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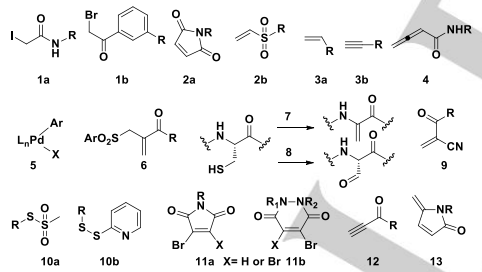
Highly Reactive and Tracelessly Cleavable Cysteine-Specific Modification of Proteins via 4-Substituted Cyclopentenone

Jian Yu, Xiaoyue Yang, Yang Sun and Zheng Yin*

Abstract: A rapid and cysteine-specific modification of proteins using 4-substituted cyclopentenone via a Michael addition tandem elimination reaction was developed. Compared to the classical method, this reaction featured fast kinetics with a stable product. More importantly, this conjugation could be tracelessly removed by exchange with a Michael addition donor. The conjugation and regeneration process not only exhibited little change to the structures or conformations of the proteins but also exhibited little disturbance to their biological functions, such as their enzymatic activities.

Chemical modification of proteins is an important tool for monitoring, modulating, and tracking proteins in living systems for biological research.[1-4] Among the natural 20 amino acids, cysteine is perhaps one of the most attractive and convenient targets for site-specific chemical modification due to its high nucleophilicity, relatively low natural abundance[5] and the ease of its introduction into a specific site by site-directed mutagenesis.[6] Electrophiles including α-halocarbonyls (**1**) and Michael acceptors (**2**) were classical approaches for the modification of cysteine (Scheme 1). Reagents and approaches recently developed for cysteine-specific modifications include thiol-alkene (**3a**),[7-8] thiol-alkyne (**3b**),[9] allenamide (**4**),[10] organometallic palladium (**5**)[11], bis-alkylation (**6**)[12] and functional transformation to dehydroalanine (**7**)[13] or aldehyde (**8**).[14]



Scheme 1. Reagents for the modification of cysteine.

Despite various methodologies in the toolbox, in general, the expectation of a rapid, highly efficient, highly selective, and biocompatible reaction for protein modification has not yet been fully met. In many cases, a high concentration of reagent (1~100 mM) and long incubation time (hours to overnight) are required for protein modification, which limit the further practical application of these reactions in biological studies at the cellular level, in organisms and in living animals.[15] The electrophilicity of the reagent is one of the important parameters for cysteine modification. Compounds having low electrophilicity with only one electron withdrawing group (EWG) such as iodoacetamide (**1a**, **IAA**) or vinyl sulfone (**2b**)[16] only exhibited relatively mild activity towards cysteine at physiological conditions. Increasing the pH may accelerate the reaction but consequently may result in side products.[17] The electrophilicity was enhanced with more EWGs on alkenes such as maleimide (**2a**) or α-cyano-α,β-unsaturated carbonyl compounds (**8**), which surely resulted in better reactivity but caused more side effects including poor selectivity or the occurrence of the instability of both reagents and products. For example, maleimides tend to undergo ring-opening hydrolysis yielding maleamic acids[18] and may also possibly contaminate other functionalities such as the amino group. Alkene **9** with two EWGs on one side showed a reversible reaction, and similar molecules such as Bardoxolone methyl or MCE-23[19] were used as a reversible covalent inhibitor/candidate/drug against cysteine-containing proteins.

The non-cleavable nature of the ligation prevents any possibility for regenerating the unmodified protein through the disassembly of the conjugate.[20] Cleavable or reversible conjugations that are biological compatible have become useful tools for biological research[21] since cleavable conjugations are required in many research fields such as protein immobilization, drug development,[22-23] and proteomics.[24-25] Only a few methods are available for cleavable cysteine-specific modification, including Ellman's reagent (**10**),[26-27] bromo-maleimides (**11a**),[28] bromo-pyridazinediones (**11b**),[29] electron deficient acetylenes (**12**),[30] and recently reported 5-methylene pyrrolones (**13**).[31]

Herein, the cysteine-specific protein modification with 4-substituted cyclopentenone was reported. Compared to many previous reports,[32] this ligation could achieve high reactivity (with a rate constant over 100 $M^{-1}s^{-1}$ at the peptide level) and stable product at physiological conditions with little impact on the function and structure of the protein. More importantly, this modification could be tracelessly cleavable to regenerate the protein with a Michael addition donor.

4-Acetoxy cyclopentenone (**14**) (Scheme 2) could be deemed as the predecessor of cyclopentadienone (**16**),[33-34] an extremely unstable antiaromatic electrophile, which had a high tendency to dimerize.[35] The literature survey showed that **14** was significantly more stable than common β-acetoxy ketone and resistant to β-elimination.[33] Hypothetically, 4-acetoxy cyclopentenone (**14**) could be used for cysteine protein modification through the mechanism of Michael addition (**15a**) with rapid elimination (**15b**) that is similar to reported small molecule reactions.[36-38] β-Elimination could occur rapidly on **15a** only after Michael addition due to the unfavored antiaromatic nature of **16,** which would provide selectivity for cysteine. The conjugation product (**15b**) is expected to be very stable due to the same reason. In addition, the modified protein could potentially be regenerated (**15c**) in the presence of nucleophilic reagents.

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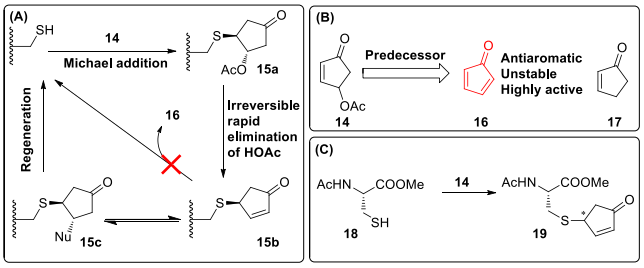

Scheme 2. (A) Possible mechanism of the modification and removal reaction. (B) **14** could be deemed as a predecessor of the highly unstable compound (**16**). (C) Model study of small molecules.

The initial study was performed using a cysteine derivative (**18**) (**Supporting information part 3**) that reacted with 4-acetoxy cyclopentenone (**14**). The reaction of **18** and **14** in a concentration of 10 mM in PBS with no other additives completed in 10 minutes at room temperature. This reaction was much faster than common unsaturated carbonyl compounds such as cyclopent-2-enone.[39] The overall reaction was confirmed to be "substitution" instead of addition. The product was identified as 1:1 diastereomers (**19**).

A model peptide with a sequence of GTSWCYNQKRHDGP (**20**) was used for further studies (Figure 1A). Peptide (**20**) contains all natural amino acids with nucleophilic functionalities for the purpose of the selectivity study. The cysteine was rapidly modified by **14** in PBS buffer (pH = 7.4) at room temperature to afford the sole adduct with a precise molecular weight increase corresponding to cyclopentendione (+80.026) (Figure 1C). Intermediate (**15a**) was observed (Figure 1B) at 2 min right after the beginning of the reaction, followed by rapid elimination to afford the product, which matched the possible mechanism.

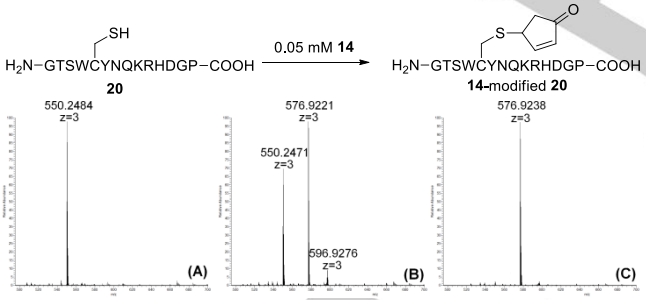

Figure 1. Mass spectrum of peptide (5 μM) modified by **14** (50 μM) in PBS buffer (pH = 7.4) at room temperature. The reaction was monitored by LCMS. (A) t = 0 min; (B) t = 2 min; (C) t = 12 min.

Further study showed that 4-acetoxy cyclopentenone (**14**) had much higher reactivity than cyclopent-2-enone (**17**) in the reaction with peptide (**20**). The screening of substituted cyclopentenones illustrated that the leaving groups were vital to the activity. (**Supporting Information part 4.1**). Further mechamism investigation suggested that the high activity of **14** was derived from the antiaromatic property of **16**. As intramolecular elimination was fast enough, we presumed the peptide modification to be a pseudo-first-order Michael addition and measured the rate constant accordingly (**Table S5**).

Subsequently, we sought to apply this reaction to the modification of proteins. UBXD (**Supporting information part 5.1**), containing one cysteine and 6 lysines, was selected as a model protein. The reaction was quantitatively analyzed[40] using the protein mass spectrum (Q-Exactive orbitrap). As a result, the cysteine at UBXD could be rapidly modified in the conditions of 10 μM protein and 0.5 mM **14** for 60 min at room temperature in PBS with little by-product (Figure 2AB). The excess of **14** was likely to be stable without any reaction in the reaction media (**Figure S8**). Equivalent **14** reacting with peptide in 50 μM could finally consumed in stoichiometric fashion (**Figure S9**). In comparison, **17** had no reaction and **IAA** only gave 21% yield (Table 1). Accordingly, rate constants of relevant reagents for modification of UBXD were messured (**Table S6**). LC−MS^2 analysis of the resulting peptide fragments from trypsin digestion showed that UBXD modification by **14** occurred only on cysteine with high selectivity, and no modification was observed on lysine or the N-terminal amine (**Figure S7**). The modification had little impact on the structure or conformation of UBXD according to the UV-*vis* and CD (circular dichroism) spectra (**Figure S6**). Various buffers were studied for their impact. The reaction was hardly affected by buffers under the physiological environment. However, when the pH was lower than 6, the reaction could not proceed efficiently (**Table S7**).

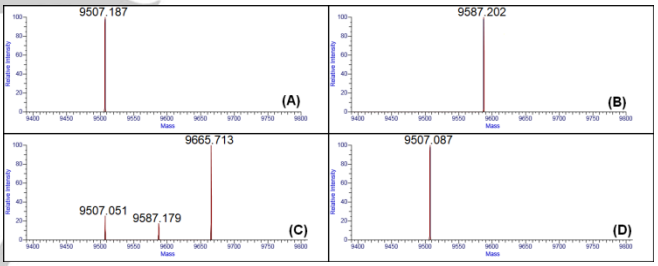

Figure 2. Deconvolouted spectra of UBXD (Please see full mass spectrum in the **Figure S4**). (A) Unmodified UBXD observed with 9507.187; (B) completely modified UBXD by **14** observed with 9587.202; (C) t = 10 min; modified UBXD with a mercaptoethanol Michael adduct intermediate observed with 9665.713; (D) t = 180 min; regenerated UBXD observed 9507.087.

After the chemical modification of UBXD, the small molecule was removed via spin concentration, and the product was stored in PBS buffer at room temperature. Cyclopentenone (**17**)- and bromo-maleimide (**11a**)-modified UBXD were used as controls. Similar to the previous publication,[18] maleimide-modified UBXD tended toward ring-opening hydrolysis with considerable speed (**Figure S10**), while UBXD modified by **17** slowly decomposed via reverse Michael addition. As expected, the product of UBXD ligated with **14** was stable without noticeable degradation. Despite maleimide showed higher rate constant than **14**, the disadvantages such as instability were clearly observed.

Several cysteine-containing proteins were used to study the application potential of this reaction, while **IAA** was chosen as the control. **14** could achieve higher activity than **IAA** for the modification of UBXD (Table 1), which completed modification

with 0.5 mM **14** in 60 min at room temperature in PBS. HCP1 was a protein with one hindered cysteine, which had low activity. **14** achieved 19% and 36% modification at one hour and two hours, respectively, while **IAA** had low yield (2.5% in 2 hours). EV 71 3C protease, which contained an active cysteine and a non-active cysteine, was employed for the study. Both cysteines were modified by **14** with satisfactory yield, while **IAA** gave low activity toward the non-active cysteine. Lastly, MERS 3C-like protease containing 8 cysteines with different activities was studied. After modification by 0.5 mM small molecules for 2 hours, multiple modifications on the cysteines of **IAA** were observed from 2 to 5 modifications, while **14** gave 4 to 7 cysteine modifications. Please see the deconvolouted spectra in **Figure S5**.

Table 1. Various proteins were modified by 0.5 mM **14** or **IAA** in PBS at room temperature.

Protein 10 μM, 25 °C, PBS		Conversion / 1 h	Conversion / 2 h
UBXD[a]	0.5 mM **IAA**	21%	37%
	0.5 mM **14**	>95%	>95%
HCP1	0.5 mM **IAA**	ND[c]	2.5%
	0.5 mM **14**	19%	36%
EV 71 3C protease[b]	0.5 mM **IAA**	(+1) 44% (+2) ND	(+1) 71% (+2) 5%
	0.5 mM **14**	(+1) 34% (+2) 23%	(+1) 35% (+2) 51%
MERS 3C-like protease	0.5 mM **IAA**	(+1) 12% (+2) 38% (+3) 35% (+4) 15%	(+2) 11% (+3) 35% (+4) 47% (+5) 7%
	0.5 mM **14**	(+3) 4% (+4) 48% (+5) 38% (+6) 10%	(+4) 22% (+5) 41% (+6) 27% (+7) 10%

[a] The modification of UBXD and HCP1 was quantified by LCMS. [b] The modification of EV 71 3C protease and MERS 3C-like protease was quantified by protein deconvolution software. [c] Not detected.

A one-pot click reaction[41] was studied using this methodology to demonstrate its application in protein imaging. Terminal alkynes were introduced into **14** to give reagent **21**, which was used for the modification of cysteine in proteins including UBXD, EV 71 3C protease and BSA with different molecular weights (9.5 kD, 22.3 kD and 66.4 kD). Myoglobin, which has no cysteines, was used as a control. **IAA-yne** (**22**) was studied in parallel to **21** for comparison. After modification by **21** and **22** respectively (1.0 mM, 60 min, room tempurature), all modified proteins further proceeded through a one-pot click reaction, which conjugated with an azide rhodamine derivative. The SDS-PAGE gel analysis was shown in Figure 3. Only the modified cysteine-containing proteins emitted fluorescence. As expected, the imaging capability of **21** was better. The stability of **UBXD-20-dye** in PBS and humam plasma was determined (**Supporting information part 8.4**). The modified UBXD remained stable in PBS and slowly decomposed in human plasma which might be caused by the existence of nucleophile in human plasma.

As discussed in Scheme 2a, an unsaturated ketone was newly formed in the product of the Michael addition tandem elimination reaction (**15b**). It was envisaged that the Michael addition tandem elimination reaction could be used to remove the modification on cysteine and regenerate the protein (Figure 2 **15c**). After complete modification of UBXD by **14**, excess Michael donor, mercaptoethanol (BME), was added. As shown in Figure 2, a Michael adduct intermediate (Figure 2C) was observed (**Figure S12**). Cyclopentenone was tracelessly removed in 3 hours (Figure 2D). Other Michael addition donors such as glutathione (GSH) or Edaravone could achieve the similar result. The regeneration kinetic curve and $\tau_{1/2}$ (half protein regeneration time) are given in (**Figure S13**).

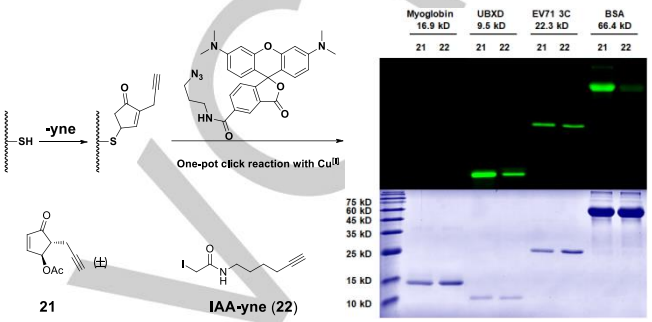

Figure 3. SDS-PAGE gel analysis of four proteins: myoglobin with no cysteine as a control; modified UBXD; EV 71 3C protease and BSA emitted fluorescence (excitation wavelength: 365 nm), which were modified by a one-pot click reaction.

EV 71 3C protease was a cysteine protease and could be assayed according to our previous report.[42] The activated cysteine at the catalytic domain was modified by **14** or **IAA**, which inactivated 3C protease. After BME was added, EV 71 3C protease was revived in exchange of cyclopentenone, while **IAA** modified 3C protease still remained inactivated. The process of ligation and the regeneration protocol not only demonstrated little change in the structure or conformation but also had little disturbance on the enzymatic activity (**Supporting information part 9.2**).

The modification of **14** was irreversible in the presence of other cysteine conjugation compounds such as **IAA** or **2a**. Therefore, we proposed that cyclopentenone, could have further application as a protecting group for cysteine. Many Michael acceptors could modify the N-terminal amine of proteins at pH = 6, which was obviously incompatible with cysteine. In such cases, **14** could well serve as protecting group for cysteine in the process of the N-terminal modification by **2a**. It was investigated by UBXD as a model protein. After complete modification of the cysteine in UBXD by **14** (Figure 5A), 20 mM **2a** was added for N-terminal modification in PB (pH = 6.0). The cyclopentenone was not found to be exchanged by **2a**. After complete modification of the N-terminal of UBXD by **2a** (Figure 5B), BME was added (Figure 5C) to tracelessly remove the cyclopentenone (Figure 5D). This exemplary application illustrated that this method could serve as a good cysteine protecting group.

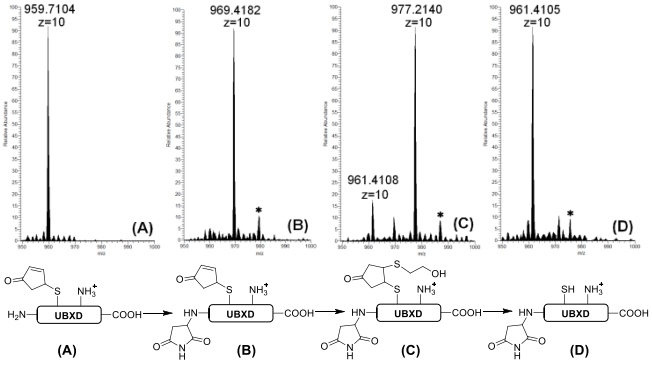

Figure 5. After protection, N-terminal modification by maleimide could be achieved without being influenced by the cysteine. However, inevitably a little (10%) lysine-modified adduct by maleimide (denoted by ∗) was present. Please see the deconvolouted spectra in **Figure S15**.

Conclusions

In this study, 4-acetoxy cyclopentenone (**14**) was developed as a reagent to achieve rapid, cysteine-specific modification of proteins. This reaction featured fast kinetics with a stable product. In addition, this conjugation could be tracelessly removed by exchange with a Michael donor. The conjugation and regeneration procedure not only had little effect on the structure or conformation of the protein but also had little disturbance on the enzymatic activity. Several applications of this methodology were successfully demonstrated.

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Keywords: protein modification; cysteine specific; tracelessly cleavable

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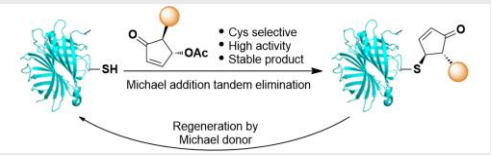

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Title

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