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Stereochemical effects of all-hydrocarbon tethers in *i*,*i*+4 stapled peptides

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ABSTRACT

The stereochemical effects of the hydrocarbon crosslink on the conformation and cellular uptake of *i*,*i*+4 stapled peptides were studied. Compared to its *S*,*S*-configurated counterpart, the crosslink bearing the *R*,*R*-configuration provided a significantly diminished helix stabilizing effect and conferred less efficient cellular uptake on the stapled peptides. These results suggest that the vesicular trafficking pathway employed by cells to take up stapled peptides is sensitive to the extent of helical character in the peptide, with greater helicity conferring increased cellular uptake.

The α-helix is the most abundant secondary structure in proteins and is a frequent participant in functionally important protein–protein interactions. Synthetic strategies to enforce the α-helical conformation in synthetic peptides have aroused considerable interest as a means of generating potential therapeutic agents or probes for exploring protein–protein interactions.[1] We have previously reported that the incorporation of a 'staple' an all-hydrocarbon crosslink flanked by α-methyl groups along one face of an α-helical peptide can greatly increase its helix content, binding affinity for a target receptor, serum stability, and in vivo half-life.[2] Of particular significance has been the observation that the presence of the staple promotes cellular uptake by endocytic vesicle trafficking by an uptake pathway that is remarkably permissive toward diversity in sequence, length, and staple location. The combination of these pharmacologically attractive features has raised the prospect that stapled peptides may represent a new class of bioactive agents for targeting so-called 'undruggable' intracellular proteins.[3]

A staple is introduced into a peptide through the incorporation of two α-methyl, α-alkenyl amino acids at positions separated by one (*i* and *i*+4) or two (*i*,*i*+7) helical turns, followed by ruthenium-mediated ring-closing metathesis (RCM)[4] to form the macrocyclic hydrocarbon crosslink. In an earlier study, we showed that an *i*,*i*+7 staple dramatically stabilized the α-helical conformation of a model sequence from RNase A (EWAETAAAKFLAAHA, **1**).[2a] The *i*,*i*+7 staple also proved effective in stabilizing an α-helix from the tumor suppressor p53, leading to a 1000-fold increase in the potency of binding to the p53 negative regulator Hdm2.[2c]

The helix stabilizing effect of *i*,*i*+4 staples incorporated into RNase peptide has also been investigated, and with this particular crosslink the additional structural variable of staple stereochemistry was investigated.[2a] Staples attached at positions 8 and 12 of the RNase peptide were found to form readily by RCM, using two units of either the *S*-configurated amino acid $\boldsymbol{S_5}$ [$S_{i,i+4}S(8)$ staple, Fig. 1A] or its enantiomer $\boldsymbol{R_5}$ [$R_{i,i+4}R(8)$ staple]; these peptides differed only in the opposite stereochemical configuration of the staple they bear (Fig. 1B and C).[5]

In our original study, neither *i*,*i*+4 stapled RNase A peptide exhibited appreciable helix stabilization attributable to macrocyclic ring-closure. Notwithstanding that initial result, when the $S_{i,i+4}S(8)$ staple was later incorporated into the BH3 domains of the apoptotic effector proteins BID[2b] and BAD,[2d] it provided substantial helix induction, indeed greater than that provided by an *i*,*i*+7 staple. This has led us to develop SAHBa (for **s**tabilized α-**h**elix of the **B**H3 domain of BID), which we found to suppress the growth of highly aggressive human leukemia cells xenotransplanted into mice.[2b] Following this in vivo demonstration of therapeutic potential for a stapled peptide, the $S_{i,i+4}S$ stapling system has been applied successfully to several other systems by our laboratory and others.[6] The growing body of positive results obtained using the $S_{i,i+4}S(8)$ staple prompted us to re-investigate the stereoisomeric $R_{i,i+4}R(8)$ staple. Here we show that the $R_{i,i+4}R(8)$ staple is significantly less helix stabilizing than $S_{i,i+4}S(8)$, and we further show that the extent of helix stabilization by these staples correlates positively with the efficiency of endosomal uptake of the peptides into cells.

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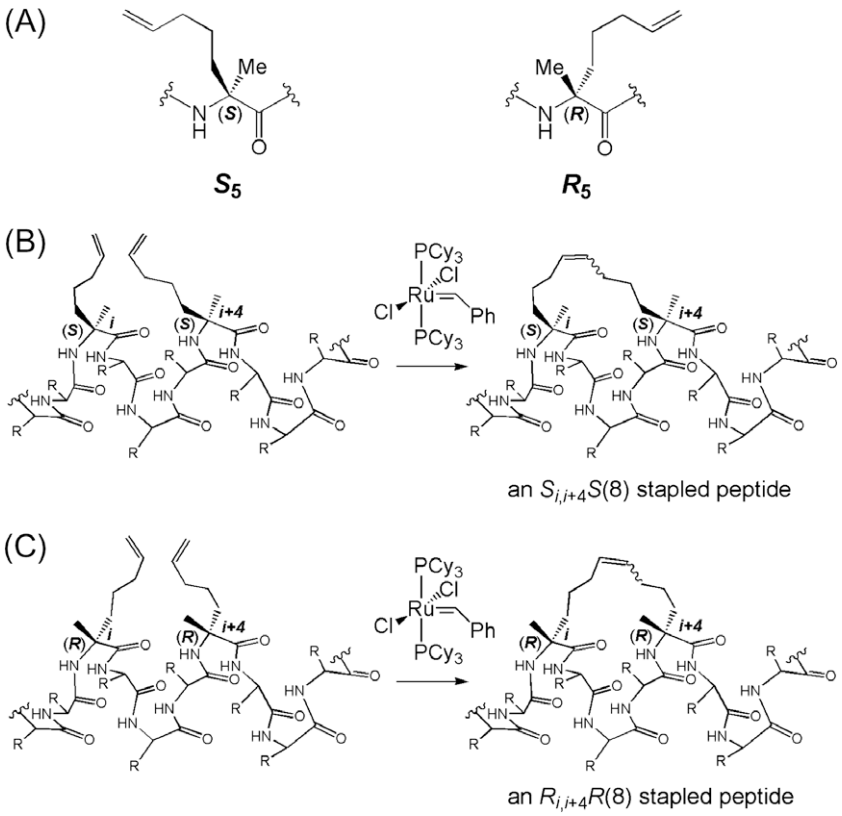

Figure 1. (A) Structures of the two enantiomeric α-methyl, α-pentenyl amino acids used to form the *i*,*i*+4 staple. (B) and (C) Schematic representation of the two stereochemically opposite *i*,*i*+4 staples, $S_{i,i+4}S(8)$ and $R_{i,i+4}R(8)$, formed using $\boldsymbol{S_5}$ and $\boldsymbol{R_5}$, respectively. The nomenclature $S_{i,i+4}S(8)$ refers to an 8-carbon metathesized crosslink with *S*-configuration at *i* and *i*+4 positions; $R_{i,i+4}R(8)$ is of opposite stereochemical configuration.

We first chose to compare the $R_{i,i+4}R(8)$ and $S_{i,i+4}S(8)$ staple configurations again on the RNase A peptide, but now at positions 4 and 8.[2a] Peptides incorporating either $\boldsymbol{S_5}$ or $\boldsymbol{R_5}$ (Fig. 1A) at both attachment positions EWA$\boldsymbol{S_5}$TAA$\boldsymbol{S_5}$KFLAAHA (***S,S*-2**) and EWA$\boldsymbol{R_5}$-TAA$\boldsymbol{R_5}$KFLAAHA (***R,R*-2**) were prepared. The fully-protected, resin-bound peptides were subjected to RCM using 20 mol % of Grubbs first generation catalyst in 1,2-dichloroethane at room temperature (Fig. 1B and C). At certain time-points during the 2 h RCM reaction, an aliquot of resin was taken from each reaction, the peptide material released and deprotected, and the products were analyzed by LC/MS. Substrates **2** and **3** showed similar reaction profiles, with both substrates undergoing efficient RCM; conversion to the stapled products (***S,S*-3** and ***R,R*-3**) reached ~90% by the 2 h time-point (Fig. 2A).[7]

To investigate the impact of staple stereochemistry on the extent of helix induction in the present peptides, we measured their circular dichroism (CD) spectra, in which the negative peak at 222 nm provides a quantitative signature for α-helix content.[8] The unstapled peptide ***S,S*-2**, bearing two $\boldsymbol{S_5}$ residues at the positions 4 and 8 positions, showed an ~8% increase in helical content over that of the corresponding unmodified peptide, while the $\boldsymbol{R_5}$-containing version ***R,R*-2** showed a slight (~5%) decrease in helicity (Fig. 2B and C). A similar trend had been observed in our earlier study of RNase peptides substituted at positions 8 and 12.[2a]

Formation of the staple substantially increased helix content for both the *S,S* and *R,R* peptides, from 36% to 72% in going from ***S,S*-2** to ***S,S*-3**, and from 24% to 40% in going from ***R,R*-2** to ***R,R*-3**. Accounting for the net effect of incorporating the staple (i.e., relative to the unmodified peptide **1**), the $S_{i,i+4}S(8)$ staple increased helical character by 44%, while $R_{i,i+4}R(8)$ provided only 11% (Fig. 2B and C). These results are consistent with the emerging consensus that the $S_{i,i+4}S(8)$ staple is a powerful helix stabilizer in most sequence contexts, though not in all. Furthermore, even in sequences that are stabilized by $S_{i,i+4}S(8)$, stereochemical inversion to $R_{i,i+4}R(8)$ causes a substantial loss of helix induction.

The mechanism by which stapled peptides trigger their own vesicular transport into cells is poorly understood at present, as are the structural features in these peptides that are responsible for uptake. As both *i*,*i*+4 and *i*,*i*+7 staples can be taken up efficiently, the uptake machinery does not appear to respond to a specific staple structure. On the other hand, there is no evidence that simply making a peptide helical enables endosomal uptake in the absence of a staple. The *S,S* and *R,R*-configurated peptides would seem to offer a valuable structure–activity comparison, because their staples are constitutionally identical, but owing to differences in staple stereochemistry, the peptides differ substantially in their α-helicity. We therefore used flow cytometry to examine the effects of the *i*,*i*+4 staple stereochemical configuration on cellular uptake. HeLa cells were treated with 2 μM fluorescein-labeled **1**, ***R,R*-3** and ***S,S*-3** in Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM) at 37 °C for 4 h. Fluorescence-activated cell sorting (FACS, Fig. 1D) analyses revealed, as expected, that the unmodified peptide **1** did not increase the fluorescence of HeLa cells over the background level, hence it was not cell-permeable. The ***R,R*-3** stapled peptide increased cell fluorescence modestly (evidenced by a rightward shift of the red versus green curve in Fig. 2D), suggesting modest uptake. However, ***S,S*-3** strongly increased cell fluorescence, to a level consistent with robust cell uptake.

To test whether the inferior helix stabilization of the $R_{i,i+4}R(8)$ staple is observed for sequences other than the model RNase A peptide, we next prepared an analog of the aforementioned SAHBa in which its *S,S*-configurated staple [$S_{i,i+4}S(8)$] was replaced by an

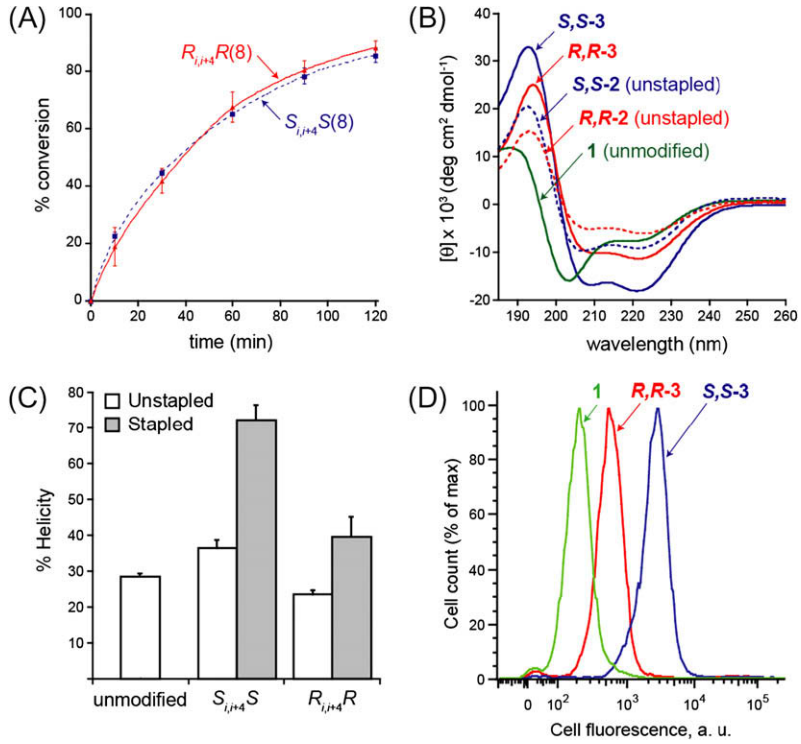

Figure 2. (A) Time-course comparison of $S_{i,i+4}S(8)$ and $R_{i,i+4}R(8)$ ring-closure by olefin metathesis. (B) Circular dichroism spectra of $S_{i,i+4}S(8)$ (blue line) and $R_{i,i+4}R(8)$ (red line) stapled peptides, their unstapled precursors (dotted lines in the matching color), and the unmodified RNase A peptide (green line). (C) Comparison of percentage of helicities exhibited by $S_{i,i+4}S(8)$ and $R_{i,i+4}R(8)$ stapled peptides (solid bars) with their respective unstapled precursor and the unmodified peptide (open bars). Percentage of helicities were calculated from molar ellipticities at 222 nm ($[\theta]_{222}$) using $-31{,}500(1-2.57/n)$ and 0 deg cm^2 dmol^{-1} as the values for 100% and 0% helicity, respectively; n is the number of amino acid residues in the peptide.[8] (D) Flow cytometry profile of fluoresceinated $S_{i,i+4}S(8)$ (blue) and $R_{i,i+4}R(8)$ (red) stapled peptides in comparison with unmodified control (green).

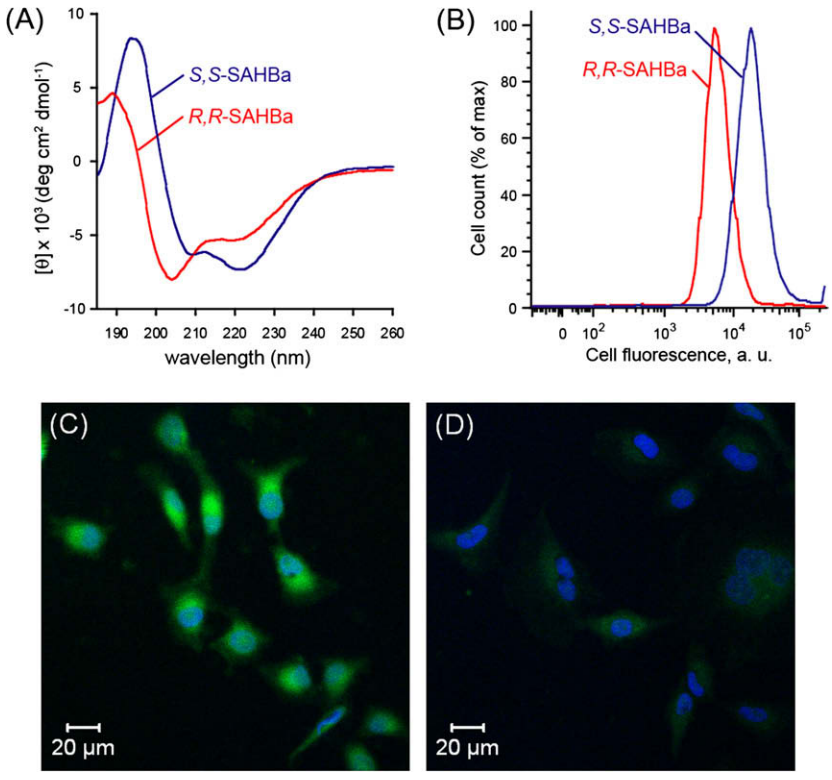

Figure 3. (A) Circular dichroism spectra of $S_{i,i+4}S(8)$ staple-containing SAHBa (*S,S*-SAHBa, blue) and its $R_{i,i+4}R(8)$ staple-containing analog, *R,R*-SAHBa (red) (B) Flow cytometry profile of fluoresceinated *S,S*-SAHBa (blue) and *R,R*-SAHBa (red). (C) and (D) Confocal microscope images of HeLa cells treated with 10 μM of fluoresceinated *S,S*-SAHBa (C) and *R,R*-SAHBa (D) (green fluorescence) with the nuclei being co-stained by 4′,6-diamidino-2-phenylindole (DAPI, blue fluorescence).

R,R-configurated one [$R_{i,i+4}R(8)$], (i.e., Ac-EDIIRNIARHLA-$\boldsymbol{R_5}$VGD$\boldsymbol{R_5}$$N_L$DRSIW-$NH_2$, in which N_L = norleucine). The RCM of this substrate also proceeded smoothly to afford *R,R*-SAHBa. As shown in Figure 3A, *R,R*-SAHBa and *S,S*-SAHBa exhibited dramatically different CD spectra; while the CD spectrum of *S,S*-SAHBa has the appearance typical of that for a right-handed α-helix, *R,R*-SAHBa displayed a CD spectrum similar to that of that the corresponding unmodified BH3 peptide.[9] Cellular uptake of *R,R*-SAHBa also appeared to be less effective than that of *S,S*-SAHBa, as indicated by flow cytometry analysis (Fig. 3B), consistent with the results obtained with RNase A peptides. The decreased cellular uptake of *R,R*-SAHBa versus *S,S*-SAHBa was also observed in their confocal microscopy images (Fig. 3C and D).

The data presented here, taken together with data reported previously in the literature, help to formulate a consensus view on helix stabilization by hydrocarbon-stapled peptides. The extent of helix stabilization by both *i*,*i*+4 and *i*,*i*+7 staples varies from peptide to peptide, and from position to position within a given peptide sequence. Computational folding simulations of stapled peptides have indicated that the existence of quasi-stable 'dummy' states in the unfolded peptide diminishes helix stability;[10] as these states are not trivial to predict, synthesis and screening of panels of staple-permuted peptides appears to the most efficient route to identify a candidate having optimal helix stability. Notwithstanding such sequence-dependent effects, the magnitude of helix stabilization by the $S_{i,i+4}S(8)$ staple is so much greater than that provided by the stereochemically opposite $R_{i,i+4}R(8)$ staple, and the superiority of the former seems likely to hold true for most, if not all, sequence contexts. The detrimental effect of $\boldsymbol{R_5}$ incorporation on helix induction might be attributable to unfavorable steric interactions involving its alkenyl side-chain, an effect related to that observed for D-amino acids containing long or bulky side-chains.[11] We also demonstrated that $R_{i,i+4}R(8)$ stapled peptides were less effective in cell-penetration than their $S_{i,i+4}S(8)$ counterparts. These results suggest a model wherein the presence of the staple promotes endosomal uptake, but that the helix content of the peptide *per se* also exerts an important influence.

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