Protein Modifications

DOI: 10.1002/anie.201403121

Allenamides as Orthogonal Handles for Selective Modification of Cysteine in Peptides and Proteins**

Ata Abbas, Bengang Xing, and Teck-Peng Loh**

Abstract: *In this study, a remarkably simple and direct strategy has been successfully developed to selectively label target cysteine residues in fully unprotected peptides and proteins. The strategy is based on the reaction between allenamides and the cysteine thiol, and proceeds swiftly in aqueous medium with excellent selectivity and quantitative conversion, thus forming a stable and irreversible conjugate. The combined simplicity and mildness of the process project allenamide as robust and versatile handles to target cysteines and has potential use in biological systems. Additionally, fluorescent-labeling studies demonstrated that the installation of a C-terminal allenamide moiety onto various molecules of interest may supply a new methodology towards the site-specific labeling of cysteine-containing proteins. Such a new labeling strategy may thus open a window for its application in the field of life sciences.*

Selective chemical modification of protein structure is of significant importance to directly visualize protein dynamics, clearly understand their interaction mechanisms, and precisely mediate protein–protein interactions under single-cell and molecule resolution. Among the various remarkable labeling strategies, the sulfhydryl group in peptides and proteins has remained an attractive target for site-selective modification[1] owing mainly to its higher nucleophilicity and relatively lower natural abundance.[2] Briefly, the direct modification of cysteine can be mainly represented by two typical chemical pathways, that is, the nucleophilic substitution of a leaving group through the thiol of cysteine, as is the case for α-halocarbonyl derivatives (e.g. 2-iodoacetic acid and related variants), and the Michael addition of a thiol group to Michael acceptors like α,β-unsaturated systems. Another less common strategy is the metal-catalyzed cystein modification where most notable works involve cross-metathesis of allyl sulphides using the ruthenium catalyst reported by the group of Davis,[3] and thiol–allene coupling using a gold catalyst reported by the group of Che.[4] In recent years, some notable efforts to successfully target cysteine residues can be summed up as cross-metathesis of allyl sulfides,[3] and conjugation of thiol groups with allyl selenosulfate salts,[5] electron-deficient alkynes,[6] or bromomaleimides,[7] dithiomaleimide[8] derivatives, and PEGylation of native disulfide bond using a double cysteine alkylating reagent.[9] However, most strategies suffer from one or more drawbacks and the search for a versatile methodology remains elusive. While the cross-reactivity of classical reagents, including α-halo acyl analogues and maleimides, with other amino acids such as histidine and lysine residues potentially affects the selectivity, their reversibility/irreversibility is a matter of interest in newer findings. For example, bromomaleimides, dithiomaleimides, and electron-deficient alkynes form a bond with cysteine thiols, and can be reversed with excess glutathione, DTT, and other reactants. This reversibility, though extremely useful, may pose a limitation when in vivo application is required, because of the presence of albumin in blood serum and glutathione in the intracellular environment. Therefore, an urgent need exists to find promising orthogonal handles and related labeling strategies which can selectively and irreversibly bind with cystein. The groups of Ovaa and Mootz have recently demonstrated, simultaneously and independently, that terminal alkynes (in modified ubiquitin) can be used to inhibit cysteine proteases irreversibly through in situ thiol–alkyne coupling,[10] which is quite impressive and surprising as alkynes have largely been considered inert under biological conditions. Activation of alkynes by a positively charged protein pocket (oxoanion hole) has been proposed to be responsible for this unexpected reactivity.[11]

With the long-term goal to understand and regulate protein functions through their chemical modification, our group has previously reported modification of the N-terminus of peptides and proteins using Mukaiyama aldol condensations,[12] and herein we, for the first time, present an efficient way to utilize C-substituted terminal allenamide moieties as an "orthogonal handle" to selectively and irreversibly modify cysteine residues in fully unprotected peptides and proteins which have other reactive amino-acid side chains. The reaction is characterized by quantitative yields, very high selectivity, mild reaction conditions, and high reaction rates. In a typical reaction, simple pipette mixing of a cysteine-containing peptide or protein with allenamide in aqueous buffer of pH 8.0 at room temperature gives the conjugation product with quantitative conversion within 10–20 minutes. LCMS was used to confirm quantitative conversion while

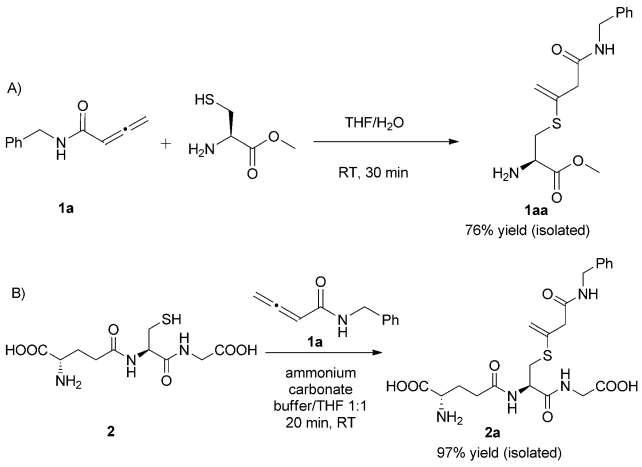
[*] Prof. T. P. Loh
Department of Chemistry
University of Science and Technology of China
Hefei, Anhui 230026 (China)
A. Abbas, Prof. B. Xing, Prof. T. P. Loh
Division of Chemistry and Biological Chemistry
School of Physical and Mathematical Sciences
Nanyang Technological University
21 Nanyang Link, Singapore-637371 (Singapore)
E-mail: teckpeng@ntu.edu.sg

[**] Nanyang Technological University and the Singapore Ministry of Education Academic Research Fund. This research was supported financially by Nanyang Technological University (New Initiative Funding) and the National Environment Agency (NEA-ETRP Project Ref. No. 1002 111).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201403121.

NMR spectroscopy and MS/MS data were used for detailed characterization of the conjugation products to confirm the site selectivity. The advantages of using an allenamide moiety are that it is easily prepared, can be installed on relatively complex molecules, is stable at room temperature, is not prone to hydrolysis or polymerization upon standing for long time, gives a simple addition product with thiol without removal of any group, and does not react with hydroxy groups, amines, or carboxylate ions, which are the most commonly encountered nucleophiles in biological systems. Such a simple and effective cysteine-labeling strategy may provide great possibilities for many in vivo and in vitro applications, including protein imaging and others.

During our study with C-substituted terminal allenamides, we found that they are much less reactive, than their keto and ester counterparts, towards [3+2] cycloaddition reactions, probably because they are less electron deficient than allenones and allenoates. However, they are attacked quickly by thiol,[13] but not by amines, to give vinyl sulphides, and the reaction proceeds well in water. Our study began with the synthesis of the model benzyl allenamide **1a** and its subsequent reaction with cysteine methyl ester which contains free thiol and amino groups (Scheme 1 A). No reaction was



Scheme 1. Reactions of the allenamide **1** with cysteine methyl ester (A) and glutathione (B). THF = tetrahydrofuran.

observed at the amino group, even when **1a** was used in excess. Only the thiol group was found to be involved in the reaction, thus giving the product **1aa**. At the same time, no product was obtained when **1a** was reacted with lysine, serine, and methionine amino acids under similar reaction conditions. The naturally occurring tripeptide, glutathione (GSH; **2**), was next reacted with **1a** in ammonium carbonate buffer (pH 8.0) and THF (20 %) solution (Scheme 1 B). The desired product **2a** was obtained in 97 % yield upon isolation and characterized by NMR spectroscopy and LC-MS/MS data, thus confirming the modification at the cysteine residue instead of at the amino groups in the peptide residues, and suggesting the excellent reaction selectivity of C-substituted terminal allenamides with the specific cysteine conjugation (see the Supporting Information).

To further demonstrate selectivity, cysteine modification of three other peptide sequences including Cys-Gly-Lys-Ser-Arg-Phe (**3**), Lys-Ser-Cys-Gly-Arg-Phe (**4**), and Tyr-Asp-Ser-Gln-Cys-Phe-His-Arg-Trp (**5**), having terminal as well as internal cysteine residues, was also investigated. In all cases, quantitative conversion was obtained upon incubating the peptide (250 μM) with **1a** (10 equiv) for 10 minutes in ammonium carbonate buffer (pH 8.0) at room temperature. LCMS and LC-MS/MS analysis confirmed the modification to have taken place selectively at cysteine. Notably, other amino acid residues including histidine, lysine, tyrosine, and other reactive side chains did not give any reaction with **1a** even after standing for overnight. The irreversibility of the conjugation product was tested with the peptide **5** and GSH (**2**). No reversion to the starting **5** was observed when the conjugation product of **5** and **1a** was incubated overnight at 37 °C with excess (100 equivalent) glutathione. Similarly, the treatment of **2a** with excess (100 equivalents) DTT did not produce **2** (LCMS), thus clearly suggesting the stability of the conjugated products. The good irreversibility of specific cysteine labeling may thus enable the possibility for the protein modification under biological conditions.

After promising results in simple peptides, similar modification of cysteine residues in more complicated peptide sequences was also examined. A more complex pair of peptides, generated from the DTT treatment of bovine insulin, was treated with **1a** to afford the fully modified chains A and B (Figure 1; see the Supporting Information for full procedures and MS/MS data).

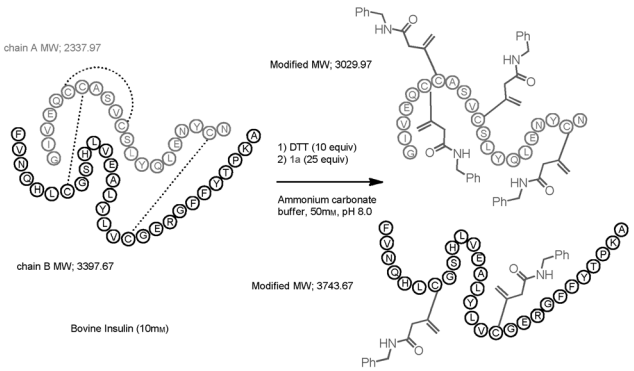
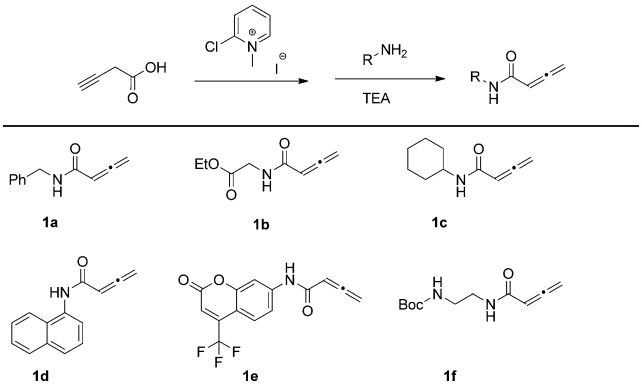


Figure 1. Modification of bovine insulin with **1a**.

To further explore the scope of the reaction, the allenamide moiety was installed on a range of molecules (**1b–f**). The standard procedure for the coupling of carboxylic acids and amines, using the Mukaiyama reagent, was slightly modified to synthesize these allenamides (Table 1) which are difficult to prepare otherwise and are rarely reported in literature. The compounds **1b–d** were then used to modify **5**, thus giving quantitative conversion and excellent selectivity in each case (see Table 2 and the Supporting Information).

We propose the mechanism of the reaction to be a straightforward 1,4-Michael addition where remarkable observation is the formation of the thermodynamically less stable product X (nonconjugated) as compared to Y (con-

Table 1: C-substituted allenamide synthesis from amines.[a]



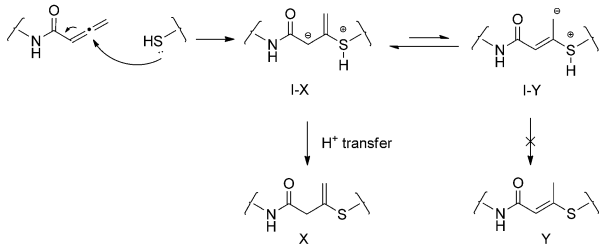
[a] A small amount of homopropargyl amide is also observed sometimes and can be isomerized to the allenic isomer by excess TEA. Boc = *tert*-butoxycarbonyl, TEA = triethylamine.

Table 2: Modification of cysteine in peptide **5** using the allenamides **1b–d**.[a]

Entry	Allenamide[b]	Peptide sequence[c]
1	**1b**	YDSQCFHRW
2	**1c**	YDSQCFHRW
3	**1d**	YDSQCFHRW

[a] Conversion was quantitative in each case (determined by LCMS). [b] Used 10 equivalents. [c] Used 250 μM.

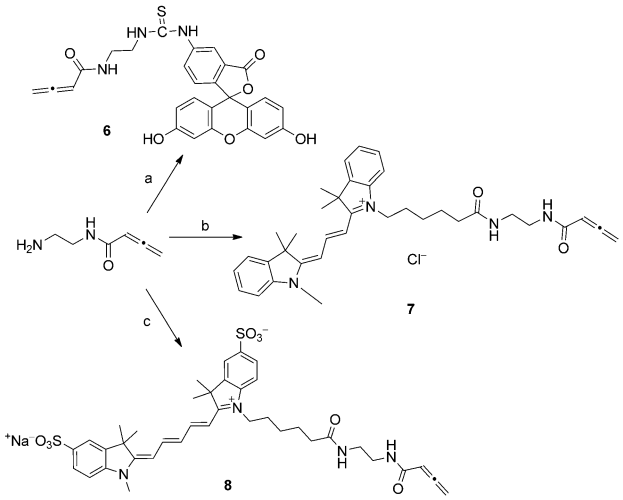
jugated). The reason for this observation may be the greater stability of the intermediate I-X than I-Y (Scheme 2). The compound X, having geminal chlorine on the γ-carbon atom, has indeed been reported to isomerize slowly to a compound corresponding to Y in the presence of strong base.[13]



Scheme 2. Proposed mechanism for addition of thiols to allenamides.

Alkylation of cysteine thiols in proteins and the stability of the resulting conjugate under peptic digestion conditions were tested on bovine serum albumin (BSA) proteins. After DTT treatment, BSA was incubated with **1a** at 37°C for 30 minutes. After purification through nano-sep filtration the protein was digested with trypsin and analyzed by LC-MS/MS. All the cysteine residues in detected peptides were found to be modified by a molecule of molecular weight corresponding to **1a** (see the Supporting Information).

Next, to test the applicability of this reaction in selective labeling of proteins at cysteine, allenamide moieties were



Scheme 3. Synthesis of labeling probes with allenamide handles. Reaction conditions: a) FITC isomer I, DMF, DIPEA, RT, 2 h; b) Cy3-NHS ester, DMF, DIPEA, RT, 2 h; c) sulfo-Cy5-NHS ester, DMF, DIPEA, RT, 2 h. DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, FITC = fluorescein isothiocyanate, NHS = *N*-hydroxysuccinimide.

installed on some probe molecules (Scheme 3). We selected fluorescein isomers I, Cy3, and Cy5 dyes as probes to be utilized in the labeling experiment. BSA, having a free thiol, was treated with **6**, **7**, and **8**. Successful labeling was confirmed by SDS PAGE analysis (Figure 2A). To show that labeling is specific for thiol groups, a well-established antibiotic resistant bacterial enzyme, TEM 1 β-lactamase (Bla), having only disulfide bonded cystein (no free cysteine) was chosen. The compound **7** was mixed with Bla without DTT treatment and no labeling was observed (lane 5, Figure 2B), whereas successful labeling was observed when Bla was treated with DTT prior to mixing with **7** (lane 3 Figure 2B). These results establish allenamide as an efficient handle to target cysteine residues selectively in the complex milieu of the protein environment and opens an alternative approach for imaging applications.

In conclusion, we have found a new orthogonal handle, allenamide, to modify thiol groups in peptides and proteins

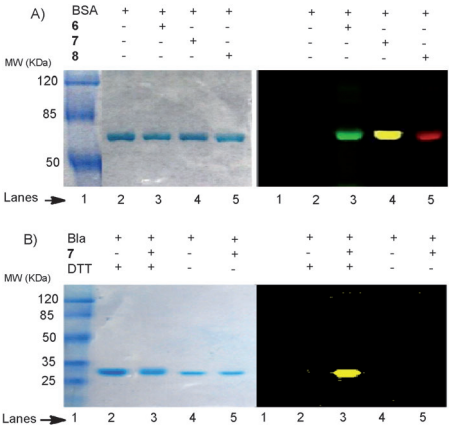


Figure 2. Labeling of BSA (A) and TEM1 β-lactamase (B).

selectively. The quick reactivity of C-substituted allenamides towards thiols, their simultaneous inertness towards amine and hydroxy nucleophiles, and their stability in aqueous buffers make them an attractive handle for targeting cysteine residues in peptides and proteins. Irreversibility of this process can be exploited in many in vivo applications such as the inhibition of cysteine proteases. The conjugation product, as shown, is stable and detectable under tryptic digestion conditions. We have demonstrated the usefulness of the allenamide handle through its installation on various molecules and the successive labeling of proteins with high selectivity.

Received: March 8, 2014
Revised: March 27, 2014
Published online: May 30, 2014

Keywords: allenes · dyes/pigments · peptides · protein modifications · sulfur

[1] J. M. Chalker, G. J. L. Bernardes, Y. A. Lin, B. G. Davis, *Chem. Asian J.* **2009**, *4*, 630 – 640.
[2] M. N. Fodje, S. Al-Karadaghi, *Protein Eng.* **2002**, *15*, 353 – 358.
[3] Y. A. Lin, J. M. Chalker, N. Floyd, G. a. J. L. Bernardes, B. G. Davis, *J. Am. Chem. Soc.* **2008**, *130*, 9642 – 9643.
[4] A. On-Yee Chan, J. Lui-Lui Tsai, V. Kar-Yan Lo, G.-L. Li, M.-K. Wong, C.-M. Che, *Chem. Commun.* **2013**, *49*, 1428 – 1430.
[5] D. Crich, V. Krishnamurthy, T. K. Hutton, *J. Am. Chem. Soc.* **2006**, *128*, 2544 – 2545.
[6] H.-Y. Shiu, T.-C. Chan, C.-M. Ho, Y. Liu, M.-K. Wong, C.-M. Che, *Chem. Eur. J.* **2009**, *15*, 3839 – 3850.
[7] a) L. M. Tedaldi, M. E. B. Smith, R. I. Nathani, J. R. Baker, *Chem. Commun.* **2009**, *0*, 6583 – 6585; b) M. E. B. Smith, F. F. Schumacher, C. P. Ryan, L. M. Tedaldi, D. Papaioannou, G. Waksman, S. Caddick, J. R. Baker, *J. Am. Chem. Soc.* **2010**, *132*, 1960 – 1965.
[8] M. P. Robin, P. Wilson, A. B. Mabire, J. K. Kiviaho, J. E. Raymond, D. M. Haddleton, R. K. O'Reilly, *J. Am. Chem. Soc.* **2013**, *135*, 2875 – 2878.
[9] S. Balan, J.-w. Choi, A. Godwin, I. Teo, C. M. Laborde, S. Heidelberger, M. Zloh, S. Shaunak, S. Brocchini, *Bioconjugate Chem.* **2006**, *18*, 61 – 76.
[10] a) R. Ekkebus, S. I. van Kasteren, Y. Kulathu, A. Scholten, I. Berlin, P. P. Geurink, A. de Jong, S. Goerdayal, J. Neefjes, A. J. R. Heck, D. Komander, H. Ovaa, *J. Am. Chem. Soc.* **2013**, *135*, 2867 – 2870; b) S. Sommer, N. D. Weikart, U. Linne, H. D. Mootz, *Bioorg. Med. Chem.* **2013**, *21*, 2511 – 2517.
[11] C. Arkona, J. Rademann, *Angew. Chem.* **2013**, *125*, 8368 – 8370; *Angew. Chem. Int. Ed.* **2013**, *52*, 8210 – 8212.
[12] J. Alam, T. H. Keller, T.-P. Loh, *J. Am. Chem. Soc.* **2010**, *132*, 9546 – 9548.
[13] a) A. Roedig, W. Ritschel, *Chem. Ber.* **1983**, *116*, 1595 – 1602; b) J. D. Buynak, J. Mathew, M. N. Rao, E. Haley, C. George, U. Siriwardane, *J. Chem. Soc. Chem. Commun.* **1987**, 735 – 737; c) R. R. Kostikov in *Science of Synthesis*, *Vol. 24* (Ed.: A. de Meijere), Thieme Publishing Group, Stuttgart, **2005**, pp. 17 – 35.

