A "Cross-Stitched" Peptide with Improved Helicity and Proteolytic Stability

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A new computational approach to obtain quantitative energy profiles for helix folding was used in the design of orthogonal hydrocarbon and lactam bicyclic peptides. The proteolytically stable, "cross-stitched" peptide SRC2-BCP1 shows nanomolar affinity for estrogen receptor α and x-ray crystallography confirms a helical binding pose.

Many protein-protein interactions occur when a helix binds to a groove, and these helix-groove interactions may be inhibited using constrained peptides that adopt a helical conformation. An example of these are "stapled" peptides, which are constrained by virtue of two appropriately placed side chains joined together by an olefin or other linkages. Extending this approach to bicyclic peptides has emerged as a strategy to further enhance helicity and proteolytic stability of constrained α-helical peptides. Verdine and coworkers[1] have described "stitched" peptides that contain two tandem hydrocarbon staples to enhance helicity, and multiple groups have reported peptides with enhanced proteolytic stability by spacing two hydrocarbon staples near each end of the peptide backbone.[2-4] While it is has been shown that different cyclizing constraints can imbue distinct properties,[5] combining orthogonal stapling strategies has remained relatively unexplored. Although, Fairlie and coworkers recently described potential advantages for lactam and olefin staples at the ends of a helical peptide targeting Bcl2A1 and Mcl-1.[6]

In our work to inhibit estrogen receptor/coactivator interactions, we saw an opportunity to prepare orthogonally stapled peptides and to study their potential benefits on helicity, stability, and affinity. The estrogen receptor is a clinically validated target in estrogen receptor-positive breast cancer, but, because of issues of resistance to current endocrine therapies, new mechanisms of antagonizing the estrogen receptor are needed. A proposed alternative mechanism for blocking the action of estrogen receptor involves using small molecules and constrained helical peptides to directly inhibit binding of the coactivator LXXLL motif to estrogen receptor.[7-16]

Previously, we described a stapled peptide, SRC2-SP4, that inhibits the estrogen receptor/coactivator interaction. In addition to the olefin staple, SRC2-SP4 contains an i - i+4 salt bridge (Arg692-Asp696).[17] We noticed an increase in helicity, binding affinity, and proteolytic stability for SRC2-SP4 vs. a homologous stapled peptide, PFE-SP2,[11] that lacked a salt bridge because of a single amino acid change (Arg692⟶Asn). MD simulations of these peptides in solution suggested that the formation of a salt-bridge enhanced helical stability. To verify this observation, we ran prolonged simulations of SRC2-SP4 in solution and observed that breakage and formation of hydrogen bonds between the arginine and aspartate side chains were closely associated with changes in peptide helicity (Figure 1A). PFE-SP2, which was unable to form this stabilizing salt-bridge, unfolded during the course of the simulation (Figure S1), in agreement with our experimental observations. There is a rich literature on helical stabilization mediated by salt bridges,[18, 19] which well supports our observations. In this work, we describe a strategy to introduce two orthogonal staples (lactam and olefin) with differing electrostatic properties. Our design principles were guided by molecular dynamics (MD) simulations and x-ray crystallography, and, gratifyingly, they have yielded novel peptides demonstrating high helicity and stability.

To obtain a quantitative analysis of helical stability, we applied a new computational approach that profiles the potential of mean force for peptide unfolding by describing α-helicity as a summation of distance and dihedral measurements applied to Bias Exchange Umbrella Sampling (BEUS).[20] This method yielded free energy profiles to quantitatively describe

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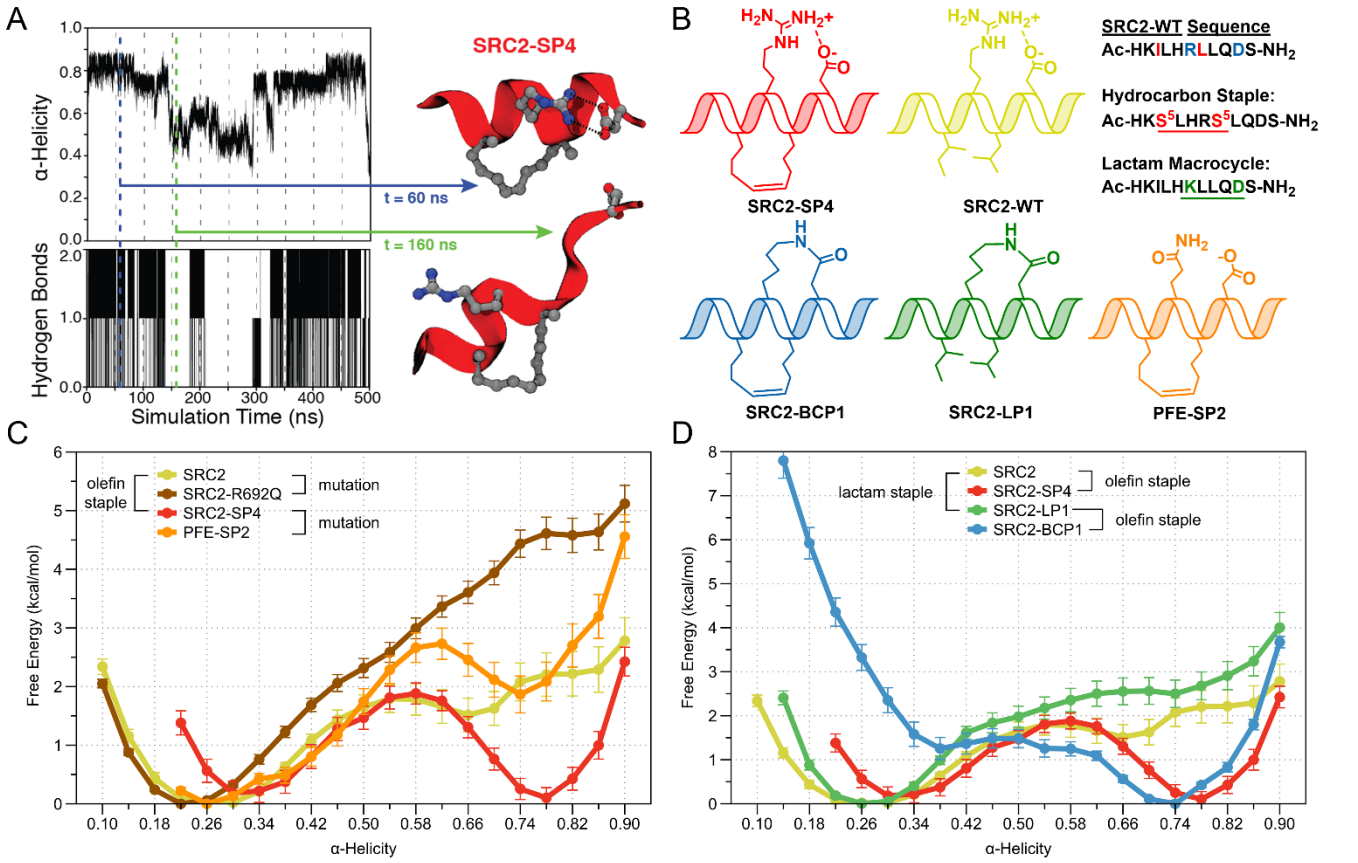
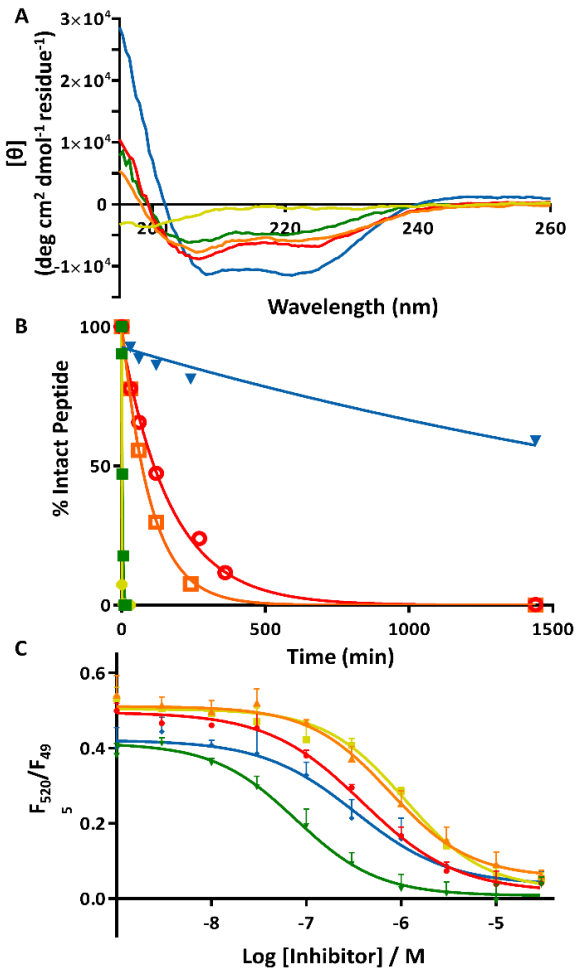


Figure 1. Computationally derived free energy profiles for peptide folding. A) Long-timescale simulations of a hydrocarbon-stapled peptide representing coactivator protein demonstrated that α-helicity correlated with presence or absence of a salt bridge between Arg692 and Asp696. B) Several additional peptides were designed to reinforce the salt bridge interaction with a covalent linkage, both in the presence and absence of the hydrocarbon staple. C) Computationally derived potential of mean force describing the relative free energy across a range of helical states indicated that the Arg692-Asp696 salt bridge provided ~2 kcal/mol of stabilizing energy. D) In silico models that install a covalent replacement for the salt bridge via an amide linkage alone were not predicted to increase helicity; however, combining the hydrocarbon and amide linkages to yield a "stitched" peptide with predicted highly stable, highly helical conformations

helical folding states for wild-type or macrocyclic peptides (Figure 1C-D). The relative free energy profile for SRC2-WT (yellow) showed a distinct minimum at <0.30 helicity, whereas incorporating an olefin-based staple, SRC2-SP4 (red), introduced a more helical character with an additional minimum at ~0.75 helicity. The increase in helicity of SRC2-SP4 relative to PFE-SP2 was associated with a ca. 2 kcal/mol reduction in free energy at high helicity (Figure 1C, red vs. orange), a finding which can be attributed to the "pseudo-stapled" nature of the Arg692-Asp696 salt-bridge. This was further supported by the ca. 2 kcal/mol reduction in free energy at high helicity seen between the comparable unstapled peptides SRC2-WT (yellow) and SRC2-R692Q (brown). Based on these data, we envisioned preparing a bicyclic peptide which would maintain the hydrocarbon staple and covalently replicate the Arg692-Asp696 salt-bridge. Computational analysis of such peptides predicted that converting the salt bridge to a covalent lactam linkage alone would not produce significant stabilization (Figure 1D, yellow vs. green); however, coupling the previously described SRC-SP4 olefin staple with an additional lactam staple to enforce the 692-696 interaction in a covalent manner should substantially increase the α-helical content of the peptide (Figure 1D, red vs. blue).

Guided by the computational results, we synthetically replaced the salt bridge with an amide-based covalent linkage. Critically, the conditions of lactam formation were orthogonal to olefin staple formation, which allowed us to avoid issues of selectivity. This approach also allowed us to replace the hydrophobic residues Ile689 and Leu693 with a hydrocarbon staple while replacing the solvent-exposed salt bridge with a relatively polar amide linkage. Moreover, Fairlie and coworkers recently showed that lactams are uniquely suited to ensuring high levels of helicity in short peptide sequences.[21] Using this orthogonal stapling approach, bicycle SRC2-BCP1 was prepared using solid phase peptide synthesis (Scheme S1). The orthogonal installation of the constraints proceeded sequentially, to near completion, and with no observed cross-reactivity.

To experimentally determine the secondary structure of the bicyclic peptides, we measured molar ellipticity by circular dichroism at several temperatures and converted $[\theta]_{222}$ readings to percent helicity (Figure 2 and S8-13) using an equation previously described by Luo and Baldwin[22] Gratifyingly, experimental measurements closely matched our computational analysis, with bicyclic peptide SRC2-BCP1 showing the highest helicity (53%), followed by hydrocarbon-



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Peptide	Olefin	Lactam	% α-Helicity at 37 °C	$t_{1/2}$ min (95% CI)	IC_{50} μM (95% CI)
SRC2-BCP1	+	+	53	2100 (1400-4300)	0.33 (0.18-0.59)
SRC2-SP4	+	-	33	120 (100-140)	0.39 (0.29-0.53)
PFE-SP2	+	-	29	69 (63-77)	0.76 (0.54-1.1)
SRC2-LP1	-	+	23	2.1 (1.3-4.3)	0.078 (0.057-0.110)
SRC2-WT	-	-	4	0.27 (0.26-0.28)	1.1 (0.80-1.5)

Figure 2. A) Circular diochroism analysis of 50 μM peptide at 37 °C. B) Proteolytic stability of peptides treated with proteinase K. C) Inhibition of estrogen receptor steroid receptor coactivator interaction measured by TR-FRET. D) The % α-helicity was calculated using $[\theta]_{222}$ values, the proteolytic $t_{1/2}$ for peptides were found using a non-linear one phase decay fit, and the IC_{50} values were found using a TR-FRET assay.

stapled peptides PFE-SP2 and SRC2-SP4 (29% and 33%) and lactam-cyclized peptide SRC2-LP1 (23%). The unconstrained SRC2-WT peptide displayed an ellipticity curve indicative of a disordered structure, and this was the only point of departure from the BEUS calculations, which predicted that the helicity of the wild-type peptide should be similar to lactam SRC2-LP1. One possible explanation for this could be that the relative contributions in the α-helicity reaction coordinate, a collective variable comprising hydrogen bonding and dihedral angle terms, may be suboptimal for reproducing the spectroscopically measured helicity values at the low end of the range. Given that this discrepancy occurred in the low-helicity case, rather than in the desired high helicity cases, the MD simulations successfully informed our staple design to prospectively yield highly stable constrained peptides.

To measure the stability of this peptide series, we used proteinase K, a serine protease with a broad spectrum of endopeptidase activity.[23] Mass spectrometric analysis of SRC-WT subjected to proteinase K showed cleavage sites at the C-terminal amide bonds of leucine, arginine, and glutamine (Figure S7 and Table S2). The unconstrained peptide was rapidly degraded under experimental conditions ($t_{1/2}$ = 0.27 min), whereas SRC2-BCP1 displayed a half-life of ~2,000 minutes, improved by nearly four orders of magnitude. For singly constrained peptides, the hydrocarbon staple provided a higher level of proteolytic stability relative to the lactam bridge. The proteolytic stability of this peptide series was correlated with both %-helicity and the computational energy barrier for peptide unfolding at low helicity scores, suggesting that reinforcing a helical conformation precluded access to peptide bonds from the active site of proteinase K. Because %-helicity tracked with proteolytic stability, our above-mentioned BEUS method may be able to predict both helicity and relative proteolytic stability within a series.

We used a TR-FRET assay to measure the peptides' ability to inhibit coactivator recruitment to estrogen receptor (Figures 2C-D and Table S3).[24] All constrained peptides had higher affinity than SRC2-WT (1.1 μM) with the most active peptide being lactam SRC2-LP1 (78 nM, 14-fold vs. WT). Combining the hydrocarbon staple with the lactam to create bicycle SRC2-BCP1 gave a peptide that was intermediate in affinity between lactam SRC2-LP1 and hydrocarbon stapled peptide SRC2-SP4, even though SRC2-BCP1 showed higher helicity. The ordering of IC_{50} values (SRC2-LP1 < SRC2-BCP1< SRC2-SP4 < SRC2-WT) suggested that stabilizing the helical peptide with a constraint had a positive impact on binding affinity, but that replacing Ile689 and Leu693 with a hydrocarbon staple was slightly deleterious for binding affinity. Phillips et al.[11] previously reported a stapled peptide that had a hydrocarbon staple in place of the lactam of SRC2-LP1. Its binding affinity was poor (>15 μM), suggesting that our approach of installing a more polar staple may be advantageous at this solvent-exposed site.

To further elucidate the molecular factors at play upon binding, we solved crystal structures of bicycle SRC2-BCP1 and lactam SRC2-LP1 bound to the ligand-binding domain of estrogen receptor α (Figures 3 and S14, and Table S4). In good agreement with our computational predictions, each peptide bound in a helical conformation between charge clamp residues Lys362 and Glu542, similar to our previously reported structure of SRC2-SP4. The hydrocarbon staple of SRC2-BCP1 supplanted Ile689 and Leu693 of the ILXXLL motif, whereas the lactam staple supplanted non-interacting solvent-exposed residues Arg692 and Asp696. The differences in IC_{50} were only 4-fold, so it might be difficult to ascertain from the structure why SRC2-LP1 was more potent than SRC2-BCP1; however, it is possible that the reason that replacement of Ile689 and Leu693 with a hydrocarbon staple showed slightly lower affinity was because of sub-optimal hydrophobic interactions formed between the staple and the surface of the estrogen receptor (Figure 3). There are several examples of constrained peptides that have a

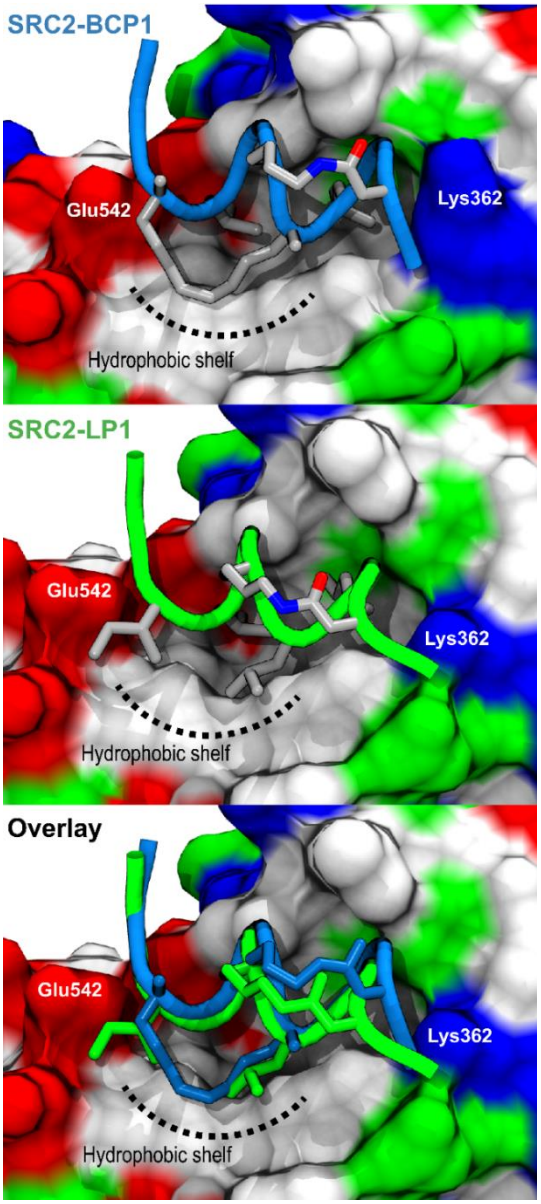

Figure 3. X-ray co-crystal structures of bicyclic peptide SRC-BCP1 (top, PDB 5WGQ) and stapled cyclic peptide SRC2-LP1 (middle, PDB 5GWD) bound to the coactivator binding cleft of the estrogen receptor α (surface: red = acidic, blue = basic, white = nonpolar, green = polar). The peptide backbone forms a helical conformation oriented between Lys 362 and Glu542. Overlaying the two structures (bottom) highlights the expanded hydrophobic contacts between ILe689 and Leu693 with the hydrophobic shelf, relative to the unbranched hydrocarbon staple, and a different conformation of the lactam linking Arg692 and Asp696.

constraint that directly interacts with the receptor[25-27], and the results presented here may be most useful for these types of peptides. Our data suggest that a non-interacting lactam (SRC2-LP1) is most effective in enhancing binding affinity, while an interacting hydrocarbon staple (SRC2-SP4 or SRC2-BCP1) enhances proteolytic and helical stability.

In conclusion, we have developed a novel orthogonal stapling strategy to create an estrogen receptor-binding cross-stitched peptide that shows high helicity and proteolytic stability while retaining nanomolar binding affinity. Our work was informed by both computation and structural biology: a powerful biased exchange umbrella sampling approach that can be used in a prospective manner to predict helicity of short peptide sequences, and x-ray crystal structures of peptides bound to estrogen receptor confirmed our predictions of binding poses. Recent interest in stabilizing helical peptides has led to an increased arsenal of stapling chemistries.[5, 28-29] Given the straightforward methods using commercially available amino acids to prepare these peptides, an array of orthogonal bicyclic strategies could be readily applied to estrogen receptor and other protein-protein interactions.

Conflicts of interest

There are no conflicts to declare.

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Peptide “cross-stitching” maintains binding affinity and can enhance helical and proteolytic stability.