De Novo Design and Their Antimicrobial Activity of Stapled Amphipathic Helices of Heptapeptides

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In this study we designed and synthesized several heptapeptides that are enforced to form an amphipathic helix using all-hydrocarbon stapling system and evaluated their antimicrobial and hemolytic activities. The antimicrobial activity showed clear structure-activity relationships, confirming the importance of helicity and amphipathicity. Some stapled heptapeptides displayed a moderate antimicrobial activity along with a low hemolytic activity. To our best knowledge, although not highly potent, these stapled peptides represent the shortest helical amphipathic antimicrobial peptides reported to date. The preliminary data obtained in this work would serve as a good starting point for further developing short analogs of amphipathic helical antimicrobial peptides.

Introduction

Antimicrobial peptides (AMPs) are naturally occurring antibiotics that play a significant role in the innate immune system of various organisms.[1-3] Because AMPs exert potent broad spectrum antimicrobial activities *via* various distinctive modes of action, this specific class of molecules has recently gained spotlight as a potential alternative to combat the growing problems of antibiotic-resistant bacteria.[4]

Membrane-lysis is one of the most common modes of action for many AMPs. The AMPs in this class have multiple cationic residues as well as multiple hydrophobic residues in their sequences and adopt an amphipathic helical conformation, positioning the hydrophobic residues on one face of the helix and the hydrophilic residues on the opposite side.[5-10] Despite their promising potential as new antimicrobial therapeutics, several undesirable properties of the AMPs in this class have been major hurdles for their practical applications as therapeutics.[11] For instance, these AMPs are highly susceptible to proteolytic degradation and therefore suffer from a short *in vivo* half-life and poor bioavailability. In addition, they could interact with various unintended biological targets due to the inherent flexibility, which could lead to many undesired side-effects.[12] Furthermore, in many cases the AMPs in this class consist of more than 25 amino acid residues, making their preparation challenging and costly.

Recently, our laboratory has reported a truncated and conformationally constrained analog of esculentin-2EM as a potent AMP.[13] Esculentin-2EM is a well-studied, 37-amino acid-containing antimicrobial peptide isolated from Korean frog *Glandirama emeljanovi*.[14] Previously, the shortest active analog of esculentin-2EM was its *N*-terminal 23-residue fragment bearing aspartic acid at position 16 replaced with a tryptophan residue.[15] In our aforementioned study, by applying Verdine's all-hydrocarbon stapling system,[16-20] we success-

fully demonstrated that a 15-residue analog of esculentin-2EM can adopt an α-helical conformation in an aqueous environment and thus display highly potent antimicrobial activities along with significantly enhanced proteolytic resistance. This all-hydrocarbon stapled 15-residue peptide represents the shortest yet active analog of esculentin-2EM reported thus far. Encouraged by the powerful helix-stabilizing potential of the peptide stapling system, we became interested in examining the possibility of developing shorter amphipathic helices as antimicrobial peptides. Here we report *de novo* design, synthesis, and biological evaluation of a series of stapled heptapeptides.

Experimental

General. Commercially available solvents and reagents were used as received. All Fmoc-protected α-amino acids (except Fmoc-(S)-α-methyl, α-petenylglycine which was purchased from Okeanos Tech Co. Ltd.), 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylamino-morpholino)]-uranium hexafluorophosphate (COMU), and Rink Amide MBHA resin were purchased from NovaBiochem. Piperidine, *N*-methyl-2-pyrrolidinone (NMP), dimethylformamide (DMF), *N,N*-diisopropylethylamine (DIEA), Grubbs 1st generation catalyst (bis(tricyclohexylphosphine)benzylidine ruthenium (IV) dichloride), 1,2-dichloroethane (DCE), triisopropylsilane (TIS), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich.

Peptide Synthesis. All the peptides were prepared using Fmoc chemistry on Rink Amide MBHA resin with a loading capacity of 0.6 mmol/g. The dry resin (50 mg, 30 μmol) was swelled in NMP for 10 min before using. The Fmoc protecting group was removed by treatment with 25% piperidine in NMP (2 × 10 min). Amino acids were coupled for 30 min using COMU as an activating agent (4.75 equiv.), 5 equiv. of

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Fmoc-protected amino acid, and 10 equiv. of DIEA in NMP. The coupling of Fmoc-(S)-α-methyl,α-petenylglycine was conducted for 2 h with Fmoc-protected amino acid (3 equiv.), COMU (2.85 equiv.), and DIEA (6 equiv.). After each coupling or deprotection reaction, the resin was washed with dichloromethane (DCM) (1 × 2 min), NMP (1 × 2 min), DCM (1 × 2 min), and NMP (1 × 2 min).

Metathesis and Purification. Ring-closing metathesis of resin-bound *N*-Fmoc, side-chain protected peptides was performed using 20 mol % of Grubbs I catalyst in degassed DCE for 2 h at room temperature. The reactions were monitored by liquid chromatography-mass spectrometry (LC/MS) after cleavage of the peptides from a resin aliquot. After draining the reaction solution, the resin was washed with DCE (3 × 2 min) and then with DCM (3 × 2 min). After the final Fmoc-deprotection reaction, the *N*-terminal amino group was treated with 30 equiv. of acetic anhydride and 60 equiv. of DIEA in NMP for 45 min. Resin was washed with DCM (3 × 2 min) and DMF (3 × 2 min) and dried *in vacuo* overnight. The peptides were deprotected and cleaved from the resin by treating them with a mixture of TFA/TIS/water (95/2.5/2.5) for 2 h, and precipitated by adding a 1:1 mixture of *n*-pentane and diethyl ether. The precipitate was collected by centrifugation, dissolved in a 1:1 mixture of acetonitrile and water, and filtered to remove resin. The products were purified through reverse phase high-performance liquid chromatography using a Zorbax C18 column (Agilent, 5 μm, 9.4 × 250 mm), and then LC/MS (Agilent, API4000).

Peptide S1. ESIMS *m/z* for $C_{48}H_{78}N_{12}O_8$ [M+2H]$^{2+}$/2 calcd 476.31, found 476.45.

Peptide S2. ESIMS *m/z* for $C_{48}H_{78}N_{12}O_8$ [M+2H]$^{2+}$/2 calcd 476.31, found 476.45.

Peptide S3. ESIMS *m/z* for $C_{51}H_{81}N_{12}O_8$ [M+2H]$^{2+}$/2 calcd 497.33, found 497.45.

Peptide S4. ESIMS *m/z* for $C_{51}H_{81}N_{12}O_8$ [M+2H]$^{2+}$/2 calcd 497.33, found 497.45.

Peptide S5. ESIMS *m/z* for $C_{45}H_{68}N_{11}O_8$ [M+2H]$^{2+}$/2 calcd 447.78, found 447.80.

Peptide S6. ESIMS *m/z* for $C_{45}H_{68}N_{11}O_8$ [M+2H]$^{2+}$/2 calcd 447.78, found 447.80.

Peptide U1. ESIMS *m/z* for $C_{50}H_{82}N_{12}O_8$ [M+2H]$^{2+}$/2 calcd 490.33, found 490.45.

Peptide U2. ESIMS *m/z* for $C_{50}H_{82}N_{12}O_8$ [M+2H]$^{2+}$/2 calcd 490.33, found 490.45.

Peptide U3. ESIMS *m/z* for $C_{53}H_{85}N_{12}O_8$ [M+2H]$^{2+}$/2 calcd 511.35, found 511.50.

Peptide U4. ESIMS *m/z* for $C_{53}H_{85}N_{12}O_8$ [M+2H]$^{2+}$/2 calcd 511.35, found 511.50.

Peptide U5. ESIMS *m/z* for $C_{47}H_{72}N_{11}O_8$ [M+2H]$^{2+}$/2 calcd 461.80, found 461.85.

Peptide U6. ESIMS *m/z* for $C_{47}H_{72}N_{11}O_8$ [M+2H]$^{2+}$/2 calcd 461.80, found 461.85.

Circular dichroism. The peptides were dissolved in a 25 mM potassium phosphate buffer solution (pH 6.5). The concentrations were determined by absorbance spectroscopy at 280 nm (extinction coefficient for tryptophan, λ_{280} = 5690 cm^{-1}). Circular dichroism spectra were collected on a Chirascan

HP dual polarization circular dichroism spectrometer with a temperature controller using the following standard measurement parameters: 1 nm step resolution, 3 accumulations, 0.5 sec response, 1 nm bandwidth, and 0.1 cm path length. All spectra were converted to a uniform scale of molar ellipticity after background subtraction. The curves were smoothed using standard parameters.

Antibacterial Assay. Antimicrobial activity was evaluated by the standard broth microdilution method[1] with a slight modification measuring the minimal inhibitory concentration (MIC) values. The antimicrobial assay was conducted against three strains of Gram-positive (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538p, *Staphylococcus epidermis* ATCC 12228) and six strains of Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Shigella dysentariae* ATCC 9752, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumonia* ATCC 10031, *Proteus mirabilis* ATCC 25933, *Pseudomonas aeruginosa* ATCC 27853). These strains of bacteria were incubated in 2 mL Luria-Bertani (LB) broth overnight at 37 °C.

The peptides dissolved in phosphate-buffered saline (PBS) were prepared with 2-fold dilutions from 0.2 μM to 100 μM in 96-well round-bottom microtiter plates. The peptide solution and the bacterial inoculums were diluted using LB broth. Bacterial suspension (10^6-10^8 colony-forming units (CFU)/mL) was then dispensed into the peptide-containing wells. After 24 h of incubation at 37 °C, the wells were examined to determine MICs, the lowest peptide concentration which completely inhibits cell growth. The antimicrobial assay was repeated twice. All bacterial strains were obtained from the Korean Collection for Type Culture (KCTC) at the Korean Research Institute of Bioscience and Biotechnology (KRIBB, Korea).

Hemolysis Assay. Peptides were dissolved in PBS. 10 μL of serially diluted peptides (0.8-200 μg/mL final concentration) was added to 190 μL of suspensions of human red blood cells (10% v/v in PBS) and incubated for 30 min at 37 °C. After centrifugation, the supernatants were diluted with 10 fold PBS and the absorbance at 405 nm was measured for each solution. The blood suspension treated with 0.2% Triton X-100 was used as a control for 100% hemolysis. The percentage of hemolysis was determined using following equation:

$$\% \text{ Hemolysis} = \frac{\text{OD}_{405\,nm} \text{ sample}}{\text{OD}_{405\,nm} \text{ positive control}} \times 100$$

The tests were performed with duplicate samples, and the average values of the two independent measurements were recorded.

Results and Discussion

The main goal of this study was to develop short amphipathic helical peptides consisting of only minimal amino acid residues as new antimicrobial peptides. Although five amino acids are enough to form a single turn of helix, it might be too short to effectively secure amphipathicity required for

(a)

(b)

a stapled heptapeptide
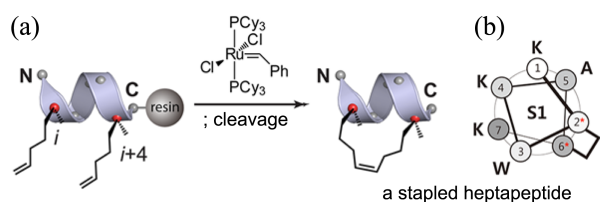
Figure 1. (a) Schematic presentation of the all hydrocarbon stapling chemistry, cross-linking two pentenyl side-chains at positions 2 and 6 for the preparation of stapled heptapeptides (b) A helical wheel projection of a representative stapled heptapeptide **S1**. The peptide was designed to be an amphipathic helix upon stapling. Lines connecting positions 2 and 6 represent the oct-4-enyl hydrocarbon cross-link.

the antimicrobial activity. Therefore we chose to focus on heptapeptides, in which an additional residue at each end of one helical turn would provide supplementary options to manipulate their physical properties. As heptapeptides are still too short to form an α-helix *per se*,[16] we took advantage of the powerful helix-stabilizing effects of Verdine's all-hydrocarbon stapling system. All-hydrocarbon peptide stapling is a powerful cross-linking system that effectively stabilizes the α-helical conformation of a peptide by incorporating an all-hydrocarbon tether formed *via* ruthenium-mediated ring-closing olefin metathesis.[17-21] For the hydrophobic face, we placed the oct-4-enyl staple between position 2 and 6 (Figure 1). For the hydrophillic face, a lysine was incorporated at positions 1, 4, and 7 each, which reside on the opposite side of the staple. The remaining positions 3 and 5, located in the interface between the hydrophobic and hydrophilic faces, were equipped with tryptophan and alanine, respectively (peptide **S1**). Positioning tryptophan in the interface between hydrophobic and hydrophilic faces is known to facilitate the interactions between amphipathic helices and the bacterial membrane surface.[15] Alanine was chosen because of its helix-promoting property.[16]

In addition to peptide **S1**, we also prepared a series of its derivatives (Figure 2). Peptide **S2**, which incorporates the same set of the amino acids, but with positions of tryptophan and alanine switched, was designed to investigate the posi-

(a)

(b)
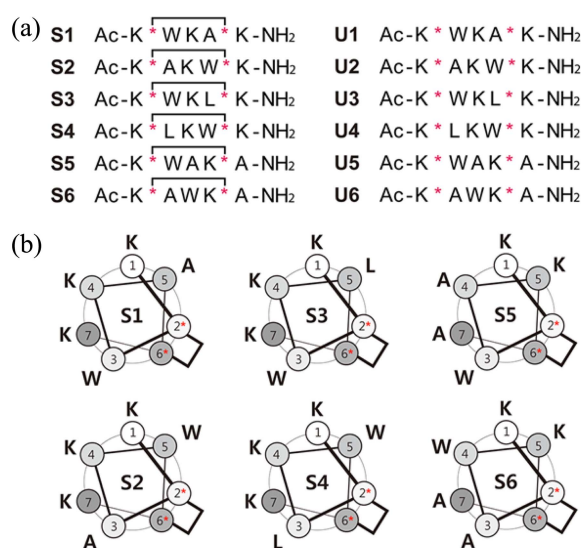
Figure 2. (a) Sequences of stapled heptapeptides **S1-S6** and their unstapled analogs **U1-U6**. Asterisks represent pentenylalanine residue. Lines represent the oct-4-enyl staple cross-linking residues at positions 2 and 6. (b) Helical wheel projections of the stapled heptapeptides. Each peptide was designed to be an amphipathic helix upon stapling. Lines connecting positions 2 and 6 represent the oct-4-enyl hydrocarbon staple.

tional effects of these two residues on antimicrobial activities. By substituting alanine with leucine, we constructed peptides **S3** and **S4** to explore the effects of an extended hydrophobic area on the activities. With peptide **S5**, which bears only two lysine residues in proximity to the hydrophobic staple, we examined the consequences of the reduced cationic patch and its different location. Peptide **S6**, the analog of **S5**, has the tryptophan residue adjacent to the hydrophilic face, rather than the hydrophobic face. Finally, peptides **U1-U6** are 'unstapled' analogs of peptides **S1-S6**, respectively.

As expected, in the far ultraviolet circular dichroism (CD) experiment, the stapled heptapeptides displayed enhanced helical contents compared to their corresponding unstapled counterparts (Figure 3). The stapled analogs clearly showed increased intensities of two minima at 208 and 222 nm and a

Table 1. Antimicrobial activities of stapled heptapeptides against selected bacteria

Bacteria line	Minimum Inhibitory Concentration (μM)[a]												
	U1	**S1**	**U2**	**S2**	**U3**	**S3**	**U4**	**S4**	**U5**	**S5**	**U6**	**S6**	**C**[b]
Gram (+)													
Bacillus subtilis	>100	25	>100	50	25	12.5	25	12.5	>100	50	>100	>100	3.1
Staphylococcus aureus	>100	25	>100	100	25	25	25	12.5	>100	50	>100	>100	6.3
Staphylococcus epidermis	>100	>100	>100	>100	100	100	100	100	>100	>100	>100	>100	6.3
Gram (−)													
Escherichia coli	100	25	>100	50	25	12.5	25	25	>100	50	>100	100	12.5
Shigella dysentariae	>100	100	>100	>100	100	25	>100	25	>100	>100	>100	>100	25
Salmonella typhimurium	>100	>100	>100	>100	>100	50	>100	50	>100	>100	>100	>100	50
Klebsiella pneumonia	>100	50	100	100	50	25	50	25	>100	100	>100	>100	12.5
Pseudomonas aeuginose	>100	50	>100	>100	100	25	100	25	>100	>100	>100	>100	12.5

[a]Minimum inhibitory concentration was defined as the lowest peptide concentration (μM) that completely inhibits the cell growth after 24 h of incubation at 37 °C. The experiment was performed in duplicate. [b]Control peptide C is a 11-mer peptide reported in a previous study.[23]

miximum at 190 nm. In the antimicrobial assay, a close correlation was observed between the helical conformation and the activity: most stapled analogs displayed improved antimicrobial activity compared to their respective unstapled counterparts (Table 1). This result suggests that this series of peptides exert their antimicrobial activity in a similar manner to other amphipathic helical AMPs. Interestingly, the Gram+ microbes were more sensitive to this series of peptides than the Gram- species, except *Staphylococcus epidermis* which was not affected by any of the peptides in this experiment.

Peptide **S2** showed significantly reduced activity compared to peptide **S1**, in which the positions of tryptophan and alanine are switched, suggesting the importance of tryptophan's position for antimicrobial activity. Peptide **S3**, an analog of peptide **S1** containing leucine in place of alanine, exhibited a 2-fold increase in antimicrobial activity. Considering the fact that most naturally-occurring AMPs are composed of about 50% hydrophobic amino acids in their sequences,[5-10] this result indicates the oct-4-enyl staple is not large enough to provide sufficient hydrophobicity for the activity. Interestingly, unlike peptide **S2**, peptide **S4** was just as potent as peptide **S3** despite the positional change of tryptophan and leucine residues. This result suggests that the positional effects of tryptophan on the antimicrobial activities are sequence-dependent. Peptides **S5** and **S6**, which only incorporate two lysine residues in their sequences, were much less active than the other stapled analogs. In particular, peptide **S6**, which is the most helical analog in this series, was almost inactive within the tested concentration range, suggesting that the helical conformation is not the only factor for the antimicrobial activity, but the number and position of the hydrophilic and hydrophobic amino acids in the sequence are also very important.

In the hemolytic assay, most of the peptides tested in this study were not highly active against human red blood cells in the given concentration range (Table 2). However, peptide **S3** and **S4**, which displayed the most potent antimicrobial activity, were slightly more hemolytic than others. This can be explained by two aspects. Peptides **U3** and **U4**, their unstapled analogs, and peptide **S1** and **S2**, their alanine-containing analogs in place of leucine, were not hemolytic. Therefore, like their antimicrobial activity, the increased hemolytic activities of peptides **S3** and **S4** must have resulted from the
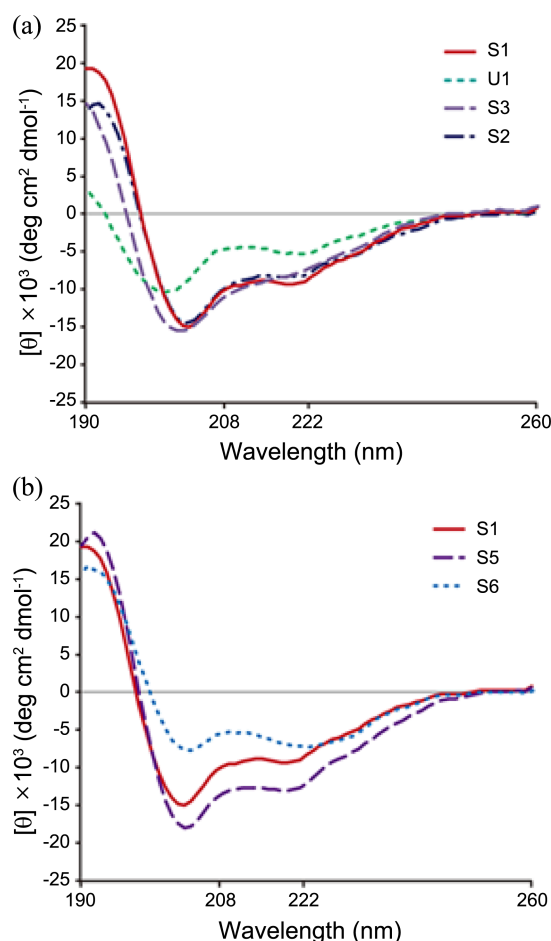
Figure 3. Circular dichroism spectra measured at 20 °C of some selected heptapeptide analogs tested in this study. (a) Comparison of circular dichroism spectra of stapled peptides **S1**, **S2**, and **S3** with peptide **U1** as a representative unstapled counterpart. (b) Comparison of circular dichroism spectra of the inactive stapled analogs **S5** and **S6** with peptide **S1**.

combinational effects of the enforced helix formation and the increased hydrophobicity. This is in agreement with the literature that has demonstrated the enhancement of hemolytic activity upon stapling.[22] However, it should be noted that peptide **S4** is ~2-fold less hemolytic than **S3**. Since the only difference between these two peptides is the relative positions of two amino acid residues, these results indicate that there

Table 2. Hemolytic activities (%) of stapled heptapeptides against human red blood cells[a]

Concentration (μM)	U1	S1	U2	S2	U3	S3	U4	S4	U5	S5	U6	S6	C[b]
100.0	0.99	1.21	0.88	1.24	1.12	7.75	1.22	4.50	1.10	1.04	1.23	0.84	75.75
50.0	0.75	1.17	0.96	1.75	1.03	3.81	1.19	2.46	1.03	0.88	0.91	0.61	67.16
25.0	0.90	1.36	0.93	0.82	0.98	2.52	1.19	1.87	0.87	0.76	1.01	0.70	58.21
12.5	0.88	0.83	0.97	0.63	0.75	2.13	0.97	1.08	0.94	0.83	0.72	0.64	34.89
6.3	0.91	0.66	1.05	0.99	1.25	0.94	0.81	0.68	0.82	0.76	0.70	0.87	7.84
3.1	0.70	0.67	0.84	0.66	0.90	1.02	1.28	0.71	0.75	0.59	0.73	0.75	1.68
1.6	0.76	0.53	0.86	1.01	0.72	0.65	1.98	0.90	0.78	0.60	0.67	0.66	0.59
0.8	0.91	0.53	0.76	0.91	0.88	0.87	0.80	0.71	0.86	0.65	0.60	0.65	0.51

[a]Percent hemolysis is relative to that by 0.1% Triton X-100. The experiment was performed in duplicate. [b]Control peptide C is a 11-mer peptide reported in a previous study.[23]

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is room for an effective separation of antimicrobial and hemolytic activities. The related study is actively underway to improve pharmacological properties *via* sequence modifications of these heptapeptides.

Conclusion

In this study, we evaluated a series of all-hydrocarbon stapled heptapeptides along with their unstapled analogs as potential antimicrobial agents. The stapled heptapeptides that were designed to form amphipathic helices displayed reasonable antimicrobial activity along with low hemolytic activity compared to their unstapled or less amphipathic counterparts. Overall, the antimicrobial activity showed clear structure-activity relationships, confirming the importance of the helicity and amphipathicity. To our best knowledge, these stapled peptides represent shortest, although not highly potent, helical amphipathic antimicrobial peptides reported to date and will likely serve as a good starting point for further development of short antimicrobial peptides to combat the rising problem of antibiotic resistance.

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