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Authors

Gharakhanian, Eric G
Deming, Timothy J

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Chemoselective synthesis of functional homocysteine residues in polypeptides and peptides†

Eric G. Gharakhanian^a and Timothy J. Deming^{*ab}

A methodology was developed for efficient, chemoselective transformation of methionine residues into stable, functional homocysteine derivatives. Methionine residues can undergo highly chemoselective alkylation reactions at low pH to yield stable sulfonium ions, which could then be selectively demethylated to give stable alkyl homocysteine residues. This mild, two-step process is chemoselective, efficient, tolerates many functional groups, and provides a means for creation of new functional biopolymers, site-specific peptide tagging, and synthesis of biomimetic and structural analogs of peptides.

Practical methods for selective conversion of natural amino acids in peptides, polypeptides and proteins into different functional residues are desirable for many areas including chemical biology,¹ materials science,² and pharmaceuticals.³ The introduced functionality can provide probes for tracking,^{1,4} mimicking of post-translational modifications,¹ or a means to adjust biological and physical properties of biomacromolecules.² Both biological⁵ and chemical synthesis¹ methods have been developed to either replace or convert natural residues using highly selective processes. In order to introduce functionality at unique sites, it is essential that the natural residues are present in low abundance, which has focused much attention on cysteine,⁶ methionine,^{5,7} and N-terminal residues.⁸ Most chemical strategies focus on the modification of highly nucleophilic cysteine residues.⁶ While many excellent methods are available for chemoselective cysteine modification, some are potentially limited by racemization and moderate yields.^{6,9} Here, we have developed methodology for efficient, chemoselective transformation of methionines in peptides and polypeptides into stable, functional homocysteine derivatives. This process uses easily handled, readily available reagents, and allows

facile incorporation of a wide range of functional modifications for different uses.

Methionine residues (**M**) are a good choice for site-specific peptide and protein modification, as well as for post-polymerization polypeptide functionalization, since they occur in low abundance in proteins, are easily introduced into peptides and polypeptides usually without protecting groups, and can undergo highly chemoselective alkylation reactions at pH < 3 in high yield (eqn (1)).⁷ While alkyl methionine sulfonium (**M^R**) products themselves are potentially valuable as functional derivatives,⁷ they can be unstable toward nucleophiles,¹⁰ and their cationic nature may be undesirable for some uses. Reactions of **M^R** salts with nucleophiles can yield up to three different products (Scheme 1).¹⁰ The demethylation pathway is attractive as it leads to stable alkyl homocysteine residues (**R-CH**), where the initial alkylating reagent reacted with **M** becomes the functional group in **R-CH** through a two-step transformation.

However, pioneering studies on **M^R** salts showed that dealkylation, demethylation and substitution reactions all can occur. Substitution occurs primarily when the nucleophile is intramolecular.¹¹

Scheme 1 Possible products for reaction of **M^R** sulfonium residues with nucleophiles. **Nu** = nucleophile; **A^H** = L-homoalanine.

^a Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90095, USA

^b Department of Bioengineering, University of California, Los Angeles, CA 90095, USA. E-mail: demingt@seas.ucla.edu

† Electronic supplementary information (ESI) available: Fig. S1–S7, experimental procedures and spectral data for all new compounds, and procedures for alkylation and demethylation reactions. See DOI: 10.1039/c6cc01253a

Our lab has shown that $\mathbf{M}^{\mathbf{R}}$ salts containing labile $\mathbf{R}$ groups, *e.g.* benzyl, can be readily and selectively dealkylated, but this only leads back to the starting material $\mathbf{M}$.¹⁰ Selective demethylation to give an $\mathbf{R}-\mathbf{C}^{\mathbf{H}}$ product was obtained for $\mathbf{R} = t\text{Bu}$,¹² but required a complex procedure due to instability of the sulfonium intermediate, which dealkylates under most conditions.¹³ In effort to develop a versatile, selective process for conversion of $\mathbf{M}$ to $\mathbf{R}-\mathbf{C}^{\mathbf{H}}$ in peptides and polypeptides, we studied the reactions of $\mathbf{M}^{\mathbf{R}}$ salts with nucleophiles in greater detail.

Our lab has reported that formation of $\mathbf{M}^{\mathbf{R}}$ salts from $\mathbf{M}$ residues in peptides, polypeptides and proteins can be accomplished chemoselectively and quantitatively using a variety of functional alkylating reagents (eqn (1)).^{7,10,14} To favor demethylation, as opposed to dealkylation or substitution (Scheme 1), the methyl substituent needs to be the most electrophilic site in the $\mathbf{M}^{\mathbf{R}}$ group. We found that labile $\mathbf{R}$ groups were readily removed from $\mathbf{M}^{\mathbf{R}}$ salts using thione and thiol nucleophiles, yet these were unable to demethylate $\mathbf{M}^{\mathbf{R}}$ salts when $\mathbf{R} = \text{Me}$.¹⁰ We reasoned that more potent nucleophiles should be able to demethylate $\mathbf{M}^{\mathbf{R}}$ salts, and that use of more sterically demanding, non-labile $\mathbf{R}$ groups should favor the demethylation pathway over dealkylation. Since $\mathbf{M}^{\mathbf{R}}$ salts prepared by reaction of $\mathbf{M}$ with functional epoxides were shown to be stable against dealkylation under a variety of conditions,⁷ we studied the reactions of a model poly(L-methionine sulfonium), poly(*S*-(2-hydroxy-4,7,10,13-tetraoxatetradecyl)-L-methionine sulfonium chloride)₆₀, $\mathbf{M}^{\text{EG}_3}_{60}$,⁷ with different nucleophiles in NaOAc buffered 95% EtOH (see Fig. S1, ESI†). We chose to use $\mathbf{M}^{\text{EG}_3}_{60}$ since it allows for facile purification and isolation of products *via* precipitation and dialysis, and the uniform sequence of $\mathbf{M}^{\text{EG}_3}_{60}$ also allows for facile product characterization by NMR. While KI and 2-mercaptopyridine gave no reaction, and sodium thioglycolate gave 7% dealkylation, the more potent nucleophiles sodium thioacetate and ammonium pyrrolidinedithiocarbamate (APDC) were found to give selective and quantitative demethylation at 24 h to the corresponding fully functionalized poly(L-homocysteine) derivative, $\text{EG}_3-\mathbf{C}^{\mathbf{H}}_{60}$ (see Fig. S1, ESI†). At a shorter reaction time of 3 h, APDC gave higher conversion to $\text{EG}_3-\mathbf{C}^{\mathbf{H}}$ groups compared to less nucleophilic thioacetate (see Fig. S2, ESI†), and hence was used for all subsequent studies.

The successful selective demethylation of $\mathbf{M}^{\text{EG}_3}_{60}$ to $\text{EG}_3-\mathbf{C}^{\mathbf{H}}_{60}$ was found to be highly dependent on both the choice of nucleophile as well as the solvent used. The combination of resonance stabilized anionic nucleophiles (thioacetate or APDC) with less polar, EtOH rich solvent mixtures was found to be optimal for efficient demethylation. Use of EtOH/water mixtures with lower EtOH content led to much slower, albeit selective demethylation reactions (see Fig. S3, ESI†). These results agree with early studies on sulfonium hydrolysis that show ion-pairing occurs in low dielectric constant solvent mixtures, *i.e.* >75% EtOH, which accelerates the reaction of sulfonium ions paired with anionic nucleophiles.¹⁵ In addition, the electron delocalization in APDC may provide additional demethylation rate enhancement similar to that seen with sulfonium hydrolysis in the presence of acetate ions.¹⁶ Using APDC and 75% EtOH, we found that complete

Fig. 1 Selectivity for demethylation *versus* dealkylation. Conditions: **1a–6a** and 5 eq. APDC in 75% EtOH for 24 h at 22 °C. **1**, **5**, **6** dialyzed. **2–4** washed with MeOH. *a* = **M** is the only possible product. Yields are of isolated, purified, fully converted polypeptides.

conversion of $\mathbf{M}^{\text{EG}_3}_{60}$ to $\text{EG}_3-\mathbf{C}^{\mathbf{H}}_{60}$ occurred in *ca.* 8 h at 22 °C (see Fig. S4 and S5, ESI†).

To further examine the selectivity of the demethylation reaction, we prepared a series of fully functionalized poly(L-methionine sulfonium)s, $\mathbf{M}^{\mathbf{R}}_{60}$, where $\mathbf{R}$ were alkyl substituents of different size and electrophilicity (samples **1a–6a**, Fig. 1).⁷ Under optimized reaction conditions from above, we found that poly(*S*-methyl-L-methionine sulfonium), **1a**, could be converted completely back to poly(L-methionine) in high yield. Notably, poly(*S*-ethyl-L-methionine sulfonium), **2a**, gave the fully demethylated product with 93% selectivity in high yield, showing that the steric difference between ethyl and methyl is enough to strongly favor the demethylation pathway. Larger *n*-alkyls, **3a** and **4a**, gave exclusively the fully demethylated $\mathbf{R}-\mathbf{C}^{\mathbf{H}}$ products. These reactions allow straightforward conversion of $\mathbf{M}$ residues to known analogs such as ethionine and buthionine.¹⁷ Sulfoniums with activated alkyls, such as allyl (**5a**) and benzyl (**6a**), were found to give exclusively the fully dealkylated product poly(L-methionine),¹⁰ confirming that demethylation does not occur if $\mathbf{R}$ has enhanced electrophilicity.

As seen above with $\mathbf{M}^{\text{EG}_3}_{60}$, $\mathbf{M}^{\mathbf{R}}$ residues derived from epoxide alkylations of $\mathbf{M}$ strongly favor demethylation when treated with APDC, with possible enhanced selectivity due to the presence of the β -OH substituents on these $\mathbf{R}$ groups.¹⁸ To test the functional group tolerance of $\mathbf{M}$ to $\mathbf{R}-\mathbf{C}^{\mathbf{H}}$ conversions, a variety of fully functionalized $\mathbf{M}^{\mathbf{R}}_{60}$ derivatives were prepared in high yield using readily obtained, functional epoxides (samples **7a–12a**, Fig. 2).⁷ All the examples shown (Fig. 2) gave exclusively the fully demethylated products in high yields after treatment with APDC. Reactive azido groups were readily incorporated (**7**), as well as charged (**8,9**) and zwitterionic (**10**) groups. Polar, non-ionic oligoethylene glycol (**11**) and monosaccharide (**12**) functionalized $\mathbf{R}-\mathbf{C}^{\mathbf{H}}$ were also selectively prepared in high yield,

Fig. 2 Complete conversion of M^{R}_{60} to $\text{R-C}^{\text{H}}_{60}$. Conditions: **7a–12a** and 5 eq. **APDC** in 75% EtOH for 24 h at 22 °C, then dialyzed. a = Washed with MeOH in lieu of dialysis. b = Starting material protected, and product deprotected by treating with K_2CO_3 before dialysis (see ESI†). Yields are of isolated, deprotected, and purified polypeptides.

providing an economical route to functional polypeptides with desirable properties.¹⁹ It is worth noting that samples **7–12** all possessed good solubility ($>10 \text{ mg mL}^{-1}$) in water at 22 °C, which was enhanced by the presence of the hydroxyl groups.

The characteristic solubility and conformational changes that occur in the complete transformation of an M_{60} polymer to an M^{R}_{60} polymer then to the $\text{R-C}^{\text{H}}_{60}$ product are shown by the example in Fig. 3. Poly(L-methionine) is a hydrophobic polypeptide with poor water solubility that adopts a rigid α -helical conformation (M_{60} , Fig. 3).²⁰ After alkylation, resulting M^{R}_{60} polymers (**11a** ($\text{M}^{\text{EG}_3}_{60}$), Fig. 3) are highly charged polyelectrolytes with good water solubility and disordered chain conformations. After demethylation, the $\text{R-C}^{\text{H}}_{60}$ polymers contain non-ionic thioether linkages, and are hydrophilic, water soluble polymers that can adopt stable α -helical conformations if the **R** substituents are not charged (**11** ($\text{EG}_3\text{-C}^{\text{H}}_{60}$), Fig. 3). The ability of $\text{EG}_3\text{-C}^{\text{H}}_{60}$ to adopt a predominantly helical conformation also shows that ordered chain conformations can be obtained, which are beneficial for tuning polypeptide properties, and may allow for improved biomimicry in peptide derivatives.²

To show the conversion of **M** to R-C^{H} residues is not only applicable to polypeptides, we also studied this conversion in a model bioactive peptide, met-enkephalin amide (**13**) (Fig. 4A and B). Enkephalins are natural, endogenous opioid peptides that are conformationally flexible and tolerate substantial chemical modification.^{3b} Numerous structure–activity relationship studies have been conducted on enkephalins to improve and understand their selectivity for binding to different opioid receptors, and are aimed at developing better treatment of neuropathic pain.^{3b,21}

Fig. 3 Effect of chemical transformations on polypeptide properties. (A) Scheme for complete conversion of helical, water-insoluble M_{60} to disordered, water soluble **11a** ($\text{M}^{\text{EG}_3}_{60}$), then to helical, water soluble **11** ($\text{EG}_3\text{-C}^{\text{H}}_{60}$) via **M** alkylation followed by demethylation. (B) CD spectra of M_{60} , **11a** and **11** in either THF (M_{60}) or DI water (**11a** & **11**). For M_{60} , no data are presented below 204 nm due to solvent absorption that prohibits data collection. **11** found to be 85% α -helical. All CD spectra recorded at 0.5 mg mL^{-1} , 20 °C.

In addition to the use of conformational restraints, glycosylation and addition of lipophilic and aromatic groups have been used to modify enkephalins to improve their activity, receptor selectivity, and bioavailability.^{3b,21,22} Hence, we reasoned that conversion of **M** residues to functional R-C^{H} analogs in met-enkephalin amide may have potential value for such studies.

Similar to our prior work on **M** alkylation in peptides,^{7,10} treatment of **13** with glycidyl azide in glacial AcOH gave a dominant product (**14**), where the **M** residue was chemoselectively alkylated. The identity of **14** was determined using ESI-MS (Fig. 4A and C), where the parent ion $[\text{14}]^+$ showed addition of a single 100 Da 3-azido-2-hydroxypropyl group to each peptide. The presence of a fragment corresponding to the loss of a thioether group $[\text{14-RSMe}]^+$, which commonly occurs during MS analysis of M^{R} ions,²³ also confirmed that alkylation was exclusively occurring at the **M** residues. Subsequent demethylation of **14** using **APDC** gave the desired product **15**, which was confirmed by observation of the $[\text{15+H}]^+$ and $[\text{15+Na}]^+$ adducts by ESI-MS (Fig. 4A and D). Expanded range ESI-MS data for all samples are shown in Fig. S6 (ESI†). High conversion in the demethylation reaction of **14** was seen by LC-MS analysis of the crude reaction mixture, which showed **15** as the predominant product (84% purity, see Fig. S7, ESI†).

Here, we described methodology for complete conversion of **M** residues in peptides and polypeptides into functionalized R-C^{H} derivatives. This mild process is chemoselective, tolerates a variety of functional groups, and gives excellent yields of products. Previously, **M** residues, mainly as the amino acid, have been converted to R-C^{H} analogs through use of Na/NH_3 ,²⁴ which is incompatible with some functional groups and can lead to racemization,²⁵ resulting in this method being rarely used for peptides. We envision our methodology for facile preparation of stable R-C^{H} derivatives from natural **M** residues will provide many opportunities for creation of

Fig. 4 Chemoselective modification of met-enkephalin amide (**13**). (A) Reaction scheme for modification of **M** residues in **13** to yield azido functionalized **R-CH** residues. ESI-MS spectra of (B) starting peptide **13**, with the proton $[13+H]^+$ and sodium $[13+Na]^+$ adducts labeled; (C) product after alkylation, with molecular ion $[14]^+$ and characteristic fragment $[14-RSMe]^+$ labeled; and (D) product after demethylation, with proton $[15+H]^+$ and sodium $[15+Na]^+$ adducts labeled. **R** = 3-azido-2-hydroxypropyl.

new functional biopolymers, site-specific peptide tagging, and synthesis of biomimetic and structural analogs of peptides.

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