** *64*, 5057.
14. Chen, Y.; Ludescher, R.; Montville, T. *Appl. Environ. Microbiol.* **1997,** *63*, 4770.

15. Hastings, J. W.; Sailer, M.; Johnson, K.; Roy, K. L.; Vederas, J. C.; Stiles, M. E. *J. Bacteriol.* **1991,** *173*, 7491.
16. Fleury, Y.; Dayem, M. A.; Montagne, J. J.; Chaboisseau, E.; Caer, J. P. L.; Nicolas, P.; Delfour, A. *J. Biol. Chem.* **1996,** *271*, 14421.
17. Fregeau Gallagher, N. L.; Sailer, M.; Niemczura, W. P.; Nakashima, T. T.; Stiles, M. E.; Vederas, J. C. *Biochemistry* **1997,** *36*, 15062.
18. Derksen, D. J.; Stymiest, J. L.; Vederas, J. C. *J. Am. Chem. Soc.* **2006,** *128*, 14252.
19. Derksen, D. J.; Boudreau, M. A.; Vederas, J. C. *ChemBioChem* **2008,** *9*, 1898.
20. Trevino, S. R.; Schaefer, S.; Scholtz, J. M.; Pace, C. N. *J. Mol. Biol.* **2007,** *373*, 211.
21. Lee, J.; Dubey, V. K.; Longo, L. M.; Blaber, M. *J. Mol. Biol.* **2008,** *377*, 1251.
22. Kaur, K.; Andrew, L. C.; Wishart, D. S.; Vederas, J. C. *Biochemistry* **2004,** *43*, 9009.
23. Hackenberger, C. P. R.; Schwarzer, D. *Angew. Chem. Int. Ed.* **2008,** *47*, 10030.
24. Dawson, P. E.; Muir, T. W.; Clarklewis, I.; Kent, S. B. H. *Science* **1994,** *266*, 776.
25. Clark, R. J.; Craik, D. *Biopolymers* **2010,** *94*, 414.
26. Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999,** *121*, 11369.
27. Backes, B. J.; Dragoli, D. R.; Ellman, J. A. *J. Org. Chem.* **1999,** *64*, 5472.
28. Flemer, S. *J. Pept. Sci.* **2009,** *15*, 693.
29. Escher, S. E.; Klüver, E.; Adermann, K. *Lett. Pept. Sci.* **2001,** *8*, 349.
30. Johnson, E. C. B.; Kent, S. B. H. *J. Am. Chem. Soc.* **2006,** *128*, 6640.
31. Yan, L. Z.; Gibbs, A. C.; Stiles, M. E.; Wishart, D. S.; Vederas, J. C. *J. Med. Chem.* **2000,** *43*, 4579.
32. Sit, C. S.; Lohans, C. T.; van Belkum, M. J.; Campbell, C. D.; Miskolzie, M.; Vederas, J. C. *ChemBioChem* **2012,** *13*, 35.
33. Bhugaloo-Vial, P.; Douliez, J.-P.; Mollé, D.; Dousset, X.; Boyaval, P.; Marion, D. *Appl. Environ. Microbiol.* **1999,** *65*, 2895.
34. Salvucci, E.; Saavedra, L.; Sesma, F. *J. Antimicrob. Chemother.* **2007,** *59*, 1102.
35. Humphrey, W.; Dalke, A.; Schulten, K. *J. Mol. Graph.* **1996,** *14*, 33.
36. Soliman, W.; Wang, L.; Bhattacharjee, S.; Kaur, K. *J. Med. Chem.* **2011,** *54*, 2399.
37. Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. *Science* **1994,** *266*, 776.
38. Boyne, A. F.; Ellman, G. L. *Anal. Biochem.* **1972,** *46*, 639.
39. Gill, S. C.; von Hippel, P. H. *Anal. Biochem.* **1989,** *182*, 319.

40. Soliman, W.; Bhattacharjee, S.; Kaur, K. *Biochim Biophys Acta* **2007,** *1774*, 1002.
41. Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. *J. Mol. Biol.* **1990,** *215*, 403.
42. Pearson, W. R. *Methods Enzymol.* **1990,** *183*, 63.
43. Spoel, D. V.; vanBuuren, A. R.; Apol, E.; Meulenhoff, P. J.; Tieleman, D. P.; Sijbers, A. L. T. M.; Hess, B.; Feenstra, K. A.; Lindahl, E.; vanDrunen, R.; Berendsen, H. J. C. *Gromacs User Manual Version 3.1.1*. **2002**, The Netherlands. Internet: *www.gromacs.org*.
44. Lindahl, E.; Hess, B.; van der Spoel, D. *J. Mol. Model.* **2001,** *7*, 306.
45. Van Der Spoel, D.; Lindahl, E.; Hess, B.; Groenhof, G.; Mark, A. E.; Berendsen, H. J. C. *J. Comput. Chem.* **2005,** *26*, 1701.
46. Fioroni, M.; Burger, K.; Mark, A. E.; Roccatano, D. *J. Phys. Chem. B* **2000,** *104*, 12347.
47. Guex, N.; Peitsch, M. C. *Electrophoresis* **1997,** *18*, 2714.

Graphical Abstract

KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW
(LeuA)

KYYGNGVHKPVNWGEAFSAGVHRLANGGNGFW
(1)