An All-Hydrocarbon Cross-Linking System for Enhancing the Helicity and Metabolic Stability of Peptides

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Peptides that bind macromolecular receptors in an extended conformation can often be converted to mimetics that retain binding but have improved protease resistance and membrane permeability.[1] However, peptides that must fold upon themselves in order to bind a receptor have proven difficult to improve by similar approaches, because of their larger size and the difficulty of mimicking functionality presented on a complex folded molecular surface. One such folded peptide structure that participates widely in biomolecular recognition events is the α-helix.[2,3] Most peptides that bind their receptors in an α-helical conformation have little helical structure when free in solution. Stabilizing the helical form of such peptides is thus expected to favor receptor binding by virtue of preorganization. Furthermore, the intramolecular hydrogen bonding associated with helix formation reduces the exposure of the polar amide backbone, thereby reducing the barrier to membrane penetration and increasing the resistance to protease cleavage.

A number of approaches for covalent helix-stabilization have been reported,[4] but most involve cross-links that are both polar and pharmacologically labile, such as disulfides[5] and lactam bridges.[6,7] An important conceptual advance on this front is the development by Grubbs and co-workers of chemistry for olefinic cross-linking of helices through *O*-allyl serine residues located on adjacent helical turns, via ruthenium-catalyzed ring closing metathesis (RCM).[8] The particular cross-links analyzed in that study, however, showed no evidence of enhancing helical stability, highlighting the difficulty of this problem from a design standpoint. Here we have taken an alternate metathesis-based approach, namely to screen multiple configurations of all-hydrocarbon cross-links differing in position of attachment, stereochemistry, and cross-linker length. Where some configurations impart significant helix-stabilization, others actually destabilize the helix. We show that stabilizing an α-helix in this way leads to markedly increased resistance to proteolysis.

The actual structure of cross-links positioned on one face of an α-helix is very dependent upon the stereochemistry at the attachment points (Figure 1). We therefore designed unnatural amino acids **1** having either *R* or *S* stereochemistry at the α-carbon and bearing alkyl tethers of various lengths (Figure 1). To avoid the intrinsic helix-destabilizing effect of D-configured amino acids while capitalizing on the helix-stabilizing effect of α,α-disubstituted amino acids we introduced an α-methyl group into **1**. We incorporated these synthetic amino acids across either one or two turns (*i* and $i + 4$, or $i + 7$ position, respectively; Figure 1) of the C-peptide sequence from RNAse A;[9] this particular peptide was chosen because it exhibits partial helicity in water, allowing us to observe both increases and decreases in helical content owing to modifications.[10]

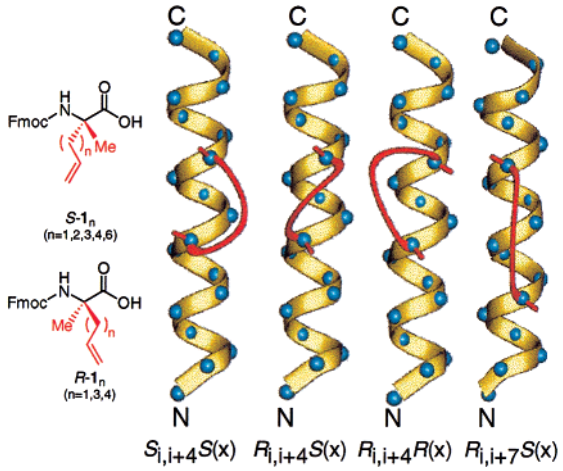

Figure 1. Strategy for stabilizing α-helices through an all-hydrocarbon cross-linking system. The key components of the system are α-methylated amino acids **1**, bearing olefinic side-chains of varying length and configured with either *R* or *S* stereochemistry. These are incorporated into peptides at the *i* and either $i + 4$ or $i + 7$ position, and then connected via olefin metathesis to cross-link one or two turns, respectively, of the helix. The overall side-chain length of $\mathbf{1} = n + 2$, and of the cross-links $= n + n + 2$. The nomenclature $R_{i,i+7}S(11)$ refers to a peptide with an *R* and an *S* configurated amino acid at positions "*i*", and "$i + 7$" respectively, and 11 carbons in the metathesized cross-link.

None of the peptides in the $R_{i,i+4}S(x)$ series ($x = 5, 6, 7$) underwent metathesis to any measurable extent. In the $R_{i,i+4}R(x)$ series, the peptide having a 6-carbon cross-link ($x = 6$) failed to metathesize, but that having a 7-carbon cross-link ($x = 7$) formed to the extent of 17%, and the metathesis reaction leading to the 8-carbon cross-linked peptide ($x = 8$) went to completion (>98%) (Table 1). In the $S_{i,i+4}S(x)$ series, the shortest member ($x = 6$) again failed to undergo RCM, but the longer versions, $x = 7$ and 8, underwent 68% and >98% conversion, respectively. In the $R_{i,i+7}S(x)$ series the cross-links were again formed with increasing efficiently as they became longer ($x = 8$, <5%; $x = 9$, 51%; 10, 77%; 11, >98%; 12, >98%). Two general trends are evident from these reactions. First, the conversions by RCM increase as a function of increasing ring size in the macrocyclic cross-link. Indeed, the 34-membered macrocycle in $S_{i,i+7}R(12)$ is formed rapidly and efficiently, despite being one of the largest macrocycles closed by RCM to date.[11] Second, small changes in ring

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(1) Gante, J. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1699−1720.
(2) Sattler, M.; Liang, H.; Nettesheim, D.; Meadows, R. P.; Harlan, J. E.; Eberstadt, M.; Yoon, H. S.; Shuker, S. B.; Chang, B. S.; Minn, A. J.; Thompson, C. B.; Fesik, S. W. *Science* **1997**, *275*, 983−6.
(3) Kussie, P. H.; Gorina, S.; Marechal, V.; Elenbaas, B.; Moreau, J.; Levine, A. J.; Pavletich, N. P. *Science* **1996**, *274*, 948−53.
(4) Andrews, M. J. I.; Tabor, A. B. *Tetrahedron* **1999**, *55*, 11711−11743.
(5) Jackson, D. Y.; King, D. S.; Chmielewski, J.; Singh, S.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 9391−9392.
(6) Phelan, J. C.; Skelton, N. J.; Braisted, A. C.; McDowell, R. S. *J. Am. Chem. Soc.* **1997**, *119*, 455−460.
(7) Bracken, C.; Gulyas, J.; Taylor, J. W.; Baum, J. *J. Am. Chem. Soc.* **1994**, *116*, 6431−6432.
(8) Blackwell, H. E.; Grubbs, R. H. *Angew. Chem., Int. Ed.* **1998**, *37*, 3281−3284.
(9) Bierzynski, A.; Kim, P. S.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2470−2474.
(10) The wild-type peptide has the sequence: Ac-EWAETAAAKFLAAHA-NH_2. The peptides synthesized in the $R_{i,i+7}S(x)$ series have the general sequence: Ac-EWAEyAAAKFLzAHA-NH_2 where (*y*, z) were substituted with the unnatural amino acid pairs (R-**1**$_3$, S-**1**$_3$), (R-**1**$_3$, S-**1**$_4$), (R-**1**$_4$, S-**1**$_4$), (R-**1**$_3$, S-**1**$_6$), and (R-**1**$_4$, S-**1**$_6$) for the peptides $R_{i,i+7}S(8)$, $R_{i,i+7}S(9)$, $R_{i,i+7}S(10)$, $R_{i,i+7}S(11)$, and $R_{i,i+7}S(12)$, respectively. The peptides synthesized in the $S_{i,i+4}S(x)$, $R_{i,i+4}R(x)$, and $R_{i,i+4}S(x)$ series have the general sequence: Ac-EWAETAAyKFLzAHA-NH_2 where (*y*, z) were substituted with the unnatural amino acid pairs (S-**1**$_1$, S-**1**$_3$), (S-**1**$_1$, S-**1**$_4$), (S-**1**$_3$, S-**1**$_3$), (R-**1**$_1$, R-**1**$_3$), (R-**1**$_1$, R-**1**$_4$), (R-**1**$_3$, R-**1**$_3$), (R-**1**$_1$, S-**1**$_2$), (R-**1**$_1$, S-**1**$_3$), and (R-**1**$_1$, S-**1**$_4$) for the peptides $S_{i,i+4}S(6)$, $S_{i,i+4}S(7)$, $S_{i,i+4}S(8)$, $R_{i,i+4}R(6)$, $R_{i,i+4}R(7)$, $R_{i,i+4}R(8)$, $R_{i,i+4}S(5)$, $R_{i,i+4}S(6)$, and $R_{i,i+4}S(7)$, respectively. Ac and NH_2 represent N-terminal acetylation and a C-terminal primary amide, respectively.
(11) Clark, T. D.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1995**, *117*, 12364−12365.

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Table 1. Percent Conversions for a 2 h Metathesis Reaction Performed on Solid Support with 10 mM Grubbs Catalyst in 1,2-Dichloroethane

cross-link	% conversion[a]	cross-link	% conversion[a]	cross-link	% conversion[a]
$R_{i,i+7}S(8)$	0	$S_{i,i+4}S(6)$	0	$R_{i,i+4}R(6)$	0
$R_{i,i+7}S(9)$	51	$S_{i,i+4}S(7)$	68	$R_{i,i+4}R(7)$	17
$R_{i,i+7}S(10)$	77	$S_{i,i+4}S(8)$	>98	$R_{i,i+4}R(8)$	>98
$R_{i,i+7}S(11)$	>98				
$R_{i,i+7}S(12)$	>98				

[a] Percent conversion product/(product + starting material) as determined by reverse phase HPLC.

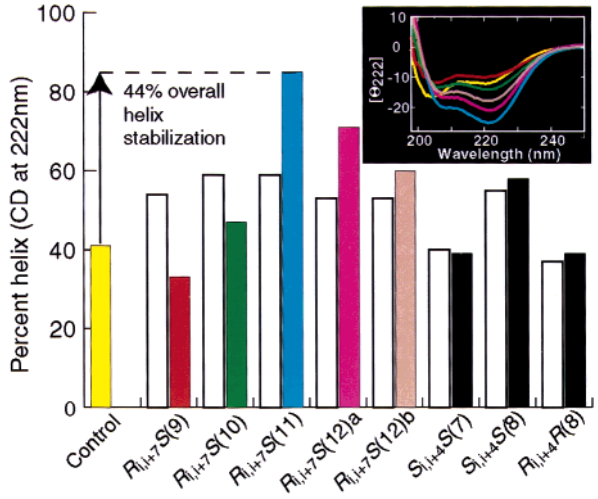

Figure 2. In the $R_{i,i+7}S$ series α-methyl amino acids increase helical structure by ~15%. Inducing a cross-link using olefin metathesis has an effect on helicity that depends on the cross-link length. $R_{i,i+7}S(11)$ is the best helix stabilizer. The uncertainties in these measurements are no greater than ±5%. (Open bars, unmetathesized; solid bars, metathesized.) Inset: CD spectra for $R_{i,i+7}S(x)$ series, color matched to histogram. [θ_{222}] units are ($\times 10^3$ deg cm^2 dmol^{-1}).

size can cause dramatic effects on the efficiency of cross-linking; for example, the 30-membered macrocycle in $R_{i,i+7}S(8)$ fails to form appreciably, whereas the 31-membered ring of $R_{i,i+7}S(9)$ forms to the extent of 50%. We believe both effects can be explained by templating of the RCM reaction through helix induction of the unmetathesized precursor peptides on the solid support in the solvent dichloroethane. According to this explanation, tethers that are too short to span the gap along the face of the templating helix are not metathesized efficiently.

To determine the effect of olefinic cross-linking on the helical propensity of the peptides, we used circular dichroism to provide a quantitative measure of helical content[12] (Figure 2). As a benchmark, the control unmodified RNase A peptide is ~40% α-helical in water containing 0.1% trifluoroacetic acid at 4 °C. All peptides that underwent RCM to the extent of ~50% or more were measured in both un-cross-linked and cross-linked forms. In most cases, and as expected,[13] inclusion of the two α,α-disubstituted amino acids into the peptide increased its helical content with respect to the unmodified control. In the i, $i + 4$ peptide series, cross-linking neither stabilized nor destabilized the helix with respect to the corresponding un-cross-linked modified peptide; the reasons for this effect are not apparent from inspection of models. RCM cross-linking of the modified i, $i + 7$ peptides produced effects ranging from 21% destabilization to significant stabilization of α-helical structure. Specifically, the helical content of the $R_{i,i+7}S(9)$ and (10) peptides decreased by 21 and 12% following RCM, whereas that of the $R_{i,i+7}S(11)$ peptide increased by 26%. Cross-linking of the $R_{i,i+7}S(12)$ peptide produced cis and trans double bond isomers one of which was more stabilizing than the other (18% vs 7%).[14] The overall trends seen in the $R_{i,i+7}S$ series can be rationalized as follows: cross-links of 9 and 10 carbons are too short to permit the formation of an unstrained helix, a cross-link of 11 carbons provides the optimal fit, and that of 12 carbons is longer than necessary and therefore does not constrain the helix as effectively as the 11-carbon cross-link. Importantly, the introduction and cross-linking of two modified amino acids as an 11-atom hydrocarbon chain stabilizes the helix by 44% when compared to an unmodified control peptide, an extent that is comparable to the best seen with other cross-linking systems.[6] As determined by sedimentation equilibrium, all of the peptides were monomeric under the conditions of the circular dichroism experiments, indicating that the helix induction is not due to aggregation.[15] Reduction of the olefins using transfer hydrogenation on solid support has no effect on the helical content as determined by CD (refer to Supporting Information).

Table 2

cross-link	cleavage rate constant (M^{-1} s^{-1}) unmetathesized	cleavage rate constant (M^{-1} s^{-1}) metathesized and hydrogenated
control	2.39	
$R_{i,i+7}S(9)$		0.37
$R_{i,i+7}S(10)$		0.34
$R_{i,i+7}S(11)$	0.50	0.058
$R_{i,i+7}S(12)$		0.12

Cleavage by proteases is one of the main pathways for inactivation of peptides in a biological setting. As all known proteases bind their substrates in an extended rather than helical conformation, inducing helical structure is expected to confer protease stability, leading to increased potency in vivo. As an in vitro test of this concept, we took advantage of the fact that the cross-linked stretch of our peptides contains a lysine residue, which can be targeted by the protease trypsin. As expected, the unmodified control peptide is highly susceptible to cleavage by trypsin ($k = 2.38$ M^{-1} s^{-1}) (Table 2). Incorporation of the two unnatural amino acids at the i and $i + 7$ positions, without cross-linking, decreases the cleavage rate by almost 5-fold, consistent with the helix-stabilizing effects noted above. Metathesis and subsequent hydrogenation produced a further stabilization, the magnitude of which is markedly dependent on the length of the cross-link. The extent of this cross-link-dependent stabilization precisely mirrored the extent of helix induction, being most pronounced for the $R_{i,i+7}S(11)$ peptide. Overall, the incorporation of the cross-link unit stabilizes this peptide toward trypsin digestion by 41-fold.

The major goal of this research program is to improve the pharmacological properties of α-helical peptides through synthetic modification. The present report is an important first step toward that end. Here we show that an all-hydrocarbon cross-linking system can greatly increase the helical propensity and metabolic stability of peptides.

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Supporting Information Available: Synthesis, ^{1}H NMR, ^{13}C NMR, and HRMS of unnatural amino acids. Synthesis, metathesis, and characterization of peptides. Description of the transfer hydrogenation procedure. Description of circular dichroism, analytical centrifugation, and trypsin digestion of peptides (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108−4116.
(13) Kaul, R.; Balaram, P. *Bioorg. Med. Chem.* **1999**, *7*, 105−117.

(14) C18 reverse phase HPLC of $R_{i,i+7}S(12)$ shows two separable product peaks (a) and (b) that have identical molecular masses (1760 Da). Peak (a) has an NMR peak at ($\delta = 5.49$) and peak (b) has an NMR peak at ($\delta = 5.52$). Hydrogenated $R_{i,i+7}S(12)$ is a single peak by reverse phase C18 HPLC.
(15) See "Analytical Centrifugation" in Supporting Information.