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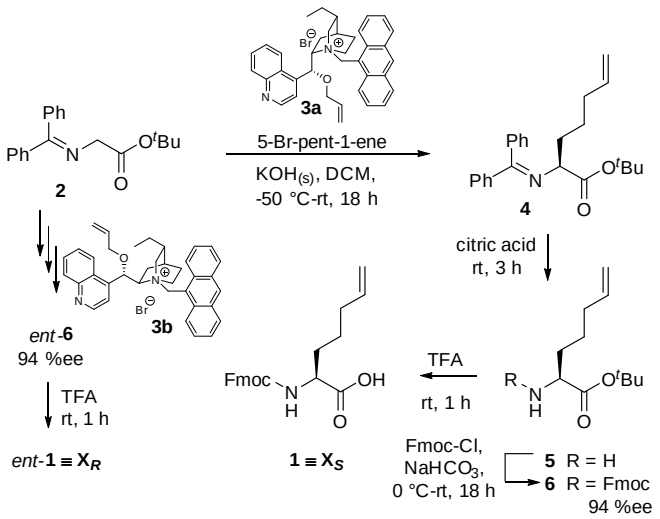
Mix and Match: Mono-substituted Hydrocarbon Diastereomer Combinations Reveal Stapled Peptides with High Structural Fidelity

Fergus S. McWhinnie,[a],[b] Kristel Sepp,[a] Charlotte Wilson,[a] Tilo Kunath,[b] Ted R. Hupp,[c] Terry S. Baker,[d] Douglas R. Houston[e] and Alison N. Hulme*,[a]

Abstract: Modified peptides such as stapled peptides, which replicate the structure of α-helical protein segments, represent a potential therapeutic advance. However, the 3D solution structure of these stapled peptides is rarely explored beyond the acquisition of CD data to quantify bulk peptide helicity; the detailed backbone structure which underlies this is typically undefined. Diastereomeric stapled peptides based on helical sections of three proteins (αSyn, Cks1 and CK1α) were generated; their overall helicity was quantified by CD; and the most helical peptide from each series was selected for structural analysis. Solution-phase models for the optimised peptides were generated using NMR-derived restraints and a modified CHARMM22 force field. Comparing these models with PDB structures allowed deviation between the stapled peptides and critical helical regions to be evaluated. These studies demonstrate that CD alone is not sufficient to assess the structural fidelity of a stapled peptide.

The development of biorthogonal 'staples' which confer stable, α-helical secondary structure on peptides has reinvigorated research into the field of proteomimetics.[1] Short helical sequences are frequently found at protein-protein interfaces, suggesting that these interactions may be effectively modulated by stapled peptides.[2] Typically, staples bridge from amino acid residues *i* to *i* + 3, *i* + 4 and *i* + 7, and can be formed using a variety of different chemistries.[3] Stapled peptides are generally more cell permeable and resistant to proteolysis than their native counterparts.[4] With the synthesis of all-hydrocarbon α,α'-di-substituted staples almost routine using recently-established protocols,[5] stapled peptides have already been validated as important tools in medicinal chemistry and their therapeutic potential is currently being explored.[6] However, anecdotal evidence suggests that following published protocols for amino acid substitution can fail to produce helical, bioactive peptides, even with extensive refinement of staple position.[7] Furthermore, studies in this field often lack robust structural comparison of the engineered helical peptides to their native protein, which might provide vital clues where only modest bioactivity is observed.[8]

Peptide fragments amenable to a stapling strategy were extracted from three disease-relevant proteins which have ill-defined functions *in vivo*: a 14-mer from the protein alpha-synuclein (αSyn) key to the pathogenesis of Parkinson's disease (Figure 1A);[9] a 12-mer from the accessory protein cyclin-dependent kinase regulatory subunit 1 (Cks1) part of the E3 ubiquitin ligase SCF^{Skp2}, implicated in cancer (Figure 1B);[10] and a 10-mer from casein kinase 1 alpha (CK1α) a serine/threonine kinase involved in phosphorylation of the oncogenic protein MDM2 (Figure 1C).[11] Analysis of published biological data for each these targets enabled the identification of non-essential residues which could be substituted without perturbing functional activity.[12] Whilst screening using the *de novo* secondary structure predictor PEP-FOLD[13] allowed the extent to which these residues conferred helical peptide structure to be assessed. Using the results of these biological and conformational analyses, appropriate *i* to *i* + 4 stapling sites for a standard 8-atom linker were identified for each peptide.[3,14b]



Scheme 1. Robust, facile and highly stereospecific monomer production.

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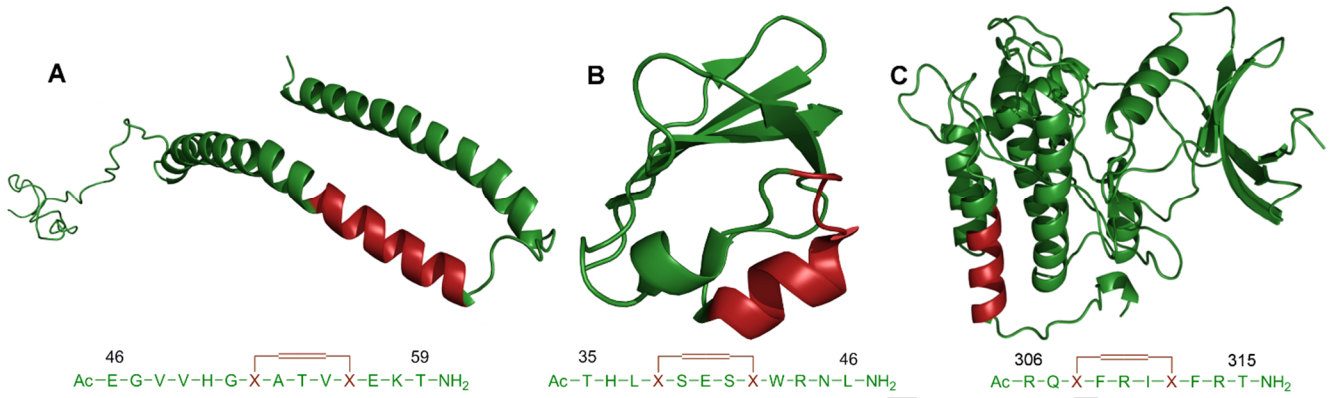
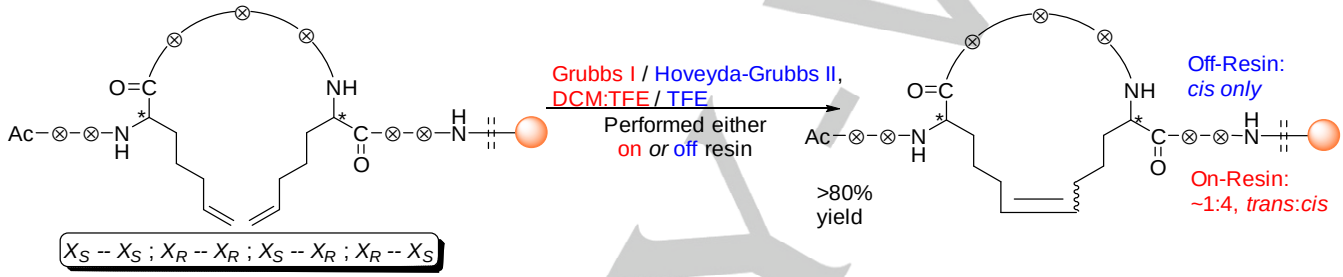


Figure 1. Proteins featured in the stapling campaign. Regions shown in red are those chosen for staple incorporation. (**A**) αSyn shown in the horse-shoe conformation which results from its interaction with small unilaminar vesicles (PDB ID: 1XQ8). (**B**) Cks1 from its crystal structure co-bound with the E3 ubiquitin ligase SCF^{Skp2} (PDB ID: 2AST). (**C**) Homology model for CK1α based on the protein CK1δ (PDB ID: 1CKI_A) produced using SWISS-MODEL.



Scheme 2. Routes to ring closure

An attractive alternative to α,α'-disubstituted ring closing metathesis (RCM) staple precursors is the use of simpler mono-substituted analogues.[14] To generate the most helical stapled peptide analogue of each of the targets using solid phase peptide synthesis (SPPS), ready access to Fmoc-protected precursor X_S (**1**, Scheme 1) and its enantiomer X_R (***ent*-1**) was required. Current syntheses of **1** are lengthy,[15] are not readily applied to the synthesis of ***ent*-1**, and are not always reproducible.[16] Phase-transfer catalyzed (PTC) alkylation of a glycine Schiff base using *pseudo*-enantiomeric pairings of catalysts derived from cinchona alkaloids has been used for the synthesis of a range of non-natural α-amino acids and offers a viable alternative.[17] Glycine Schiff base **2** was alkylated in the presence of cinchona based PTC **3a** at low temperature to give alkene **4** in quantitative yield; PTC **3b** provided the enantiomeric adduct. Treatment of **4** with mild aqueous acid gave the free amine **5** which was Fmoc-protected under basic conditions. The enantiopurity of intermediates **6** (94 %ee), and *ent*-**6** (94 %ee), was established by reference to a racemic standard. Deprotection of the *tert*-butyl ester gave the required monomers **1** and *ent*-**1** in 89% and 87% overall yield respectively.

For each of the three peptide candidates (αSyn, Cks1 and CK1α; Figure 1), all possible diastereomers of staple precursor (X_S,X_S; X_R,X_R; X_S,X_R; X_R,X_S) were incorporated by SPPS at the staple sites identified. Attempted on-resin RCM staple formation with Grubbs I catalyst was unsuccessful in dichloroethane.[18] However, switching to a more helix-promoting solvent mixture (CH_2Cl_2:CF_3CH_2OH)[19] gave excellent conversion for cyclisation (>80% as determined by HPLC following resin micro-cleavage) across the series (Scheme 2). Under these on-resin conditions the stapled peptide was produced as a readily separable mixture of *cis* and *trans* isomers, favoring the *cis* isomer (*cis:trans* typically 80:20).[20] Alternatively, the RCM reaction could be performed in comparable isolated yields on the fully-deprotected, cleaved peptides using the 2nd generation Hoveyda-Grubbs catalyst in trifluoroethanol. These solution phase RCM conditions gave only the *cis* isomer of the staple, however they were not compatible with cysteine or methionine residues.

With the *cis*-stapled peptides in hand we assessed the effect of the stapling stereo-relationships on secondary structure. Circular dichroism in pure water gave only random coil structures. In contrast, in the presence of 25% trifluoroethanol, typically used as a co-solvent to mimic microsolvation effects from protein tertiary structure and known to induce formation of the micelle-bound, helical form of the amphipathic protein αSyn,[21] marked differences in structure were observed between the native and diastereomeric stapled peptides (Figure 2). As expected, both the 14-mer αSyn and 12-mer Cks1 native peptides were poorly helical under these conditions. However, the 10-mer CK1α native peptide gave unexpectedly high helicity. For the αSyn stapled peptides, the X_S,X_R relative configuration was most helical, X_S,X_S

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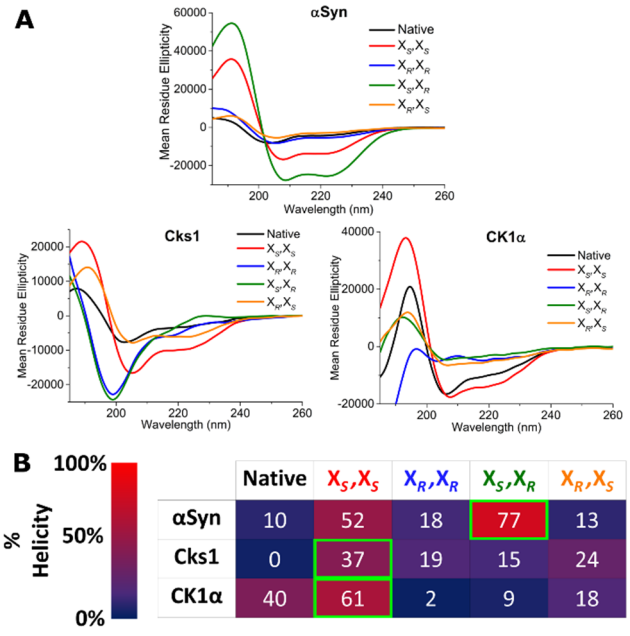

	Native	X_S,X_S	X_R,X_R	X_S,X_R	X_R,X_S
αSyn	10	52	18	77	13
Cks1	0	37	19	15	24
CK1α	40	61	2	9	18

Figure 2. Effect of stapling stereo-relationships on peptide helicity. (**A**) CD spectra of peptide diastereomers in 25% $TFE_{(aq)}$ at 25 ºC for each peptide candidate. (**B**) Heat map of percentage helicity for each peptide calculated using DichroWeb algorithms.[22]

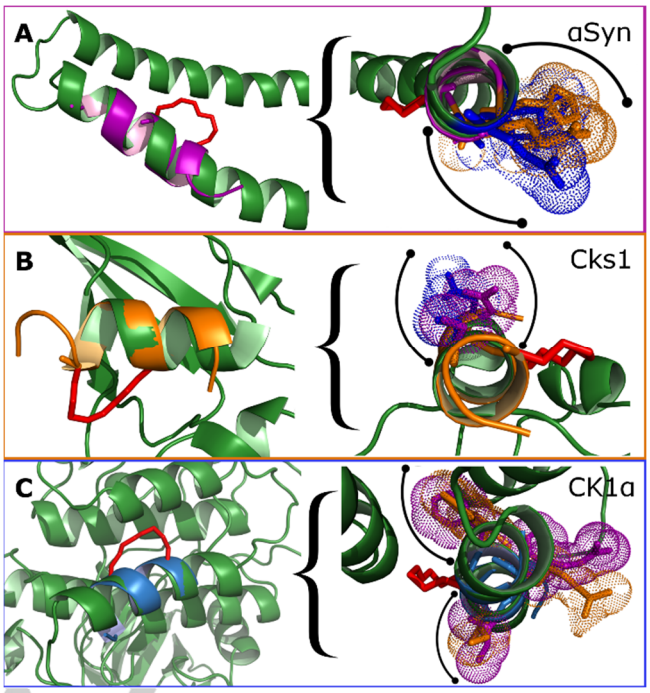

Figure 3. NMR refined solution structures of (**A**) αSyn, (**B**) Cks1 and (**C**) CK1α peptides. Left: the averaged models were backbone aligned with their parent protein and their deviation defined, RMSD (**A**) αSyn 1.3 Å; (**B**) Cks1 0.4 Å; (**C**) CK1α 0.4 Å. Right: side-chains known to be critical to protein bioactivity are indicated by space-filling and do not appear to clash sterically with the peptide staple.

gave moderate helicity, while other configurations gave very little enhancement. More modest helicities were observed for the Cks1 stapled peptides, where in contrast to the αSyn series, the most helical combination was X_S,X_S, while X_S,X_R was least helical. Finally, looking at the stapled peptides from CK1α we found that the X_S,X_S diastereomer was the most helical, whilst both the X_R,X_R and X_S,X_R peptides were more structurally disordered than the native peptide. This empirical synthetic approach, allowed us to select stapled peptides with moderate to good helicity for subsequent NMR and modelling studies.

To acquire an accurate backbone model of these stapled peptides (αSyn, X_S,X_R; Cks1 & CK1α, X_S,X_S), high concentration samples (7 mM) were used in natural abundance ^1H-^{15}N HSQC and COSY which allowed the unambiguous assignation of backbone NH signals. In tandem with NOESY, these spectra allowed the identification of ~100 distance and angle restraints per peptide (SI Figure 2), many arising from classic α-helical relationships. An ensemble of energy minimized backbone structures was produced in Xplor-NIH using a modified CHARMM22 force field to accommodate the mono-substituted staple,[23] in combination with these experimentally determined restraints.[24] Averaging these ensembles showed only modest deviation of peptide backbone configuration between the 10 lowest energy models (RMSD; αSyn 1.4 Å, Cks1 1.0 Å, CK1α 1.3 Å), giving us reasonable confidence in these structures.[25] Computational modelling of helical stapled peptides based on NMR solution data has been accomplished previously, however, these models have not been compared with data for their parent protein structures to confirm the production of an accurate helical mimic.[26] Therefore, NMR-refined solution structures of the helical peptides were compared with the corresponding PDB data to assess backbone homology (Figure 3 (left)).[27] Using full backbone alignment, we found close structural agreement between the stapled peptides and parent proteins.[28] Of note, for the Cks1:Skp2 interaction, the interacting sequence from Cks1 is comprised of 60% helix and 40% loop residues, and the backbone alignment shows that a moderately helical stapled peptide (only 37% by CD analysis) can be highly homologous with a critical helical region of its parent protein (RMSD 0.4 Å over aa's 39-46). These data demonstrate that screening using coarse-grained techniques such as CD alone is not sufficient to assess the potential of a stapled peptide. Moreover, using space filling models we could demonstrate both that the peptides display critical residues in the desired relative orientation with comparatively low RMSD values (SI Table 1 and SI Figure 3),[29,30] and that interference from the unnatural staple in these peptides was minimized (Figure 3 (right)). Thus, this rapid acquisition method produces a reliable backbone representation of these constrained peptides and would provide a structural basis to inform any later observed bioactivity.

In conclusion, this rapid and accessible protocol facilitates the identification of stapled peptides with backbone structures which replicate that of the parent protein with high fidelity. These techniques could be applied to any helical protein target enabling pre-validation of synthesized material before *in vitro* or *in vivo* analysis. We anticipate this approach will enable a deeper understanding of specific protein-protein interactions, and provide a structural rationale for the observed effects of stapled peptides.

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Conflict of interest

The authors declare no competing financial interest.

Keywords: stapled peptides • peptides • ring closing metathesis • NMR solution structure

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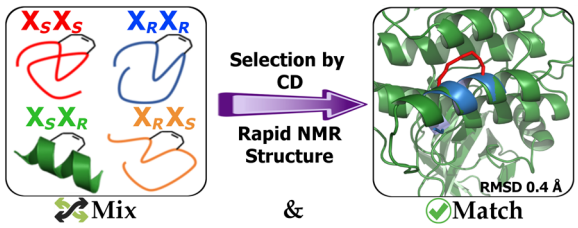

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Mix and Match: Mono-substituted Hydrocarbon Diastereomer Combinations Reveal Stapled Peptides with High Structural Fidelity

Match Point: Using a reliable and scalable monomer synthesis, different diastereomeric combinations of mono-substituted hydrocarbon peptide staples are shown to deliver varied helicities to synthetic peptides. Quantification of helicity by CD, rapid analysis of solution structure by NMR and comparison of the resultant models to published structural data shows the extent to which these peptides mimic their native substrates. This approach will allow a deeper understanding and structural rationale for the effects of stapled peptides.