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Thiol Specific and Tracelessly Removable Bioconjugation via Michael Addition to 5-Methylene Pyrrolones

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Abstract

5-Methylene pyrrolones (5MPs) are highly thiol-specific and tracelessly removable bioconjugation tools. 5MPs are readily prepared from primary amines in one step. 5MPs exhibit significantly improved stability under physiologically relevant conditions and cysteine specificity compared to commonly used analogues, maleimides. Michael addition of thiol to 5MPs occurs rapidly, cleanly and does not generate a stereocenter. The conjugates efficiently release thiols via retro-Michael reaction in alkaline buffer (pH 9.5) or via thiol exchange at pH 7.5. This unique property makes 5MPs valuable for the controlled release of conjugated cargo and temporary thiol protection. The utilization of 5MPs for protein immobilization and pull-down of active complexes is illustrated using *E. coli.* acetohydroxyacid synthase isozyme I.

Graphical abstract

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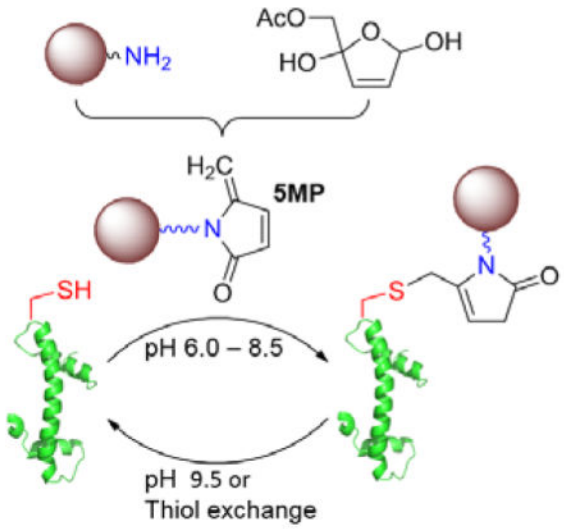
These authors contributed equally.

Notes

The authors declare no competing financial interest.

Supporting Information. General experimental methods, spectroscopic data for all new compounds, HPLC profiles and gel pictures. This material is available free of charge via the Internet at http://pubs.acs.org.



INTRODUCTION

Site-specific chemical modification is an invaluable approach for producing conjugated peptides and proteins, which are widely used as therapeutics and as tools for biochemical and biophysical studies.[1] Among the 20 natural amino acids, cysteine is especially attractive for site-specific chemical modification owing to its high nucleophilicity at physiological conditions and relatively low natural abundance.[2] Common reagents used for conjugation to cysteine include maleimides, iodoacetamides, alkyl halides, and pyridyl disulfides.[3,4] Recently developed cysteine bioconjugation reagents include dehydroalanine,[5,6] allyl sulfones,[7] diene,[8] exocyclic olefinic maleimides,[9] perfluoroaromatic reagents,[10,11] and organometallic palladium reagents.[12]

In some cases, reversible modification of a target protein is desired. For instance, once inside cells, release of the active drug from an antibody-drug conjugate (ADC) is necessary for the drug to fulfill its function.[13,14] In another example, enzymatic tagging and removal of epigenetic modifications on histones is widely involved in gene expression regulation.[15] Introducing cysteine by site-directed mutagenesis followed by cysteine specific tagging is a popular approach to generate epigenetic modifications.[16] Development of chemically reversible bioconjugation reagents will help to illustrate the functions of epigenetic modifications.[17] In addition, reversible targeting of noncatalytic cysteines was recently shown to be an advantageous strategy for developing kinase inhibitors.[18]

Despite the technique's potential utility, only a few methods are available for reversible cysteine-specific bioconjugation. Pyridyl disulfides are commonly used.[14,19] They react with cysteine to form disulfide linkages, which can be cleavaged by thiols such as β-mercaptoethanol (BME), dithiothreitol (DTT), or glutathione (GSH). Bromomaleimide is another reversible bioconjugation molecule whose cysteine conjugate is reversed by thiols.[20,21] Beyond these, dichlorotetrazine[22] and 2-napthoquinone-3-methides (oNQMs),[23] have been used to release cysteine upon photolysis of the corresponding conjugates.

Recently, during the mechanistic studies of cleavage of the C4′-oxidized abasic site (C4-AP) in a nucleosome, we found that the sugar moiety of C4-AP is transferred to the ε-amine of lysine, resulting in a 5-methylene pyrrolone (5MP) modification on the histone.[24,25] Here we report that 5MPs are thiol-specific, reversible bioconjugation reagents (Scheme 1), that are superior to commonly used, structurally similar maleimides. 5MPs are easy to prepare, stable under physiologically relevant conditions, and are highly thiol-specific. The conjugates formed between 5MPs and cysteines are stable at neutral pH, but tracelessly regenerate native peptides/proteins in either pH 9.5 buffer or by thiol exchange.

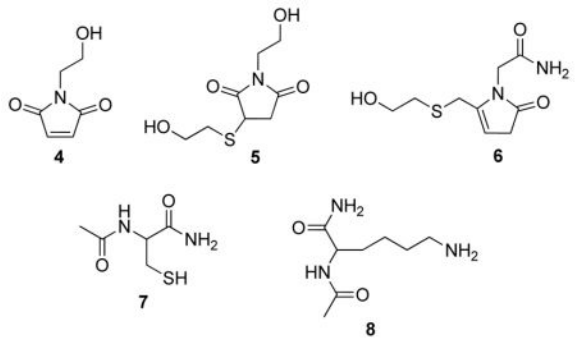
RESULTS AND DISCUSSION

5-Methylene pyrrolone (5MP) preparation

5MPs are readily prepared by coupling primary amines with **1′** in neutral aqueous solution (Scheme 2).[26] Treatment of readily available **1**[24,27] with 0.1 M HCl resulted in the assumed intermediate **1′**, which after neutralization with $NaHCO_3$, reacted with amino acid derivative **2a** at room temperature for 1 h to give **3a** in 90% yield. Other primary amine substrates, including 2-aminoethanol (**2b**), propargylamine (**2c**), biotin hydrazide (**2d**), 5-aminofluorescein (**2e**), and anti-cancer drug doxorubicin (**2f**) were also efficiently transformed to 5MPs **3b**–**f**, suggesting this coupling reaction is mild and compatible with a variety of functional groups that are commonly used for protein bioconjugation.

5-Methylene pyrrolones (5MPs) are more stable than *N*-alkyl maleimides

Maleimides are chemically unstable, especially in alkaline solutions. Ring-opening hydrolysis yields maleamic acids, which are unreactive toward thiols.[28] For instance, *N*-hydroxyethyl maleimide (**4**), decomposed completely via ring-opening hydrolysis in 2 h at pH 7.5 and 9.5 (Figure S19). In contrast, no decomposition was observed by UPLC-MS when **3a** or **3b** was incubated between pH 6.0 and 9.5 for up to 72 h (Figure S17 & 18). Hence, replacing one carbonyl with methylene in maleimide significantly improves its resistance to ring-opening hydrolysis.



Highly selective conjugation of 5MPs to thiols

The reactivity of 5MP with thiol was studied using **3a** as a model. Treatment of **3a** with excess BME at pH 7.5 rapidly produced **6** as the sole product (Figures S20–24). Furthermore, reaction of **3a** with a protected cysteine (**7**) was also rapid and clean (Figure

S27). Competitive nucleophilic attack between N-terminal amine of peptides or the ε-amine of lysine with cysteine is a limitation of maleimides as bioconjugation reagents.[29,30] However, **3a** did not react with the ε-amine of a protected lysine (**8**) between pH 6.0 and 9.5 (Figure S28), suggesting that 5MPs have superior selectivity toward cysteine compared to maleimides. Finally, unlike a maleimide, thiol addition to a 5MP (e.g. **3a**) does not generate a stereocenter, which can introduce complications.

The superior thiol specificity of 5MPs was further illustrated with reaction between **3a** and peptide **9** (Figure 1B). Peptide **9** was completely transformed to a single product (**10**) after incubation with 10 eq. of **3a** in HEPES buffer (pH 7.5) for 1 h at 37 °C. The product exhibits a $[MH]^+$ of 1906.7963 Da, which is consistent with a conjugate with one molecule of **3a**, despite the presence of other nucleophilic amino acids and the N-terminal amine. MS/MS mapping of this conjugate revealed that **3a** was exclusively attached to the side chain of cysteine (Figure S30). In contrast, treating **9** with *N*-hydroxyethyl maleimide (**4**) under the same conditions resulted in a complex mixture (Figure 1C). In addition to reaction at cysteine, conjugates between the maleimide and the N-terminal amine, lysine, as well as ring-opened hydrolysis products were also observed (Figure 1D and S31).

To evaluate the conjugation efficiency and specificity of 5MP at different pH values, **9** was incubated with 10 eq. of **3a** in acidic (pH 6.0), neutral (pH 7.5) or alkaline (pH 8.5, 9.5) buffer. UPLC-MS analysis of these reactions showed that they were completed in 10 min, and produced a sole product at pH 6.0–8.5 (Figure S33). At pH 9.5, the bioconjugation was still cysteine specific but accompanied by formation of peptide dimer. Thus, cysteine bioconjugation using 5MPs is highly specific and efficient between pH 6.0–8.5.

Cysteine specific bioconjugation on proteins by 5MPs

We evaluated the reactivity and selectivity of 5MPs with a histone H4 mutant (H4-R45C), which contains 11 lysines but only one cysteine at position 45.[31] ESI-MS analysis revealed that H4-R45C almost quantitatively reacted with 5MPs **3a**–**f** in HEPES buffer (pH 7.5) in 2 h (Figure 2 and S34). Trypsin digestion of the **3a**/**3b** modified proteins, followed by peptide mapping using UPLC-MS/MS showed that the 5MP reacted only on cysteine (Figure S35 & 36). In contrast, treating H4-R45C with maleimide **4** under the same conditions resulted in a mixture containing 1–4 modifications per molecule (Figure S37). Conjugation on lysine was identified except on the cysteine (Figure S38).

In another example, we conjugated 5MPs to *E. coli.* acetohydroxyacid synthase isozyme I (AHAS I) which catalyzes the conversion of pyruvate to acetolactate during the biosynthesis of branched-chain amino acids in plants and microorganisms.[32,33] AHAS I is composed of a regulatory subunit (ilvN) and a catalytic subunit (ilvB) that contain two and nine free cysteines, respectively. These protein subunits were employed as substrates to evaluate the efficiency of multiple cysteine bioconjugation by 5MPs. ESI-MS analysis showed that 1 mM **3a** yielded singly modified ilvN, and almost completely bis-modified protein at 25 mM (Figure 3 and S39); whereas a more complex mixture of modified ilvB was obtained upon incubation with **3a** (Figure S40). Modification of ilvN by more hydrophobic and larger **3e**

was also efficient (Figure S41), similar to **3a** yielded bis-modified protein at higher concentration (25 mM).

Traceless thiol regeneration from thiol-5MP conjugates

Thiol-maleimide conjugates decompose mainly via ring-opening hydrolysis, accompanied by a small amount of thiol exchange,[34,35] which adds to the complexity of their application *in vitro* and *in vivo*. In contrast, no trace of ring-opening product was observed when thiol-5MP conjugate (**6**) was incubated between pH 6.0 and 9.5. However, UPLC-MS analysis revealed that **6** reverted over time to **3a** and BME (Figure S42). The half-lives of decomposition of **6** in pH 6.0, 7.5, 8.5 and 9.5 buffers at 37 °C were 104.9, 16.9, 4.3 and 0.6 h, respectively. We propose that the thiol-5MP conjugate (e.g. **6**) undergoes a retro-Michael reaction, which is base catalyzed because of the acidic property of the proton at position 3 in **6** (Figure 4A). The reversible reaction between BME and **3a** was further explored by titrating **3a** (1 mM) with increasing concentrations of BME in different buffers. Fitting the titration data provided apparent K_ds[18] of ~1 mM (Figure S43) over a pH range from 6.0 to 8.5, suggesting 5MP has high activity toward thiol addition over a rather broad pH range. At pH 9.5, the K_d value is slightly smaller, suggesting that high pH facilitates the addition and elimination reactions.

These results indicate that thiol-5MPs could be useful as reversible and traceless cleavable conjugates at pH 9.5. However, triggering the retro-Michael reaction under alkaline conditions would be incompatible with intracellular and some *in vitro* applications. We speculated that the retro-Michael reaction of thiol-5MPs could be promoted at neutral pH by excess thiol, through a known thiol exchange mechanism.[35] Consequently, conjugate **6** was incubated with 20 eq. (20 mM) of GSH at pH 7.5. UPLC-MS analysis of the reaction showed that **6** was almost quantitatively transformed to **15** in less than 30 min (Figure 4B & S44), demonstrating that thiols can efficiently regenerate native proteins from 5MP-protein conjugates under mild and physiologically relevant conditions.[36] In contrast, thiol-maleimide conjugate **5** did not release any thiol when incubated in the presence of GSH, but instead decomposed exclusively through ring-opening hydrolysis (Figure S45 & 46).

The stability of a 5MP-protein conjugate was examined by incubating purified H4-R45C-**3e** at 37 °C in pH 7.5 buffer. Aliquots at different incubation times were analyzed by SDS-PAGE (Figure 5). Fluorescence quantification of the protein bands indicates that the half-life for fluorescein cleavage from protein is 28.5 h (Figure S47). Addition of 10 mM GSH to the incubation buffer decreased the half-life to 16.8 h. Addition of 100 mM DTT significantly accelerated fluorescein release from the protein (Figure 5C). The cleavage reaction was almost complete in 6 h and MS analysis showed that the released protein was intact (Figure S48), suggesting that thiol release occurs in a clean and traceless manner.

Protein immobilization and active complex pull-down using 5MPs

The simplicity of 5MP synthesis suggested that they could be readily employed to functionalize primary amine containing solid supports. 5MP modified supports could be useful for immobilizing proteins and for purifying cysteine containing proteins. As an example, the ability of 5MPs to immobilize a protein for pull-down of an active complex

was illustrated using ilvN and ilvB. We immobilized ilvN to a 5MP functionalized support and used it to fish out ilvB, with the aim to ultimately releasing the complex in a controlled manner (Figure 6A).

5MP functionalized solid supports were prepared by treating commercially available long chain aminoalkyl controlled porosity glass (LCAA-CPG)[37] with freshly prepared **1′** at pH 7.5 for 4 h. Quantitation of free amines on the support before and after the reaction using 4-chloro-7-nitrobenzofurazan[38] revealed that 5MP functionalization proceeded essentially to completion. Mixing ilvN with 5MP-CPG resulted in quantitative protein immobilization in less than 10 min (Figure S49). Blocking the 5MP-CPG with BME before adding ilvN substantially suppressed ilvN immobilization, suggesting that protein immobilization occurs through covalent bond formation between an ilvN cysteine and a 5MP on the support. After ilvN immobilization, excess 5MP was blocked by incubating with 5 mM BME for 10 min (Thiol exchange is negligible under these conditions). The ilvN-CPG was incubated with a solution of ilvB at 4 °C for 1 h. SDS-PAGE analysis of the supernatant indicated that ilvB was completely pulled-down by the solid support (Figure S50).

Various conditions were then testing for removing the protein(s) from the support. For instance, incubation with SDS-PAGE loading buffer (SDS LB: 50 mM Tris, pH 6.8, 100 mM DTT, 2% (w/v) SDS) at 95 °C for 5 min efficiently released ilvN and ilvB (Figure 6B). Treatment with the same buffer at room temperature (5 min) selectively eluted the non-covalently bonded ilvB. Several conditions were also examined for releasing active ilvB-ilvN complex. SDS-PAGE analysis of supernatants following incubation of supports using buffers of pH varying from 6.0 to 9.5 at 4 °C for 6 h revealed that the proteins were removed only at the most alkaline pH employed (Figure 6B). Protein elution was also examined using various BME concentrations (Figure S51), and 700 mM BME at 4 °C for 2 h almost quantitatively released ilvB-ilvN from the support (Figure 6B).

The ilvB-ilvN proteins eluted using pH 9.5 buffer and BME were subjected to dialysis to remove salt or BME. MS analysis revealed that the eluted ilvN and ilvB were intact (Figure S52). Moreover, reconstituted ilvN-ilvB complex converted two pyruvate molecules to an acetolactate in the prescence of thiamin diphosphate (ThDP) and flavin adenine dinucleotide (FAD) cofactors. The enzymatic activity of the eluted proteins was almost the same as that of reconstituted complex (Figure 6C).

Finally, we demonstrated that ilvN-CPG could be used for efficiently capturing ilvB from cell lysate (Figure 6D). Thus, 5MP functionalized solid supports are good choices for protein immobilization and pull-down of active complexes, which could be applicable in protein purification, as well as protein-protein, and protein-nucleic acid inter-action studies.

CONCLUSIONS

The above experiments demonstrate that 5-methylene pyrrolones (5MPs) are a new type of thiol-specific and tracelessly removable bioconjugation tools. 5MPs have a number of merits, including ease of synthesis. 5MPs are prepared from primary amines through a mild coupling reaction using readily available chemicals. 5MPs exhibit significantly improved

stability and thiol specificity compared to maleimides, which are widely used for thiol bioconjugation. Cysteine specific bioconjugation of protein by 5MP occurs rapidly, cleanly, and the Michael addition does not generate a new stereocenter, which simplifies product analysis.

5MP-protein conjugates decompose slowly at neutral pH (half time ~ 1 day), but tracelessly and rapidly release native proteins at pH 9.5 or via a thiol exchange reaction. The slow degradation of 5MP-protein conjugates could limit their applications in some fields, such as *in vivo* delivery conjugated drugs. However, the properties of 5MPs, especially compared to maleimides, will make them very valuable in cases where reversible and traceless cleavage of bioconjugates under physiological conditions or temporary thiol/cysteine protection is required.

In addition, the mild and efficient generation of 5MPs from primary amines could be further utilized to produce 5MPs on lysine residues and N-terminal positions of proteins,[39,40] which could be subjected to further functionalization or to generate inter/intramolecular lysine-cysteine cross-links. Research in this area is ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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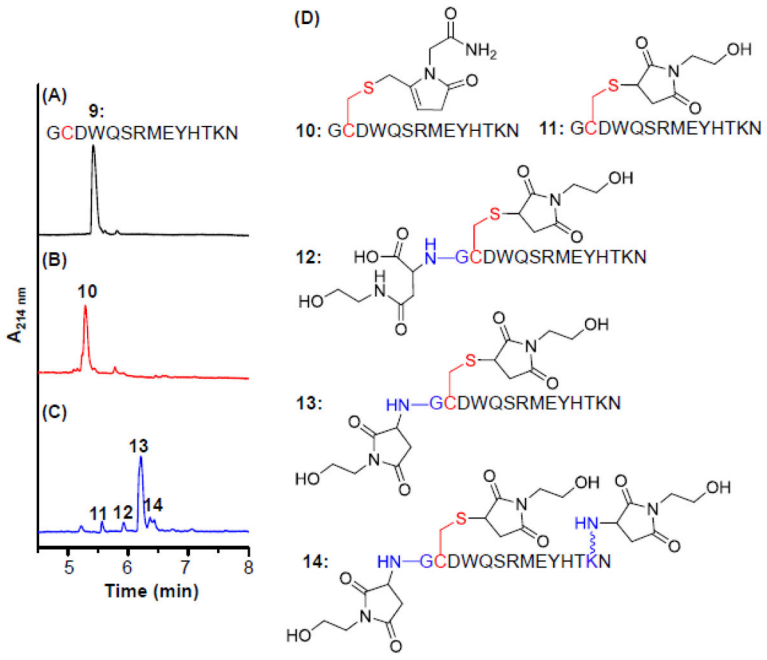


Figure 1.

UPLC analysis of the reaction between peptide **9** (A) with **3a** (B) or **4** (C). Reaction conditions: **9** (0.4 mM) and **3a** or **4** (4 mM, 10 eq.) in HEPES (50 mM, pH 7.5), 100 mM NaCl, 37 °C, 1 h.

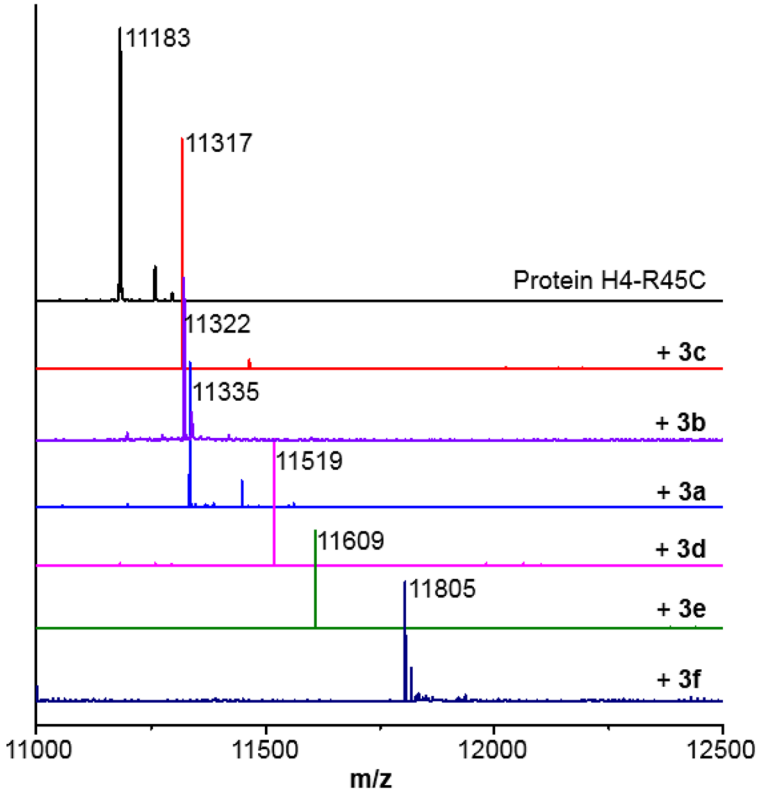


Figure 2.

MS analysis is of reactions between protein H4-R45C and **3a**–**f.** Reaction conditions: H4-R45C (50 μM) and **3a**–**f** (10 mM) in HEPES (10 mM, pH 7.5), 100 mM NaCl, 37 °C, 2 h.

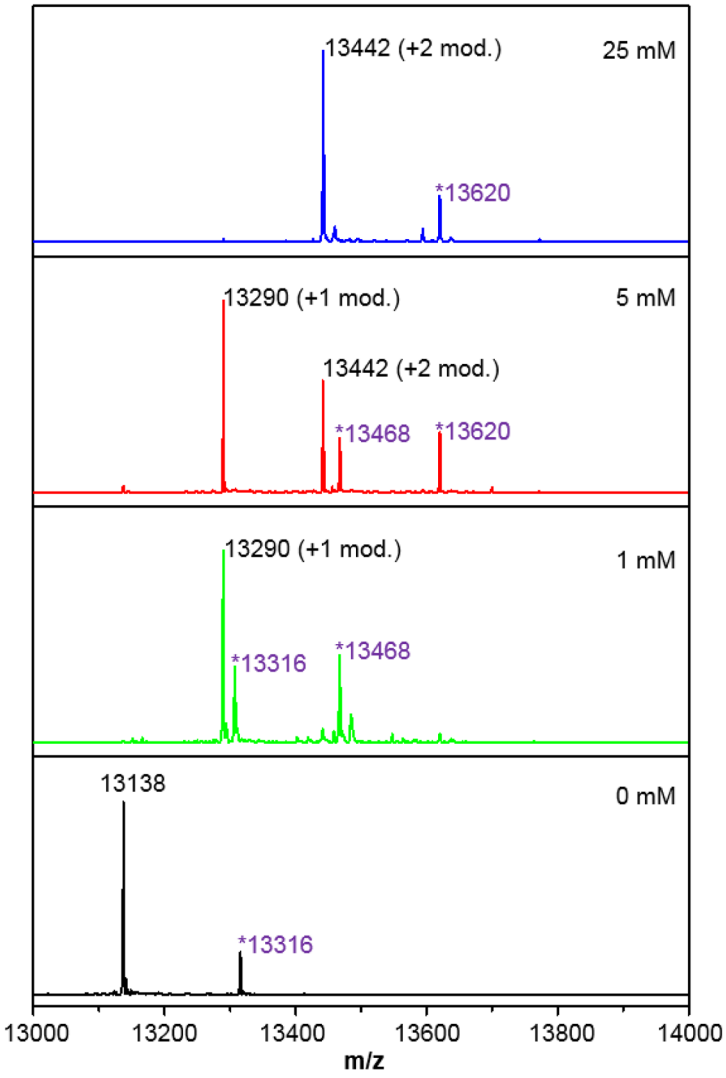


Figure 3.

MS analysis of the reaction between protein ilvN with different concentrations of **3a**. A +178 Da adduct (denoted by*) was present during the ESI-MS analysis for protein ilvN.

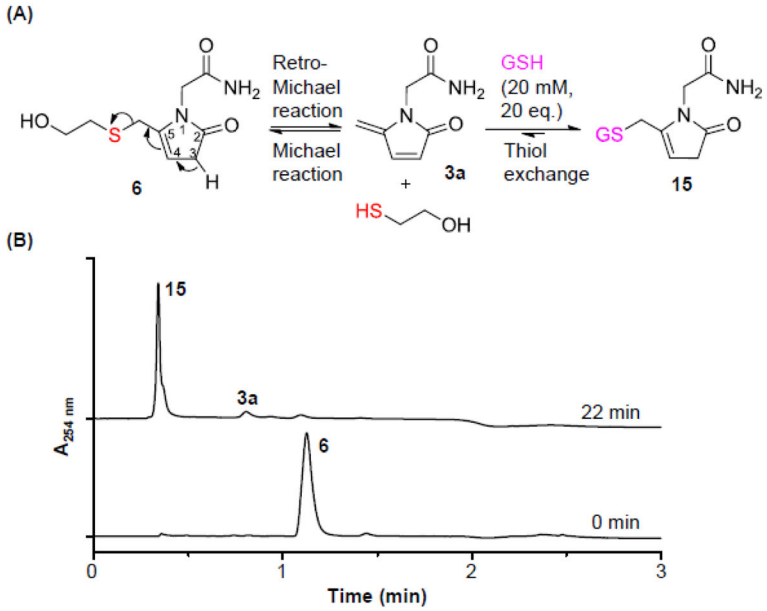


Figure 4.

Proposed mechanism (A) and UPLC analysis of the thiol exchange reaction of **6** with GSH. Reaction conditions: **6** (1 mM) and GSH (20 mM, 20 eq.) in HEPES (20 mM, pH 7.5), 100 mM NaCl, 37 °C.

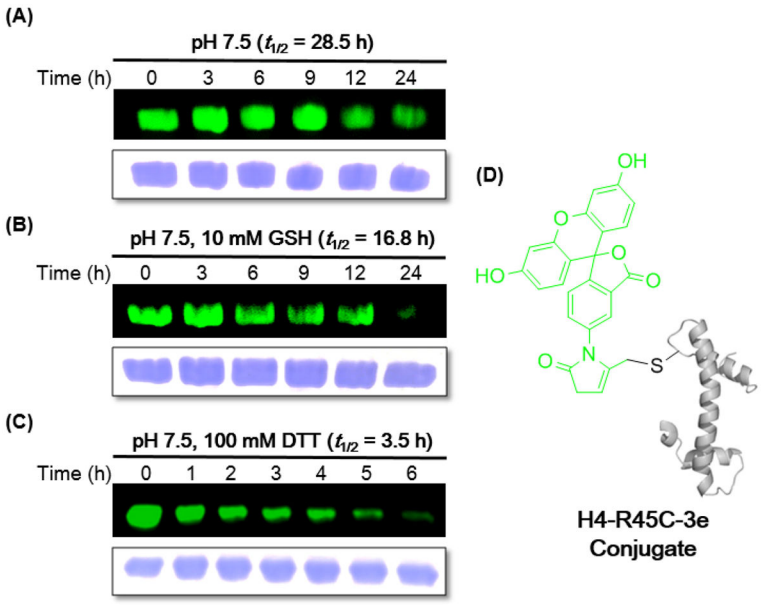


Figure 5.

SDS-PAGE gels showing the release of fluorescein moiety from H4-R45C-**3e** in pH 7.5 buffer (A), with 10 mM GSH (B) or 100 mM DTT (C) at 37 °C. Top gels: Fluorescence. Bottom gels: Coomassie staining.

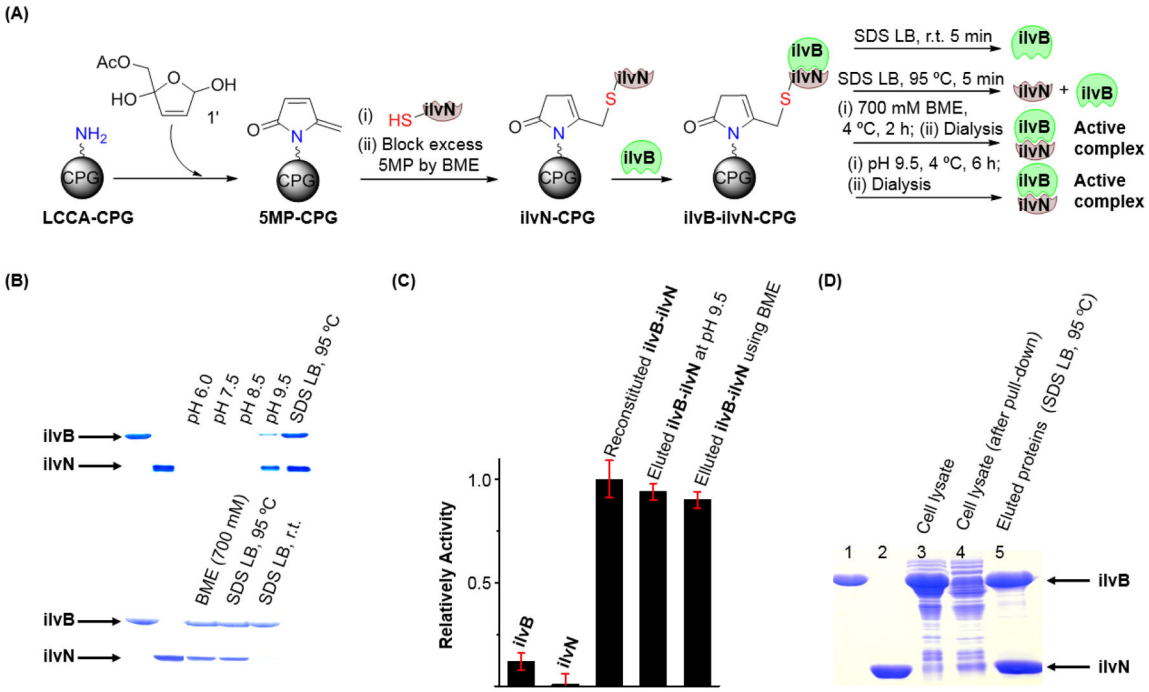
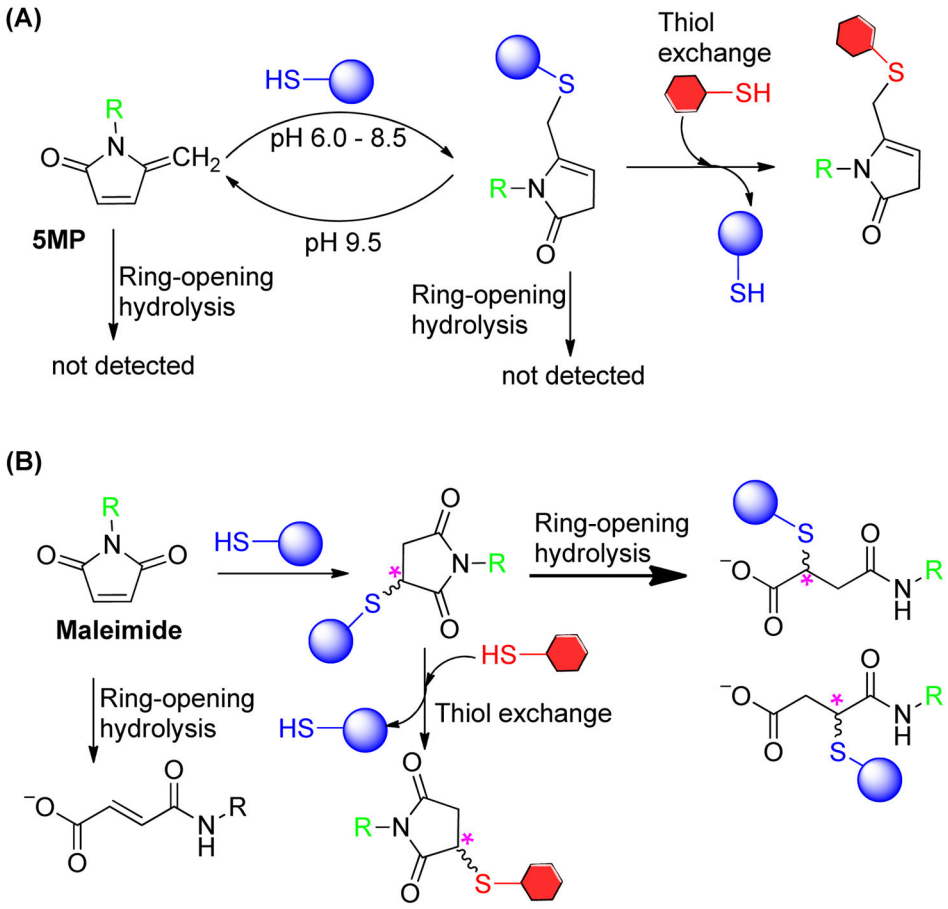
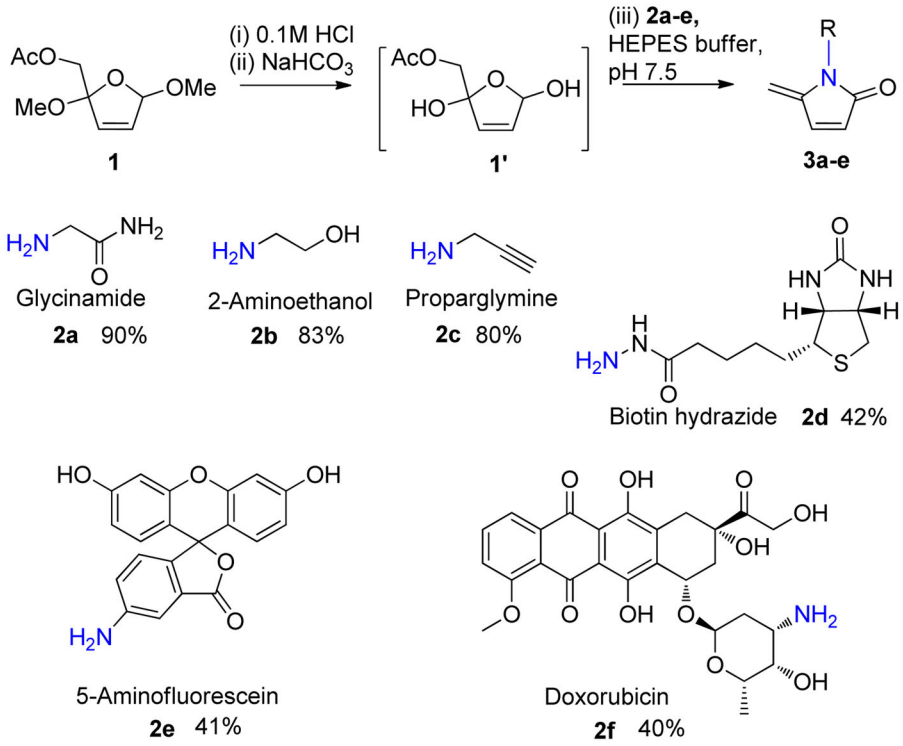


Figure 6.

Preparation and utility of a 5MP immobilized protein. (A) 5MP functionalized solid support used for protein immobilization and pull-down of active protein complex. (B) SDS-PAGE analysis of the eluted proteins from ilvB-ilvN-CPG under different conditions. (C) The relative activity of eluted ilvB-ilvN complex released under different conditions. (D) SDS-PAGE showing pull-down of ilvB from cell lysate using ilvN-CPG.



Scheme 1.



Scheme 2.