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RESEARCH ARTICLE

Macrocyclic Antimicrobial Peptides Engineered from ω-Conotoxin

Xinya Hemu and James P. Tam*

School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 03s-71, Singapore 637551, Singapore

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Abstract: The potent calcium channel blocker ω-conotoxin MVIIA is a linear cystine-knot peptide with multiple basic amino acids at both termini. This work shows that macrocyclization of MVIIA linking two positive-charge terminal clusters as a contiguous segment converts a conotoxin into an antimicrobial peptide. In addition, conversion of disulfide bonds to amino butyric acids improved the antimicrobial activity of the cyclic analogs. Ten macrocyclic analogs, with or without disulfide bonds, were prepared by both Boc and Fmoc chemistry using native chemical ligation. All cyclic analogs were active against selected Gram-positive and Gram-negative bacteria with minimal inhibitory concentrations in a low μM range. In contrast, MVIIA and its linear analog were inactive at concentrations up to 0.5 mM. The cyclic analogs also showed 2 to 3-fold improved chemotactic activity against human monocytes THP-1 compared with MVIIA. Reduction of molecular stability against thermal and acid treatment due to the reduced number of disulfide crosslinks can be partly restored by backbone cyclization. Together, these results show that macrocyclization and side chain modification of a linear conopeptide lead to a gain-of-function, which brings a new perspective in designing and engineering of peptidyl therapeutics.

Keywords: Macrocyclization, cyclic conotoxin, antimicrobial peptide, chemotaxis.

1. INTRODUCTION

Bioactive peptides with large foot prints are potential drug candidates that offer advantages of high on-target specificity and low off-target adverse effect than small molecules with small foot prints. However, they suffer a major limitation of poor metabolic stability. Advances in designing highly stable peptide therapeutics using structural constraints such as backbone macrocycles have begun to address this limitation [1-3]. Successful examples include those guided by structures such as stapled peptides [4] and polycyclics [5] and those inspired by nature such as cyclic peptides [6] and lasso peptides [7]. In addition, recent examples have exploited the cyclic peptide scaffolds for drug design to obtain potent and orally-active bioactive peptides by inserting a bioactive peptide into an inter-cystine loop of a disulfide-rich cyclotide [3, 8].

End-to-end cyclization of a linear peptide improves metabolic stability by protecting peptide from exopeptidase degradation and, in some instances, improves biological activity. Our laboratory previously showed that the advantages of designing macrocyclic antimicrobials through the backbone cyclization of linear antimicrobial peptides (AMPs) such as human defensins, protegrins, and tachyplesins [9-12]. For these disulfide-rich host-defense peptides, backbone cyclization not only increases stability but also their membranolytic selectivity and tolerance to high-salt conditions. In this study, we show that macrocyclization of a linear peptide, ω-conotoxin MVIIA [13-15], results in converting a calcium channel inhibitor into an antimicrobial.

MVIIA possesses three disulfide bonds arranged as a cystine-knot and six basic residues at both termini [16-18]. The cationic- and Cys-rich features of MVIIA are also found in AMPs, such as defensins and cyclotides, however, MVIIA is not known to kill microbes. Killing microbes through membranolysis mediated by these cationic AMPs are often initiated by the selective interaction of the cationic residues with the negatively charged phospholipids on microbial membranes, followed by insertion of hydrophobic side chains into lipid bilayer or induction of potential on two sides of lipid bilayer, leading to electroporation [19]. Thus, we envisioned that upon end-to-end cyclization, two cationic clusters of MVIIA will join to form an extended basic-charged epitope that could gain membranolytic activity on microbes.

By chemical synthesis, we prepared a series of MVIIA and its derivatives, including a linear analog with an extra dipeptide tail, and ten cyclic analogs with a reduced number of disulfide bonds. All synthetic conotoxins were examined for the gain or loss of functions, including stability, antimicrobial activity, and changes in membranolytic selectivity as a consequence of backbone cyclization and side-chain modifications. The channel inhibitory activity of MVIIA is closely related with the native cystine-knotted, disulfide-stabilized structure, as deletion of any individual disulfide linkage is known to result in about a 100-fold decrease of channel binding activity [20]. Therefore, the channel-related activity of cyclic ω-conotoxins was not investigated in this report.

2. MATERIALS AND METHOD

2.1. Synthesis of Cyclic Conotoxin Precursors on Mercaptopropionic Acid (MPA)-linked MBHA Resin

3-mercaptopropionic acid (MPA) was coupled to MBHA (4-methylbenzhydrylamine) resin **1** with HATU/DIEA (2/2 eq.) as a linker to convert the C-terminal acid into a thioester. Manual coupling of amino acid sequences was done with Boc-AA/BOP/DIEA (4/4/6, mol/mol). After assembly, peptide thioesters **2a-f** were cleaved by hydrogen fluoride (HF) /p-cresol (9/1, v/v) in Teflon-sealed apparatus for 60 min at -5 °C. HF was then removed by vacuum and peptide-MPA thioesters **2a-f** were precipitated by diethyl ether and subjected to the reversed-phase C18 HPLC purification.

2.2. Synthesis of Cyclic Conotoxin Precursors on Thioethylbutylamido (TEBA) Resin Using Cl-Trt(2-Cl) Resin

Chloro-Trt(2-Cl) polysterene resin (0.5 g, 0.6 mmol) was swollen for 30 min in dichloromethane (DCM,10 mL) and the resin was filtered and washed with DCM (1 min x 2). (2-butylamino) ethanethiol (0.3 mmol, 44 μL) in DCM (20 ml) was added, and the suspension was shaken for 1 h at room temperature**.** 3.6 mmol DIEA (3.6 mmol, 627 μL) in methanol was added and the reaction

*Address correspondence to this author at the School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 03s-71, Singapore 637551, Singapore; E-mail: JPTam@ntu.edu.sg

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mixture shaken for 10 min to quench the unreacted resin. The resin was washed with DCM and DMF, respectively, to give TEBA resin **2**. A mixture of Fmoc-Arg(Pbf) (1.2 mmol, 779 mg), 1-[Bis (dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 1.2 mmol, 456 mg) in dimethylformamide (DMF) and *N,N*-Diisopropylethylamine (DIEA, 1.8 mmol, 314 μL) was added to resin **2**. The suspension was shaken for 1 h and coupling procedure was duplicated to give Fmoc-Arg(Pbf)-TEBA resin. The remaining peptide sequence was synthesized manually to give peptide-TEBA resin. A mixture of trifluoroacetate (TFA)/triisopropylsaline (TIS)/H_2O (95/2.5/2.5, v/v) was added to peptide resins and the reaction mixture was shaken for 2 h. After dropwise addition of the reaction mixture to a chilled diethyl ether for precipitation, the precipitate was dried *in vacuo* and purified by C18-reversed phase-high performance liquid chromatography (RP-HPLC) to give linear conotoxin precursors M1B-TEBA **4a**, M2B-TEBA **4b**, M3B-TEBA **4c** and MVIIA-GS-TEBA **4d**.

2.3. Synthesis of Conotoxin MVIIA Precursor on Rink Amide Resin

The native MVIIA sequence was synthesized on Rink amide resin (300 mg, 0.34 mmol/g) by Fmoc chemistry. The Fmoc-Cys(Trt) (0.4 mmol) was coupled to deprotected Rink amide resin by N,N'-Diisopropylcarbodiimide (DIC, 0.4 mmol) and Hydroxybenzotriazole (HOBt, 0.4 mmol) in DMF for 1 h to give Fmoc-Cys(Trt)-NH-Rink amide resin. After deprotection of Fmoc by 20 % piperidine solution in DMF, the remaining sequence was coupled stepwise to resin support using a microwave-assisted synthesizer and a protocol of Fmoc-AA /benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) /DIEA (5/5/10 eq.). After the final Fmoc deprotection, the protected MVIIA-NH-Rink resin (786 mg) was obtained. A mixture of TFA/TIS/H_2O (95:2.5:2.5, v/v, 5 ml) was added to 200 mg peptide-resins to remove the side chain protecting groups and to cleave peptides from the resin support. The cleaved peptides were precipitated in chilled diethyl ether and purified by RP-HPLC to give the linear conotoxin precursor MVIIA-NH_2 **9** (87 mg crude, 36 mg purified peptide, isolated yield 13.6%) (Supplement Fig. S2A).

2.4. Synthesis of a Linear Conotoxin Precursor MVIIA-GS on Wang Resin

The linear conotoxin precursor MVIIA-GS **11** was synthesized on Wang resin (167 mg, 0.1 mmol) by Fmoc chemistry using a microwave-assisted synthesizer. The coupling and deprotection methods were the same as above to give M-GS-resin (484 mg). A 5-ml-mixture of TFA/TIS/H_2O (95:2.5:2.5, v/v) was added to resins to remove side chain protection groups and cleave peptides from the resin support. The deprotected peptide M2B-OH was precipitated in chilled diethyl ether and purified by RP-HPLC to give the linear conotoxin precursor MVIIA-GS **11** (119 mg crude and 55 mg purified peptide, isolated yield 20%).

2.5. TEBA-Mediated Cyclization of Cyclic Conotoxins

Peptide-S-MPA thioesters **2a-f** and peptide-TEBA precursor **4a-d** were dissolved in 0.1 M sodium phosphate buffer (pH 3) at a concentration of 1 mM with a 100 fold excess. MESNa and incubated at 40°C for 16 h. The formation of TEBA-S-form **5a-d**, MES thioesters **6a-j** and thiolactones **7a-j** were monitored by RP-HPLC and MALDI-TOF mass spectrometry. In the absence of MESNa, only thiolactones **7a-j** were formed and the reaction took a longer time to complete. Tris(2-carboxyethyl)phosphine (TCEP, 15 mM) were added to the reaction mixture to prevent unexpected disulfide formation. After adjusting the pH to 7.5 by the addition of 2 N NaOH, thia zip cyclization proceeded at room temperature with gentle stirring for 1-2 h to give reduced cyclic conotoxins cM1B **8a**, cM2B **8b**, cM3B **8c**, cM1B2Acm **8d**, cM1B3Acm **8e**, cM2B1Acm **8f**, cM2B3Acm **8g**, cM3B1Acm **8h**, cM3B2Acm **8i** and cMVIIA-GS **8j**. The completion of cyclization was monitored by MALDI-TOF and RP-HPLC.

2.6. Oxidative Folding of Cyclic and Linear Peptide Precursors

The crude mixtures containing the reduced and cyclic MVIIA analogs **8a-c** were diluted 50 times with the folding buffer 0.1 M NH_4OAc and 2 M $(NH_4)_2SO_4$ to reach 20 μM. Redox reagents reduced and oxidized glutathione were added in a molar ratio of peptide:GSSG:GSH: 1:10:100 (mol/mol). Oxidative folding was performed at 4°C with magnetic stirring for two to three days. The folding reactions of reduced cyclic MVIIA analogs **8d-i** were conducted by adding 10% dimethyl sulfoxide (DMSO) to the cyclization mixture and incubated overnight at room temperature. Folded products were purified by RP-HPLC. Peptides with a single disulfide bond (1*SS*) were dissolved in 10% acetic acid in deoxygenated water to the final concentration of 2 mM. HCl (10 mM) was added to the reaction solution to prevent sulfoxide formation at methionine. A stock solution of iodine in methanol (0.1 M) was added into peptide solution dropwisely to reach 10 mM. Concurrent deprotection of Acm and oxidation of the free thiols was performed for 60 min at room temperature to form the second disulfide bond. Excessive iodine was removed by dropwise addition of the ascorbic acid solution (1 M) until the brown color changed to colorless.

2.7. Radical-mediated Global Desulfurization

Peptide cMVIIA-GS **8j** (0.1 μmol) was exposed to phosphine source TCEP (0.1 mmol), proton source glutathione (0.2 mmol), and initiator 2,2'-azobis(2-(2-imidazolin-2-yl)propane)dihydrochloride (VA-044) (1 μmol), in a 0.1 M phosphate buffer at pH 6.5. The desulfurization reaction took about 3 h at 40°C to convert all unprotected Cys into Ala and cM6A **15**.

2.8. Disulfide Mapping of 2*SS*-cyclic Conotoxins

Peptides eluted from HPLC were mixed with equal volume of 200 mM citric buffer, pH 3.0. 5 mM TCEP was added to reduce peptide at room temperature for about 5-8 min. Partial reduction condition was monitored by MALDI-TOF mass spectrometry. N-ethylmaleimide (NEM, 20 mM) was added to the reduction mixture to perform alkylation at room temperature. Alkylation of NEM usually took 1 to 1.5 h. The partially reduced and alkylated species 1*SS*+2S-NEM was isolated by RP-UHPLC (Shimadzu, highest pressure NEXARA). 1*SS*+2S-NEM peak eluted from UHPLC was mixed with 100 mM ammonium acetate buffer, pH 7.0. Approximately 50x chymotrypsin was added and digest peptide at room temperature for 1 h. Digestion was monitored by MALDI-TOF. Upon complete digestion, linear peptide was analyzed by MS/MS. Second digestion by trypsin was performed by adding 50x trypsin into the chymotrypsin digestion mixture for additional 10 min incubation. Peptide fragments obtained by trypsin/chymotrypsin double digestion were characterized by MALDI-TOF MS and MS/MS (Applied Biosystems, ABI 4800).

2.9. Stability Tests

Heating stability of cyclic peptides was examined at 100°C by performing a constant heating on a water solution of peptides (0.1 mM) for 1-24 h. The resultant peptide and degraded products were analyzed on a reverse-phase analytical-grade HPLC. Acid hydrolysis test was performed in a 200 mM HCl solution with peptide concentration of 0.1 mM. The resultant peptide and hydrolysis products were analyzed on a reverse-phase analytical-grade HPLC.

2.10. Antimicrobial Assay

The antimicrobial activities of peptide analogs were tested by Lehrer's two-stage radial diffusion assay [21]. *E. coli*, *S. aureus*, and *C. tropicalis* were tested. Bacteria culture with $4x10^6$ CFU was mixed with 10 mL agarose gel with minimal nutrient (30mg TSB/mL, 10 mM phosphate buffer) and pour on petri dish to form

underlay gel. Equal size wells (2 mm diameter) were punched in the solidified underlay gel by 1ml pipette tip connected to a vacuum pump. An aliquot (3 μL) of peptide samples with stack of dilution from 100 μM to 3.125 μM was loaded into each well. The peptide and the underlay gel with bacteria were incubated at 37 °C for 3 h with gel-side up. A nutrient-rich overlay gel (6 mg TSB (tryptic soy broth)/mL) was added on top of underlay gel, and the bacteria were allowed to grow overnight. The minimal inhibitory concentration (MIC) was calculated by measuring the diameter of the clear zone against different peptide concentrations. Each experiment was repeated three times and the average diameter was used.

2.11. Hemolytic Assays

Fresh human erythrocytes taken from a healthy volunteer was washed with PBS three times and resuspended to a 0.5% suspension in PBS. 50 μL was added to each well of a 96-well plate. Linear and cyclic conotoxins MVIIA, cM1B, cM2B and cM3B were serially diluted in 50 μL PBS. Each peptide concentration was tested in triplicate. A solution of 0.1% Triton X-100 without any test peptides was used as the positive control. PBS without any test peptides was used as negative control. The 96-well plate was incubated at 37 °C for 1 h, and the plate with erythrocytes were centrifuged at 1000 rpm for 10 min. Aliquots of 50 μL supernatant from each well was transfer to a new 96-well plate and measured by ELISA reader at 415 nm. The level of hemolysis was calculated as the percentage of maximum lysis by Triton X-100 after adjusting for minimum lysis in PBS. The peptide concentration caused for 50% hemolysis (HD_{50}) was calculated.

2.12. Chemotaxis Assays

Mouse leukemic monocyte macrophage RAW 264.7 was seeded in a 96-well Boyden chamber plate. A series of peptide solutions with concentration from 1 nM to 10 mM were loaded in the feeder tray. After a 6 h-incubation, detachment medium was added in the harvest tray, and upper chamber plate was transfer to harvest tray for 30 min incubation to release membrane-bound cells. Cells were lysed by the lysis-buffer (1% Triton X-100 in 10 mM Tris-Cl, pH 7) for 10 min. Excreted actin were labeled by fluorescent dye for measurement using 96-well plate reader (TECAN, Infinite M200Pro).

3. RESULTS

3.1. Design

Both N- and C-terminal residues of MVIIA are cysteine residues, C1 and C25, and exist as disulfide bonds of a cystine knot network. To ligate these two ends, an linker peptide is needed to form an end-to-end macrocycle. Using computer modeling to guide the design of cyclic MVIIA analogs, we initially added a tetrapeptide GGPG linker that sufficed to form a thermodynamically-stable 29-residue 3*SS*-cyclic analog (cCG29). In practice, we encountered low yield in the oxidative folding of cCG29 which inhibited producing sufficient sample for characterization and various bioassays [22]. We then re-designed the cyclic analogs to increase structural flexibility and side chain diversity by reducing the number of disulfide bonds and converting a disulfide bond into two hydrophobic aminobutyric acid (Abu) residues, a Cys isostere, or two hydrophilic S-acetamidomethyl (Acm)-protected Cys residues, or two Ala residues (Table **1**).

Ten cyclic analogs with side chain modifications are grouped into three series, including (1) three two-disulfide 2*SS*-cyclic MVIIA analogs linked by a Gly-Ser dipeptide to provide minimal spacing between the two basic amino acid clusters of native ω-conotoxin, of which one pair of Cys residues were substituted with Abu and designated cM1B, cM2B, and cM3B **13a-c**; (2) six one-disulfide (1*SS*)-cyclic MVIIA analogs of which one pair of Cys residues substituted by Abu and another pair substituted by Cys(Acm), designated cM1B2Acm, cM1B3Acm, cM2B1Acm, cM2B3Acm, cM3B1Acm, and cM3B2Acm **14a-f**; (3) a disulfide-free (0*SS*) cyclic MVIIA analogs, designated cM6A **15**, in which all Cys residues were converted to Ala by a radical-assisted desulfurization [23] of a cyclic S-reduced precursor, cM-GS **12j**. For comparison, a linear analog MVIIA-GS **4** containing a dipeptide GS-tail with three native disulfide bonds was also prepared.

Table 1. ω-conotoxin MVIIA and its synthetic analogs.

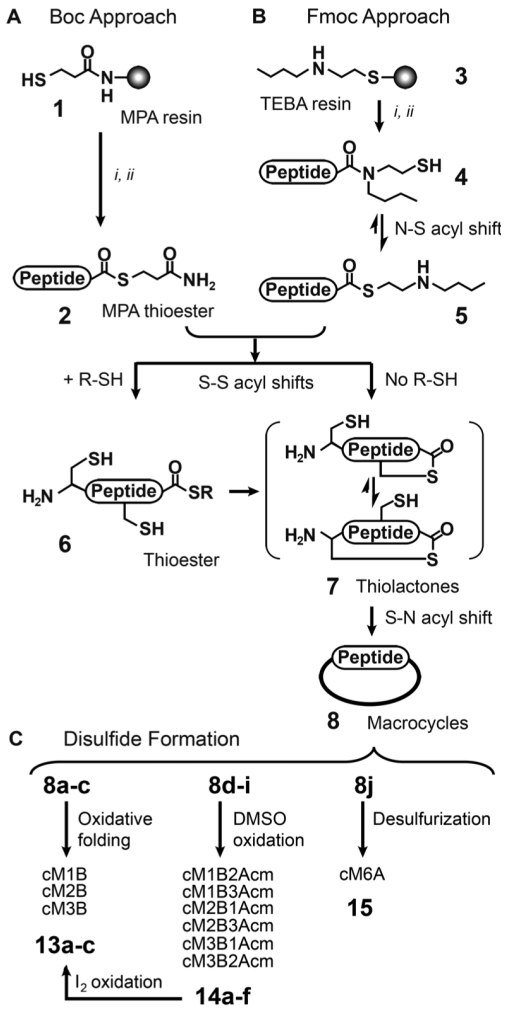
Peptides	No.	Sequences
MVIIA	10	CKGKGAKCSRLMYDCCTGSCRSGKC-NH_2
MVIIA-GS	12	CKGKGAKCSRLMYDCCTGSCRSGKC-GS
cM1B*	13a	Cyclo-BKGKGAKCSRLMYDCBTGSCRSGKC-GS
cM2B	13b	Cyclo-CKGKGAKBSRLMYDCCTGSBRSGKC-GS
cM3B	13c	Cyclo-CKGKGAKCSRLMYDBCTGSCRSGKB-GS
cM1B2Acm	14a	Cyclo-BKGKGAK(Acm)CSRLMYDCBTGSC(Acm)RSGKC-GS
cM1B3Acm	14b	Cyclo-BKGKGAKCSRLMYDC(Acm)BTGSCRSGKC(Acm)-GS
cM2B1Acm	14c	Cyclo-C(Acm)KGKGAKBSRLMYDCCTGSBRSGKC(Acm)-GS
cM2B3Acm	14d	Cyclo-CKGKGAKBSRLMYDC(Acm)CTGSBRSGKC(Acm)-GS
cM3B1Acm	14e	Cyclo-C(Acm)KGKGAKCSRLMYDBCTGSC(Acm)RSGKB-GS
cM3B2Acm	14f	Cyclo-CKGKGAK(Acm)CSRLMYDBCTGSC(Acm)RSGKB-GS
cM6A	15	Cyclo-AKGKGAKASRLMYDAATGSARSGKA-GS

*: peptides are designated as, c for cyclized backbone, M for MVIIA, number 1, 2, 3 represents *SS* pair C1-C16, C8-C20, C15-C25 respectively, B for Abu substitution and Acm for Acm-protected-Cys.

3.2. Peptide Synthesis by Boc and Fmoc Chemistry

For preparing cyclic peptides by a thia-zip cyclization previously developed by our laboratory requires the design of a linear precursor with a cysteine at the N-terminus and a thioester at the C-terminus [3, 8, 22, 24-29]. Both Fmoc and Boc chemistry were employed for preparing the thioester precursors (Scheme **1A** and **1B**) [24, 30, 31]. Boc approach was used to prepare linear precursors of 1*SS*-cyclic analogs and Fmoc approach was used to prepare 2*SS*- and 0*SS*-cyclic peptide precursors. Eventually, all cyclic reduced peptides underwent disulfide formation or desulfurization to obtain the final products (Scheme **1C**).

For Boc approach, peptide-S-mercaptopropionic acid (MPA) was attached to MBHA resin to give MPA-MBHA resins **1** as previously reported in the chemical synthesis of cyclotide kB1 [32]. Peptide-MPA thioesters **2a-f** cleaved from the resin support by hydrogen fluoride were used, without purification, for cyclization. For Fmoc approach, our recently developed base-resistant thioethylbutylamide (TEBA) resin **3** was exploited. The linear peptide precursors **4** with a C-terminal TEBA thioester surrogate was stable during repetitive piperidine treatment in Fmoc peptide synthesis. Under acidic conditions, including 95% trifluoroacetic acid (TFA)



Scheme 1. Synthesis schemes of cyclic conotoxins with 0-2 disulfide bonds.

cleavage solution and 0.1% TFA-water solution for reverse phase (RP)-HPLC, about 20% of the thioethylamino groups underwent a reversible *N-S* acyl shift reaction to form a peptide-S-TEBA thioester **5** (Fig. **1A**).

At pH 3, we observed and characterized thioester or thiolactone intermediates of the proposed thia zip cyclization **2**, **4** and **5,** which underwent N-S and S-S acyl shifts mediated by an external thiol or an internal Cys thiol. In the presence of an external thiol such as sodium mercaptoethane sulfonate (MESNa), thioesterification resulted in both peptide-MES thioester **6** and peptide thiolactones **7** (Fig. **1B**). In the absence of an external thiol, only the isomeric peptide thiolactones **7** were observed (Fig. **1C**). These thiolactone intermediates containing an α-amine have the same mass but more hydrophilic as compared with the expected amide-macrocycles. Consequently, the more polar thiolactones were distinguished from the late-eluting amide-macrocycles in RP-HPLC. Also thioester bond absorbs UV at 260 nm, which is another feature that helped us to identify thiolactone intermediates from the final cyclic product. Conversion of all N-forms **4** into stable thioesters or thiolactones completed after 16-24 h. In practice, the thia-zip cyclization was performed under a "one-pot" condition by adjusting pH from 3 to 7.5 and resulted in >80% reduced cyclic peptides **8** after 1 h (Fig. **1D** and Fig. **S1**). In addition, the peptide chains of linear MVIIA **9** and MVIIA-GS **11** were synthesized by standard Fmoc chemistry on Rink-amide resins (Fig. **S2**).

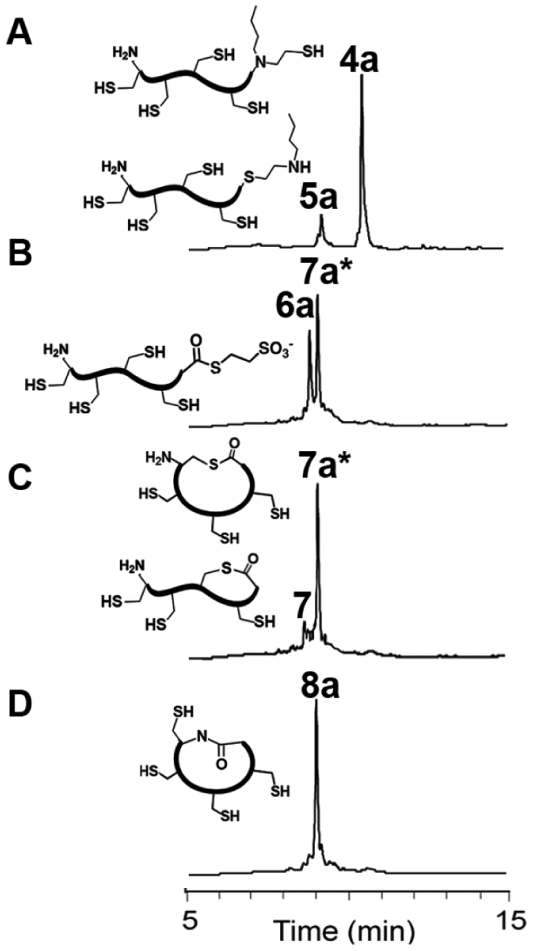


Fig. (1). Chemical synthesis of reduced cyclic cM1B 8a on TEBA resin using Fmoc chemistry.

3.3. Global Oxidative Folding vs. Chemoselective Disulfide Formation

Formation of disulfide bonds with correct connectivity for the peptides containing more than one disulfide bonds was achieved by both global oxidation and chemoselective method. Global oxidation of 2*SS*-cyclic reduced **8a-c**, linear reduced MVIIA **9** and MVIIA-GS **11,** were performed with oxidized and reduced glutathione (peptide:GSSG:GSH = 1:10:100 mol/mol) in 0.1 M ammonium acetate buffer (pH 7.8) and 2 M ammonium sulfate at 4 °C [33]. The 2*SS*-cyclic analogs cM1B-cM3B **13a-c** produced the desired disulfide isomers in 20% yield due to an increase in hydrophobicity that caused aggregation during the folding process (Fig. **S3**). Other analogs did not appear to have such an aggregation problem and were obtained in good yield. MVIIA **10** was afforded in 81% yield in 48 h (Fig. **S4**) and 3*SS*-linear MVIIA-GS **12** in 78% yield (Fig. **S5**).

DMSO oxidization converted the 2Cys2Acm-cyclic peptides **8d-i** into 1*SS*-cyclic products **14a-f**. To form the second disulfide bond by a chemoselective approach, **14a-f** were further treated with iodine to deprotect and oxidize Cys(Acm) to form 2*SS*-cyclic peptides **13a-c** (Fig. **S6**). These Boc chemistry-derived 2*SS*-cyclic products were used as standards for the corresponding products in the global oxidative folding prepared by the Fmoc chemistry. In addition, disulfide mapping of the 2*SS*-cyclic conotoxins were also performed to confirm their correct disulfide connectivity as described in Experimental.

The disulfide-free cyclic conotoxin cM6A **15** was obtained by desulfurizing all six Cys of cyclic and reduced **8j** into Ala with a

water-soluble radical initiator VA-044, a phosphine source (TCEP) and a proton donor (GSH) [23]. The reaction was conducted in a phosphate buffer (pH 6.5) at 40 °C for 3 h to afford cM6A in 84% yield (Fig. **S7**).

3.4. Antimicrobial Activity

Radial-diffusion assays were performed with MVIIA and analogs against three bacterial strains, *Escherichia coli* (Gram-negative), *Pseudomonas aeurginosa* (Gram-negative), *Staphylococcus aureus* (Gram-positive), and two fungal strains, *Candida kefyr* and *Candida tropicalis*. The microbicidal potency of each peptide was evaluated by the minimum inhibitory concentration (MIC) (Table **2**). MVIIA (up to 500 μM) was inactive against all three bacterial strains but exhibited moderate antifungal activity against *C. kefyr* and *C. tropicalis* with MICs of 28.8 μM and 39.8 μM, respectively. The linear analog MVIIA-GS gave similar results, indicating the GS linker has no effect on antimicrobial activity. In contrast, all ten cyclic conotoxins derived from MVIIA were active against the selected three bacterial strains with MICs from 3.3-90.2 μM. Most of these analogs exhibited improved antifungal activity with MICs from 2.1-18.2 μM against *C. kefyr* and 2.0-11.4 μM against *C. tropicalis*, with the exception of cM1B3Acm, which had a 42.2 μM MIC against C. tropicalis. Similar to membrane-active human and plant defensins, the microbicidal activity of cyclic conotoxins was inactive under the physiological condition with high salt content (0.1 M NaCl).

Table 2. Minimal inhibitory concentration (MIC) of antimicrobial activities of MVIIA and its analogs.

Peptide	*SS*	MIC (μM)				
		EC	PA	SA	CA	CT
MVIIA	3	> 500	>500	> 500	28.8	39.8
MVIIA-GS	3	> 500	ND	> 500	ND	ND
cM1B	2	8.9	78.4	7.9	2.3	11.2
cM2B	2	8.4	90.2	8.0	2.4	2.0
cM3B	2	8.6	11.8	4.2	2.1	2.3
cM1B2Acm	1	12.6	39.6	30.2	4.2	6.7
cM1B3Acm	1	3.3	23.4	78.2	18.2	42.2
cM2B1Acm	1	11.4	16.7	21.2	5.0	15.6
cM2B3Acm	1	39.4	72.4	18.8	6.9	4.9
cM3B1Acm	1	7.9	10.2	18.2	6.9	7.9
cM3B2Acm	1	4.1	9.4	7.4	3.4	6.9
cM6A	0	7.4	ND	8.9	ND	ND

EC, E. coli; PA, P. aeruginosa; SA, S. aureus; CA, C. kefyr; CT, C. tropicalis. ND, not determined.

3.5. Hemolytic Activity and Membranolytic Selectivity

A notable characteristic of membrane-active AMPs is their membranolytic selectivity, specifically recognizing microbial membranes *via* multiple charge-charge interactions. Hemolytic analysis of human red blood cells showed that native MVIIA and its cyclic analogs were not active against mammalian cell membranes. Conotoxin MVIIA exhibited no hemolytic activity at concentrations <1mM (HD_{50} = 4.2 mM). The HD_{50} of cyclic analogs ranged from 1.3-3.4 mM (Table **3**).

Table 3. Therapeutic index (HD_{50}/MIC) of MVIIA and its cyclic analogs.

Peptide	HD_{50} (mM)	HD_{50}/MIC Ratio				
		EC	PA	SA	CA	CT
MVIIA	4.23	-	-	-	147	106
cM1B	2.83	318	36	358	1229	252
cM2B	1.32	157	15	165	548	658
cM3B	1.62	187	136	383	766	699
cM1B2Acm	1.53	121	39	51	364	227
cM1B3Acm	3.37	1016	144	43	185	80
cM2B1Acm	2.55	223	153	120	509	163
cM2B3Acm	1.42	36	20	76	206	292
cM3B1Acm	3.07	386	301	169	444	388
cM3B2Acm	1.45	351	154	195	425	209

HD_{50}: dose for 50% hemolysis of erythrocytes

3.6. Chemotaxis

Another characteristic of cationic AMPs such as human defensins is the cytokine-like chemotactic activity [34, 35]. The chemotactic effects of native MVIIA and three 2*SS*-cyclic analogs were examined using the human monocyte cell line THP-1. All four synthetic peptides were found to be chemotactic from 10 nM to 0.1 mM, increasing cell migration from 3% (medium only) to 10% (with peptides) after a 6 h-incubation (Fig. **2**). These results showed that conotoxin MVIIA exhibited weak chemotactic activity on THP-1 cells. This chemoattractive activity increased moderately in the 2*SS*-cyclic analogs.

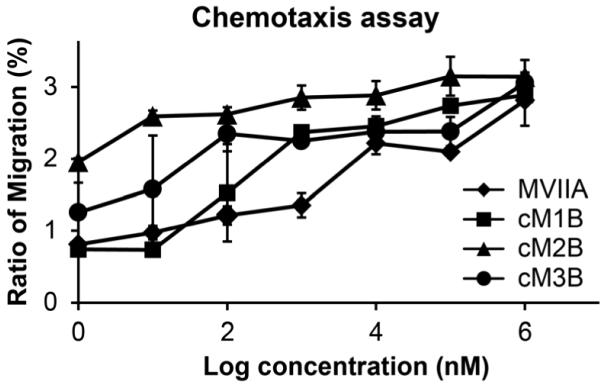


Fig. (2). Chemotactic effect of MVIIA and cyclic analogs on human monocyte cell-line THP-1.

3.7 Heat and Acid Stability

The stability against heat and acid of MVIIA and all synthetic analogs were examined. In the heat stability test (100 °C, 3 h), 60-80% of 2*SS*-cyclic analogs remained intact after heating and about 50% of native MVIIA and linear MVIIA-GS remained intact (Fig. **3A**). In the acid hydrolysis test, > 85% of MVIIA survived a 6 h-treatment with 0.25 M HCl at 37 °C whereas the 2*SS*-cyclic analogs showed improved stability with 85-95% remained intact (Fig. **3B**). The disulfide-free cyclic analog cM6A showed the lowest stability in both assays.

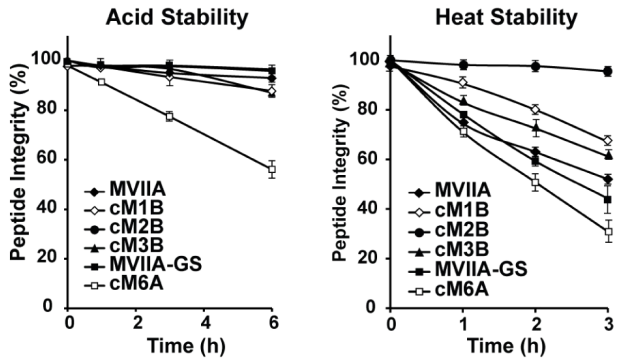


Fig. (3). Acid and heat stability of MVIIA and its linear and cyclic analogs.

4. DISCUSSION

In this work, we used both Boc and Fmoc synthesis to prepare thioesters as linear precursors of cyclic peptides. Thioesters are base labile. Whereas preparing thioester presents no problem for the acid-based Boc-chemistry, thioester becomes a major challenge in the base-repetitive Fmoc procedure. The development of an amide-based TEBA linker as a thioester surrogate allows the thioester preparation using Fmoc chemistry, which is a method of choice for peptide synthesis in most laboratories. For disulfide formation, we also used two approaches to show that the global oxidation is more efficient than the chemoselective disulfide formation. Together we demonstrate a more efficient approach of making cyclic cysteine-rich peptides.

A loss-of-function caused by converting naturally occurring cyclic peptides to acyclic peptides has been reported previously by our laboratory on linearized circular bacteriocin AS-48 [36]. AS-48 is a 70-residue antimicrobial peptide expressed by *Enterococcus faecalis*. It contains five helices and a cluster of positive charges on helix-5 underlies its antibacterial activity by promoting pore formation in cell membranes. González et al. reported that the linear form of AS-48 with an opening in the middle of helix-5 showed reduced antimicrobial activity [37]. Our work showed that opening at helix 1 eliminated antimicrobial activity by changing the overall molecular conformation. Likewise, converting a cyclotide to an acyclic form also results in loss of certain functions [38]. Interestingly, both cyclic and acyclic forms of cyclotides are found in plants and antimicrobial activities of both cyclotide forms have been reported [39, 40]. Conceptually different from these reports, we demonstrate here that converting a linear peptide (such as ω-conotoxin MVIIA) into cyclic form result in a gain-of-function as an antimicrobial. This gain-of-function by joining the N- and C-segment as a contiguous segment through cyclization should be carefully considered in engineering cyclic peptidyl therapeutics.

Although conotoxin MVIIA has a high cationic content (+6 net-charge), it is not an antimicrobial, likely due to the scattering of positive residues and lack of hydrophobic surface. We posited that backbone cyclization would enhance the membrane-associated activity of MVIIA against microbes by grouping six basic residues on one continuous epitope, thereby expanding the cationic density. This hypothesis has been proven by ten cyclized MVIIA derivatives with 0 to two disulfide bonds. They display similar antimicrobial activity as plant defensins and cyclotides against a broad-spectrum of bacteria and fungi. Cyclic conotoxins showed different antimicrobial activity against two Gram-negative bacteria *E. coli* and *P. aeurginosa* that may due to the different lipid composition of bacterial membranes [41]. Our data did not permit us to draw conclusions on the mechanisms of killing against different bacteria strains. In contrast, their linear counterpart MVIIA-GS was inactive (MIC >500 μM), indicating that the Gly-Ser linker peptides exert minimal influence on activity.

A unifying concept, referred to molecular electroporation, can be exemplified by NK-lysin, a linear antimicrobial peptide isolated from the pig small intestine with 78 residues and five α-helices cross-linked by three disulfide bonds [42]. Its reported mechanism is contributed through membrane leakage is induced by positively charged antibacterial peptides creating an electrical potential >0.2 mV on either side of the microbial membrane, leading to electrical shocks and instant pore formation. This electroporation model could explain both the antimicrobial activity of cyclic conotoxins due to the formation of the cationic-rich neo-epitope in the conotoxin sequence. Potent cyclic analogs with MICs ≤10 μM provide a safe therapeutic window, with an HD_{50}/MIC ratio ranging from 100 to >1000. A slight increase of hemolytic effect was shown for the cyclic analogs with substituted Abu residues, suggesting the increase of hydrophobicity could elevate the hemolytic activity.

Our results also suggested the relative unimportance of disulfide linkages in designing a Cys-rich AMP based on conotoxin MVIIA, as all ten cyclic analogs, even including cM6A with a complete removal of all disulfide bonds, display comparable potency in the radial diffusion assay. A similar phenomenon has been reported for human β-defensin HBD-3, as the removal of all disulfide bonds does not abolish the structural elements that confer its antimicrobial activity [43]. In addition to the cyclized backbone, the location of Abu and Cys(Acm) residues in the 1*SS*-cyclic analogs exerts certain influences on the antimicrobial potency of individual cyclic conotoxins. For instance, both cM2B3Acm and cM3B2Acm retaining the first disulfide bond between C1 and C16 have identical sequences, except for their Abu and Cys(Acm) substitution sites, suggesting that these two cyclic analogs share similar backbone structure. However, cM2B3Acm is 2- to 8-fold less potent than cM3B2Acm against all three bacterial strains. Such variations might be contributed by the hydrophobicity differences in Abu and Cys(Acm) side chains.

Increasing the number of structural constraints by cyclization of disulfides would generally lead to an increase in stability. In agreement, our results showed that stability is correlated with the increasing number of intramolecular crosslinks, including both disulfide bonds and cyclic backbone. For the 2*SS*-analogs, removal of one disulfide bond resulted in an extended inter-Cys loop of 7-13 residues. Such modification increased flexibility and reversed effects on molecular stability. After backbone cyclization, additional crosslink reconstructed the stability, and thus the 2*SS*-cyclic analogs displayed comparable or increased stability as MVIIA. The disulfide-free cM6A left single intramolecular crosslinking as a macrocycle showed the weakest molecular stability.

CONCLUSION

Cyclic CRPs are useful scaffolds for engineering bioactive peptides to enhance their chemical and metabolic stability. This report shows that macrocyclization of linear ω-conotoxin MVIIA analogs not only enhances their structural stability but also creates new functions. Linking both terminal ends as a macrocycle produces a new cationic amino-acid-rich fragment, which confers antimicrobial properties not found in the linear ω-conotoxin MVIIA. Converting the disulfide bonds to aminobutyric acid residues further enhances antimicrobial activity. Thus, our work provides new insights into the design and development of linear peptidyl therapeutics through macrocyclization.

LIST OF ABBREVIATIONS

CRP	=	Cysteine-rich peptide
HPLC	=	High-performance liquid chromatography
MS	=	Mass spectrometry
Boc	=	Tert-Butyloxycarbonyl
Fmoc	=	Fluorenylmethyloxycarbonyl
Abu	=	Aminobutyric acid

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest. This research was supported by the National Research Foundation (NRF-CRP8-2011-05) of Singapore.

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SUPPLEMENTARY MATERIAL

Synthesis schemes of conotoxin MVIIA and its linear analog MVIIA-GS. List of compounds. Additional MALDI-TOF mass spectrometry and RP-HPLC profiles of synthetic peptides.

REFERENCES

[1] Hruby V. Conformational restrictions of biologically active peptides via amino acid side chain groups. Life Sci 1982; 31: 189-199.

[2] Clark R, Fischer H, Dempster L, *et al*. Engineering stable peptide toxins by means of backbone cyclization: Stabilization of the α-conotoxin MII. Proc Natl Acad Sci USA 2005; 102: 13767.

[3] Wong C, Rowlands D, Wong C-H, *et al*. Orally Active Peptidic Bradykinin B1 Receptor Antagonists Engineered from a Cyclotide Scaffold for Inflammatory Pain Treatment. Angew Chem Int Ed, 2012; 51: 5620-5624.

[4] Verdine G, Hilinski G. Chapter one - Stapled Peptides for Intracellular Drug Targets. In: Wittrup KD, Gregory LV, ed.^eds., Methods enzymol. Academic Press, 2012; pp. 3-33.

[5] Baeriswyl V, Heinis C. Polycyclic peptide therapeutics. Chem Med Chem, 2013; 8: 377-384.

[6] Rosengren K, Daly N, Plan M, Waine C, Craik D. Twists, knots, and rings in proteins. Structural definition of the cyclotide framework. J Biol Chem 2003; 278: 8606-16.

[7] Duquesne S, Destoumieux-Garzón D, Zirah S, Goulard C, Peduzzi J, Rebuffat S. Two enzymes catalyze the maturation of a lasso peptide in *Escherichia coli*. Chem Bio 2007; 14: 793-803.

[8] Qiu Y, Taichi M, Wei N, Yang H, Luo KQ, Tam JP. An orally active bradykinin B1 receptor antagonist engineered as a bifunctional chimera of sunflower trypsin inhibitor. J Med Chem 2017; 60: 504-510.

[9] Yu Q, Lehrer R, Tam J. Engineered salt-insensitive alpha-defensins with end-to-end circularized structures. J Biol Chem 2000; 275: 3943-9.

[10] Tam J, Wu C, Yang J. Membranolytic selectivity of cystine-stabilized cyclic protegrins. Eur J Biochem 2000; 267: 3289-300.

[11] Tam J, Lu Y, Yang J. Marked increase in membranolytic selectivity of novel cyclic tachyplesins constrained with an antiparallel two-beta strand cystine knot framework. Biochem Biophys Res Commun 2000; 267: 783-90.

[12] Tam J, Lu Y, Yang J. Correlations of cationic charges with salt sensitivity and microbial specificity of cystine-stabilized beta - strand antimicrobial peptides. J Biol Chem 2002; 277: 50450-50456.

[13] Olivera B, Cruz L, Desantos V, *et al*. Neuronal Calcium-Channel Antagonists - Discrimination between Calcium-Channel Subtypes Using Omega-Conotoxin from Conus-Magus Venom. Biochemistry 1987; 26: 2086-2090.

[14] Nielsen K, Adams D, Thomas L, *et al*. Structure-activity relationships of ω-conotoxins MVIIA, MVIIC and 14 loop splice hybrids at N and P/Q-type calcium channels. J Mol Biol 1999; 289: 1405-1421.

[15] Terlau H, Olivera B. Conus Venoms: A rich source of novel ion channel-tageted peptides. Physiol Rev 2003; 84: 41-68.

[16] Saether O, Craik D, Campbell I, Sletten K, Juul J, Norman D. Elucidation of the primary and three-dimensional structure of the uterotonic polypeptide kalata B1. Biochemistry 1995; 34: 4147 - 4158.

[17] Basus V, Nadasdi L, Ramachandran J, Miljanich G. Solution structure of ω-conotoxin MVIIA using 2D NMR spectroscopy. FEBS Lett 1995; 370: 163-169.

[18] Kohno T, Kim J, Kobayashi K, Kodera Y, Maeda T, Sato K. Three-Dimensional Structure in Solution of the Calcium Channel Blocker. ω-Conotoxin MVIIA. Biochemistry 1995; 34: 10256-10265.

[19] Nguyen L, Haney E, Vogel H. The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol 2011; 29: 464-472.

[20] Price-Carter M, Hull M, Goldenberg D. Roles of individual disulfide bonds in the stability and folding of an omega-conotoxin. Biochemistry 1998; 37: 9851-9861.

[21] Lehrer R, Rosenman M, Harwig S, Jackson R, Eisenhauer P. Ultrasensitive assays for endogenous antimicrobial polypeptides. J Immunol Methods 1991; 137: 167-73.

[22] Hemu X, Taichi M, Qiu Y, Liu D, Tam J. Biomimetic synthesis of cyclic peptides using novel thioester surrogates. Pept Sci 2013; 100: 492-501.

[23] Wan Q, Danishefsky S. Free-radical-based, specific desulfurization of cysteine: A powerful advance in the synthesis of polypeptides and glycopolypeptides. Angew Chem Int Ed 2007; 46: 9248-9252.

[24] Tam J, Lu Y, Liu C, Shao J. Peptide synthesis using unprotected peptides through orthogonal coupling methods. Proc Natl Acad Sci USA 1995; 92: 12485-12489.

[25] Tam JP, Lu YA. A biomimetic strategy in the synthesis and fragmentation of cyclic protein. Protein Sci 1998; 7: 1583-1592.

[26] Tam JP, Lu YA, Yu Q. Thia zip reaction for synthesis of large cyclic peptides: Mechanisms and applications. J Am Chem Soc 1999; 121: 4316-4324.

[27] Sun Y, Lu GS, Tam JP. A thioester ligation approach to amphipathic bicyclic peptide library. Org Lett 2001; 3: 1681-1684.

[28] Lu YA, Tam JP. Peptide ligation by a reversible and reusable C-terminal thiol handle. Org Lett 2005; 7: 5003-5006.

[29] Taichi M, Hemu X, Qiu Y, Tam JP. A Thioethylalkylamido (TEA) Thioester Surrogate in the Synthesis of a Cyclic Peptide via a Tandem Acyl Shift. Org Lett 2013; 15: 2620-2623.

[30] Dawson P, Muir T, Clark-Lewis I, Kent S. Synthesis of proteins by native chemical ligation. Science 1994; 266: 776-9.

[31] Tulla-Puche J, Barany G. On-Resin Native Chemical Ligation for Cyclic Peptide Synthesis. J Org Chem 2004; 69: 4101-4107.

[32] Tam J, Lu Y. Synthesis of large cyclic cystine-knot peptide by orthogonal coupling strategy using unprotected peptide precursor. Tetrahedron Lett 1997; 38: 5599-5602.

[33] Kubo S, Chino N, Kimura T, Sakakibara S. Oxidative folding of omega-conotoxin MVIIC: effects of temperature and salt. Biopolymers 1996; 38: 733-744.

[34] Oppenheim J, Biragyn A, Kwak L, Yang D. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. Ann Rheum Dis 2003; 62: ii17-ii21.

[35] Daly N, Rosengren K, Henriques S, Craik D. NMR and protein structure in drug design: application to cyclotides and conotoxins. Eur Biophys J 2011; 40: 359-70.

[36] Hemu X, Qiu Y, Nguyen GK, Tam JP. Total Synthesis of Circular Bacteriocins by Butelase 1. J Am Chem Soc 2016; 138: 6968-71.

[37] González C, Langdon G, Bruix M, Gálvez A, Valdivia E, Maqueda M, Rico M. Bacteriocin AS-48, a microbial cyclic polypeptide structurally and functionally related to mammalian NK-lysin. Proc Natl Acad Sci USA 2000; 97: 11221-11226.

[38] Barry D, Daly N, Clark R, Sando L, Craik D. Linearization of a naturally occurring circular protein maintains structure but eliminates hemolytic activity. Biochemistry 2003; 42: 6688-6695.

[39] Wong C, Taichi M, Nishio H, Nishiuchi Y, Tam J. Optimal oxidative folding of the novel antimicrobial cyclotide from hedyotis biflora requires high alcohol concentrations. Biochemistry 2011; 50: 7275-7283.

[40] Nguyen G, Lian Y, Pang E, Nguyen P, Tran T, Tam J. Discovery of linear cyclotides in monocot plant Panicum laxum of Poaceae family provides new insights into evolution and distribution of cyclotides in plants. J Biol Chem 2013; 288: 3370-3380.

[41] Epand R, Savage P, Epand R. Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins). BBA-Biomembranes 2007; 1768: 2500-2509.

[42] Miteva M, Andersson M, Karshikoff A, Otting G. Molecular electroporation: a unifying concept for the description of membrane pore formation by antibacterial peptides, exemplified with NK-lysin. FEBS Lett 1999; 462: 155-8.

[43] Wu Z, Hoover D, Yang D, *et al*. Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3. Proc Natl Acad Sci USA 2003; 100: 8880-5.