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# Antimicrobial activity of doubly-stapled alanine/lysine-based peptides

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ABSTRACT

In this study, we examined the potential of Verdine's double-stapling system for the de novo design of amphipathic helical antimicrobial peptides. We designed, synthesized, and tested a prototypical doubly-stapled helix of an alanine/lysine based model sequence, which showed reasonable antimicrobial activities and highly increased proteolytic stability. We then show that its hemolytic activity as well as antimicrobial activities can be further manipulated through the systematic modifications. Overall, the preliminary results obtained from this study imply that the doubly-stapled helices of short peptides can serve as a highly promising scaffold for the rational design of potent, selective, and metabolically stable antimicrobial peptides that can combat against the growing problems of antibiotic-resistance.



Antimicrobial resistance to conventional antibiotics has been a serious major threat to public health. Thus, there is an urgent need for novel designer antibiotics that can combat antibiotic resistance. In this regard, antimicrobial peptides (AMPs) are considered promising alternatives as they kill bacteria via various distinctive modes of action.[1–3] Despite their great potential, the clinical use of natural AMPs has been hampered by several undesirable properties.[4] In recent decades, a great deal of research effort has been expended to improve pharmacological properties of AMPs, mainly focusing on the improvement of their antimicrobial potency and prokaryotic selectivity.[5–8] Poor bioavailability is one of the major drawbacks of AMPs due to their high proteolytic susceptibility. However, strategies to enhance proteolytic stability of AMPs is relatively understudied.

The majority of AMPs kill bacteria via leakage of the cytoplasmic contents by disrupting bacterial membrane.[9–11] Amphipathic helical conformation is one of the most common architectural features of AMPs in this class; when interacting with bacterial membranes, they adopt an α-helix, positioning multiple hydrophobic residues on one face and multiple hydrophilic residues on the other side. It is well established that the hydrophobic face plays a key role in interacting with the hydrophobic interior of the cell membrane, whereas the hydrophilic face is critical for the specific interactions with the membrane surfaces of certain microbes.[12–14]

Recently, our laboratory reported a synthetic analog of an natural AMP, esculentin-2EM.[15] Its structural and metabolic stability was significantly improved by exploiting the powerful helix-stabilizing capability of Verdine's all-hydrocarbon cross-linking strategy, termed peptide stapling system.[16–19] Incorporation of a hydrocarbon 'staple' into a significantly shortened sequence of esculentin-2EM resulted in a more active antimicrobial analog, demonstrating the highly promising potential of the peptide stapling system for designing new AMPs out of numerous natural AMPs. However, due to the tremendous diversity of natural AMPs with regard to their sequences and mechanisms of action, it would be difficult to establish reliable structure-activity relationships. Therefore, we have been interested in rational design of artificial AMPs using the peptide stapling system as a key tool.[20] As part of our continuous efforts, in this study, we applied Verdine's double-stapling system to an alanine/lysine-based 15-residue model sequence and examined its potential as a promising platform for rational design of synthetic AMPs.

Previously Kim and colleagues reported a highly stereoselective ring-closing metathesis (RCM) reaction among four electronically-identical olefinic side-chains on a peptide substrate, by which two oct-4-enyl cross-links can be simultaneously incorporated on one face of a peptide helix (Fig. 1).[21] They claimed that the double staples would further rigidify a longer stretch of α-helix and thereby further increase proteolytic stability. This has been confirmed by Chapuis and colleagues, who utilized this highly effective tandem stapling system to generate a doubly-stapled analog of lasioglossin,

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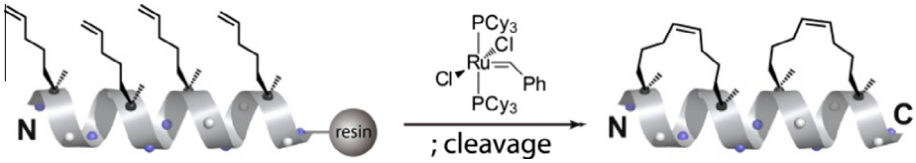


**Figure 1.** Schematic presentation of the all-hydrocarbon double-stapling chemistry incorporating two oct-4-enyl crosslinks in tandem. Previous studies indicated that a ring-closing metathesis reaction between two inner olefinic side-chains does not occur.[21] Previous studies indicated that the *i*,*i*+4 stapling exclusively yields cis olefin.[22–24]

a natural AMP.[25] However, this doubly-stapled lasioglossin failed to show any beneficial effects on antimicrobial and hemolytic activities despite its significantly increased helicity and proteolytic stability.

In spite of discouraging results from Chapuis' study, we envisioned that the doubly-stapled helix can provide an effective scaffold for the design of artificial AMPs. Firstly, as the proteolytic instability is one of the most serious drawbacks of most peptide drugs including natural AMPs, robustness against proteolytic degradation must be recognized as a great benefit of doubly-stapled peptides. Secondly, two hydrocarbon staples incorporated on one side of helix could *per se* form the hydrophobic face of an amphipathic helix (Fig. 2B). Therefore, thirdly, the amphipathic feature, which is commonly required for antimicrobial activity of many natural AMPs, can readily be constructed by placing multiple cationic residues on the opposite side of helix (Fig. 2B).

To examine the double stapling system as a general platform for metabolically stable antimicrobial peptides, we designed a doubly-stapled peptide **Ac-DS-14W** based on an alanine/lysine-based model sequence (Fig. 2A, entry 3). This peptide was designed to have two oct-4-enyl staples, which cross-link residues at positions 2 and 6 and positions 9 and 13, respectively. These positions were chosen to place the hydrocarbon staples on one face of helix, allowing them to serve as the hydrophobic face of the amphipathic helix as well as helix-stabilizing elements (Fig. 2B). At positions 1, 4, 7, 8, 11, and 15, six lysine residues are incorporated to form the cationic face of the amphipathic helix upon stapled. At positions 3, 5, 10, and 12, located in the interface between the hydrophobic and hydrophilic faces of the helix, helix-promoting alanine residues were introduced.[26] At position 14 was placed a tryptophan residue as incorporating tryptophan in the interface between hydrophobic and hydrophilic faces is known to facilitate interactions with bacterial membrane.[27] Finally, to maximize the helix-stabilizing effects by providing an additional hydrogen-bonding partner at each end, the N-terminus of the peptide was acetylated and its C-terminus amidated.[28] For comparison, we also designed peptides **Ac-SS-14W** and **Ac-UM-14W**, a singly-stapled and an unmodified analog of **Ac-DS-14W**, respectively (Fig. 2A, entries 1 and 2).

The peptides were prepared using the published synthetic protocols.[17] Briefly, linear peptide substrates were synthesized via typical Fmoc/*t*-Bu-based solid-phase peptide synthesis. RCM of each olefin-containing substrate under the typical reaction conditions cleanly yielded a respective corresponding stapled product as analyzed by LC/MS. Especially, as Kim and colleagues demonstrated in their previous study,[21] the substrate for the synthesis of **Ac-DS-14W**, bearing four olefinic side-chains at positions 2, 6, 9, and 13, underwent a smooth RCM reaction affording a doubly-stapled product as an exclusive product (100% conversion after two 2 h-RCM reactions). After Fmoc-deprotection, the N-terminus of each peptide was acetylated. The global deprotection and cleavage reactions gave rise to the final products, which were further purified by semi-preparative high-performance liquid chromatography (HPLC).

We first examined conformational preferences of this peptide series using far ultra-violet circular dichroism (CD) spectroscopy (Fig. 3). Helix formation of unmodified analog **Ac-UM-14W** would be unfavorable due to the potential electrostatic repulsion among cationic side-chains of lysine residues in its helical conformation. On the other hand, singly-stapled analog **Ac-SS-14W** exhibited a typical CD spectrum for α-helix, characterized by two minima near 208 and 222 nm and a maximum near 190 nm. This result again confirms the highly effective helix-stabilizing power of the hydrocarbon staple. Doubly-stapled **Ac-DS-14W** showed the most enhanced helical contents among this series of peptides. As revealed by the ratio of the CD intensities at 208 and 222 nm, the helical conformation of the doubly-stapled peptide appeared to be the most rigid.

Next, we evaluated antimicrobial activities of this peptide series against selected bacteria. Under the assay conditions employed in this study, **Ac-UM-14W** was completely inactive against both classes of bacteria (Table 1, entry 1). **Ac-SS-14W**, however, showed significantly enhanced antimicrobial activities against all the


**Figure 2.** Sequences (A) and a wheel diagram (B) of peptide analogs. X and black circles represent the residues cross-linked by an oct-4-enyl staple (line). Ac and $NH_2$ at each end represent the N-terminal acetyl group and the C-terminal primary amide, respectively. Blue circles represent lysine residues forming a cationic, hydrophilic face of helix.

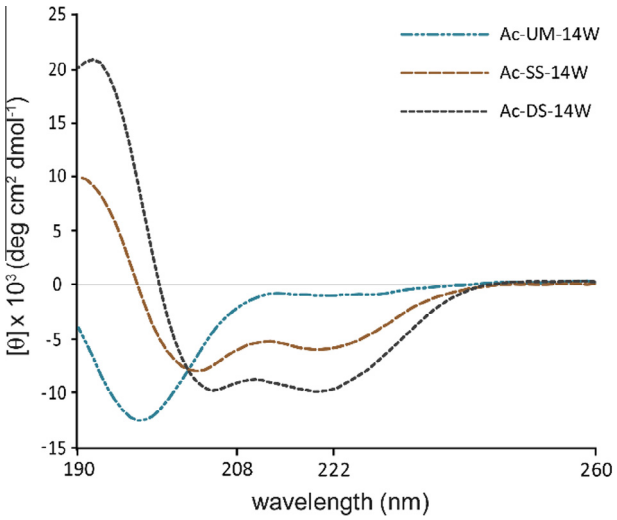


**Figure 3.** Circular dichroism spectra of **Ac-UM-14W**, **Ac-SS-14W**, and **Ac-DS-14W** (30–80 μM) in a 25 mM potassium phosphate buffer solution at 20 °C. These peptides carry a net charge of +6.