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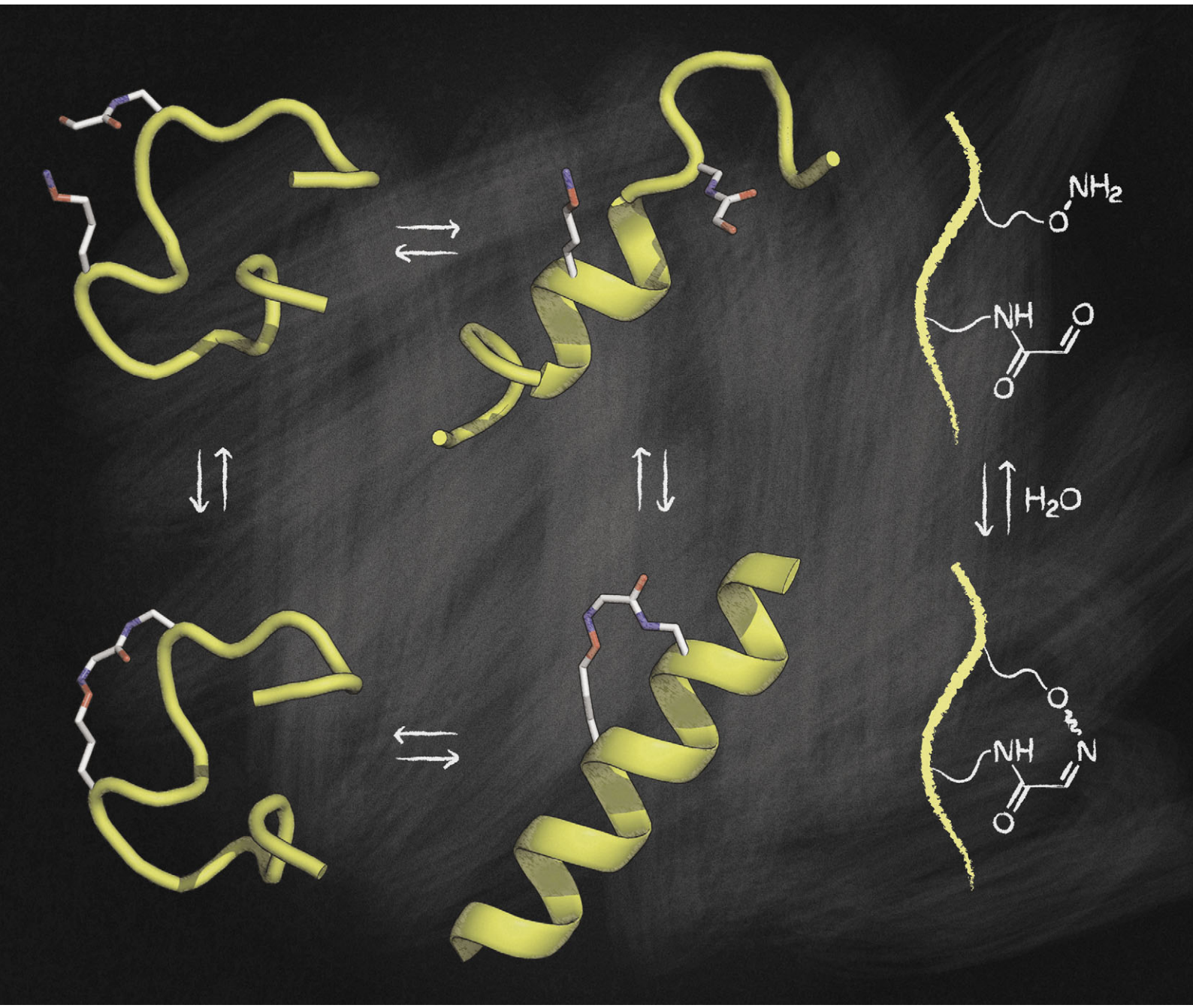

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COMMUNICATION

Promoting peptide α-helix formation with dynamic covalent oxime side-chain cross-links†

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Covalent side-chain cross-linking has been shown to be a viable strategy to control peptide folding. We report here that an oxime side-chain linkage can elicit α-helical folds from peptides in aqueous solution. The bio-orthogonal bridge is formed rapidly under neutral buffered conditions, and the resulting cyclic oximes are capable of dynamic covalent exchange.

α-Helical secondary structures are commonly found at protein-protein binding interfaces.[1] The need for chemical agents that selectively disrupt protein-protein interactions has motivated extensive efforts to mimic α-helical segments of proteins with peptides, peptide mimetics and small molecules.[2] The use of short peptide sequences derived from natural proteins for this purpose is limited by their tendency to be minimally structured when removed from the native protein context. One of the first chemical methods examined for promoting α-helix formation in short peptides was the introduction of a covalent lactam bridge.[3] Recent years have seen significant developments in covalently-constrained helical peptides, including new side-chain cross-linking chemistries[4] and complementary strategies based on replacement of backbone hydrogen bonds with covalent isosteres.[5] These technologies have been applied to produce potent, bioactive helix mimics.[6]

A subset of linkages examined to date as side-chain "staples" can be formed chemoselectively in physiological conditions.[4a,d–f] We are interested in exploring the ability of reactions that are (1) bio-orthogonal and (2) dynamically reversible in water to control peptide folding. Such technologies have the potential to enable the discovery of bioactive cross-link topologies in polycyclic peptides by combining the benefits of peptide stapling[4–6] with template-assisted amplification as demonstrated in the field of dynamic combinatorial chemistry.[7] As a first step toward this goal, we report here the ability of dynamic covalent oxime side-chain cross-links to promote α-helix formation in aqueous solution. Oximes are widely used in chemoselective ligations involving proteins[8] and have been used in dynamic combinatorial libraries.[7,9] A few oxime-based cyclic peptides have been reported;[10] however, neither the dynamics of the linkage in that context nor its ability to influence folding has been studied.

We selected peptide **1** (Fig. 1) as a model system to explore the ability of oxime side-chain cross-links to promote an α-helical conformation in aqueous solution. This 17-residue sequence, partially helical at pH 7, has previously been used to examine the energetic contributions of non-covalent interactions (*e.g.*, salt bridges) to α-helix formation.[11] Peptides **2–5** are derivatives of **1** designed to probe the relationship between the structure of an oxime bridge (residue spacing, side-chain length) and its ability to stabilize the α-helix fold. Residues **X** and **Z** (isosteres of Orn and Lys, respectively) bear aminooxy groups attached to the peptide backbone by either 2 or 3 carbons. Residue **U** has a Ser-acylated amine side chain that can be efficiently converted to a glyoxyl aldehyde in neutral aqueous buffer by treatment with a mild oxidant.[10a,b,12]

Peptides **1–5** were prepared by standard microwave-assisted Fmoc solid-phase peptide synthesis (SPPS). Monomers for incorporation of **X** and **Z** were synthesized by modification of known routes to related derivatives (Scheme S1†).[13] A protected form of residue **U** suitable for use in SPPS is known.[10b] Two synthetic observations about the aminooxy-functionalized peptides are noteworthy. We found that TFA-mediated cleavage of peptides containing **X** or **Z** from a Rink-linker-based resin without addition of ethanedithiol as a nucleophilic scavenger led to no isolatable product. Addition of the nucleophilic scavenger provided yields and crude purities similar to those obtained for the natural sequence **1**

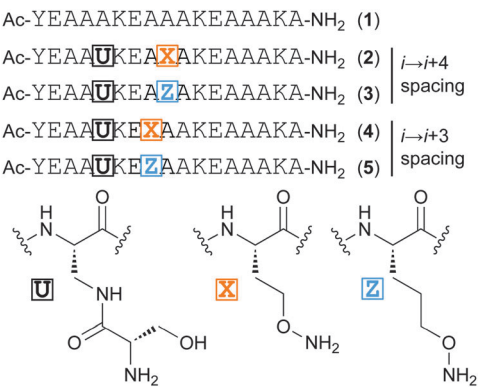

Fig. 1 Structures of peptides **1–5** and residues **U**, **X** and **Z**.

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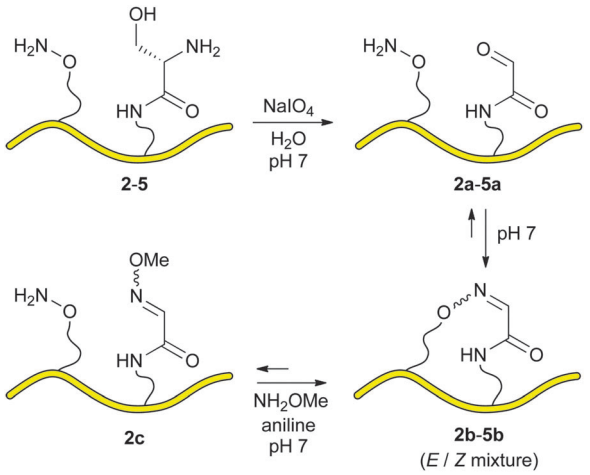
† Electronic supplementary information (ESI) available: Fig. S1–S6, Table S1–S2, Scheme S1 and experimental methods. See DOI: 10.1039/c1cc12010g



Scheme 1 Conversion of peptides **2–5** to cyclic oximes **2b–5b**, and exchange of **2b** to linear oxime **2c**.

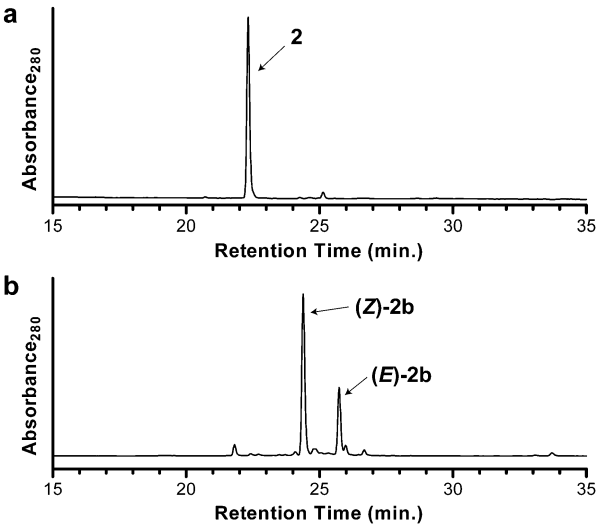


Fig. 2 HPLC analysis of the oxidation and cyclization of peptide **2**. The reaction, 100 μM peptide in 100 mM phosphate pH 7, was monitored (a) before and (b) 10 min after addition of 2 equiv. $NaIO_4$.

prepared by the same method. We attribute loss of material in the absence of ethanedithiol to the irreversible reattachment of peptide to resin during cleavage by linker alkylation of the aminooxy side chain.[14] We also found that some C_{18} HPLC stationary phases irreversibly bind aminooxy-functionalized peptides (*i.e.*, injected material does not elute). We were able to find C_{18} analytical and preparative columns free of this problem (see supporting information).

With peptides **1–5** in hand, we next sought to test the efficiency of the oxidation of the **U** residue in **2–5** to an aldehyde and subsequent oxime formation (Scheme 1). We carried out cross-linking reactions in conditions that are typical for biophysical characterization of peptide folding or receptor binding. We prepared a 100 μM solution of each peptide in 100 mM phosphate buffer at pH 7 and added sodium periodate to cleave the 1,2-aminoalcohol in each **U** residue to the corresponding glyoxyl aldehyde.[10a,b,12] HPLC/MS analyses of the reaction mixtures minutes after addition of periodate showed complete consumption of starting material in all cases (Fig. 2, S1).

The oxidized peptides are capable of establishing an equilibrium between the linear aminooxy-aldehyde (**2a–5a**) and cyclic oxime (**2b–5b**) forms. While the oxime is thermodynamically favored,[8,9,15] the kinetics of intermolecular oxime ligation reactions are typically extremely slow at neutral pH; acidic conditions and/or nucleophilic catalysts are used to achieve reasonable rates.[16] No evidence was observed for the linear peptides **2a–5a** at the first HPLC time point taken to monitor the reaction (~10 min). Instead, the majority (85–90%) of the starting material was converted to two new species with mass corresponding to oximes **2b–5b**. We assign the two major products observed by HPLC in each case as resulting from the *E*- and *Z*-oxime isomers; the *E*/*Z* ratio varied among **2b–5b**. For peptide **2b**, we assigned the absolute configuration of the two oxime isomers by ^{1}HNMR spectroscopy (Fig. 2b, S2†).

The distribution of products observed at 10 min was unchanged after 24 h. For peptide **2b**, we isolated the *E*- and *Z*-oxime isomers by HPLC and examined the isomerization of each. The isomers are able to interconvert in pH 7 phosphate, albeit slowly; isomerization is faster under acidic conditions (Fig. S3†). The above data suggest that the system is in dynamic exchange in pH 7 buffer but is kinetically trapped at a non-equilibrium state.

In order to further characterize the dynamic covalent nature of the cyclic peptide oximes, we prepared an equilibrating mixture of **2a**/**2b** at pH 7 as described above and added 1000 equiv. of buffered *O*-methylhydroxylamine to compete with the peptide **X** residue aminooxy group for reaction with the glyoxyl aldehyde; aniline was added to a final concentration of 10 mM to facilitate exchange.[16] The above conditions set up a dynamic equilibrium between linear peptide **2a**, cyclic oxime **2b** (*E* and *Z* isomers) and linear oxime **2c** (*E* and *Z* isomers). HPLC/MS analysis after 24 h showed formation of a small amount of product with mass corresponding to the linear oxime **2c** (Fig. S4†); the amount of **2c** increased slightly at 48 h to a final conversion of ~3%, suggesting the system is slow to reach equilibrium under these conditions. At pH 4, exchange was much faster, and ~10% of the material converted to linear oxime **2c** after 24 h.

We employed circular dichroism (CD) spectroscopy to compare the α-helicity of control peptide **1** to that of linear peptides **2–5** and cyclic analogues **2b–5b** (Fig. 3). All samples were measured at 100 μM peptide concentration in pH 7 phosphate buffer. Control peptide **1** showed modest helicity under the conditions of the experiment, indicated by the negative peaks at 208 and 222 nm. CD spectra of peptides **2–5** prior to oxidation showed almost no α-helical secondary structure (Fig. 3a). The loss of helicity in **2–5** relative to **1** is likely due to the introduction of the bulky, cationic **U** residue side chain in place of an Ala in parent sequence **1**.

Addition of 2 equiv. of periodate to peptides **2–5**, conditions where the cyclic oximes **2b–5b** are the predominant species by HPLC/MS (*vide supra*), led to significant changes in the CD spectra (Fig. 3b). The α-helical population among **2b–5b** varied as a function of the structure of the oxime bridge (Table S1†): an $i \rightarrow i+4$ spacing of aldehyde and aminooxy residues in the sequence (**2b**, **3b**) leads to more helical population than the corresponding $i \rightarrow i+3$ spacing (**4b**, **5b**).

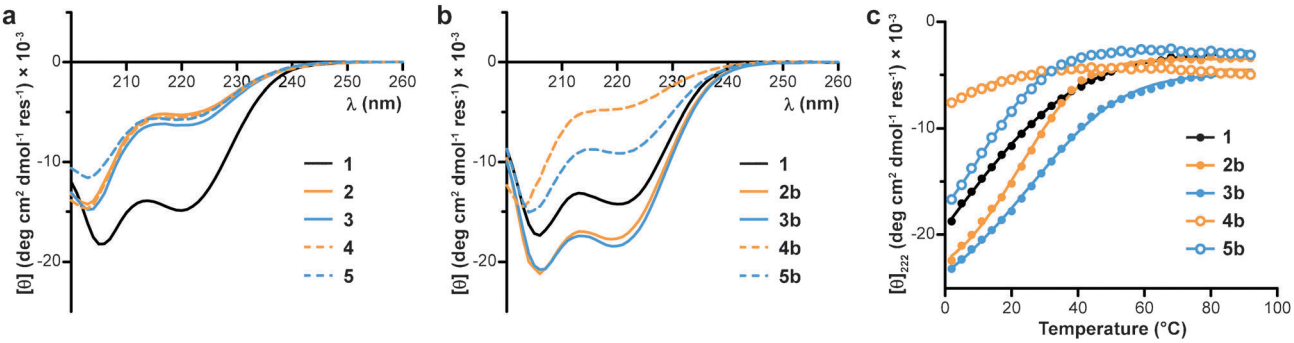


Fig. 3 Circular dichroism scans at 20 °C (a,b) and thermal melts (c) for peptides **1–5** and oxime-bridged cyclic peptides **2b–5b**. Measurements were carried out on 100 μM concentration peptide solutions in 100 mM phosphate pH 7 in absence (a) or presence (b,c) of 200 μM sodium periodate. Measurements of **2b–5b** were carried out on unseparated *E*/*Z* oxime mixtures.

Molecular modelling suggests that the oxime macrocycles in **4b** and **5b** cannot be accommodated into an idealized α-helix without significant side-chain distortion (Fig. S5†). The best folded oxime-constrained peptides **2b** and **3b** are more helical than the control sequence **1** under identical conditions. Replacement of the **Z** residue in **3** with Lys (*i.e.*, O → CH_2 substitution on the side chain) abolished the enhanced helicity observed for **3** after periodate addition (Fig. S6†), supporting the essential role of the oxime bridge in the observed CD data. In thermal melts monitored at 222 nm, **2b** and **3b** show a ~15 °C increase in T_m relative to **1** (Fig. 3c, Table S1†). Interestingly, the oxime side-chain linkages impacted the reversibility of the folding process; neither **2b** nor **3b** recovered their initial helicity after cooling a thermally unfolded sample. The unfolding of control peptide **1** was fully reversible under these conditions.

In summary, we have demonstrated that a bio-orthogonal oxime side-chain linkage can promote α-helical folding in a medium-length peptide in aqueous solution. The covalent bridge is formed rapidly and in high yield under neutral buffered conditions. Spacing and side-chain length of the residues bearing the aminooxy and aldehyde precursor functional groups are important determinants of α-helicity in the cyclic oxime. We see evidence that the oxime peptides are capable of isomerization and dynamic exchange with an aminooxy-functionalized small molecule; however, the rate of exchange is slow at neutral pH.[16] It will be interesting to see how the rate of oxime exchange compares when the competing aminooxy groups reside on the same molecule (*i.e.*, a longer peptide with n **U** residues and $n+1$ **Z** residues). We see such species as being useful for the template-assisted discovery of new bioactive cross-link topologies and folding patterns in polycyclic peptides and proteins. Efforts to analyse the kinetics and thermodynamics of folding and oxime exchange in these and more complex systems are ongoing.

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