ORGANIC LETTERS

2010
Vol. 12, No. 13
3046−3049

Introduction of All-Hydrocarbon *i*,*i*+3 Staples into α-Helices via Ring-Closing Olefin Metathesis

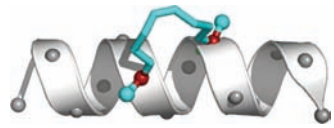
Young-Woo Kim, Peter S. Kutchukian, and Gregory L. Verdine*

Departments of Chemistry and Chemical Biology, Stem Cell and Regenerative Biology, Harvard University, Cambridge, Massachusetts 02138, and Program in Cancer Chemical Biology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115

gregory_verdine@harvard.edu

Received May 6, 2010

ABSTRACT



The introduction of all-hydrocarbon *i*,*i*+3 staples into α-helical peptide scaffolds via ring-closing olefin metathesis (RCM) between two α-methyl,α-pentenylglycine residues incorporated at *i* and *i*+3 positions, which lie on the same face of the helix, has been investigated. The reactions were found to be highly dependent upon the side-chain stereochemistry of the amino acids undergoing RCM. The *i*,*i*+3 stapling system established here provides a potentially useful alternative to the well-established *i*,*i*+4 stapling system now in widespread use.

Conformational restriction through synthetic modification serves as an attractive strategy to enforce the bioactive α-helical structure upon peptides to create functional probes and potential next-generation therapeutics. Among the various strategies employed to stabilize the α-helical conformation in peptides, with few exceptions,[1] those that have shown the greatest promise involve the incorporation of a covalent cross-link between the side chains of amino acid residues that are separated by approximately one (*i* and *i*+4 positions), two (*i*,*i*+7), or three turns (*i*,*i*+11) of the helix.[1]

For example, we have shown that the incorporation of an all-hydrocarbon "staple"—a macrocyclic hydrocarbon cross-link bearing an α-methyl group at each terminus—into peptides at *i*,*i*+4 and *i*,*i*+7 positions can greatly increase α-helical content, chemical and thermal stability, protease resistance, target-binding affinity, and cell permeability.[2] This hydrocarbon stapling system has been successfully applied to a number of biological systems by our laboratory and by others.[3,4]

Recently, we have encountered certain systems in which the pattern of amino acid utilization in binding to a target does not permit the introduction of an *i*,*i*+4 staple without disruption of binding. Furthermore, we have become interested in incorporating two or more independent hydrocarbon staples into peptides, as we reasoned that such multiply stapled peptides might possess enhanced structural stability, relative to the monostapled counterparts. For both these

(1) (a) Chapman, R. N.; Dimartino, G.; Arora, P. S. *J. Am. Chem. Soc.* **2004**, *126*, 12252–12253. (b) Wang, D.; Liao, W.; Arora, P. S. *Angew. Chem., Int. Ed.* **2005**, *44*, 6525–6529.

(2) For recent reviews, see: (a) Henchey, L. K.; Jochim, A. J.; Arora, P. S. *Curr. Opin. Chem. Biol.* **2008**, *12*, 692–697. (b) Gamer, J.; Harding, M. M. *Org. Biomol. Chem.* **2007**, *5*, 3577–3585. (c) Fletcher, S.; Hamilton, A. *J. R. Soc. Interface* **2006**, *3*, 215–233.

(3) (a) Schafmeister, C. E.; Po, J.; Verdine, G. L. *J. Am. Chem. Soc.* **2000**, *122*, 5891–5892. (b) Walensky, L. D.; Kung, A. L.; Escher, I.; Malia, T. J.; Barbuto, S.; Wright, R. D.; Wagner, G.; Verdine, G. L.; Korsmeyer, S. J. *Science* **2004**, *305*, 1466–1470. (c) Kim, Y.-W.; Verdine, G. L. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2533–2536.

(4) (a) Bernal, F.; Tyler, A. F.; Korsmeyer, S. J.; Wlaensky, L. D.; Verdine, G. L. *J. Am. Chem. Soc.* **2007**, *129*, 2456–2457. (b) Danial, N. N.; Walensky, L. D.; Zhang, C. Y.; Choi, C. S.; Fisher, J. K.; Molina, A. J.; et al. *Nat. Med.* **2008**, *14*, 144–153. (c) Gavathiotis, E.; Suzuki, M.; Davis, M. L.; Pitter, K.; Bird, G. H.; Katz, S. G.; Tu, H.-G.; Cheng, E. H.-Y.; Tjandra, N.; Walensky, L. D. *Nature* **2008**, *455*, 1076–81. (d) Moellering, R. E.; Cornejo, M.; Davis, T. N.; Bianco, C. D.; Aster, J. C.; Blacklow, S. C.; Kung, A. L.; Gilliland, D. G.; Verdine, G. L.; Bradner, J. E. *Nature* **2009**, *462*, 182–188. (e) Zhang, H.; Zhao, Q.; Bhattacharya, S.; Waheed, A. A.; Tong, X.; Hong, A.; Heck, S.; Curreli, F.; Goger, M.; Cowburn, D.; Freed, E. O.; Debnath, A. K. *J. Mol. Biol.* **2008**, *278*, 565–580.

10.1021/ol1010449
Published on Web 06/07/2010

applications, it would be useful to have the option of installing a staple at the *i* and *i*+3 positions. Like the well-studied *i*,*i*+4 all-hydrocarbon staple, the *i*,*i*+3 system would place the macrocyclic hydrocarbon chain on one face of the α-helix (Figure 1); the former bridges 1.11 turns of the helix,

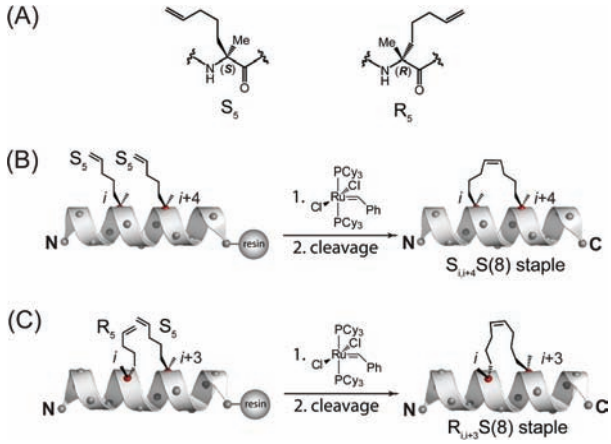


Figure 1. (A) Structures of the two enantiomeric α-methyl,α-pentenylglycine units used for hydrocarbon cross-linking. (B and C) $S_{i,i+4}S(8)$ and $R_{i,i+3}S(8)$ stapling as a representative *i*,*i*+4 and *i*,*i*+3 hydrocarbon cross-linking, respectively. The nomenclature $S_{i,i+4}S(8)$ refers to an 8-carbon metathesized cross-link with *S*-configuration at *i* and *i*+4 positions; $R_{i,i+3}S(8)$, *R*-configuration at *i* and *S*- at *i*+3 position, respectively.

whereas the latter bridges 0.83 turns. In the case of *i*,*i*+4 stapling, both the efficiency of RCM and the helical content of the stapled peptides thus produced were found to be highly dependent upon the stereochemistry of the α-methyl,α-pentenylglycine residues that form the staple. Here we report that *i*,*i*+3 staples are also highly stereochemically dependent for both formation and helix stabilization. The *i*,*i*+3 system provides a potentially useful alternative to its well-studied *i*,*i*+4 counterpart.

For *i*,*i*+4 stapling, the most stereochemically favorable cross-link is formed using two units of (*S*)-α-methyl,α-pentenylglycine ($\boldsymbol{S_5}$), with ruthenium-mediated RCM being used to form an $S_{i,i+4}S(8)$ staple (Figure 1B). RCM between two of the corresponding *R*-configurated amino acids ($\boldsymbol{R_5}$) to give an $R_{i,i+4}R(8)$ staple also proceeds efficiently, but this particular staple neither stabilizes helices well nor provides peptides that are highly cell-penetrant.[2a,c] RCM reactions using one *R*- and one *S*-configurated amino acid are very inefficient.[3a] It is well established that in proteins side-chain configurations required for helix stabilization via *i*,*i*+3 interactions are different from those via *i*,*i*+4 interactions, and therefore we expected that *i*,*i*+3 staples would show stereochemical behavior distinct from *i*,*i*+4.[5] To examine the stereochemical effects on RCM in *i*,*i*+3 cross-linking, we prepared peptide substrates based on a model sequence from RNase A (EWAETAAAKFLAAHA, **1**) incorporating all four possible combinations of either $\boldsymbol{S_5}$ or $\boldsymbol{R_5}$ (Figure 1A) at both positions 4 and 7 (EWA$\boldsymbol{X_1}$TA$\boldsymbol{X_2}$AKFLAAHA, in which $\boldsymbol{X_1}$, $\boldsymbol{X_2}$ = $\boldsymbol{S_5}$ or $\boldsymbol{R_5}$). In addition, a peptide containing $\boldsymbol{S_5}$ at positions 4 and 8 was prepared as an *i*,*i*+4 stapled control (EWA$\boldsymbol{S_5}$TAA$\boldsymbol{S_5}$KFLAAHA). These staple positions were chosen to maintain the same amino acid composition in the *i*,*i*+3 and *i*,*i*+4 stapled analogues. The fully protected, resin-bound peptides were then subjected to RCM. As monitored by LC/MS, the four *i*+3 substrates showed significantly different reaction profiles: the RCM of $S_{i,i+3}S$ and $S_{i,i+3}R$ was slow, $R_{i,i+3}R$ moderate, and $R_{i,i+3}S$ fast, even faster than $S_{i,i+4}S$ (Figure 2). Relocation of the *i*,*i*+3 staple

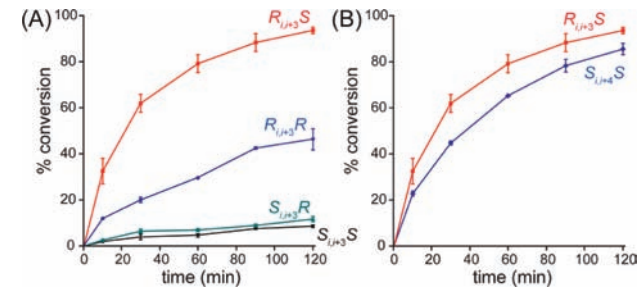


Figure 2. Time course analysis of metathesis reaction. (A) Stereochemical effects of *i*,*i*+3 stapling. (B) Comparison of RCM reaction profiles for $R_{i,i+3}S(8)$ and $S_{i,i+4}S(8)$ staplings.

to another site in the peptide (EWAETA$\boldsymbol{X_1}$AK$\boldsymbol{X_2}$LAAHA, in which $\boldsymbol{X_1}$, $\boldsymbol{X_2}$ = $\boldsymbol{S_5}$ or $\boldsymbol{R_5}$) gave similar results (Table 1).

Table 1. % Conversion after 2 h RCM

i,*i*+3	EWA$\boldsymbol{X_1}$TA$\boldsymbol{X_2}$AKFLAAHA[a]	EWAETA$\boldsymbol{X_1}$AK$\boldsymbol{X_2}$LAAHA[a]
$\boldsymbol{X_1}$, $\boldsymbol{X_2}$	product (% conversion[b])	product (% conversion[b])
$\boldsymbol{R_5}$, $\boldsymbol{R_5}$	$\boldsymbol{R_{i,i+3}R}$**-2** (46.4 ± 4.6)	$\boldsymbol{R_{i,i+3}R}$**-3** (39.8 ± 1.8)
$\boldsymbol{R_5}$, $\boldsymbol{S_5}$	$\boldsymbol{R_{i,i+3}S}$**-2** (93.7 ± 1.3)	$\boldsymbol{R_{i,i+3}S}$**-3** (90.5 ± 2.9)
$\boldsymbol{S_5}$, $\boldsymbol{R_5}$	$\boldsymbol{S_{i,i+3}R}$**-2** (11.7 ± 1.2)	$\boldsymbol{S_{i,i+3}R}$**-3** (26.6 ± 1.3)
$\boldsymbol{S_5}$, $\boldsymbol{S_5}$	$\boldsymbol{S_{i,i+3}S}$**-2** (8.7 ± 0.5)	$\boldsymbol{S_{i,i+3}S}$**-3** (13.6 ± 1.1)
i,*i*+4	EWA$\boldsymbol{X_1}$TAA$\boldsymbol{X_2}$KFLAAHA[a]	EWAET$\boldsymbol{X_1}$AAK$\boldsymbol{X_2}$LAAHA[a]
$\boldsymbol{X_1}$, $\boldsymbol{X_2}$	product (% conversion[b])	product (% conversion[b])
$\boldsymbol{S_5}$, $\boldsymbol{S_5}$	$\boldsymbol{S_{i,i+4}S}$**-2** (85.6 ± 2.4)	$\boldsymbol{S_{i,i+4}S}$**-3** (82.0 ± 0.9)

[a] Side chains of all substrates were protected during the metathesis reaction. [b] Product/(product + starting material) as determined by reverse-phase HPLC following cleavage from resin.

To better understand these trends, we performed a Monte Carlo conformational search of the cross-link with the peptide constrained in the α-helical state, and we identified the global minimum structure for each cross-link (see Supporting Information). Since the RCM reaction is carried out in a helix-stabilizing nonpolar solvent,[6] and may be under thermodynamic control,[7] we reasoned that the energetic effect

(5) McGregor, M. J.; Islam, S. A.; Sternberg, J. E. *J. Mol. Biol.* **1987**, *198*, 295–310.

(6) Urnes, P.; Doty, P. *Adv. Protein Chem.* **1961**, *16*, 401–544.
(7) Monfette, S.; Fogg, D. E. *Chem. Rev.* **2009**, *109*, 3783–3816.

of staple introduction in the folded state might correlate with RCM yield trends. The computational results indicated that the $R_{i,i+3}S(8)$ staple is the most stabilizing, in agreement with our experimental findings. The other systems are less stable by 15−37 kcal/mol, due in part to *syn*-pentane interactions engendered between the α-methyl group and the cross-link (Figure 3).

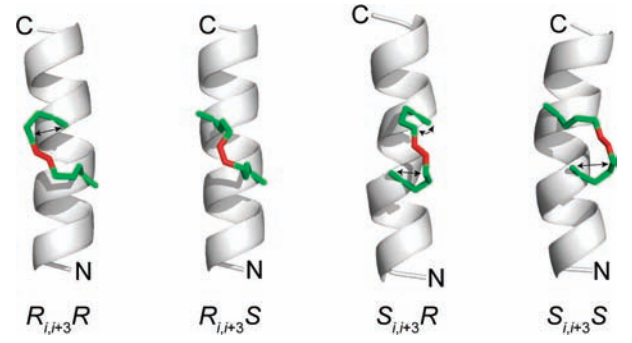


Figure 3. Graphical representation of the global minimum *i*,*i*+3 stapled peptides. The olefin moiety is colored red. Unfavorable *syn*-pentane interactions are highlighted by arrows. Only the $R_{i,i+3}S$ staple is free from *syn*-pentane interactions.

In protein structures, nonpolar, hydrogen-bonding, or ion-pairing interactions between side chains at *i* and *i*+3 positions are often identified as an important contributor for helix stabilization.[8] Not surprisingly then, covalent cross-linking between the *i* and *i*+3 residues via disulfide or lactam bridge formation has been found to stabilize α-helical structure in peptides.[9] In most cases, however, the *i*,*i*+3 cross-linking strategy has shown to be less effective than *i*,*i*+4 approaches for α-helix stabilization but has shown greater promise in stabilizing β-turns or 3_{10}-helices.[10] To assess the impact of the staple in our system on helix stability, we analyzed the most favorable staple, $R_{i,i+3}S(8)$, using circular dichroism (CD) spectroscopy. Both *i*,*i*+3 stapled peptides, ***R*$_{i,i+3}$*S*-2** and ***R*$_{i,i+3}$*S*-3**, showed CD spectra that are typical of an α-helical peptide, with a minimum at 208 and 222 nm and a maximum near 195 nm at 20 °C (Figure 4A). The helical content of these peptides, measured by mean residue ellipticity at 222 nm and 20 °C, were 67 and 49%, respectively, both substantially more helical than the unmodified analogue **1** (34%). Indeed, these *i*,*i*+3 stapled peptides were only slightly less helical than the corresoponding *i*,*i*+4 stapled counterparts, ***S*$_{i,i+4}$*S*-2** (70%) and ***S*$_{i,i+4}$*S*-3** (56%). In other peptide sequences, *i*,*i*+4 outperformed *i*,*i*+3 by a somewhat larger margin (see Supporting Information). The structural stability of *i*,*i*+3 stapled peptides was also measured as a function of concentration of the denaturant guanidinium hydrochloride. Here, ***R*$_{i,i+3}$*S*-2** exhibited a substantially greater resistance to chemical denaturation than the unstapled peptide, an effect that was similar to that of the *i*,*i*+4 stapled counterpart, ***S*$_{i,i+4}$*S*-2** (Figure 4B).

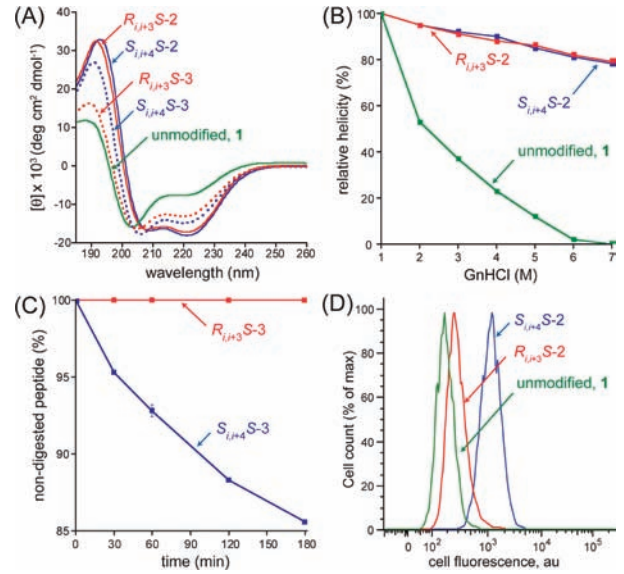


Figure 4. Circular dichroism spectra (A), guanidinium hydrochloride denaturation (B), tryptic digestion (C), flow cytometry profile (D) of the unmodified RNase A peptide (green) and its $R_{i,i+3}S$ (red) and $S_{i,i+4}S$ (blue) stapled analogues.

In tryptic digestion assays, the peptides showed a surprisingly strong dependence of protolytic stability on the position of the staple. After 2 min incubation with trypsin at a substrate/enzyme ratio (S/E) of 80:1, 94% of ***R*$_{i,i+3}$*S*-2** and 89% of ***S*$_{i,i+4}$*S*-2** had undergone tryptic cleavage; the unmodifiend analogue **1** was completely digested during the same period of time. However, both ***R*$_{i,i+3}$*S*-3** and ***S*$_{i,i+4}$*S*-3** underwent no detectable protease degradation under these conditions, even when treated with 4-fold more trypsin (S/E = 20:1) (data not shown). When an overwhelming amount of trypsin was used (S/E = 2:1), ***S*$_{i,i+4}$*S*-3** did show appreciable digestion ($t_{1/2}$ of 829 min, Figure 4C). Remarkably, however, ***R*$_{i,i+3}$*S*-3** remained completely resistant to tryptic proteolysis under such aggressively digestive conditions.

The marked difference between product series **2** and **3** in tryptic resistance appeared to be attributable to the location of the scissile bond; peptides ***R*$_{i,i+3}$*S*-2** and ***S*$_{i,i+4}$*S*-2** have the lysine residue outside of the stapled segment, whereas ***R*$_{i,i+3}$*S*-3** and ***S*$_{i,i+4}$*S*-3** contain the lysine residue within the staple region. It may be that the stapled region is simply incapable of being inserted into the active site of the protease, whereas the flanking region is protected to some extent by helix nucleation but is nevertheless capable of undergoing

(8) Klingler, T. M.; Brutlag, D. L. *Pro. Sci.* **1994**, *3*, 1847–1857.
(9) (a) Luo, P.; Braddock, D. T.; Subramanian, R. M.; Meredith, S. C.; Lynn, D. G. *Biochemistry* **1994**, *33*, 12367–12377. (b) Gulyas, J.; Rivier, C.; Perrin, M.; Koerber, S. C.; Sutton, S.; Corrigan, A.; Lahrichi, S. L.; Craig, A. G.; Vale, W.; Rivier, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10575–10579. (c) Leduc, A.; Trent, J. O.; Wittliff, J. L.; Bramlett, K. S.; Briggs, S. L.; Chirgadze, N. Y.; Wang, Y.; Burris, T. P.; Spatola, A. F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11273–11278.
(10) (a) Marepalli, H. R.; Antohli, O.; Becker, J. M.; Naider, F. *J. Am. Chem. Soc.* **1996**, *118*, 6531–6539. (b) Schievano, E.; Bisello, A.; Chorev, M.; Bisol, A.; Mammi, S.; Peggion, E. *J. Am. Chem. Soc.* **2001**, *123*, 2743–2751. (c) Boal, A. M.; Guryanov, I.; Moretto, A.; Crisma, M.; Lanni, E. L.; Toniolo, C.; Grubbs, R. H.; O'Leary, D. J. *J. Am. Chem. Soc.* **2007**, *129*, 6986–6987.

active site insertion. The extended conformation is known to be a prerequisite for recognition by proteases.[11] The substantial difference between $\boldsymbol{R_{i,i+3}S}$**-3** and $\boldsymbol{S_{i,i+4}S}$**-3** in tryptic resistance, on the other hand, implies perhaps that the region constrained by the $R_{i,i+3}S(8)$ staple might be more rigid than that by the $S_{i,i+4}S(8)$ staple. In addition, the closer proximity of the scissile bond to α,α-dialkylated amino acids in $\boldsymbol{R_{i,i+3}S}$**-3** might be another key feature responsible for its higher protease stability compared to $\boldsymbol{S_{i,i+4}S}$**-3**.[12]

Next, we examined the impact of the $R_{i,i+3}S(8)$ staple on cellular uptake. Fluorescence-activated cell sorting analyses at 37 °C using HeLa cells showed that the i+3 stapled peptide $\boldsymbol{R_{i,i+3}S}$**-2** displayed modestly increased cell fluorescence compared to its unmodified analogue **1**, and the enhancement of cell penetration was not as pronounced as observed with the i+4 stapled counterpart, $\boldsymbol{S_{i,i+4}S}$**-2** (Figure 4D). The reasons for this divergence are not clear, as the mechanism of endocytic uptake of stapled peptides remains unelucidated.

Incorporation of multiple staples can be beneficial in several respects, e.g., to rigidify a longer stretch of an α-helix and thereby to further increase protease stability. For instance, for two i,i+4 staples to be formed with two intervening residues, thereby allowing them to reside on a single face of the helix, it is important for the two $S_{i,i+4}S$ staples to form while avoiding formation of the possible $S_{i,i+3}S$ staple. Therefore, in addition to the potential usefulness of the $R_{i,i+3}S(8)$ staple as an alternative helix-stabilizing strategy, the unfavorable i,i+3 stapling systems identified above can also be of practical use for the design of multiply stapled peptides. To demonstrate this experimentally, we designed two peptide substrates, EW$\boldsymbol{S}_5$ETA$\boldsymbol{S}_5$AK$\boldsymbol{S}_5$LAA$\boldsymbol{S}_5$A (**4**) and EWA$\boldsymbol{S}_5$TAA$\boldsymbol{S}_5$KF$\boldsymbol{R}_5$AA$\boldsymbol{S}_5$A (**5**) (Figure 5). RCM of each substrate under the typical reaction conditions cleanly gave rise to a double-stapled product as an exclusive product as analyzed by LC/MS (>98% conversion after two 2 h RCM reactions). The two pentenyl side-chain residues in the middle of each sequence are in a $S_{i,i+3}S$ or $S_{i,i+3}R$ relationship, respectively, which represents two of the most unfavorable combinations of the side-chain configuration for i,i+3 stapling (Table 1). Therefore, structures **6** and **7** in Figure 5 are virtually certain to represent those formed via double-RCM in these reactions. Indeed, in a time-course analysis of an alanine-based substrate ($\boldsymbol{S}_5$AAA$\boldsymbol{S}_5$AA$\boldsymbol{S}_5$AAA$\boldsymbol{S}_5$A, **8**) using LC/MS, only two single-RCM intermediates, highly likely those with an $S_{i,i+4}S$ staple at either end, were observed during metathesis (Figure 5C). These intermediates then coalesced cleanly into a double-RCM product as the reaction progressed. The structures of the tandem-stapled products were confirmed by MS/MS analysis, which definitively establishes the positions of staple incorporation.

In summary, in this letter we demonstrated that ring-closing metathesis reactions of olefin-bearing side chains at i and i+3 positions occur in a highly stereoselective manner on α-helical peptide scaffolds. Incorporation of the favorable $R_{i,i+3}S(8)$ staple appears to stabilize the α-helical conformation, though perhaps to a lesser extent than the $S_{i,i+4}S(8)$ counterpart. In addition, by performing double stapling reactions, we demonstrated the usefulness of newly established stereochemical preference rules governing i,i+3 stapling. Extended studies on $R_{i,i+3}S$ stapling and multiple stapling approaches are underway. This new stapling strategy represents an additional asset in our toolbox for the rational design of stapled peptides that hold promise as a new therapeutic modality to modulate the functions of macromolecules currently considered "undruggable".

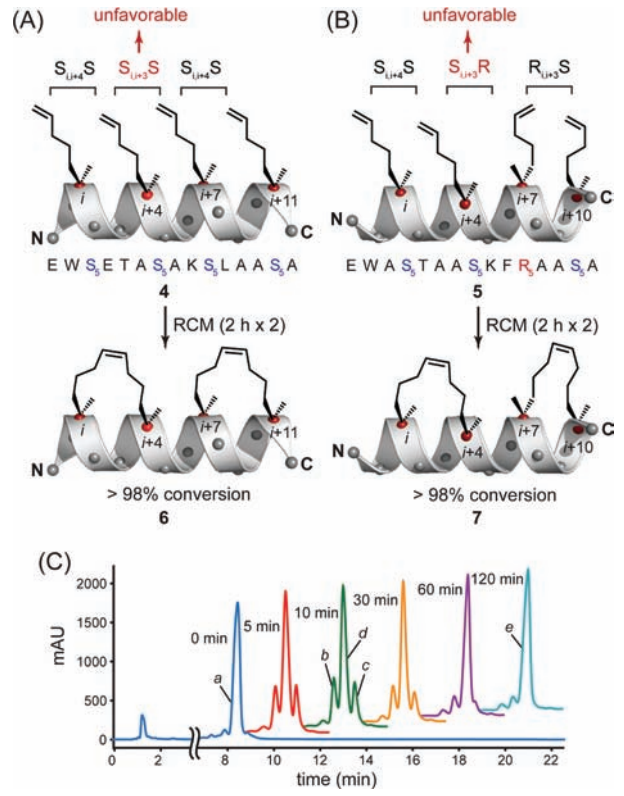


Figure 5. (A and B) Schemetic presentation of tandem ring-closing metathesis reactions of tetraolefinic peptide substrates **4** and **5** and their double-RCM products. (C) Time-course monitoring of double RCM reaction of peptide **8**, $\boldsymbol{S}_5$AAA$\boldsymbol{S}_5$AA$\boldsymbol{S}_5$AAA$\boldsymbol{S}_5$A. Only two single-RCM intermediates (b and c) were observed. The subtrate (a) and its double-RCM product (e) were eluted together (d).

Acknowledgment. We thank Tom Grossmann (Harvard University) for assistance with MALDI TOF/TOF analysis. This research was supported by the Harvard & Dana-Farber Program in Cancer Chemical Biology and an NSF graduate research fellowship (to P.S.K.).

Supporting Information Available: Synthesis and characterization of peptides. Computational modeling study. Description of circular dichroism, trypsin digestion, and flow cytometry analysis of peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

OL1010449

(11) Tyndall, J. D.; Nall, T.; Fairlie, D. P. *Chem. Rev.* **2005**, *105*, 973–999.

(12) Yamaguchi, H.; Kodama, H.; Osada, S.; Kato, F.; Jelokhani-Niaraki, M.; Kondo, M. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 2269–2272.