Synlett Z. E. Paikin et al. Letter

Metabolite-Mediated Protein Macrocyclization

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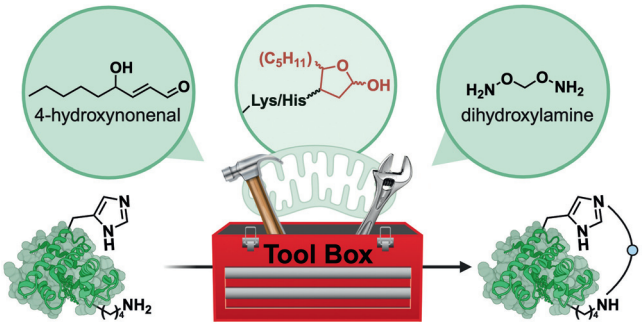

Received: 26.01.2025
Accepted after revision: 17.02.2025
Published online: 19.03.2025 (Version of Record)
DOI: 10.1055/s-0043-1775459; Art ID: ST-2025-01-0043-L

ABSTRACT Protein macrocyclization is a pivotal process in the stabilization of protein structures, significantly enhancing their proteolytic stability and thermostability. While nature elegantly accomplishes this through a diverse family of ligases, laboratory methods typically rely on recombinant proteins engineered with unnatural amino acids and cysteine crosslinkers. Herein, we present a biological metabolite 4-hydroxynonenal (4-HNE) to selectively modify nucleophilic amino acids, cysteine (Cys), histidine (His), and lysine (Lys) into electrophilic hemiacetals followed by cyclization via oxime chemistry. This reaction demonstrates a broad substrate scope, enabling the modification and cyclization of proteins with a wide range of three-dimensional structures and molecular weights, from 5.8 to 60 kDa. The resulting cyclized proteins exhibit greater proteolytic stability and enhanced thermal stability at elevated temperatures compared to their uncyclized counterparts. This clearly underscores the critical role of cyclization in preserving the intricate 3D structures of proteins and opens new avenues for advanced protein engineering.

Key words proteins, cyclization, chemoselectivity, biomimetic synthesis, bioorganic, chemistry

Protein macrocycles have gained significant recognition in recent years, with numerous examples identified in both prokaryotic and eukaryotic organisms.[1,2] These cyclic proteins are distinguished by their remarkable proteolytic and thermal stability.[3–5] Unlike previously known cyclic peptides, such as the immunosuppressant cyclosporin and other cyclic peptides from microorganisms, these new cyclic proteins are conventional gene products rather than products of nonribosomal synthesis.[6–9] Additionally, this new class of cyclic proteins differs from nonribosomal synthesized peptides in size and composition. While traditional cyclic peptides are typically limited to 5–12 amino acids and may include modifications like D-amino acids or N-methylation, these newly discovered cyclic proteins range from 14 to 78 amino acids and consist solely of naturally occurring amino acids without such modifications.[2–5]

The pharmaceutical industry has shown increasing interest in cyclic peptides due to their superior properties, such as enhanced bioactivity, lower toxicity, improved target affinity, and greater resistance to proteolysis.[10–13] This has led to their exploration as potential scaffolds for designing protein-based therapeutics. A notable example is cyclic insulin, which not only resists degradation by carboxypeptidase Y (CPY) but also maintains potency equivalent to that of linear human insulin.[14]

Current methods for protein cyclization typically involve the use of enzymes like ligases or cyclases,[15–21] the incorporation of unnatural amino acids, or the introduction of cysteine crosslinks (Figure 1).[18–20] However, these methods are generally limited to proteins with fewer than 80 amino acids. Herein, we introduce a novel biocompatible chemical approach for protein cyclization utilizing the metabolite 4-hydroxynonenal (4-HNE) generated in the mitochondria.[22,23] This method enables selective conversion of His and Lys into electrophilic hemiacetals by Michael addition, followed by cyclization of proteins using oxime chemistry (Figure 1).

Our approach is versatile and effective, capable of modifying proteins of varying three-dimensional structures and molecular weights (5.8–80 kDa) under mild physiological conditions with quantitative conversions. The cyclic proteins produced through this method exhibit high thermostability at elevated temperatures, surpassing their uncyclized counterparts. This new method for protein cyclization represents a significant advancement and has the potential to revolutionize the field of protein therapeutics.

In our efforts to develop a method for the cyclization of wildtype proteins using the 4-HNE metabolite [for synthesis see Figure 1 in the Supporting Information (SI)] and to apply this chemistry to protein engineering, we began by

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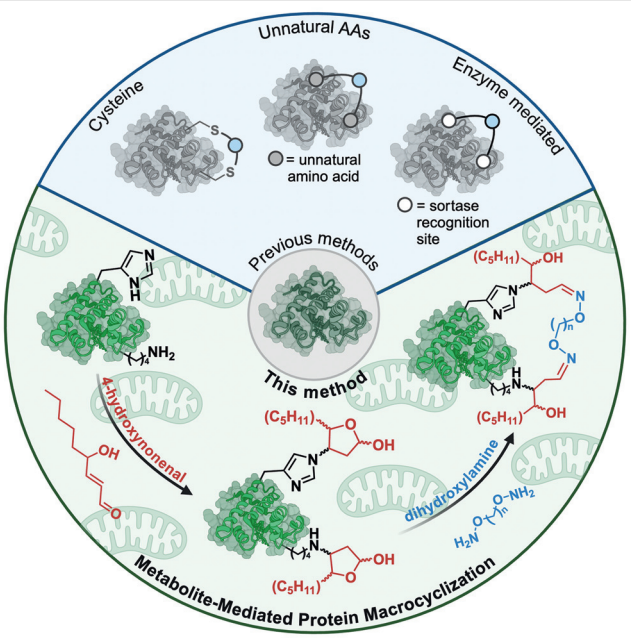
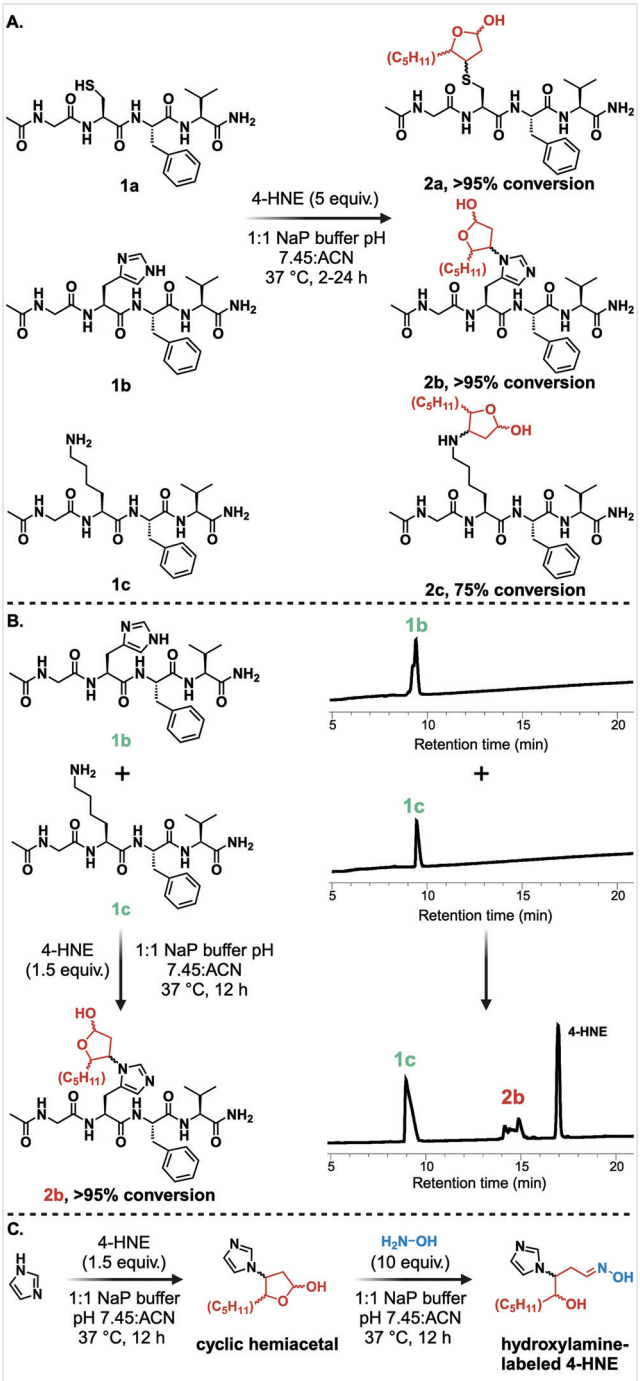


Figure 1 Metabolite-mediated protein macrocyclization. Previous methods focused on cysteine crosslinks, unnatural amino acids, and enzyme catalysis. This method: protein cyclization by biological metabolite, 4-HNE, converting His and Lys nucleophiles to electrophilic hemiacetals followed by oxime ligation with a dihydroxylamine linker.

optimizing the reaction with model peptides containing nucleophilic residues. We used peptides AcGXFV, where X represents Cys (C), His (H), or Lys (K) (**1a–c**). Each peptide was subjected to 5 equivalents of 4-HNE under physiological conditions (pH 7.45) at room temperature. Our results revealed complete conversion (>95%) of Cys in peptide AcGCFV (**1a**) to the Michael addition product **2a** within 2 h. The His in peptide AcGHFV (**1b**) was fully converted (>95%) into product **2b** within 12 h, while Lys in peptide AcGKFV (**1c**) achieved 75% conversion into product **2c** over 24 h (Scheme 1A and Figure 2 in the SI). To further investigate the reactivity of His compared to Lys, we incubated equal amounts of peptides AcGHFV (**1b**) and AcGKFV (**1c**) with a limited quantity (1.5 equivalents) of 4-HNE. Modification occurred exclusively in the His-containing peptide, with no 1,4-addition products observed with Lys within 12 h (Scheme 1B and Figure 3 in the SI).

In an analogous experiment, we observed the formation of the 1,4-single addition product **2a** with the Cys peptide AcGCFV (**1a**) in the presence of either His peptide AcGHFV (**1b**) or Lys peptide AcGKFV (**1c**) (see Figure 3 in the SI). Given that Cys is less abundant and often involved in stabilizing disulfide bonds in protein tertiary structures, significant chemoselectivity issues are not anticipated, and we anticipate this modification to be largely selective for His and Lys. Additionally, no modification of other reactive amino acids was observed with peptide Ac-KQYWRMES (**1d**), which includes Tyr, Arg, Ser, Met, Trp, Glu, and Gln, further demonstrating the selectivity for His and Lys (see Figure 4 in the SI). For the characterization of the product

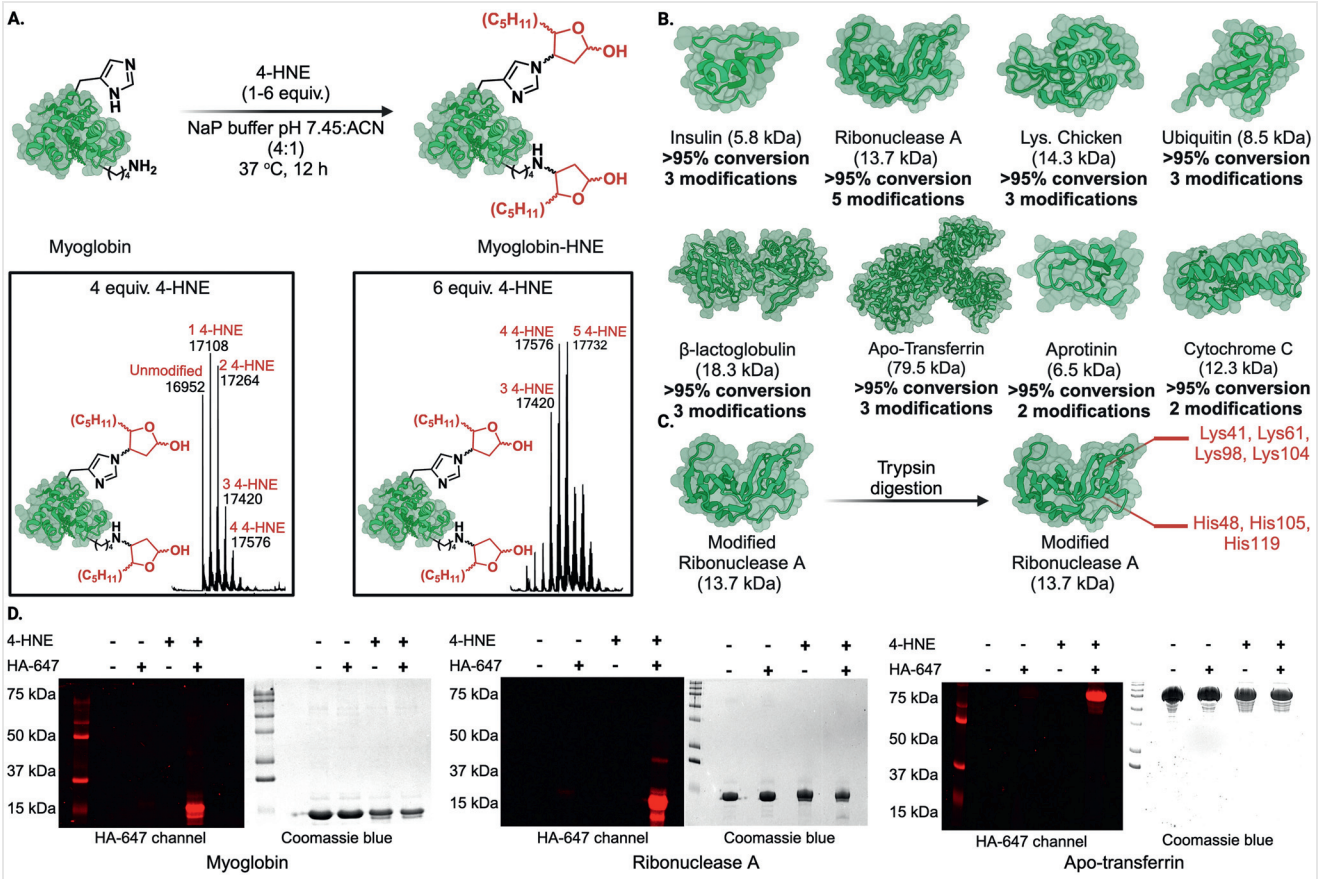


Scheme 1 Hemiacetal formation with 4-HNE. (A) Reaction of peptides containing nucleophilic residues with 4-HNE. (B) Comparison of the reactivity of His vs Lys with 4-HNE. Broad **2b** peak represents multiple diastereoisomers of cyclic hemiacetal. (C) Imidazole reacts with 4-HNE to form cyclic hemiacetal followed by reaction with hydroxylamine to generate oxime product.

with 4-HNE, we carried out a large-scale reaction with imidazole and observed the formation of the hemiacetal product under the reaction conditions, obtained by the 1,4 Michael addition followed by the cyclization between hydroxy group and aldehyde as analyzed by NMR spectroscopy (see Figure 5 in the SI). The electrophilicity of the hemiacetal was demonstrated by the addition of hydroxylamine, as full conversion to the oxime product was observed, with verification by HPLC and HRMS (Scheme 1C and Figure 5 in the SI).

We next assessed the effectiveness of this reaction for protein modification by using myoglobin as a model protein and varying the equivalents of 4-HNE from 1 to 6 equivalents. Remarkably, we achieved efficient modification of myoglobin (>75%) using just 4 equivalents of 4-HNE (Scheme 2A and Figure 6 in the SI). At higher concentrations (6 equivalents), quantitative modification of myoglobin was observed, with a maximum of five modifications (Scheme 2A and Figure 6 in the SI).

We then applied our method to a diverse range of commercially available proteins, including insulin, ribonuclease A, lysozyme chicken, ubiquitin, β-lactoglobulin, apo-transferrin, aprotinin, and cytochrome C, spanning molecular weights from 5,000 Da to over 79,000 Da and various three-dimensional structures. Our method achieved over >95% modification with excellent selectivity for His or Lys, even at low concentrations (100 μM, 10 examples, Scheme 2B and Figures 7 and 8 in the SI). The mild reaction conditions preserved critical disulfide bonds, as seen with insulin (Scheme 2B and Figure 8 in the SI). We proceeded to conduct MS/MS analysis of a ribonuclease A sample with seven 4-HNE modification sites, which were found to be on both His and Lys (Scheme 2C and Figure 9 in the SI). Encouraged by the high labeling efficiency and the generation of an electrophilic hemiacetal warhead, we demonstrated the application of 4-HNE for selective labeling of native proteins with varying warheads. We added alkyne-functionalized hydroxylamine and biotin-functionalized hydroxylamine to 4-HNE-labeled β-lactoglobulin and observed full conversion to the expected oxime conjugation products (see Figure 10

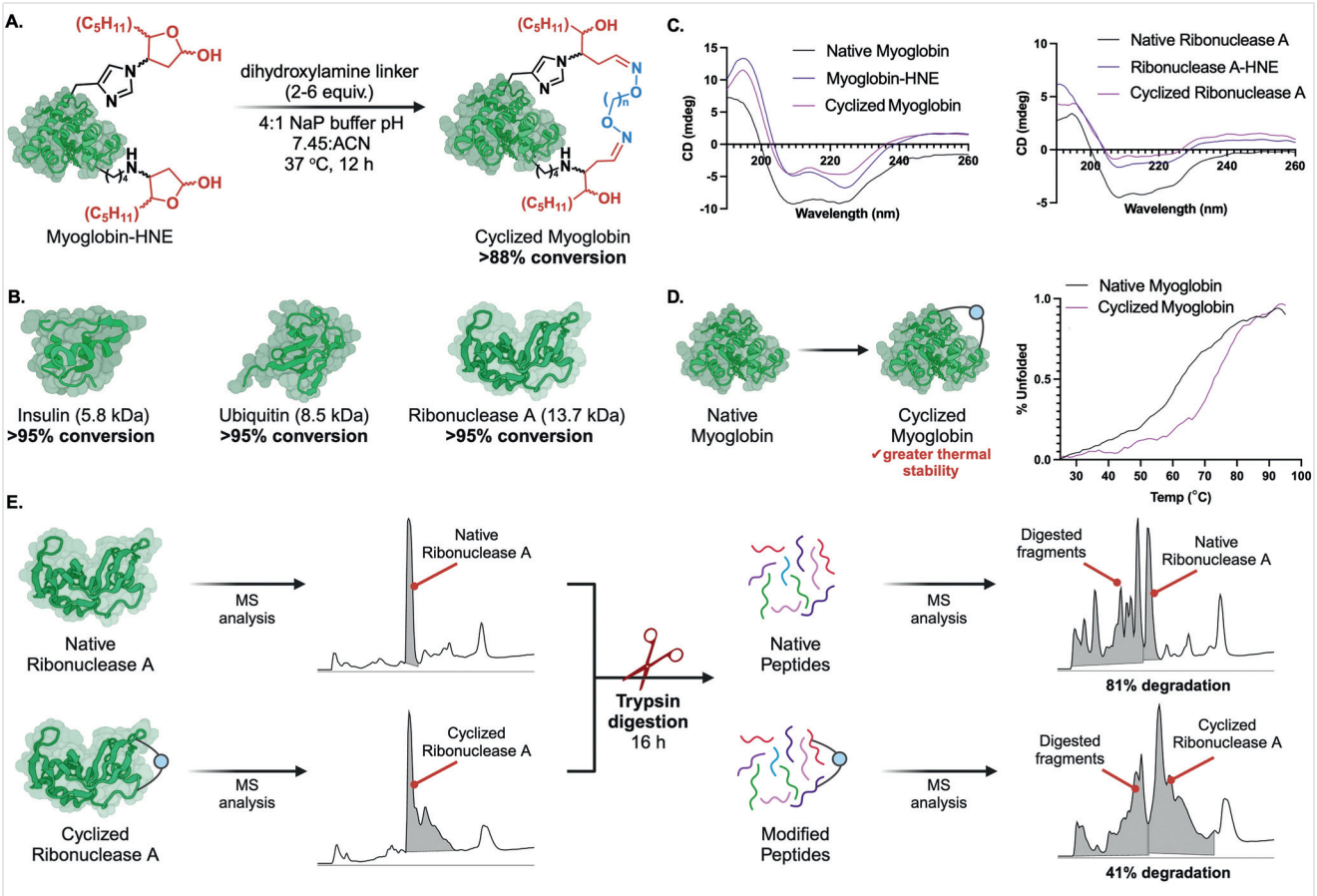


Scheme 2 Protein optimization. (A) Optimization conditions for myoglobin and intact masses for modified myoglobin using 4 and 6 equivalents of 4-HNE. (B) Protein substrate scope. (C) Site of modification is determined by MS/MS analysis of ribonuclease A. (D) Fluorophore labeling of 4-HNE-modified proteins myoglobin, ribonuclease A, and apo-transferrin and their analysis by in-gel fluorescence.

in the SI). Next, we incubated native myoglobin, ribonuclease A and apo-transferrin with 4-HNE for 16 h at room temperature, followed by reaction with a hydroxylamine fluorophore, and analysis by SDS-PAGE with in-gel fluorescence. The results clearly showed successful fluorophore labeling of proteins in the presence of all the three reaction components (lanes 4, Scheme 2D and Figure 11 in the SI). No fluorophore labeling was observed in control experiments lacking any of the components (lanes 1–3, Scheme 2D).

Given the high efficiency and robustness of 4-HNE chemistry in forming oxime conjugates with hydroxylamine molecules, we investigated its potential for the selective in situ cyclization of proteins. Our goal was to create cyclic proteins by linking two 4-HNE-modified residues, thereby enhancing the stability of protein tertiary structures. Traditional protein-cyclization methods often rely on the incorporation of unnatural amino acids, cysteine residues, or enzyme-recognized peptide tags at specific positions within the protein.[15–21] In our approach, we incubated 4-HNE doubly modified native myoglobin with varying amounts of dihydroxylamine linker (2–6 equivalents). We observed successful crosslinking between the two hemiacetal groups generated by 4-HNE, achieving ca. 88% conversion into monocyclic protein with 6 equivalents of dihydroxylamine linker (Scheme 3A and Figure 12 in the SI). The optimized conditions for this protein cyclization were determined to be 6 equivalents of dihydroxylamine in 100 mM phosphate buffer (pH 7.5) with ACN at room temperature for 12 h.

We next applied this cyclization approach to a diverse range of commercially available protein substrates, including insulin, ubiquitin, and ribonuclease A. Our method successfully achieved full cyclization for these proteins (Scheme 3B and Figure 12 in the SI). Notably, the use of the dihydroxylamine linker exclusively resulted in cyclization



Scheme 3 Protein cyclization and CD data. (A) Optimization of cyclization on doubly HNE-modified myoglobin using dihydroxylamine linker. The optimized condition was found to be the addition of 6 equivalents of linker, resulting in >88% conversion into the cyclized product. (B) Scope of cyclized proteins with varying size and three-dimensional structure. (C) CD spectra of unmodified, HNE-modified, and cyclized myoglobin and ribonuclease A. Secondary structure and helical content is overall maintained. (D) Thermal denaturation curves collected from 25 °C to 95 °C at λ = 222 nm in 10 mM pH 7.45 NaP buffer for both native myoglobin and cyclized myoglobin. E. Assessment of proteolytic stability of both native myoglobin and cyclized myoglobin by trypsin incubation and subsequent analysis of degradation by LC-MS.

without forming monoaddition products. This absence of monoaddition is likely due to the reversibility of single addition products in the presence of the nearby hydroxylamine. These results underscore the potential of this chemistry for the selective cyclization of native proteins, enhancing their stability against chemical denaturation while preserving their catalytic activity.

We conducted MS/MS analysis of ribonuclease A, insulin, and myoglobin samples with two HNE modifications, which we subsequently cyclized, to pinpoint the sites of cyclization on these proteins. Ribonuclease A cyclization occurred between modified K31 and H105, and insulin cyclization was driven by modification of H10 on the B chain, while myoglobin modification appears to have been less site-specific, with various 4-HNE-modified residues observed (see Figure 13 in the SI).

To validate that our method does not alter the protein's three-dimensional structure, we took CD spectra of 4-HNE-modified and cyclized myoglobin. Both modifications left the helical structure of myoglobin largely unperturbed compared to the native protein, with helical content decreasing marginally upon modification and cyclization (Scheme 3C and Figure 14 in the SI). We also took CD spectra of 4-HNE-modified and cyclized ribonuclease A and observed α-helix and β-sheet character to be retained in the modified samples. As with myoglobin, helical content did not decrease substantially upon modification and cyclization (Scheme 3C and Figure 14 in the SI).

We proceeded to evaluate the thermal stability of our cyclized proteins by recording the CD signal of both native and cyclized myoglobin as a function of temperature from 25 °C to 95 °C at λ = 222 nm in 10 mM pH 7.45 NaP buffer. Generation of a thermal denaturation curve revealed a higher melting temperature (T_m) for the cyclized myoglobin (T_m = 75 °C) relative to the native protein (T_m = 63 °C, Scheme 3D and Figure 15 in the SI). In effect, the secondary structure of myoglobin is stabilized by the crosslink generated in 4-HNE-mediated cyclization, with helicity maintained at higher temperatures. These findings reveal the efficacy of this cyclization chemistry in enabling efficient and selective protein cyclization while preserving structural integrity and bioactivity at elevated temperatures.

Finally, to assess the proteolytic stability of our cyclized proteins, we incubated native, and cyclized ribonuclease A with equal quantities of trypsin under physiological conditions (37 °C). Based on LC–MS, after 16 h, native ribonuclease A was 81% degraded, while cyclized ribonuclease A was only 41% degraded, demonstrating an enhancement in stability against proteases upon introduction of the unnatural 4-HNE-mediated crosslink (Scheme 3E and Figure 16 in the SI).

In summary, we have developed a robust and highly efficient chemoselective reaction for modifying histidine (His) or lysine (Lys) residues using mitochondrial-derived biological metabolite, 4-hydroxynonenal (4-HNE). We effectively modified nine different proteins of varying sizes and three-dimensional structures, highlighting 4-HNE's ability to discern subtle reactivity differences among His and Lys on native proteins. This capability is particularly valuable for protein engineering and antibody–drug conjugate (ADC) synthesis. This reaction introduces an electrophilic hemiacetal warhead, which facilitates further functionalization with a variety of nucleophiles. Leveraging these electrophilic warheads, we performed selective fluorophore labeling and achieved successful protein cyclization using orthogonal oxime chemistry. Our approach demonstrates a broad substrate scope, enabling clean and efficient conversion into stable macrocyclic proteins. CD studies revealed the maintenance of secondary structure upon modification with 4-HNE and subsequent cyclization with the dihydroxylamine linker. Cyclization of the proteins enhanced the thermal stability of protein three-dimensional structure, as evidenced by an increase in melting temperature relative to native protein. An increase in proteolytic stability upon cyclization was also revealed by exposing native and cyclized protein to trypsin digestion conditions.

Conflict of Interest

The authors declare no conflict of interest.

Funding Information

This research was supported by National Institutes of Health (NIH) grants (1R01HG012941-01) and a Research Scholar Grant (RSG-22-025-01-CDP) from the American Cancer Society to M.R.

Acknowledgment

Z.E.P. and A.V.G. acknowledge Abhinaba Das for assisting in collection of CD spectra and John Talbott for help in graphing thermal denaturation curves.
Author Contributions: Z.E.P., A.V.G and M.R. designed the study. Z.E.P performed small molecule experiments, peptide modification, and protein structure experiments. Z.E.P. and A.V.G. performed the protein modification experiments and MS/MS. A.V.G. optimized the cyclization method. Z.E.P., A.V.G. and M.R. wrote the manuscript.

Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0043-1775459. General experimental procedures and characterization details, including HPLC, HRMS, and ^{1}H and ^{13}C NMR spectra of all reported compounds are included in the supporting information.

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