Published in final edited form as:

Angew Chem Int Ed Engl. 2015 July 20; 54(30): 8665–8668. doi:10.1002/anie.201502607.

ACE-linked peptides: a convergent approach for peptide macrocyclization and labeling

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Abstract

Macrocyclization is a broadly applied approach for overcoming the intrinsically disordered nature of linear peptides. For example, significant efforts have been developed to stabilize α -helical structures, through tethering proximal side chains. While these approaches successfully mimic protein α -helices, the structural requirements of the tether typically prevent further synthetic modifications to the non-binding face of the helix. Here we demonstrate the utility of dichloroacetone (DCA) to enhance helical secondary structure when introduced between peptide nucleophiles, such as thiols, to yield an acetone (ACE)-linked bridge. In addition to stabilizing helical structures, the ketone moiety embedded into the linker can be modified using oxime ligation with diverse molecular tags. Insights into the structure of the tether were obtained through co-crystallization of a constrained S-peptide in complex with RNase S. The scope of ACE-linked peptides was further explored through the generation of N-terminus to side chain macrocycles and a new approach for generating fused macrocycles (bicycles). Together, these studies suggest that ACE-linking is generally applicable to peptide macrocycles with a specific utility in the synthesis of stabilized helices that incorporate functional tags.

Keywords

Tether; Oxime Ligation; Bioconjugation; Macrocyclization; Labelling

Despite being a simple element of secondary structure, single α -helices are frequently found at the interface of protein complexes.^[1] In these cases, structural mimics of the helix can be used to disrupt the protein-protein interaction. The design and synthesis of side chain “tethered” or “stapled” helical peptides has yielded potent, cell permeable inhibitors for targets that are often considered undruggable using small molecules^{[2–4][5]} For example,

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Fesik and co-workers demonstrated the cell permeability and selective binding of a stapled peptide to replication protein A.^[6]

Side chain tethered peptides are a member of a broader family of peptide macrocycles and a multitude of synthetic strategies have been developed for their synthesis.^[7–10] Many chemical links have been explored to achieve ring closure including, lactam bridges,^{[11,12][13]} disulfide bonds,^[14] alkenes^{[15],[16,17]} and triazoles.^[18–20] The utility of each linkage is based on a combination of compatibility with solid phase peptide synthesis (SPPS), effectiveness for ring closure, and compatibility with the peptide structure being stabilized. Typically, the peptide is prepared via SPPS where the functionalized amino acids are integrated within the peptide chain, followed by on-resin or in-solution macrocyclization. Many of these strategies require the synthesis of elaborate unnatural amino acids or orthogonal protection strategies. An alternative approach is to cross-link two naturally occurring amino acid side chains via a linker using a chemoselective reaction. For example, cyclization between two cysteine amino acids placed in the i and i+4 or i+7 positions using a cross-linker offers significant helix stabilization.^{[21],[22]} Regardless of the methodology, peptide cyclization can significantly stabilize the preorganization of peptide sequences into desired secondary structures by destabilizing the denatured state.^{[15],[16,23]}

Helical stabilization through bisalkylation of thiol containing side chains is appealing due to the ease of generating the linear peptide precursors.^[21,22] There are a variety of different cysteine and homocysteine derivatives described in the literature that can be used to add diversity to the staple.^[24,25] Many practical applications require subsequent labelling of the constrained peptide, with fluorophores, affinity tags, lipids or PEG polymers. Proper placement of the tag can be critical for conserving target binding and gaining biological activity such as binding affinity or cell penetration. However, since most peptide side chains are either directly involved in ligand binding or are engaged in forming the tether, only the termini are left for further diversification. While there are a few examples where tags have been built into the peptide staple,^{[26,27],[28]} the option for chemoselective diversification after formation of the staple affords significant synthetic advantages. For example a 1,2,4,5-tetrabromodurene scaffold for CLIPS macrocyclization has been previously reported.^[28] Herein, we demonstrate the use of DCA, a known protein crosslinker,^[29,30] as an effective tool for synthesizing cyclic and helical peptides primed for chemoselective oxime ligation within the tether.

To validate the approach, we used a previously described model peptide.^[22] Two sequences were synthesized, Ac-YGGEAAREAXAREXAARE-CONH₂, where X= Cys **1a** or homo-Cys (hC) **1b**. Each peptide was dissolved at 0.1 mM in 50 mM NH₄HCO₃, pH 8 and reduced with TCEP (1.1 eq). The cyclization was initiated with DCA in DMF (1.5 eq) and was complete in 3 hr to give products **2a** and **2b** in > 90% conversion by HPLC. In a scaled up synthesis, cyclization of **1b** (hC) with DCA proceeded cleanly to yield the desired product **2b** in a 70% recovered yield after preparative HPLC.

We determined the degree of helical secondary structure of each peptide in solution using CD spectroscopy. The linear peptide **1b** displayed only 22% helicity and gave a largely random coil spectra with a strong minima at ~ 200 nm. In contrast, the hC constrained

peptide **2b**, was 53% helical with minima at 208 nm and 222 nm, while the Cys constrained peptide **2a** was found to be only 35% helical. Additionally, the CD spectra of the methyloxime derivative of peptide **2b** was superimposable with the parent ketone **2b** (Figure S1).

Previously, DeGrado and Greenbaum treated peptide **1a** with a series of bis-alkylators and found that the (α,α' -dibromo-*m*-xylene) cross-linker (9-atom linker) provided greater helix stabilization than the *o*-xylene (8-atom linker) or *p*-xylene (10-atom linker) based cross-linkers.^[22] Our results are consistent with this optimal linker length, with the highly helical ACE-linked hC peptide **2b** having a 9-atom linker, while the unstructured ACE-linked Cys peptide **2a** has a 7-atom linker.

To evaluate the utility of the macrocyclic ketone as a chemoselective ligation handle, typical conditions for aniline catalyzed oxime ligation were examined.^[31] The cyclized peptide **2b**, (0.5 mM) in 50 mM NH₄OAc, 100 mM aniline, pH 4.5 was treated with the aminooxy tag (2 eq) at 25 °C. Full conversion to the desired oxime, as a mixture of *cis* and *trans* isomers, was observed in 16 h (Figure 2). A variety of aminooxy tags could be ligated to the ACE-linker, including fluorophores such as Alexafluor 488 (**4**) and Alexafluor 647 (**7**). Biotinylation was also successful using aminooxy-PEG4-biotin (**6**). A bisaminooxy ethane linker can be used to make dimers (**5**), or to simply add an aminooxy group (**8**), which can be used to link the helical peptide onto the surface of carrier proteins or virus-like particles.^[32] Furthermore, N-terminal aminooxy peptides can be used to introduce additional functionality, such as polyarginine (**3**) to aid in cell penetration,^[33] or a FLAG epitope tag (**9**) for immunological assays. Ligation on to the ACE-linker leaves the N-terminus of the helix open for structural or functional modifications.

The synthesis of peptide bicycles have attracted significant interest, since they can serve as mimics of complex protein structures including pairs of protein loops.^[34] The orthogonal reactivity of the ACE-linker inspired the design of a bis-homocysteine peptide with an N-terminal aminooxy group positioned to form a second macrocycle. The reaction with DCA was selective towards the hC thiolates and subsequent intramolecular oxime ligation yielded the bicyclic peptide scaffold (**10**). Interestingly, oxime macrocyclization was sluggish (>24 h) and we observed a covalent aniline intermediate that was stable to HPLC. The use of dichloroacetone highlights a tool, which can be used to display multiple peptide loops.

To explore the utility in a peptide/protein binding system, an analog of the S-peptide was synthesized to bind to the S-protein. It has previously been reported that the first 20 amino acids on RNase A (S-peptide) form a helix and can be cleaved off using subtilisin A to give RNase S.^[35,36] Although the S peptide does not form a helix in solution, it can still bind to RNase S at high affinity.^[37] An analog of the S-peptide was synthesized to facilitate incorporation of the ACE-linker between homocysteine residues (Ac-KETAAhCKFEhCQHMDs-NH₂). The homocysteine residues were subsequently constrained with DCA. Importantly, the S-peptide mimic contained a Lys residue, demonstrating the ability to crosslink with DCA in the presence of alternative nucleophiles. We determined the crystal structure of the constrained S-peptide : RNase S complex at 2.2 Å resolution. As shown in Figure 3, the tethered S-peptide binds RNase S in a helical

conformation with the ACE ketone moiety protruding away from the RNase S surface – a positioning that should easily accommodate tagging. The overall conformation of our cross-linked peptide is highly conserved with the S-peptide in complex with RNase A (ref, PDBID 3OQY) as evidenced by an rmsd of 0.18 Å for all main-chain atoms within the helical region of the peptide (Figure S2).

In the absence of a second thiol group, dichloroacetone is effective at cyclizing peptides from the cysteine side chain to the N-terminus. This reaction is illustrated by the synthesis of peptides **11** and **12** in which a 16-membered ring forms smoothly between the side chain and N-terminus (Figure 4), and suggests an expanded scope for DCA macrocyclization.

In conclusion, we have demonstrated the utility of DCA crosslinking to stabilize alpha helices. A crystal structure of a cross-linked S-peptide in complex with RNase S highlights that the linker supports a helical secondary structure and presents the conjugation site away from the surface of the protein. DCA crosslinking can facilitate multiple loop peptide scaffolding, as well as peptide macrocyclization. The ability of the ketone to undergo subsequent oxime ligation allows for the diversification of the peptide in a convergent manner, and offers the opportunity for placement of important tags in the middle of constrained peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the National Institutes of Health (F32 GM103162 (NA), R01 GM098871 (PED)) for financial support.

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Figure 1.

CD spectra of the peptide **1b** compared to ACE-linked peptide **2a** and **2b**. CD spectra were taken with 25 μ M peptide in 50 mM phosphate buffer, pH = 7.4 at 25 C.

Figure 2. Substrate scope of the oxime ligation with the DCA linker. *Two-step yield from linear peptide.

Figure 3.
Co-crystal structure of ACE-linked modified S-peptide bound to RNase S (PDB ID: 4YGW)

*HPLC conversion (isolated yield)

Figure 4.
Head-to-side chain cyclization with DCA.

Scheme 1.
Peptide cyclization with DCA