Highly Chemo-, Regio- and Stereoselective Cysteine Modification of Peptides and Proteins with Ynamides

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ABSTRACT: Chemoselective modification of peptides and proteins has wide applications in chemical biology and pharmaceutical development. An efficient cysteine (Cys) precise modification protocol via rationally designed *β*-addition of ynamides is reported. The strong electron-withdrawing triflyl group on the nitrogen atom of ynamides plays a crucial role for controlling the chemo-, regio- and stereoselectivities of this protocol. Another substituent of the terminal ynamides offers a handle for functionality diversification. This Cys modification with ynamides proceeds efficiently in a slightly basic aqueous media (pH 8) to provide a series of *Z*-isomer of the corresponding conjugated products with excellent stereoselectivity (> 99%) and superior stability. All the reactive peptide side chain functional groups such as amino, carboxyl, primary amide, and hydroxyl groups, as well as the unprotected imidazole and indole rings are compatible. This method displays a broad substrates scope including linear and cyclic peptides and proteins. The potential application of this method in peptide and protein chemical biology was exemplified by Cys-bioconjugation with ynamides containing different functional molecules, including small molecule drug, fluorescent and affinity tags. In addition, this strategy is also compatible with click chemistry (performed in one pot), which remarkably extends the applications of this tool in chemical biology. Furthermore, the chemoselective biotinylation of ubiquitin(G47C) variant with a biotinylated ynamide, as well as the regioselective modification of Cys14 and Cys38 in bovine pancreatic trypsin inhibitor (BPTI) could be accomplished smoothly under the optimized reaction conditions without perturbation of the other two disulfide bonds.

INTRODUCTION

Protein functions are greatly diversified and adjusted by various post-translational modifications (PTMs), such as phosphorylation, glycosylation, acetylation, and ubiquitylation, which are intricately mediated by natural enzymes.[1] However, it is still challenging to produce proteins with precise PTMs using biological techniques, hence chemical protein synthesis has turned out to be highly valuable approach to accomplish this goal.[2-3] In addition, chemists have developed a variety of chemical tools to mimic the function of some protein PTMs[4-7], and chemical modifications of peptides and proteins have made enormous impact for the developments of biotherapeutics[8-10] as well as for basic biological studies[11-14]. Despite the progress in the chemoselective bioconjugation of previously unexplored amino acid side chains, including serine (Ser)[15], selenocysteine (Sec)[16-17], histidine (His)[18], methionine (Met)[19-22], selenomethionine (Sem)[23], tyrosine (Tyr)[24-28] and tryptophan (Trp),[29-31] lysine (Lys)[32-36] and cysteine (Cys)[37-41] are still the preferred residues for chemical protein modification due to their unique nucleophilicity[42-43]. While Lys is highly common in protein sequences (~6% human proteins sequences[44]), many of which are solvent exposed, Cys is one of the least frequent amino acids in proteins (~ 1.7%)[45], especially those in the reduced form on the protein surfaces, which are readily accessible for chemical modifications. Different strategies of Cys-specific modification have been developed, including Michael addition[46-47], metal-mediated reactions[48-51], nucleophilic substitution[37, 52] and radical transformations[53-54]. Among these reactions, Michael addition is a widely used strategy to conjugate small molecules containing *α,β*-unsaturated systems, such as maleimides,[55] to the proteins of interest both *in vivo* and *in vitro*.[38, 56-58] Although the rapid kinetics of these reactions has attracted considerable attention,[59] two major drawbacks including the low chemoselectivity (see the Supporting Information for detailed chemoselectivity experiments on peptide models with maleimide and iodoacetamide, Figure S63-S73) and poor stability of the conjugated products in the presence of external thiols (like glutathione) plagued their applications in chemical biology (Figure 1a)[8, 60-61]. Even recent progresses employing innovative Michael acceptors with electron-deficient alkynes, including phosphonamidate[62] and diethynyl phosphinates[63] have been explored, challenges in the stereo- and regioselectivities, and the stability

of the conjugated products, which are important for drug discovery[64-65], still remain (Figure 1b)[38, 60, 66].

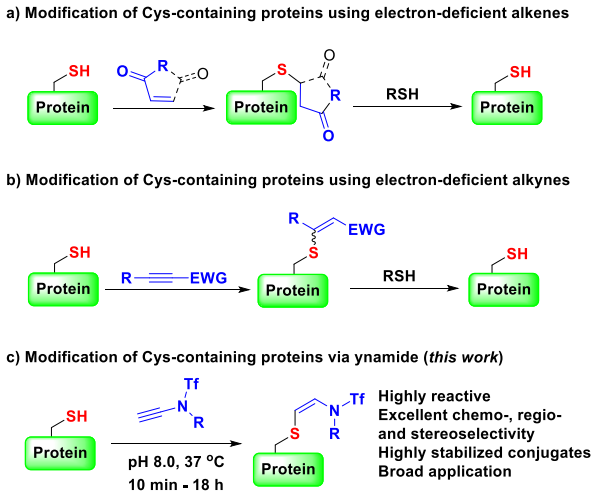


Figure 1. Cys-specific modifications with electron-deficient alkenes/alkynes.

Ynamides are strongly polarized alkyne derivatives due to the direct attachment of the electron-donating nitrogen atom to the triple bond and the electron-withdrawing group (EWG) on the nitrogen atom, which provides an optimal balance between the stability and reactivity.[67] Thus, ynamides display unique reactivities owing to the flexible modulation of EWG and have evolved into versatile synthons and building blocks.[68-71] We have recently disclosed that ynamides could be used as novel coupling reagents for facilitating amide[72-73] and ester[74-75] bond formation, which were accomplished by taking advantage of the α-addition of carboxylic acid to ynamides (Figure 2a).[71] Furthermore, we have found that the regioselectivity could be reversed by employing basic reaction conditions as exemplified by the regio- and stereoselective hydroamidation[76] and hydrophosphorylation[77] of ynamides. These reactions proceeded smoothly via a base-promoted *trans*-selective β-addition with excellent regio- and stereoselectivity, which was rarely exploited compared with the extensively studied α-addition of ynamides (Figure 2b)[78-80]. Meanwhile, the radical hydrothiolations of internal[80-81] and terminal[79] ynamides with thiyl radicals through β-addition were reported. Although these radical reaction conditions are relatively complex and not compatible with multiple sensitive functional groups of peptides and proteins, such pioneering contributions inspired us to develop a Cys-specific modification using ynamides. According to our studies on ynamide chemistry, while α-addition of ynamides (Figure 2a) is preferred under acidic conditions, basic reaction conditions favor the β-addition (Figure 2b). We rationalized that selective Cys modification via a nucleophilic β-addition of mercapto group to ynamides would be feasible by employing the basic reaction conditions, in which α-addition of carboxylic acid to ynamide could be prevented. Guided by such proposal, we have developed an efficient Cys precise modification protocol by taking advantage of the less studied β-addition of ynamides (Figure 1c). The excellent chemo- and regioselectivity of this protocol enabled Cys to be selectively modified in the presence of all other amino acid residues with reactive functional groups such as amino, carboxyl, amide, and hydroxyl groups, in addition to the imidazole and indole rings to give only the Z-isomer of the corresponding conjugated products (Figure 1c).

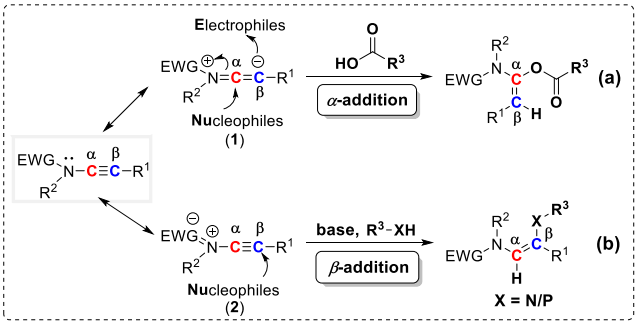


Figure 2. Nucleophilic α-addition and β-addition of ynamides.

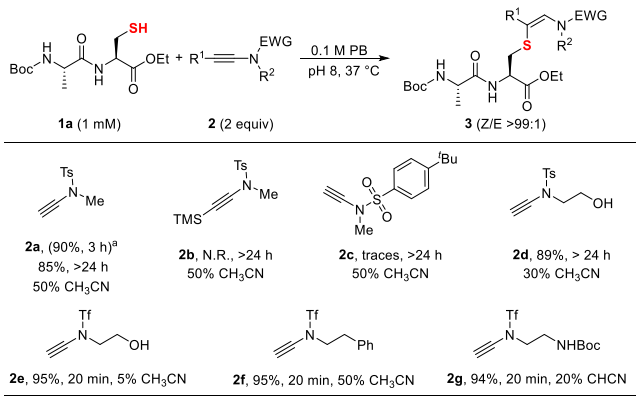
RESULTS AND DISCUSSION

Rational Design of Ynamide. To assess the feasibility of our proposal, a model dipeptide Boc-Ala-Cys-OEt (**1a**, 1 mM) was treated with Na_2CO_3 (2 equiv) and *N*-methylynetoluenesulfonamide (MYTsA, **2a**, 2 equiv) in acetonitrile (MeCN). Delightfully, quantitative conversion was observed after 3 h, and only *Z*-isomer of product (**3a**) was detected (see the Supporting Information for details), indicating an excellent stereoselectivity (*Z*:*E* > 99:1). Further optimization was performed to extend the application of this method to peptide and protein modifications, in which milder reaction conditions, including aqueous media, near-neutral pH (6-8), and tolerance to other reactive peptide functional groups, are required.

When **1a** (1 mM) and **2a** (2 equiv) were mixed in MeCN/phosphate buffer (PB) (1:1, pH 8), the target conjugated product **3a** could be obtained in 85% yield but with a prolonged reaction time (24 h), which suggested that a rational design of the ynamide reagent is necessary (Table 1). Next, internal ynamide (**2b**) was evaluated but did not provide any conjugated product. This result indicated that the R^1 substituent has a significant negative effect on the reactivity of ynamide for this transformation. Then, terminal ynamide (**2c**) containing a *p*-^{t}Bu on the phenyl group was used but also was inert under the same reaction conditions, which might be attributed to its poor solubility in aqueous media. To address this issue, R^2 substituent (methyl group in **2a**) was replaced by a 2-hydroxyethyl group (**2d**) with a hope of improving the solubility of the ynamide in aqueous media. Indeed, the reaction of **2d** proceeded smoothly in phosphate buffer with 30% MeCN as a co-solvent and afforded the target product **3d** in 89% isolated yield. Obviously, the EWG on the nitrogen atom of ynamide plays a crucial role for controlling the reactivity of ynamide, and a stronger EWG is beneficial to strengthen its activity for acting as a Michael acceptor for nucleophiles (Figure **2b**). Thus, the *p*-toluenesulfonyl (Ts) was replaced by a stronger electron-withdrawing trifluoromethylsulfonyl group (triflyl, Tf) to offer a novel ynamide with a more electron-deficient C-C triple bond (**2e**). Using this ynamide (**2e**), the conjugation reaction was completed within 20 min in phosphate buffer with only 5% MeCN as a co-solvent, giving the desired product in 95% yield and excellent stereoselectivity (*Z*:*E* > 99:1). Similarly, ynamides (**2f**) and (**2g**) showed comparable efficiency, which further implied the potential diversification of ynamides via functionalization of R^2 substituent.

Optimization of the Reaction Conditions. With the optimal ynamide (**2e**) in hand, we started to study its reactivity under different reaction conditions (Table 2). Varying pH conditions (entries 1−4, Figure S22) showed that the reaction was sluggish

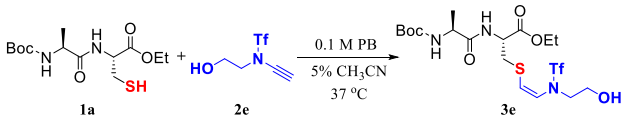
Table 1. Rational design of ynamides for cysteine modification.



[a]Na_2CO_3 (2 equiv), 100% MeCN. All reactions were monitored by HPLC, and the yields presented were isolated yields. PB = phosphate buffer.

under neutral conditions (pH 6-7.5), but highly efficient at pH 8, which was consistent with our expectation. Screening of the loading of **2e** (entries 4−6, Figure S23) suggested that the conjugation reaction could be completed within 5 min with only 5.0 equiv of **2e** (entry 6, Figure S23). Varying the concentration of the reagents (entries 8−11, Figure S24) revealed that the reaction could be performed at as low as 10 μM of peptide **1a** with 5 equiv of **2e** retaining a comparable efficiency (entry 11, Figure S24).

Table 2. Optimization of the conjugation of ynamide with cysteine-containing peptide

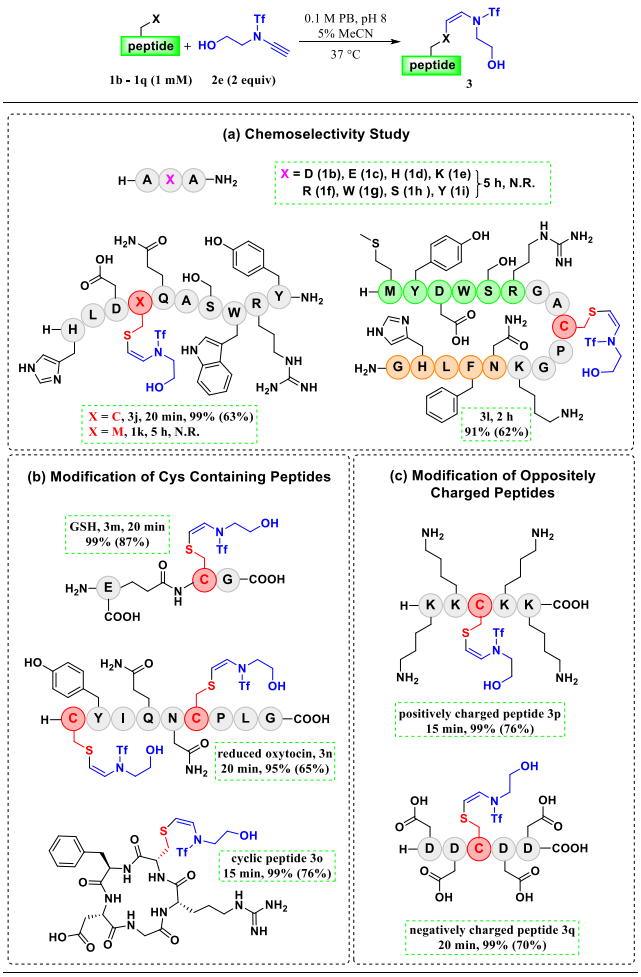


entry	pH	**1a** (mM/μM)	**2e** (equiv)	time	conversion	yield[a]
1	6.0	1 mM	2.0	1 h	< 1%	-
2	7.0	1 mM	2.0	1 h	< 1%	-
3	7.5	1 mM	2.0	1 h	10%	-
4	8.0	1 mM	2.0	20 min	> 99%	95%
5	8.0	1 mM	3.0	15 min	> 99%	92%
6	8.0	1 mM	5.0	5 min	> 99%	92%
7[b]	8.0	1 mM	2.0	50 min	> 99%	95%
8	8.0	100 μM	2.0	40 min	> 99%	92%
9	8.0	50 μM	2.0	140 min	> 99%	91%
10	8.0	10 μM	2.0	80 min	80%	72%
11	8.0	10 μM	5.0	20 min	> 99%	93%

[a]isolated yield. [b]room temperature.

Chemoselectivity Study. With the established reaction conditions in hand, we moved forward to investigate the chemoselectivity of this strategy in the presence of other unprotected amino acid side chains (Table 3a). When Asp and Glu containing tripeptides (**1b** and **1c**) were treated with **2e** under the standard reaction conditions, no reaction could be detected even after extended reaction times (5 h), indicating that the normal α-addition of carboxylic acids to ynamides did not take place.[73] This is attributed to the fact that basic reaction condition deprotonated carboxyl group and thus prohibited the protonation and α-addition of carboxylic acid to ynamide. Furthermore, considering that β-hydroamidation of ynamides with amines and amides in the presence of strong base has been reported,[76, 82] tripeptides containing histidine (His, **1d**), lysine (Lys, **1e**), arginine (Arg, **1f**) and tryptophan (Trp, **1g**) were investigated. We were delighted to find that these side chains were well tolerated in this weak basic

Table 3. Systematic study of Cys modification by ynamides in the presence of other amino acid residues.

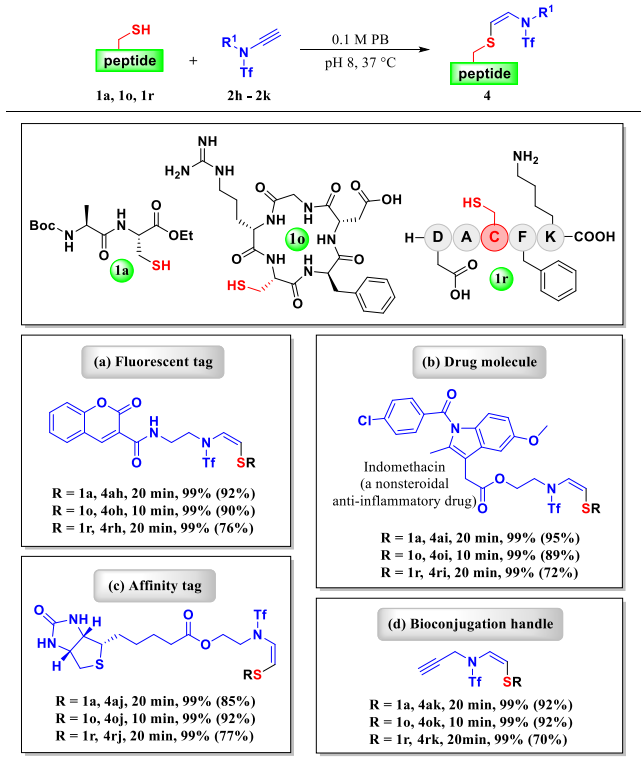


Reaction conditions: peptides (**1b** – **1q**, 1 mM), ynamide (**2e**, 2 equiv), in 0.1 M PB containing 5% MeCN, pH 8, 37 °C. All yields presented were determined by integrated areas of HPLC peaks (at 220 nm), whereas isolated yields are in parentheses. GSH = reduced glutathione; N.R. = no reaction.

reaction condition. Nucleophilic serine (Ser, **1h**) and tyrosine (Tyr, **1i**) were also compatible under our optimized reaction conditions. Next, the chemoselective modification of Cys was performed in the presence of all possible side chains (**1j** and **1l**). Notably, the reactions of **1j** and **1l** were completed within 20 min and 2 h, respectively, providing a single product in excellent yield, and the modification site was confirmed to be the Cys residue by trypsin digest of **3l** (Figure S37). As expected, no modification was detected when Cys was substituted with Met (**1k**).

The Scope of Peptides in the Reaction. The robustness of this methodology was further evaluated with other peptide substrates (**1m-1q**, Table 3b and 3c). Glutathione (GSH, **1m**) could be modified by ynamide **2e** in excellent yield under the standard reaction conditions. Further, while oxidized oxytocin was inert, the reduced oxytocin (**1n**), containing a terminal Cys and an internal Cys, could be bis-functionalized in excellent yield within 20 min, indicating that the position of Cys in the sequence did not affect the efficiency of the modification. Moreover, a potent $\alpha V\beta 3$ integrin-binding cyclic peptide[83] (**1o**) was employed in this transformation to provide the desired Cys-specific modified product in 76% yield. Finally, two peptides with highly positively (**1p**) and negatively (**1q**) charged sequences, respectively, were selected as key examples to evaluate the influence of electrostatic effect on this transformation. Both Cys-specific modifications of **1p** and **1q** proceeded smoothly under the standard conditions, illustrating that electrostatics did not have negative effect on the reaction.

Table 4. Selective Cys modification of peptides with ynamides containing various biologically relevant tags and probes.



Reaction condition: peptides **1a** (1 mM)/**1o** (0.5 mM)/**1r** (1 mM), and ynamides **2h-2k** (2 equiv), 0.1 M PB, pH 8, 37 ºC; (a-b) 50% MeCN, (c) 5% MeCN; (d) 10% DMSO. All yields presented were determined by integrated areas of HPLC peaks (at 220 nm), whereas isolated yields were in parentheses.

Peptide Modification by Ynamide-Based Biologically Relevant Molecules. To demonstrate the applicability of this strategy in peptide and protein chemical biology, different biologically relevant functional groups, including fluorescent tag (coumarin, **2h**), complex drug molecule (indomethacin, **2i**), affinity tag (biotin, **2j**) and alkyne handle for bioconjugation (**2k**), were introduced to ynamides (see SI for details) and evaluated by employing the Cys containing peptides (Table 4). The Cys-selective modification of protected peptide (**1a**), cyclic peptide (**1o**) and unprotected peptide (**1r**) with a variety of important ynamide-based functional molecules (**2h-2k**) proceeded smoothly in buffer with 10%-50% MeCN as the co-solvent (for solubility reasons) to afford the desired products in excellent yields (Table 4).

One-Pot Dual Functionalization of Cys with Click Chemistry. Considering the great importance of click reaction, the compatibility of the Cys-specific modification with click reaction was studied. As shown in Figure 3, Cys of peptide **1l** was modified efficiently by ynamide **2k** to release the *Z*-conjugated product **5k$_1$** containing an additional C-C triple bond (Figure 3b). To our delight, **5k$_1$** took part in the proceeding click reaction with biotin-PEG$_3$-azide (**2m**) readily to anchor a biotin tag to peptide **1l** in a one-pot manner furnishing the target dual functionalized product (**5k$_2$**) in 90% yield (Figure 3). The excellent efficiency of such one-pot, two-step dual functionalization unambiguously illustrated the potential application and flexibility of this strategy to anchor a biotin tag or other useful functional groups to a peptide of interest.

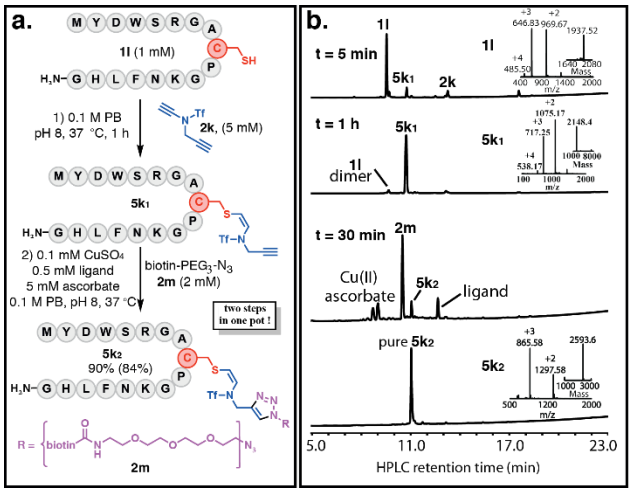


Figure 3. Cys specific dual functionalization. Reaction conditions: 1) peptide **1l** (1 mM) and ynamide **2k** (5 mM) were incubated in 0.1 M PB, pH 8, at 37 ºC for 1 h. 2), azide **2m** (2 mM), $CuSO_4$ (0.1 mM), ligand (bathophenanthroline disulfonic acid disodium salt, 0.5 mM) and sodium ascorbate (5 mM) were added to the reaction, which was incubated at 37 ºC for 30 min.

Stability Study of the Peptide Conjugates. The stability of the conjugated products is utmost important for developing a powerful peptide and protein modification strategy. To examine the stability of the ynamide-modified peptides under different conditions, we incubated product **3a** (1 mM) in four different buffer solutions at pH 2, 8, 10 and 13. The modified peptides were stable under these conditions and kept intact after 10 h (Figure S60). Moreover, the cleavage of **3a** in the presence of external thiol to regenerate the unmodified **1a**, which occur in other methods[39, 60] was not detected in our case (2-mercaptoethanol, Figure S61). Furthermore, the ynamide modified GSH peptide, **3k** (10 μM), did not undergo β-syn elimination, which is commonly encountered in other methods when treated with large excess of H_2O_2 (at pH 4, 8 and 10 at 37 ºC), even after extended time (24 h, Figure S62). The remarkable stability of modified products further supports the potential application of this chemical approach in peptide and protein modification.

Modification of Cys-Containing Proteins. Encouraged by these results, we went further to investigate the application of Cys-specific modification with ynamide derivatives on proteins. Biotinylated ynamide (**2j**) was readily prepared (see SI) and used for protein modification. A ubiquitin(G47C) variant (**6**, 250 μmol, prepared by chemical protein synthesis, see the Supporting Information for details)[84-86] was treated with 5 equiv of **2j** at pH 8 and 37 ºC, to afford the biotin labeled ubiquitin(G47C*) (**7**) in 43% isolated yield after 12 h (Figure 4a, and Figure S56 in the Supporting Information). In contrast, no modification of ubiquitin(G47A) variant was observed under the same reaction conditions even after 18 h, confirming the Cys selectivity. The efficient chemo-, regio- and stereoselective biotinylation of ubiquitin(G47C) variant (**6**) unambiguously illustrated the potential application of this strategy in the construction of protein conjugates. Furthermore, the modification of bovine pancreatic trypsin inhibitor (BPTI), a 58-residue Cys-rich protein with three disulfide bonds, was also examined.[87-88] First, BPTI (**8**, 250 μmol) was treated with 1.5 equiv of TCEP, which selectively reduced the solvent exposed Cys14-Cys38 disulfide bond,[87] leaving the other two disulfide bonds intact, followed by the addition of 10 equiv of **2e**/**2j**, separately. After 12 h, the corresponding bis-modified

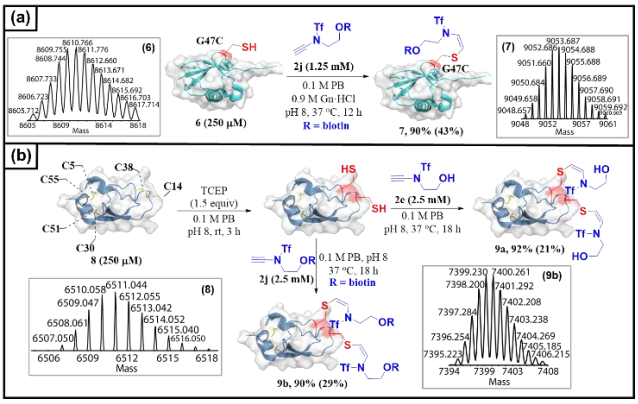


Figure 4. Chemoselective modification of ubiquitn variant and partly reduced BPTI. (**a**) ubiquitin(G47C) (**6**, 250 μM) and **2j** (1.25 mM) were incubated in 0.1 M PB (containing 0.9 M Gn•HCl, pH 8) at 37 ºC for 12 h. (**b**) Reduction of C14-C38 disulifde: BPTI (**8**, 250 μM) by a 1.5 equiv TCEP in 0.1 M PB (pH 8) at room temperature for 3 h; dual modifications of C14 and C38: **2e**/**2j** (2.5 mM) in 0.1 M PB (pH 8) at 37 ºC for 18 h.

proteins (**9a** and **9b**) were isolated in 21% and 29% yields, respectively (Figure 4b). Although 8% oxidation of the reduced Cys residues to form disulfide bonds (dimer of **6** and native **8**) were detected in both cases, no perturbation on other disulfides of BPTI was observed. The compatibility of disulfide bond in this transformation implied the potential applications of this protocol in complex biological systems.

CONCLUSION

In summary, we have developed a novel chemo-, regio- and stereoselective Cys modification of peptides and proteins by using rationaly designed ynamides. The unprecedented Cys-specific modification was achieved by taking advantage of the base promoted *β*-addition of Cys thiol to ynamides. The three modifiable sites of ynamides, including the alkynyl moiety and two substituents on the nitrogen atom, offeres great chemical space for optimization. The strong electron-withdrawing triflyl group played a crucial role, which guarantees the stability and improves the *β*-addition reactivity of ynamide towards Cys. In addition, the other substituent on the nitrogen atom offers a handle tool for functional diversification with various functional groups. This method exhibited excellent chemoselectivity for thiol group in the presence of other peptide side chains including carboxylic acid, free amino group, primary amide, hydroxyl group, and NH of imidazole and indole. Its ruboustness in chemical biology was exmplified by applications in chemical modifications of different peptides (both linear and cyclic peptides) and proteins (ubiquitin and BPTI). Furthermore, ynamide-modified peptide conjugates displayed remarkable stability under rigorous conditions, including acidic, basic, and oxidative conditions, as well as competition with external thiols, which is an obvious advantage over maleimides and other related strategies. This study provides a useful chemical tool for peptides and proteins precise modification for future drug discovery and development. Further exploration of the manipulation of peptides and proteins *in vivo* and *in vitro* with ynamides is underway in our laboratories.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Materials and methods, experimental procedures, characterization, HPLC, and LC-MS data, as well as HRMS, ^{1}H and ^{13}C NMR spectra (PDF).

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Notes

The authors declare no competing financial interest.

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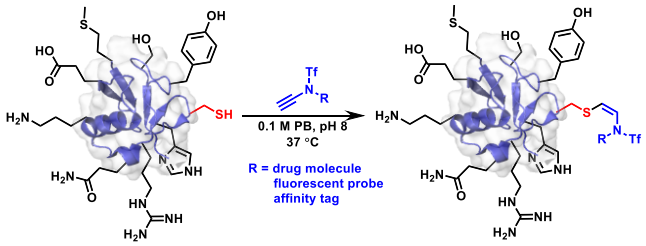
Tf
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0.1 M PB, pH 8
37 °C
R = drug molecule
fluorescent probe
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