Structure–Activity Relationship Study of Helix-Stabilized Antimicrobial Peptides Containing Nonproteinogenic Amino Acids

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ABSTRACT: Helical amphipathic peptides containing cationic and hydrophobic amino acid residues can possess potent antimicrobial activity against both Gram-positive and Gram-negative bacteria. In this study, several amphipathic peptides with enhanced helical structures containing nonproteinogenic amino acids were designed, and the relationships between the antimicrobial activity, hemolytic activity, and cytotoxicity were evaluated. In particular, the effect on the antimicrobial activity and cytotoxicity of the number and position of stapling structures introduced into the sequence was investigated. Peptide **stp1** containing **α,α**-disubstituted amino acids showed potent antimicrobial activity against multidrug-resistant bacteria (MDRP, SP45, and *Staphylococcus aureus*) without causing appreciable hemolytic activity or cytotoxicity. The cytotoxicity was found to be somewhat correlated to the hydrophobicity of the peptides.

KEYWORDS: Antimicrobial peptide, Amphipathic peptide, Helical structure, Nonproteinogenic amino acid, Gram-positive bacteria, Gram-negative bacteria

INTRODUCTION

A century after the discovery of the antibiotic penicillin, the treatment of bacterial infectious diseases is still problematic. The inappropriate use of antibiotics has led to an increase in the number of drug-resistant bacteria, which has become a major issue. In addition, antibiotics are used in large quantities in livestock feed, which contribute to the outbreaks of multidrug-resistant bacteria. It is estimated that 1.27 million people died from drug-resistant bacteria in 2019. The WHO and Drugs for Neglected Diseases founded the Global Antibiotic Research and Development Partnership in 2016 to develop new antimicrobial agents against drug-resistant bacteria. Multidrug-resistant bacteria are an obstacle in the treatment of bacterial infections. AMPs act bacteriostatically by opening pores in the bacterial cell membranes that allow cell components to leak out of the cell, which is different from the bacteriostatic mechanism of action of conventional antibiotics. AMPS have a broad antimicrobial spectrum because they do not target specific proteins, making AMPS less prone to causing drug resistance in bacteria. Representative AMPs, such as cathelicidin and magainin, are characterized by an amphipathic α-helix, in which hydrophobic and cationic amino acid residues are arranged separately in the vertical direction of the helical structure. Such a helical structure is considered essential for antimicrobial activity because the structural properties of helix provide an amphipathic environment. Our group has found that the amphipathic peptide, **stripe** (**KLKKKAG**), in which hydrophobic and cationic residues are aligned vertically along the helix surface, exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria. In particular, helix-stabilized peptides containing helical inducers, such as 2-aminobutyric acid residues or hydrocarbon stapling structures, in an amphipathic peptide have shown potent antimicrobial activity. Despite the importance of the helical structure for antimicrobial activity, **stripe** does not appear to have strong helical properties because it contains Gly residues, which destabilize the helical structure. The introduction of a hydrocarbon stapling structure into peptide sequences has been widely used to stabilize the helical structure of oligopeptides. These methods that introduce nonproteinogenic amino acids into an oligopeptide can not only stabilize the helical structure but also provide resistance to enzyme hydrolysis and enable the hydrophobicity of the peptides to be controlled. In the present study, we designed...
peptides in which the Gly residue in the stripe sequence was substituted with stapling structures and dAAs, and we evaluated the preferred secondary structure, antimicrobial activity, hemolytic activity, and cytotoxicity. The antimicrobial activity of the peptides was altered by the introduction of stapling structures and dAA residues into stripe (Figure 1).

In particular, N-terminal stapling had a tendency to have a negative effect on the antimicrobial activity, and highly hydrophobic peptides had a tendency to have reduced antimicrobial activity and increased hemolytic activity and cytotoxicity.

**MATERIALS AND METHODS**

**General.** Chemicals are purchased from Sigma-Aldrich Co. LLC, Watanabe Chemical Industries, Tokyo Chemical Industry Co. Ltd., Fujifilm Wako Pure Chemicals Co. Inc., Kanto Chemicals Co. Inc., Kishida Chemical Co. Ltd., and the CEM corporation. Purchased chemicals are used without further purification. High-resolution mass spectrometry was carried out using a Shimadzu IT-TOF MS equipped with an electrospray ionization source.

**Peptide Synthesis.** Peptide synthesis was performed on a 50 μmol scale based on Fmoc solid-phase methods. Before the reaction, Protide LL rink-amide resin (0.20 mmol/g) was soaked in DMF-CH₂Cl₂ = 1:1 solution. Amino acid (0.2 M) in DMF (3 equiv), 0.2 M of COMU in DMF (3 equiv), and DIPEA (6 equiv) were used in each coupling. Coupling was performed for 30 min. Amino acids S₅, A₀, C₂, and the following amino acid are allowed to couple in 1 h. Deprotection of the Fmoc group was performed by using 20% piperidine in DMF for 20 min. Olefin metathesis was performed in 1,2-dichloroethane using 20 mol % of Grubbs second-generation catalyst under N₂. In particular, the reaction performed in 1,2-dichloroethane using 20 mol % of Grubbs second-generation catalyst under N₂ was performed in 20 min by bubbling for 4 h without deprotection of the Fmoc group and repeated until the unreacted peptides are no longer detectable in LC-MS. After elongation, peptides were cleaved from the resin using a cleavage cocktail (TFA:TIPS:water = 95:2.5:2.5) in 2 h. TFA was evaporated under a stream of N₂ gas, and cold ether was poured into the residue. Resulting solid crude peptides were dissolved in MeCN/water and purified by reversed-phase high performance liquid chromatography using an XBridge Prep C18 OBD column (19 mm × 250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 10 mL/min). Purified peptide solution was lyophilized. The peptide purity was assessed using an analytical HPLC and Inertsil WP300 C18 column (4.6 mm × 250 mm, 5 μm, solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/ MeCN, flow rate: 1.0 mL/min, gradient: 10% → 90% A over 30 min).

**CD Spectrum Measurement.** CD spectra were recorded on a JASCO J-1100 CD spectrometer using a 1.0 mm path length cell. The data are expressed in terms of [θ], i.e., total molar ellipticity (deg cm² dmol⁻¹). 20 mM phosphate buffer solution (pH = 7.4) containing 1% of sodium dodecyl sulfate (SDS) was used as a solvent, and the peptide concentration was 100 μM.

**Antimicrobial Activity Measurement.** Bacteria strains (P. aeruginosa NRBC 13275, MDR, S. aureus NRBC 13176) were obtained from the Biological Resource Center, NITE (NRBC; Tokyo, Japan). E. coli DH5αr was purchased from BioDynamics Laboratory Inc. (Tokyo, Japan). SP45 was provided by Osaka University. The bacteria were inoculated to a 96-well plate at 90 μL/well (104 CFU/well). Peptides are diluted with a standard 2-fold dilution method from 500 to 0.975 μM (final concentration from 50 to 0.0975 μM) using phosphate-buffered saline. Then, peptides are added to a 96-well plate at 10 μL/well followed by incubation for 18 h at 37 °C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the peptide which inhibits bacteria growth completely by measurement of absorption at a wavelength of 595 nm. The MIC value was taken from the lowest peptide concentration, which inhibited bacterial proliferation. The assay was performed twice and in triplicate each time.

**Hemolysis Activity Measurement.** Human red blood cells (RBCs) were provided kindly from the Japanese Red Cross Society (Tokyo, Japan), which collects from volunteers under informed consent. Peptides are diluted with a standard 2-fold serial dilution method from 200 to 1.95 μM (final concentration from 100 to 0.975 μM) using 172 mM Tris-HCl buffer (pH = 7.6) in a 96-well plate at 100 μL/well. RBCs were centrifuged (1900 rpm, 15 min, 4 °C), and supernatant was removed. The former 2 steps were repeated. The precipitate was 100-fold diluted with 172 mM Tris-HCl buffer (pH = 7.6). Then, 100 μL/well was added to a 96-well plate at 100 μL/well and incubated for 30 min at 37 °C. The 96-well plate was centrifuged, and the collected supernatant absorption was measured at a wavelength of 535 nm. M-Lycotixin and ampicillin were used as positive and negative controls, respectively. The hemolysis activity value was taken from the highest peptide concentration, which caused full hemolysis.

**Cell Viability Assay.** Cell viability was determined using water-soluble tetrazolium WST-8 [4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1-benzene sulfonate] reagent in a spectrophotometric assay according to the manufacturer’s instructions (Dojindo). Cells treated with the compounds were incubated with the WST-8 reagent for 0.5 h at 37 °C in a humidified atmosphere of 5% CO₂. The absorbance of the medium at 450 or 490 nm was measured using an EnVision Multiblank Plate Reader (PerkinElmer).

**Electrophysiological Measurement.** In the electrophysiological measurement experiments, we used the following reagents: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; Avanti Polar Lipids, Inc., USA); 1,2-dioleoyl-sn-glycero-3-phospho-[1-¹⁴C]-glycerol) (DOPG; Avanti Polar Lipids, Inc., USA); n-decane (Wako Pure Chemical Industries, Ltd., Japan); 3-morpholinopropane-1-sulfonic acid (MOPS, Nacalai Tesque, Inc., Japan); and potassium chloride (KCl, Nacalai Tesque, Inc., Japan). DOPE and DOPG were melted in n-decane at a concentration of 10 mg/mL, and we obtained a lipid mixture of DOPE/DOPG (3:1 mol/mol). The composition of the lipid mixture was regulated so that it became 200 mM KCl, 10 mM MOPS, and pH 7.0 in the Milli-Q system (Millipore, Billerica, MA, USA).

Electrophysiological measurement using a microfabricated device was performed as previously reported. We used a micropipet (Eppendorf, Hamburg, Germany) at fabrication for lipid bilayers in each chamber. After addition of 0.9 μL of 10 mg/mL DOPE/DOPG (3:1 mol/mol) in n-decane solution to each chamber, we added 4.7 μL of 100 nM peptide liquid and 200 mM KCl liquid solution, allowing us to easily prepare the planar liquid bilayer. The peptide was added to only one chamber, to which 100 mV voltage was applied to simulate the environment of living cells.

Channel current signals were acquired using a JET patch-clamp amplifier (Tecella, CA, USA). The droplet contact device was fabricated in our laboratory. It has two electrodes: one side was connected to the JET patch-clamp amplifier and applied 100 mV of constant voltage, and the other side was grounded. As AMPs formed pores on the lipid bilayer, ions passed through the pores, resulting in...
the current signals. We measured current signals under the conditions of a 20 kHz sampling rate with a 4 kHz low-pass filter at room temperature (23 ± 1 °C). Data were analyzed using pCLAMP ver. 11.2 (Molecular Devices, CA, USA) and Excel (Microsoft, Washington, USA). The classification of current signals obtained from several minutes to around 1 h of data was performed according to the previous definition using the data numbers (N > 3, n > 150). The scoring of electrophysiologically parameters is based on our previous report, and each parameter of the score for antimicrobial activities is summarized in Table 3.

In Silico Based Peptide Modeling. The in silico based peptide modeling was performed on the MOE 2022.07 software (CCD, Canada). Peptide conformation minimization was carried out by molecular dynamics (MD) as the initial conformation set to the α-helical structure. The force fields are set to AMBER-10.

Table 1. Peptide Sequences Synthesized in This Study

<table>
<thead>
<tr>
<th>stripe</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>stp1</td>
<td>KLLKKAGKLLKKAGKLLKKAG</td>
</tr>
<tr>
<td>stp2</td>
<td>KLLKKAGKLLKKAGKLZKKAZ</td>
</tr>
<tr>
<td>stp3</td>
<td>KL-6KKAZKL-6KKAZKLLKKAG</td>
</tr>
<tr>
<td>stp4</td>
<td>KL-6KKAZKL-6KKAZKLLKKAG</td>
</tr>
<tr>
<td>stp5</td>
<td>KL-6KKAZKL-6KKAZKLLKKAG</td>
</tr>
<tr>
<td>stp6</td>
<td>KL-6KKAZKL-6KKAZKLLKKAG</td>
</tr>
</tbody>
</table>

<sup>a</sup> The N-terminus of each peptide has an amine (H), and the C-terminus of each peptide has an amide (NH<sub>2</sub>) group. Z = 1-amino-cyclohexanecarboxylic acid (Ac<sub>N</sub>C). The cyclization between the i and i + 4 residues of the two (S)-4-pentenylalanine (S<sub>i</sub>) residues is indicated by an asterisk.

**RESULTS AND DISCUSSION**

The synthesized peptides are shown in Table 1 and Figure 2. The stapling structure was introduced by replacing Gly and its i–4 position residue, Leu, with (S)-pentenylalanine (S<sub>i</sub>) in stripe (Figure 1). We did not include a peptide with a triple stapling structure because of concerns that it would be too hydrophobic. Despite our efforts, we encountered challenges in synthesizing the C-terminal stapling peptide, and the reasons for this obstacle remain unclear. We explored various approaches, including utilizing resins with different loading densities (0.41 and 0.19 mmol/g) and different base polymers (PEG and Tentagel) but were unable to achieve the desired outcome. Therefore, 1-aminocyclohexane-1-carboxylic acid (Ac<sub>N</sub>C), which has the same number of carbons as S<sub>i</sub> and is also known to stabilize helical structures, was introduced instead of S<sub>i</sub>. Two Ac<sub>N</sub>C residues have the same elemental composition as that of an S<sub>i</sub>—S<sub>i</sub> bridge. The peptide sequence of stripe, which is a repeating sequence of seven residues, was divided into N-terminal, middle, and C-terminal segments, and peptides were synthesized with original and modified segments (Table 1). stp1, stp2, and stp3 are sequences with mutations in the C-terminal, middle, and N-terminal segments of stripe, respectively. stp4, stp5, and stp6 have double mutations in the C-terminal and middle, N-terminal and middle, and N- and C-terminal segments, respectively. Each designed peptide was synthesized by using the conventional Fmoc solid-phase method. The stapling structure was achieved by ring-closing olefin metathesis using Grubbs second-generation catalyst under N<sub>2</sub>. The synthesized peptides were purified by reversed-phase, high-performance liquid chromatography. Purified peptides were detected by UV absorption and characterized by LC-MS equipped with an ESI ion source.

The secondary structures of the synthesized peptides were analyzed by circular dichroism (CD) spectroscopy. The CD spectra measurements were conducted with a 100 μM concentration of peptide in 20 mM phosphate buffer (pH = 7.4) with 1% sodium dodecyl sulfate (SDS) to imitate cell membrane surface conditions. All the peptides formed an α-helical structure, and no appreciable differences in the structures were observed (Figure 3). These results suggested that even stripe containing Gly residues can form a sufficiently stable α-helical structure in solutions containing SDS. Without SDS, some peptides formed α-helices, but stripe formed a random coil structure (Supporting Information).

The antimicrobial activity was evaluated against four Gram-negative strains, *Escherichia coli* DH5α; *Pseudomonas aeruginosa*; multidrug-resistant *P. aeruginosa* (MDRP); and a clinically isolated multidrug-resistant *P. aeruginosa* (SP45) strain, and one Gram-positive strain: *Staphylococcus aureus*. The peptides were diluted by a standard 2-fold dilution procedure and evaluated from 50 to 0.0975 μM. Peptides stp1 and stp2 and stripe showed antimicrobial activity, while the other peptides were less active (Table 2). Notably, stp1 and stp2 had greatly increased antimicrobial activity against SP45, MDRP, and *S. aureus* compared with stripe. The strong antimicrobial activity against *S. aureus* may be because of the increased hydrophobicity of stp1 and stp2, compared with stripe. The other peptides were not as active as stp1 and stp2. N-Terminal stapling (stp3, stp5, and stp6) tended to weaken the antimicrobial activity. Thus, higher hydrophobicity or the N-terminal stapling of peptides may reduce the antimicrobial activity. However, there was no clear relationship between the strength of the helical structure and the antimicrobial activity of the synthesized peptides. Then, we assessed the hemolytic activity of the peptides against human red blood cells by measuring the absorbance of hemoglobin at 535 nm. Peptide stp1, containing Ac<sub>N</sub>C residues, showed the lowest hemolytic activity, while the stapled peptides showed high hemolytic activity. When the number of stapled structures was increased, the hemolytic activity was significantly increased (Table 2). The cytotoxicity of the peptides was also evaluated in a WST-8 assay against the normal human diploid fibroblast cell line TIG3. The CC<sub>50</sub> value was calculated by constructing a 3- or 4-point calibration curve. The results are shown in Table 2; the cytotoxicity of the peptides was consistent with the trend for hemolytic activity, with stp1 being the least cytotoxic and stp5 the most cytotoxic. Notably, N-terminal stapling tended to increase the hemolytic activity and decrease the antimicrobial activity. The level of cytotoxicity was also relatively high for the stapled peptides but not for unstacked stp1, which appeared to reflect the hydrophobicity of the peptides. Peptides with fatty acids at the N-terminus tend to be toxic; thus, the N-terminal stapling might have a similar effect. Although no obvious relationship between the antimicrobial activity and cytotoxicity...
was observed, as the antimicrobial activity decreased, and the cytotoxicity tended to increase.

The cytotoxicity might be explained from Mourtada’s report, in which the intensity of the hydrophobic residue network in the peptide sequence was strongly related to the hemolytic activity. All of the peptides designed in the present study had amphipathic properties, with all of the hydrophobic residues located on one side of the helices. Therefore, we wondered if it would be possible to calculate the side-chain hydrophobic area from the structures obtained from in silico modeling simulations. First, each peptide was anchored to the α-helical structure, and molecular dynamics simulations were used to optimize the secondary structure. The calculated results showed that the side-chain hydrophobicity (the sum of the hydrophobic residue patch areas) was not correlated with the CC$_{50}$ value ($R^2 = 0.3523$) (Figure 4A) but was highly correlated with the HPLC retention time ($R^2 = 0.9553$) (Figure 4B). Also, the HPLC retention time showed a modest correlation with the CC$_{50}$ value ($R^2 = 0.6591$) (Figure 4C). In Mourtada’s report, cytotoxicity index values were calculated based on the relative HPLC retention time of model peptides containing every amino acid. The HPLC retention times might help estimate trends in the cytotoxicity. The total patch area of hydrophobic residues was strongly correlated with the HPLC retention time but not with the cytotoxicity. Peptides stp2, stp3, stp4, and stp6 exhibited similar CC$_{50}$ values even

Table 2. Antimicrobial Activity, Hemolytic Activity, and Cytotoxicity of the Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>E. coli (μM)</th>
<th>P. aeruginosa (μM)</th>
<th>MDRP (μM)</th>
<th>S. aureus (μM)</th>
<th>Hemolytic activity (μM)</th>
<th>CC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>stripe</td>
<td>1.56</td>
<td>6.25</td>
<td>25</td>
<td>25</td>
<td>12.5</td>
<td>&gt;100$^b$</td>
</tr>
<tr>
<td>stp1</td>
<td>1.56</td>
<td>3.12</td>
<td>6.25</td>
<td>3.12</td>
<td>1.56</td>
<td>50</td>
</tr>
<tr>
<td>stp2</td>
<td>3.12</td>
<td>3.12</td>
<td>6.25</td>
<td>6.25</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>stp3</td>
<td>12.5</td>
<td>25</td>
<td>50</td>
<td>50</td>
<td>12.5</td>
<td>1.56</td>
</tr>
<tr>
<td>stp4</td>
<td>12.5</td>
<td>12.5</td>
<td>25</td>
<td>25</td>
<td>12.5</td>
<td>3.12</td>
</tr>
<tr>
<td>stp5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>&gt;50</td>
<td>0.39</td>
<td>7</td>
</tr>
<tr>
<td>stp6</td>
<td>25</td>
<td>12.5</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

$^a$MDRP: Multidrug-resistant P. aeruginosa. N.D.: Not determined. $^b$Hemolytic activity was determined against human red blood cells. $^c$CC$_{50}$ values were determined by the WST-8 assay against the TIG3 cell line.
though the hydrophobicity was different between the single pair mutations (stp2 and stp3) and the double pair mutations (stp4 and stp6). One common feature of these peptides was the presence of a stapling structure at the N-terminus or in the middle of the sequence. Thus, the cytotoxicity may be greatly influenced, not only by the hydrophobicity but also by the presence of the stapling structure itself. In addition, the hydrophobicity values of Ala residues obtained in the present study were almost zero (see the Supporting Information); Ala may be required to maintain the hydrophobic network. Note that in silico calculations do not reflect the side-chain neighboring effect. Side-chain stapling in AMPs has been reported to enhance the helicity and hemolytic activity of the peptides. 21,22 The antibacterial activity of only some of the stapled AMPs in the present study was improved, and previous studies have also not been able to establish a generality of the

Figure 4. Correlation plot factors. (A) Correlation plot of the sum of the hydrophobic residue patch areas versus the CC_{50} value. (B) Correlation plot of the sum of the hydrophobic residue patch areas versus the HPLC retention time. (C) Correlation plot of the HPLC retention time versus the CC_{50} value. (D) Summary of CC_{50} values, hydrophobic residue patch areas, and HPLC retention times (solvent A: 0.1% TFA/water, solvent B: 0.1% TFA/MeCN, flow rate: 1.0 mL/min, gradient: 10%–90% A over 30 min) of the peptides.

Figure 5. (A) Preparation of the planar lipid bilayer using the droplet contact method. (B) Representative signal patterns and the indicated mode of action. (C) Typical current and time traces of stripe peptides. (D) Signal classification of peptides and their ratio.
effect of side-chain stapling. Introducing peptide residues that destabilize the helix has been reported to reduce the helicity and improve the antimicrobial activity without appreciable hemolytic activity.\textsuperscript{23} Therefore, a structure that is too rigid, such as in \textit{stp5}, may also adversely affect the cytotoxicity. Furthermore, Tian et al. reported that the side-chain stapling of peptides with the more hydrophilic triazole and lactam bridges formed helical structures without increasing the hemolytic effect.\textsuperscript{24} Implementing a hydrophilic stapling approach to stabilize helical structures may be desirable to decrease the cytotoxicity.

To investigate the underlying mechanisms responsible for the antimicrobial and hemolytic activities of the peptides, we conducted electrophysiological analyses using a lipid bilayer system (as depicted in Figure 5A). Previous studies have demonstrated that membrane-binding peptides, including AMPs and cell-penetrating peptides, display ion current signals that can be classified based on their mode of action, such as pore formation or membrane penetration (as shown in Figure 5B).\textsuperscript{11–13} Using this information, we developed a method to deduce the mode of action of \textit{stripe} peptides using DOPE/DOPG membranes that imitate the cell membranes of \textit{E. coli}. It is worth noting that while natural AMPs like magainin typically require micromolar concentrations in this experiment we used significantly lower concentrations for the \textit{stripe} peptides as they caused rapid membrane rupture. Thus, in this experiment, we needed to reduce the concentration to a peptide concentration of 100 nM. The results of the ion current measurements indicated that \textit{stp1–6} exhibited erratic current shapes suggestive of detergent-like random membrane disruption (Figure 5B). Additionally, the multilevel shape of the current suggested the formation of toroidal pores. These findings suggested that \textit{stripe}-based peptides formed pores or defects in the membrane but mainly did not form stable or rigid pore structures, which are characterized by a step current shape, as observed with barrel-stave models (Figure 5B).

Subsequently, we estimate the relationship between the membrane disruption including the pore formation and MIC. The pore formation in the membrane should have a high collation with the MIC if it is the main factor in antimicrobial activity. Two parameters of the pore stability and pore-forming activity were used to estimate the correlation with the MICs of each peptide. The definition of each parameter is given as follows: pore stability = the stable pore-forming signals (multilevel + step)/all signals (multilevel + step + erratic); pore-forming activity = penetrating signals (spike)/all signals (multilevel + step + erratic); and charge flux (CF) = total ion flux as described in Figure 6A.

However, these parameters did not have a high correlation value against the MICs (the correlation value $R^2 = 0.47$). Next, we used the other membrane disruption parameters and total ionic flux (charge flux) and investigated the correlation with the MIC value, based on our previous estimation.\textsuperscript{13}

Using these parameters, the parameters of each peptide were compared and ranked and scored to provide a comparison, called the antimicrobial (AM) score. This score could be used to roughly predict the antimicrobial activity based on the MIC value.

The scores are plotted against the MIC values of \textit{stp1–6}, as shown in Figure 6B. The results showed that the AM score was correlated to the MIC value against DH5$\alpha$ ($R^2 = 0.80$). In previous studies, the pore formation scores have also shown a trend of correlation with the MIC value, and the results of the present study followed a similar trend, suggesting that membrane disruption including the pore formation was the main antimicrobial mechanism.

Regarding the local structure, stapling on the N-terminal side generally led to a decrease in the pore stability score. On the other hand, introducing an additional mutation in the C-terminal Ac$_N$C series peptides tended to increase both the CF and pore formation scores (Table 3). Interestingly, stapling in

![Figure 6](https://pubs.acs.org/doi/10.1021/acsbiomater.3c00759)

**Figure 6.** (A) Definition of the charge flux in this study. (B) The relationship between the score and the MIC value against \textit{E. coli} DH5$\alpha$ for each peptide.

### Table 3. Scores for the Three Different Parameters for Each Peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Pore stability</th>
<th>Pore forming</th>
<th>CF</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>stp1</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>stp2</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>stp3</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>stp4</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>stp5</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>stp6</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>
CONCLUSIONS

The nonproteinogenic amino acid residue Ac can and stapling structure were introduced into the AMP stripe to evaluate the relationship between the secondary structure, antibacterial activity, and cytotoxicity. All of the peptides formed stable α-helical structures, and two peptides, stp1 and stp2, showed enhanced microbial activity against multidrug-resistant bacteria and Gram-positive bacteria (MDRP, SP45, and S. aureus). The hydrophobicity of the peptides was related to the hemolytic activity and to the cytotoxicity, with the most hydrophobic, stp5, being highly cytotoxic and stp1 being less hydrophobic and less cytotoxic. Additionally, the HPLC retention times of the peptides suggested that hydrophobic peptides were cytotoxic and were highly correlated with the side-chain hydrophobicity obtained by in silico calculations. Therefore, the calculated hydrophobicity was indirectly correlated with the cytotoxicity and might be used for a rough estimation of the cytotoxicity. The increased antimicrobial activity might be because of the substitution of Gly with hydrophobic residues. Also, the electrophysical analysis indicated that the introduction of Ac residues at the C-terminus of stripe could effectively increase the antimicrobial activity, while additional stapling structures at the N-terminus or middle of the sequence reduced the activity. We hope to design peptides with increased antimicrobial activity by focusing on hydrophobic residues and predicting the cytotoxicity using in silico calculations in future work.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.3c00759.

Additional data including CD spectra, analytical data of synthesized peptides, and statistic data of the hydrophobic residue patch area of peptides (PDF)

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Author Contributions

Takahito Ito, W.H., N.O., and T.M. performed the experiments and analyzed the results. T.I., R.K., and Y.D. designed the research and wrote the paper. All authors discussed the results and commented on the manuscript.

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ABBREVIATION

MDRP, Multidrug-resistant Pseudomonas aeruginosa; WHO, World Health Organization; AMPS, Antimicrobial peptides; K, Lysine; L or Leu, Leucine; A, Alanine; G or Gly, Glycine; IT-TOF MS, Iontrap-time of flight mass spectroscopy; DMF, N,N-Dimethylformamide; CH3Cl, Dichloromethane; COMU, 1-[1-(Cyano-2-ethoxy-2-oxethylidenamine)oxy] dimethylaminomorpholin)uronium hexafluorophosphate; DIPEA, N,N-Diisopropylethylamine; TIPS, Trisopropylsilane; Fmoc, 9-Fluorenlymethylycarbamate; TFA, Trifluoroacetic acid; HPLC, High-performance liquid chromatography; MeCN, Acetonitrile; UV, Ultraviolet; LC-MS, Liquid chromatography—mass spectroscopy; ESI, Electro spray ionization; CD, Circular dichroism; SDS, Sodium dodecyl sulfate; MIC, Minimum inhibitory concentration; S. aureus, Staphylococcus aureus; E. coli, Escherichia coli; P. aeruginosa, Pseudomonas aeruginosa; CFU, Colony forming unit; RBC, Human red blood cells; Tris-HCl, Tris(hydroxymethyl)aminomethane hydrochloride; CC50, Cytotoxic constant 50; WST-8, 4-[3-(2-Methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazol]−-1,3-benzenedisulfonate; DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine; DOPG, 1,2-Dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol); MOPS, 3-Morpholino-propane-1-sulfonic acid; KCl, Potassium chloride; MD,
Molecular dynamics; CF, Charge flux; AM, Antimicrobial; PEG, Polyethylene glycol

REFERENCES


