Macrocyclization Hot Paper

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Acetone-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. Herein, it is shown that dichloroacetone (DCA) enhances helical secondary structures when introduced between peptide nucleophiles, such as thiols, to yield an acetone-linked bridge (ACE). Aside from stabilizing helical structures, the ketone moiety embedded in the linker can be modified with diverse molecular tags by oxime ligation. 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 the acetone-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 acetone linking is generally applicable to peptide macrocycles with a specific utility in the synthesis of stabilized helices that incorporate functional tags.*

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 as "undruggable" using small molecules.[2–5] For example, Fesik and coworkers have demonstrated the cell permeability and selective binding of a stapled peptide to replication protein A.[6]

Side-chain-tethered peptides are members 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,[11–13] disulfide bonds,[14] alkenes,[15–17] 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 by 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 method, 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 the bis(alkylation) of thiol-containing side chains is appealing owing 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 labeling of the constrained peptide, for example, 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, as 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. Whereas there are a few examples where tags have been built into the peptide staple,[26–28] the option for chemoselective diversification after formation of the staple affords significant synthetic advantages. For example, a 1,2,4,5-tetrabromodurene scaffold has previously been reported for CLIPS macrocyclization.[28] Herein, we demonstrate the use of dichloroacetone (DCA), a known protein cross-linker,[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, namely Ac-YGGEAAREA**X**ARE**X**AARE-$CONH_2$, where X = Cys (**1a**) or homo-Cys (hC; **1b**). Each peptide was dissolved at a concentration of 0.1 mM in an aqueous NH_4HCO_3 solution (50 mM, pH 8) and reduced with tris(2-carboxyethyl)phosphine (TCEP; 1.1 equiv). The cyclization was initiated with DCA in DMF (1.5 equiv) and was complete in three hours to give products **2a** and **2b** in >90% conversion according to HPLC analysis (Scheme 1). In a scaled-up process, cyclization of **1b** (hC) with DCA proceeded cleanly to yield the desired product **2b** in a yield of 70% after preparative HPLC.

We determined the degree of helical secondary structure of each peptide in solution using CD spectroscopy (Figure 1).

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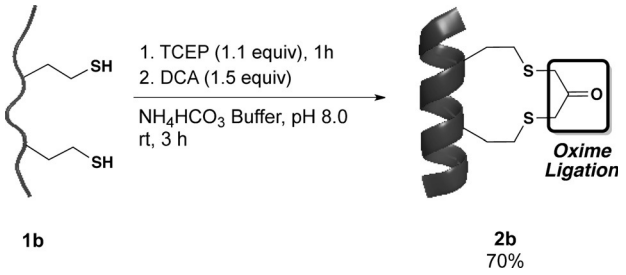

Scheme 1. Peptide cyclization with DCA.

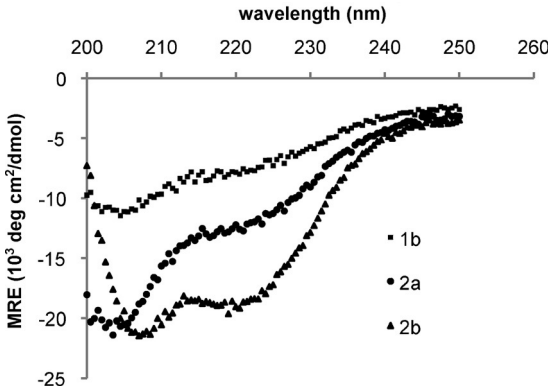

Figure 1. CD spectrum of peptide **1b** compared to those of the acetone-linked peptides **2a** and **2b**. CD spectra were recorded at a peptide concentration of 25 μM in phosphate buffer (50 mM, pH 7.4) at 25 C. MRE = mean residue ellipticity.

The linear peptide **1b** displayed only 22% helicity and gave a largely random-coil spectrum with a strong minimum at approximately 200 nm. In contrast, the hC-constrained peptide **2b** was 53% helical with minima at 208 nm and 222 nm, whereas the Cys-constrained peptide **2a** was found to be only 35% helical. Furthermore, the CD spectrum of the methyloxime derivative of peptide **2b** was superimposable with that of the parent ketone **2b** (Supporting Information, Figure S1).

Previously, DeGrado, Greenbaum, and co-workers had treated peptide **1a** with a series of bis(alkylation) reagents and found that the α,α′-dibromo-*meta*-xylene cross-linker (a 9-atom linker) provided greater helix stabilization than the cross-linkers based on *ortho*-xylene (8-atom liker) or *para*-xylene (10-atom linker).[22] Our results are consistent with this optimal linker length, with the highly helical acetone-linked hC peptide **2b** having a nine-atom linker, whereas the unstructured ACE-linked Cys peptide **2a** has a seven-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 NH_4OAc, 100 mM aniline, pH 4.5) was treated with the aminooxy tag (2 equiv) at 25 C. Full conversion into the desired oxime, as a mixture of the *cis* and *trans* isomers, was observed in 16 hours (Figure 2). A variety of aminooxy tags could be ligated to the acetone linker, including fluorophores such as Alexafluor 488 (**4**) and Alexafluor 647 (**7**). Biotinylation was also successful using aminooxy-PEG4-biotin (**6**). A bis(aminooxy) 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

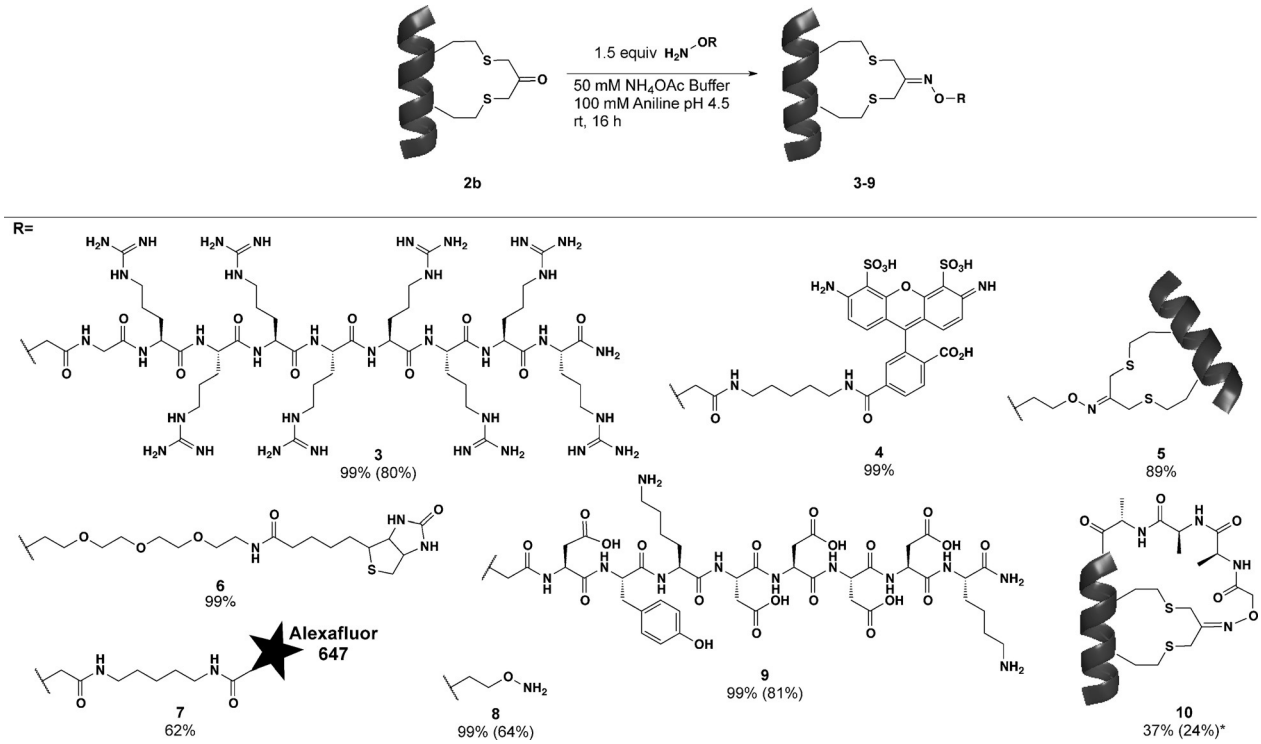

Figure 2. Substrate scope of the oxime ligation with the DCA linker. Yields are given as the conversion determined by HPLC with the yield of isolated product in parentheses. *: Two-step yield from the linear peptide.

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 onto the acetone linker leaves the N-terminus of the helix open for structural or functional modifications.

The synthesis of peptide bicycles has attracted significant interest, as they can serve as mimics of complex protein structures, including pairs of protein loops.[34] The orthogonal reactivity of the acetone 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 DCA highlights a reagent that can be used to display multiple peptide loops.

To explore the utility in a peptide/protein binding system, an analogue 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 with high affinity.[37] An analogue of the S-peptide was synthesized to facilitate incorporation of the acetone linker between homocysteine residues (Ac-KETAA**hC**KFE**hC**QHMDS-NH_2). The homocysteine residues were subsequently constrained with DCA. Importantly, the S-peptide mimic contained a Lys residue, which can be cross-linked 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 acetone 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 (PDBID No. 3OQY) as evidenced by a root-mean-square deviation 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 the N-terminus (Figure 4), and suggests an expanded scope for DCA macrocyclization.

In conclusion, we have demonstrated the utility of DCA cross-linking to stabilize α-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. Dichloroacetone cross-linking 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 an opportunity for the placement of important tags in the middle of constrained peptides.

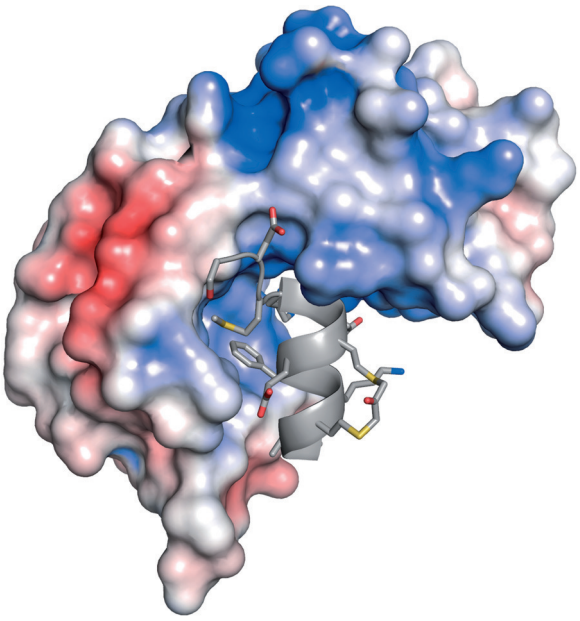

Figure 3. Co-crystal structure of the acetone-linked modified S-peptide bound to RNAse S (PDB No. 4YGW).

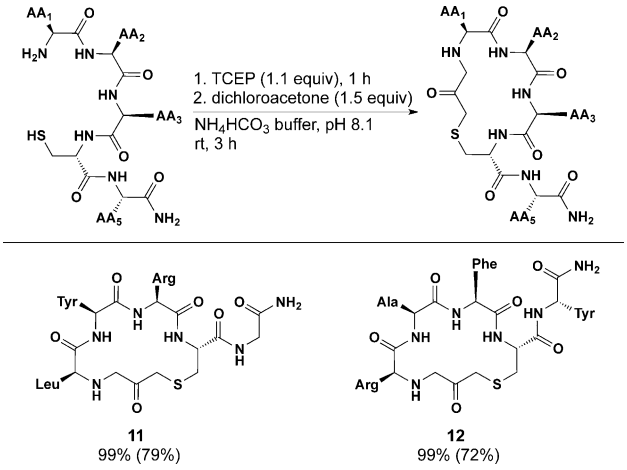

Figure 4. Head to side chain cyclization with DCA. Yields are given as the conversion determined by HPLC with the yield of isolated product in parentheses.

Keywords: bioconjugation · macrocyclization · oxime ligation · peptides · protein labeling

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