論文の内容の要旨

論文題目 ：Design, Synthesis and Functional Analysis of Ion-Channel Forming Helical Peptide Based on Gramicidin Structure

(グラミシジン A を基盤としたイオンチャネル型ヘリックスペプチドの設計・合成・機能解析)

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Abstract

Introduction

Gramicidin A (GA) is a natural ion-channel forming antimicrobial peptide isolated from *Bacillus brevis* that incorporates into lipid bilayers and facilitates the transmembrane flux of monovalent cations (e.g. Na^+, K^+) upon reversible *N,N*-dimerization in lipid bilayers.[1] From the structural point of view, GA is an intriguing molecule because it consists of alternating D and L amino acids and folds into several conformations depending on its surrounding environments, namely $\beta^{6.3}$-helical dimers in lipid bilayers and antiparallel double helix in organic solvents (Figure 1a). The former $\beta^{6.3}$-helical dimer structure is shown to predominate in lipid bilayers and serves as an ion channel structure, which is considered to be essential to bacterial killings.[2] To this date, only a limited numbers of natural occurring peptides are known to form $\beta^{6.3}$-helix.[3] The author is particularly interested in GA since it could serve as a good structural motif to develop an ion-channel based antibiotic. As an initial step toward this end, the author hypothesized that stabilization of the $\beta^{6.3}$-helical conformation would enhance its ion-channel or antimicrobial activities.

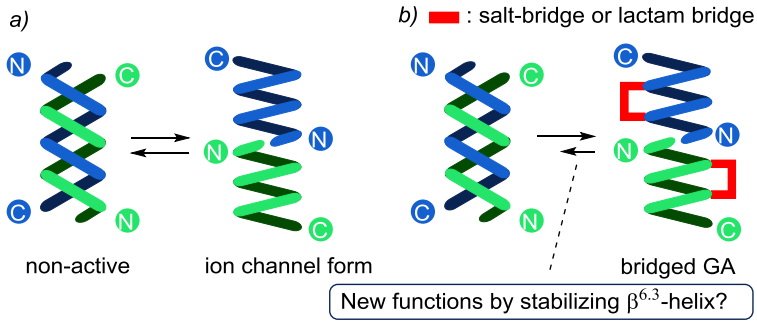


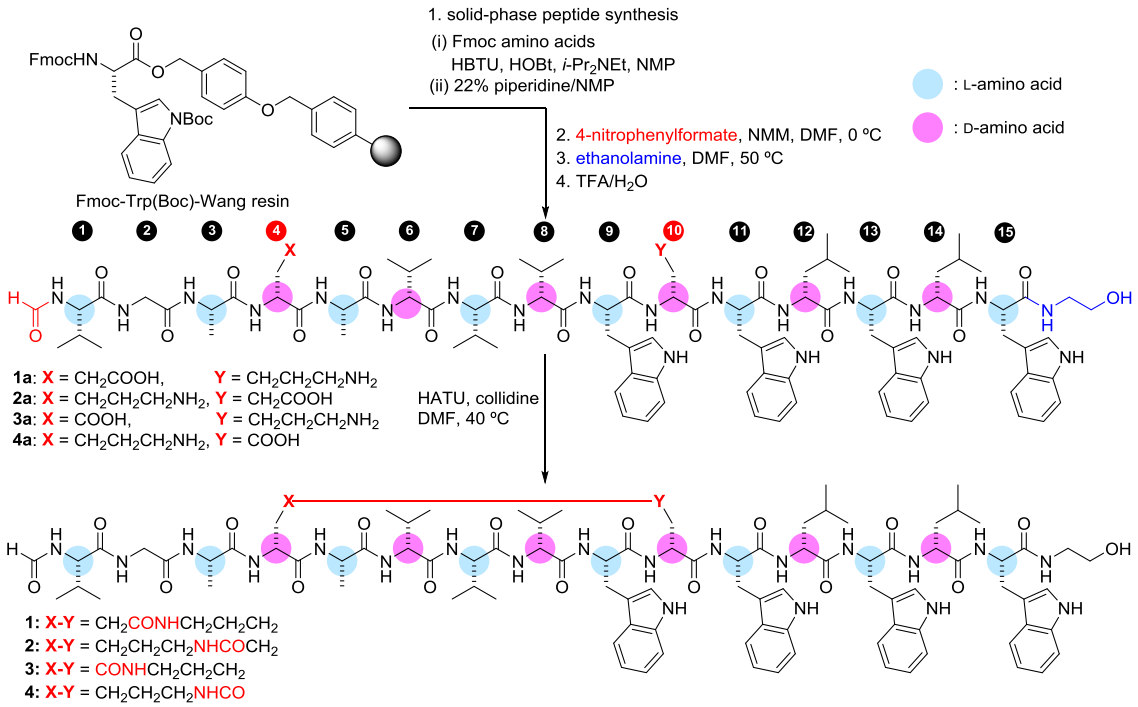
Figure 1. (a) The representative conformations of gramicidin A: double helical forms in organic solvent (left). Helical dimers in lipid bilayers (right). (b) A concept of salt-bridge or lactam-bridge strategy.

Results and Discussion

1. Synthesis of gramicidin analogues

Stabilization of $\beta^{6.3}$-helical structure was envisaged by chemical modification of side chains with salt-bridge or lactam-bridge (Figure 1b). To evaluate the importance of linkage length and substitution pattern of those chemical modifications, four salt-bridge analogues and four lactam-bridged analogues were synthesized (Scheme 1). Briefly, the standard Fmoc solid phase peptide synthesis strategy[4] was adopted to synthesize salt-bridge peptides **1a-4a**. After peptide elongation, the N-terminal formyl groups were introduced with 4-nitrophenylformate and NMM on solid support, and then the peptides were treated with ethanolamine to cleave from the resins with the amidation of their C-termini.[4c] Removal of all protecting groups with TFA, followed by reversed-phase HPLC purification afforded pure salt-bridge peptides **1a-4a** (4.3%, 13%, 1.9% and 3.2% overall yield, respectively). Finally, treatment of **1a-4a** with HATU gave rise to the lactam-bridged analogues **1-4**. After purification by reversed-phase HPLC, the lactam-bridged peptides **1-4** were obtained in 33%, 46%, 41% and 39% yield, respectively.

Scheme 1. Synthesis of gramicidin analogues. HBTU = *O*-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; HOBt = 1-hydroxybenzotriazole; NMM = N-methyl morpholine; HATU = *O*-(7-azabenzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate.



2. Structural Analysis

With synthetic peptides in hands, their conformations were investigated in model lipid bilayers by circular dichroism (CD) experiment. The CD spectra of the GA analogue/liposome mixtures were measured in buffer conditions (25 mM Na phosphate buffer and 75 mM Na_2SO_4, pH 7.5). As clearly indicated from CD spectra in EYPC/POPG (Figure 2 A, B), peptides **1**, **2** and **3** showed two distinctive positive peaks at 220 nm and 238 nm, which are the fingerprints of $\beta^{6.3}$-helical structures in lipid bilayers.[5] Peptide **4** gave distorted CD spectra, suggesting

deformation of $\beta^{6.3}$-helix. In contrast, all salt-bridge analogues showed quite different CD spectra, suggesting they formed structures other than $\beta^{6.3}$-helix. Compared with CD spectra of native GA (Figure 2 A, black line), the overall spectral intensities of **2** and **3** were stronger than those of GA, which was assumed to the stabilization of $\beta^{6.3}$-helical structure by the lactam-bridge. Further NMR analysis of GA and **2** in SDS micelle condition have confirmed that both peptides formed $\beta^{6.3}$-helical dimer structures.

3. Functional Analysis

To investigate the functions of synthetic peptides, the time-course fluorescent experiment was carried out using pH-gradient liposomes as a model membrane. In this experiment cation exchange can be monitored as an increase of fluorescence from the pH-sensitive fluorescent probes encapsulated inside the liposomes. Figure 2C summarizes the result of EYPC/POPG conditions. Of these peptides, all the lactam-bridged peptides showed faster cation exchange than salt-bridge peptides **1a**-**4a** or native GA, as was evident from a steep slope of the plot within 1000 seconds. Considering the results of CD study together, the observed faster cation exchange rate indicated an increase in population of $\beta^{6.3}$-helical structure in the lipid membrane, resulting from the stabilization of $\beta^{6.3}$-helical conformation by the lactam-bridge. To further investigate ion channel function, single channel recording experiment was performed. Under pH 4.8 conditions, the lactam-bridged peptide **2** exhibited distinctively different ion-channel properties in terms of conductance and channel open time compared to GA.

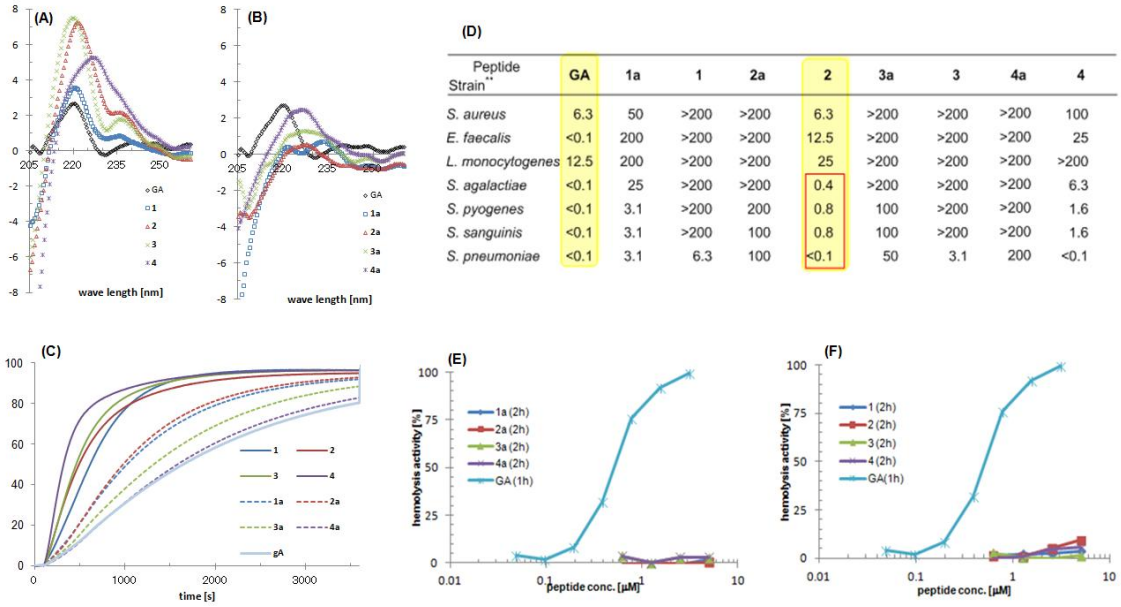
4. Bioactivity assay hemolytic activity, antimicrobial activity

To investigate biological activities of synthetic peptides, antimicrobial assay and hemolytic activity assay were performed. The antimicrobial activity was measured as a minimum inhibitory concentration (MIC) of peptides by a standard procedure. The assay was performed against various strains of gram-positive bacteria (Figure 2D). All of the salt-bridge peptides showed negligible activity against any strains of bacteria. In contrast, the lactam-bridged peptide **2** showed comparable antimicrobial activity as GA against various bacterial strains. In the next, hemolytic activity was measured using rabbit red blood cells. Surprisingly, all the synthetic peptides showed no detectable hemoglobin leakage within the tested range of concentration at 1 h of incubation. Even after 2 h of incubation, the lactam-bridged peptide **2** showed only partial hemolytic activity (<10%). This is a distinct difference from GA, which showed a HC_{50} 0.5 μM and detectable hemolytic activity already at 0.2 μM (Figure 2E, F). Furthermore, peptide **2** showed three orders of decreased cytotoxicity against P388 mouse leukemia white blood cells compared to GA.

Conclusion

The author successfully designed and synthesized the salt-bridge and lactam-bridged GA analogues**.** Their structures and functions were analyzed in model lipid bilayers, and their biological activities such as antimicrobial, hemolytic activity and cytotoxicity were also investigated. The CD, fluorescent and single channel experiment suggested that the lactam-bridged peptide **2**

retain stabilized $\beta^{6.3}$-helical conformation, acted as ion channel with distinctively different ion-channel properties and showed faster cation exchange on model lipid membrane. The lactam-bridged peptide **2** showed comparable antimicrobial activity against various gram-positive strains and exhibited at least several dozens times weaker hemolytic activity compared to native GA. Although details about the selectivity towards bacteria and decreased cytotoxicity need to be clarified, the current study clearly demonstrate utility of lactam-bridge as a modulator of function and provide us with a promising lead compound for ion-channel based antimicrobial therapeutics.



(D)

Peptide / Strain**	GA	1a	1	2a	2	3a	3	4a	4
S. aureus	6.3	50	>200	>200	6.3	>200	>200	>200	100
E. faecalis	<0.1	200	>200	>200	12.5	>200	>200	>200	25
L. monocytogenes	12.5	200	>200	>200	25	>200	>200	>200	>200
S. agalactiae	<0.1	25	>200	>200	0.4	>200	>200	>200	6.3
S. pyogenes	<0.1	3.1	>200	200	0.8	100	>200	>200	1.6
S. sanguinis	<0.1	3.1	>200	100	0.8	100	>200	>200	1.6
S. pneumoniae	<0.1	3.1	6.3	100	<0.1	50	3.1	200	<0.1

Figure 2. CD spectra of synthetic peptides in EYPC/POPG liposomes (A, B). Time course H^+/Na^+ exchange across lipid bilayers of pH-gradient liposomes (C). Antimicrobial activity of synthetic peptides (MIC in μg/mL) (D). Hemolytic activity of synthetic peptides (E, F).

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