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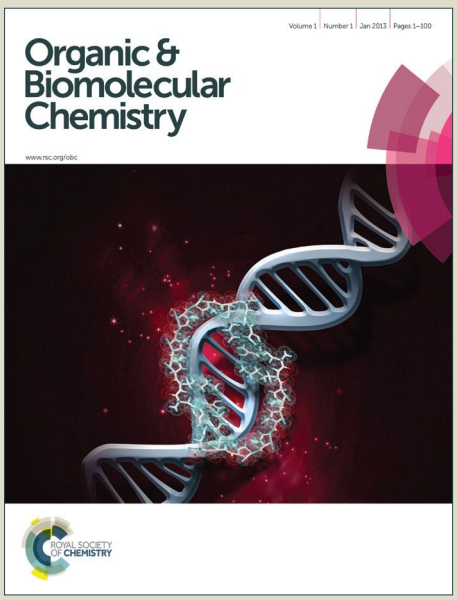

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Diaminodiacid-Based Solid-Phase Synthesis of all-Hydrocarbon Stapled α-helical Peptides

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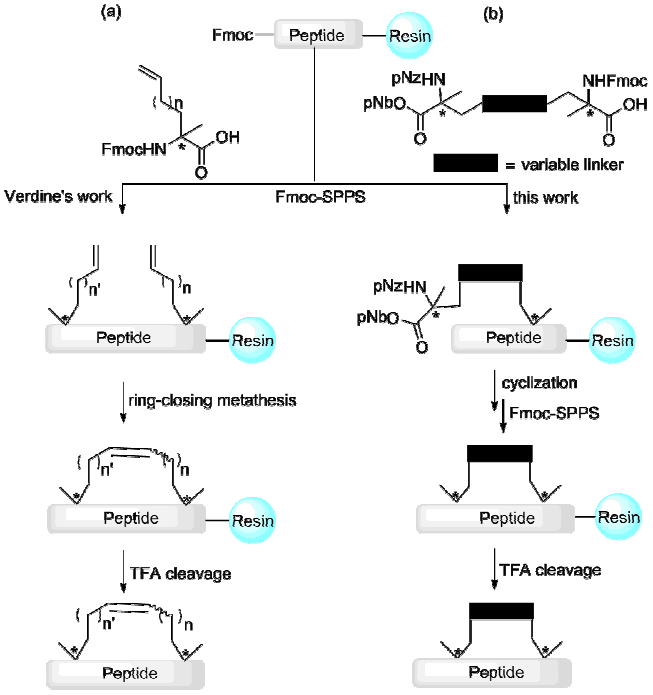
An alternative stapling strategy was described herein by using Fmoc solid-phase peptide synthesis that employed pre-prepared diaminodiacid building blocks to introduce all-hydrocarbon staples into peptides by on resin cyclization. Compared to unstapled native peptides, diaminodiacid-based stapled peptides exhibited increased α-helicity ratio and stability toward protease. Moreover, the length of linkage was found to have impact on the bioactivity of the peptides on Wnt pathway inhibition. Therefore, the new stapling method provided an alternative way to obtain stapled peptides with tunable linkers of diaminodiacids.

Protein-protein interactions (PPIs) mediate series of significant biological processes and it was regarded as next-generation of therapeutic targets.[1] In general, proteins interact with each other through secondary structure. Among them, α-helices are the most common protein secondary structures which are mostly found at the interface of a multitude of crucial PPIs.[2] It has been of great interest to explore this particular folding motif as modulator of PPIs.[3] However, small peptide segments from proteins often show poor proteolytic stability, cell permeability and decreased biological activity because they own less helical property. Thus, stabilization of the helical conformation of peptides is indispensable for regulating of PPIs.[4]

Many strategies have been developed to stabilize peptide helices,[1b,5] in which the mostly used is side-chain cross-linking, including disulfide bond formation,[6] thioether ligation,[7] lactam bridge,[8] click chemisty,[9] etc.[10] Verdine group reported an all-hydrocarbon cross-linking system by ring-closing olefin metathesis (Scheme 1a).[11] In their work, the introduction of α-methyl group benefits α-helical conformation formation and the hydrophobic of all-hydrocarbon linker facilitates cellular uptake. Despite the great success, development of alternative stapling methods remains interesting as they may enable the introduction of more types of all-hydrocarbon staples.



Scheme 1 Metathesis-based (a) versus diaminodiacid-based (b) synthesis of all-hydrocarbon stapled peptide

Here we report an alternative all-hydrocarbon stapling strategy employing pre-prepared diaminodiacids to the solid-phase synthesis of stapled peptides (Scheme 1b). Various linkers with different structures could be introduced by the diaminodiacids strategy.[12] Pre-prepared diaminoacids have well-defined structures and therefore the isomer problem could be avoided. To our knowledge, it was the first time to employ diaminodiacids

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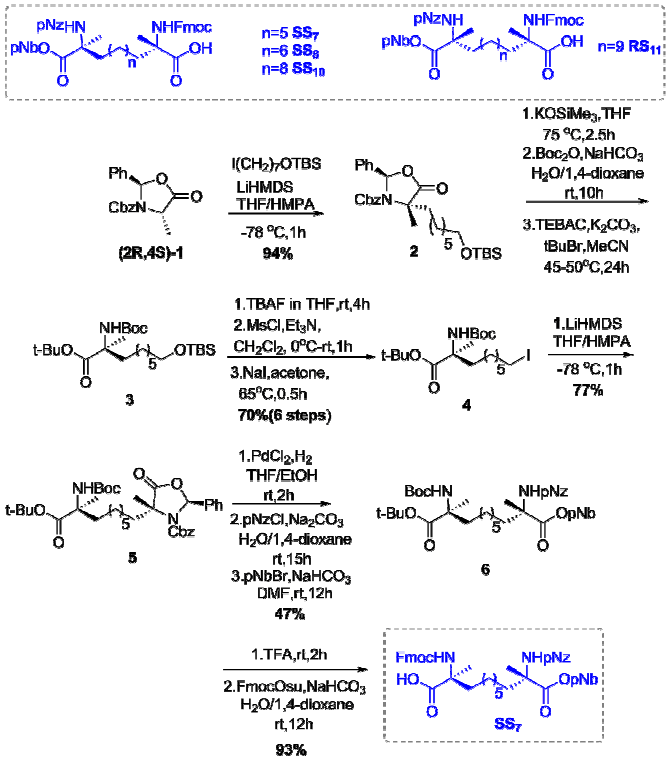
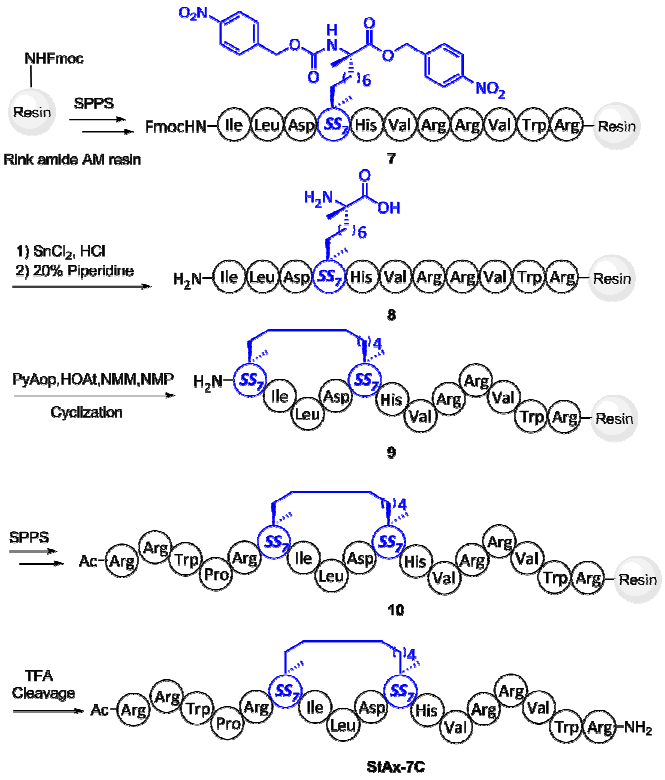
building blocks for the synthesis of all-hydrocarbon stapled peptides. To evaluate diaminodiacid stapling strategy, we synthesized series of peptide inhibitors for Wnt signaling pathway. Compared to native unstapled peptide, diaminodiacid-based stapled peptides showed increased helical propensity, proteolytic stability and inhibiting activity. Moreover, peptides with different lengths of linkers exhibited tunable inhibiting activity. Thus, our stapling strategy makes possible to increase the activity of stapled peptide by optimizing the linker of the diaminodiacids.



Scheme 2 Structure and synthesis of diaminodiacid building blocks (SS7 as an example)

Our work began with the synthesis of diaminodiacids building blocks (Scheme 2). For i,i+4 stapling, $\mathbf{SS_7}$, $\mathbf{SS_8}$, $\mathbf{SS_{10}}$ was synthesized and $\mathbf{RS_{11}}$ was obtained for i,i+7 stapling. As shown in Scheme 2 ($\mathbf{SS_7}$ as an example), alkylation of *trans*-oxazolidinone (2*R*,4*S*)-**1** with tert-butyl((7-iodoheptyl)oxy)-dimethylsilane and lithium hexamethyldisilazide (LiHMDS) afforded **2** in good yield (94%). 4-Disubstitutedoxazolidinone **2** could be cleaved with potassium trimethylsilanolate ($KOSiMe_3$) to yield free quaternary amino acid, which was reacted with di-tert-butyl dicarbonate (Boc_2O) and tert-butylbromide (tBuBr)/N,N,N'-triethylbenzenemethanaminium chloride (TEBAC) successively. Then the resulting fully-protected amino acid **3** was treated with tetrabutylammonium fluoride (TBAF) to remove tert-butyldimethylsilyl. Methylation and iodination of the resulting terminus hydroxyl gave **4** in high yield (70%, 6 steps). Then, alkylation of **1** with **4** generated **5** in 77% isolated yield. Compound **5** was subjected to hydrogenolysis and reacted with p-nitrobenzylchloroformate (pNZCl)/p-nitrobenzyl bromide (pNbBr) to afford the fully-protected diaminodiacid **6** (47%, 3 steps). We chose pNz/pNb as protecting groups because this couple was compatible with automated Fmoc-SPPS on peptide synthesizer. Deprotection of Boc/tBu and reaction with N-(9-Fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) afforded Fmoc-protected diaminodiacid $\mathbf{SS_7}$ (93%, 2 steps), which could be used for synthesis of stapled peptide. $\mathbf{SS_8}$ and $\mathbf{SS_{10}}$ were synthesized in similar procedure as $\mathbf{SS_7}$, while *cis*-oxazolidinone (2*S*, 4*S*)-**1** was used instead of (2*R*, 4*S*)-**1** in the second alkylating step for $\mathbf{RS_{11}}$. To verify the chiral purity of synthesized diaminodiacids, we tested it with o-phthalaldehyde/N-Boc-cysteine.[13]



Scheme 3 Synthesis of stapled peptide via diaminodiacids based strategy (StAx-7C as an example)

With protected diaminodiacid building blocks in hand, we next evaluated the new stapling strategy by preparing Wnt/β-Catenin signal pathway targeted peptides. The canonical Wnt pathway regulates cell differentiation, proliferation, and survival. Aberrant activation of this pathway was implicated in the development of variety of cancers.[14] β-Catenin plays a key role in Wnt signaling by participating in critical PPIs. The sequence of stapled peptide synthesized here was confirmed to inhibit oncogenic Wnt signaling by directly targeting β-catenin/TCF interaction. By insertion of staple by Verdine's method, the peptide showed enhanced α-helicity and biological activity.[15] The synthesis of stapled peptide by diaminodiacids strategy was presented in Scheme 3. Peptide **7** containing $\mathbf{SS_7}$ was first synthesized on Rink amide AM resin (0.33 mmol/g loading) following standard Fmoc-SPPS. O-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was used as

coupling reagent instead of O-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HCTU) when SS_7 was incorporated. Next, deprotection of pNz/pNb group on **7** by $SnCl_2$ (2.5 M in DMF)/HCl and removal of the N-terminus Fmoc group afforded peptide **8**. Peptide **9** was obtained by cyclization of **8** by using (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphoniumhexafluorophosphate (PyAop)/1-Hydroxy-7-azabenzotriazole (HOAt)/N-methylmorpholine (NMM). The remaining five amino acids were assembled onto **9** with a final acetylation procedure producing the N-terminus capped peptide **10**. After acidic cleavage and deprotection from the resin, crude **StAx-7C** was obtained.

High performance liquid chromatography (HPLC) was used to purify the diaminodiacid based crude **StAx-7C**. As shown in Figure 1a, crude peptide contained only a single major component and therefore can be conveniently purified to the desired stapled peptide **StAx-7C** (52% isolated yield). ESI-QTOF-MS further confirmed the identity of **StAx-7C** (Figure 1b). We conclude that the diaminodiacid based stapling strategy developed herein was efficient for the synthesis of stapled peptide. **StAx-8C**, **StAx-10C** and **StAx-11C** were prepared using the same approach with **N-35R**, while **StAx-35R** was synthesized by Verdine's RCM method for comparison (Table 1, isolated yield of each peptide is 38% for StAx-8C, 26% for StAx-10C, 37% for StAx-11C, 55% for N-35R and 35% for StAx-35R).

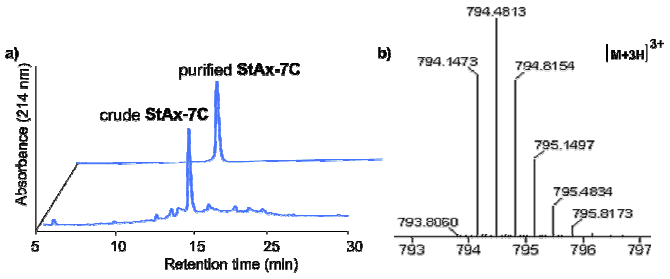


Fig. 1 (a) HPLC traces of crude and purified StAx-7C（Gradient: 20-50% of 0.08% TFA in CH_3CN. (b) ESI-QTOF-MS spectrum of StAx-7C (calc.: 2379.4141).

To explore the impact of diaminodiacid building blocks with various chain lengths for helix stability, circular dichroism (CD) spectroscopy of each peptide was tested. As shown in Figure S9, we concluded that long chain (**StAx-10C**, **StAx-11C**) exerted more impact on helix stability than short one (**StAx-7C**). Moreover, Table 1 indicated that the helical ratio of **N-35R** and **StAx-7C** were calculated to 5% and 7% according to the method described by Fairlie.[16] These values were lower than other peptides, such as 19% for **StAx-35R**, 12% for **StAx-8C**, 17% for **StAx-10C**, 20% for **StAx-11C**. The results also suggested that diaminodiacid based stapling method acts similar as ring-closing metathesis-based stapling strategy in stabilizing the α-helix structure of peptide.

Table 1. Sequences of peptides and the corresponding α helicity.

Compound	Sequence	α helicity
N-35R	Ac-RRWPRSILDQHVRRVWR-NH_2	5%
StAx-35R	Ac-RRWPRS_5ILDS_5HVRRVWR-NH_2	19%
StAx-7C	Ac-RRWPRSS_7ILDSS_7HVRRVWR-NH_2	7%
StAx-8C	Ac-RRWPRSS_8ILDSS_8HVRRVWR-NH_2	12%
StAx-10C	Ac-RRWPRSS_{10}ILDSS_{10}HVRRVWR-NH_2	17%
StAx-11C	Ac-RRWPRRS_{11}ILDQHVRS_{11}RVWR-NH_2	20%

To further study the activity of stapled peptides on inhibiting Wnt pathway, we measured the transcriptional activity of Wnt/β-catenin pathway by using Topflash reporter assay. We found that all stapled peptides treatment inhibited the reporter expression in the presence of Wnt3a (Figure 2). **StAx-10C** shows higher efficiency for the inhibition of Wnt3a-induced Top-flash reporter than **StAx-35R,** which own the same stapled position. It was worth to note that **StAx-11C,** with stapled position at i, i+7, has the highest activity (50% transcriptional suppression), possibly due to the highest ratio of α-helicity. This result indicated that the linker of stapled bridge has important impact on activity of stapled peptide. Thus, we consider that diaminodiacid based stapling strategy may lead to peptides with higher bioactivity by optimizing the linker.

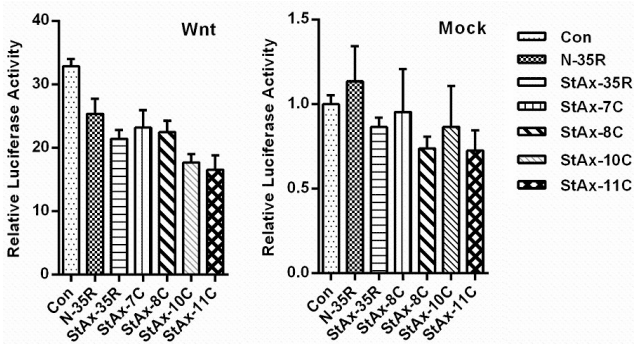


Fig. 2 Stapled peptides inhibit TOP flash luciferase reporter activity in Wnt3a-stimulated HeLa cells.

Finally, we evaluate the protease stability of **StAx-10C** and **StAx-11C** for inhibiting Wnt pathway as model. **StAx-10C** and **StAx-11C** (25 μM) were subjected to chymotrypsin (0.5 ng/μL, pH 7.4) which mostly cleaves after Phe, Tyr, Trp, Leu, Met.[17] The kinetic degradation of each peptide was monitored by HPLC (Figure 3). For **N-35R**, the half-life time was only 15 min, while **StAx-10C** and **StAx-11C** were measured to 38 and 106 min. This result was consistent with previous studies that protease stability of peptides can be increased after stapling. Compared to

N-35R, **StAx-11C** showed 7-fold increased stability against chymotrypsin-mediated degradation, **StAx-10C** exhibited only 2-fold increase. This difference could be explained that higher helicity of **StAx-11C** own more compact structure than **StAx-10C**, possibly leading to less protease cleavage site exposed. These results demonstrated that protease stability of peptide could be improved by using diaminodiacid based stapling strategy. Moreover, we identified that all three peptides were cleaved at the C-terminus Trp-Arg site (SI).

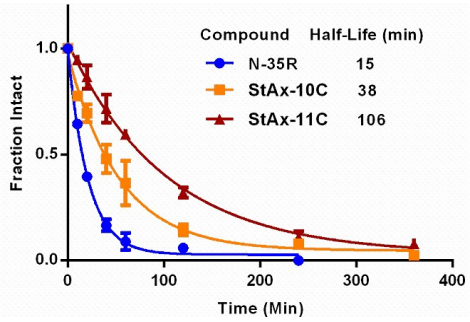


Fig. 3 Time-course degradation curve of N-35R, StAx-10C and StAx-11C under the treatment of chymotrypsin. StAx-10C and StAx-11C exhibited longer half time compared to native unstapled peptide.

In summary, we present a practical method for Fmoc-SPPS of all-hydrocarbon stapled peptides by using pre-prepared diaminodiacid building blocks. Peptides stapled exhibited increased property of helicity and protease stability than unstapled one. Moreover, its Wnt pathway inhibitory effect could be tuned by the change of linker. The main advantage of this strategy is the potential introduction of crosslinks that are not accessible via ring-closing metathesis. Therefore the diaminodiacid based stapling method provides a new way to optimize biological activity of stapled peptides by introducing different types of linkers.

Acknowledgements

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