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Ethynylation of Cysteines from Peptides to Proteins in Living Cells

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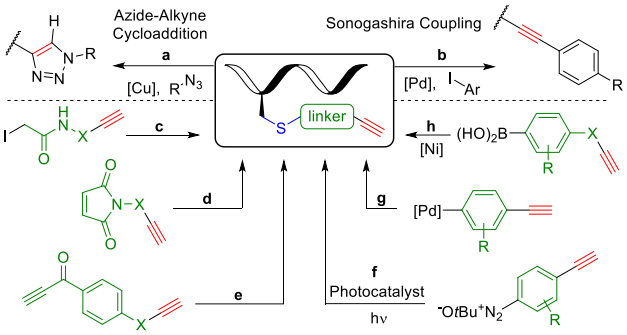
Abstract: Efficient methods to introduce bioorthogonal groups, such as terminal alkynes, into biomolecules are important tools for chemical biology. State-of-the-art approaches are based on the introduction of a linker between the targeted amino acid and the alkyne, and still present limitations of either reactivity, selectivity or adduct stability. Herein, we present an ethynylation method of cysteine residues based on the use of ethynylbenziodoxolone (EBX) reagents. In contrast to other approaches, the acetylene group is directly introduced onto the thiol group of cysteine and can be used in one-pot in a copper-catalyzed alkyne-azide cycloaddition (CuAAC) for further functionalization. Labeling proceeded with reaction rates comparable or higher than the most often used iodoacetamide on peptides or maleimide on the antibody trastuzumab. Under optimized conditions, high cysteine selectivity was observed. The reagents were also used in living cells for cysteine proteomic profiling and displayed an improved coverage of the cysteinome compared to previously reported iodoacetamide or hypervalent iodine-reagent based probes. Fine-tuning of the EBX reagents allowed optimization of their reactivity and physical properties for the desired application.

Introduction

Selective chemical modifiers, combined to bioorthogonal reactive handles, are crucial to study and alter biological systems.[1] High selectivity is key to their success. In addition, these reagents must lead to high reaction rates in aqueous media under air, at neutral pH and moderate temperature. Moreover, they should be non-toxic and non-interfering with protein structures. Amongst them, reagents that transfer terminal acetylenes to biomolecules are of particular interest. Indeed, acetylenes are apolar and unable to establish strong hydrogen bonds. They are therefore unlikely to generate significant structural alterations once attached to biomolecules. Ethylene is also the smallest suitable functional group for bioorthogonal reactions and is frequently employed in chemical biology,[2] mostly through copper-catalyzed azide-alkyne cycloadditions (CuAACs) (Scheme 1, path **a**).[3] This well-known [3+2] annulation has found numerous applications both *in vitro* and *in vivo*.[4] Other metal-assisted reactions also exploited the unique reactivity of terminal alkynes to modify biomolecules. For example, palladium-assisted Sonogashira cross-couplings were applied to protein labeling in living systems (path **b**).[5]



Scheme 1. Well-established alkyne-linker approach for cysteine functionalization.

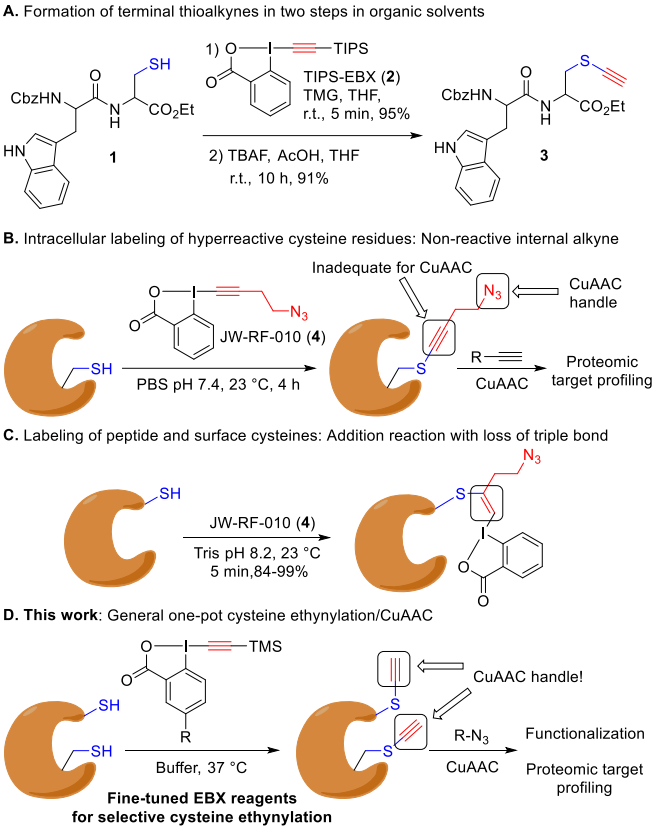
Labeling reagents that carry terminal alkynes and selectively modify cysteines are especially useful, as the latter are a long-established target in chemical biology.[6] So far, iodoacetamide-alkynes[7] and maleimide derivatives[8] are the most common reagents (Scheme 1, paths **c** and **d**). Nevertheless, there are still limitations to these approaches, such as insufficient reactivity or selectivity, as well as low adduct stability, resulting in suboptimal coverage of the cysteinome.[1] Beyond these traditional reagents, only few studies described the one-step attachment of terminal acetylenes to cysteine residues, based on addition to alkynones (path **e**) [9] or photocatalyzed arylation using diazonium salts (path **f**).[10] Recently, efficient palladium-[11] and nickel-assisted[12] arylations were described (paths **g** and **h**), but their application remains limited by solubility and biocompatibility issues.[13]

Most importantly, all these methods share a common feature: a linker between the cysteine residue and the terminal alkyne is mandatory. However, this linker may have a dramatic impact on the structure, the stability and the localization of the labeled molecule.[14] Furthermore, labeling loss can be observed with poorly stable linkers, such as succinimides[15] and vinyl ketones,[9a] in particular through hydrolysis and/or external organosulfur attack. Installing the terminal alkyne directly on the sulfur atom without any linker would constitute the minimal disturbance possible, and may lead to a uniquely reactive alkyne. However, there is currently no one-step method to introduce an ethynyl group onto cysteine under physiological conditions.

Over the last years, our group has focused on the functionalization of thiols with hypervalent iodine compounds. These reagents are particularly attractive for chemical biology, as they combine high reactivity and selectivity, low toxicity and sufficient stability.[16] We first reported a chemoselective

alkynylation of thiols such as dipeptide **1**, using tri*iso*propylsilylethynylbenziodoxolone (TIPS-EBX (**2**) Scheme 2A).[17] The resulting silylated alkyne could then be deprotected to give free terminal alkyne **3**, which could be functionalized via CuAAC. However, this multi-step procedure performed in organic solvents was not suitable for chemical biology applications. We therefore designed a reagent (JW-RF-010 (**4**)) with an additional azide handle, which was applied for *in vitro* and *in vivo* native cysteine labeling (Scheme 2B).[18,19] JW-RF-010 (**4**) displayed high chemoselectivity, outperforming iodoacetamide, the gold standard in cysteine targeting. However, the labeling by JW-RF-010 (**4**) provided only modest coverage of the cysteinome, comparable to iodoacetamide alkyne. We then investigated a selective modification of any cysteines- and other thiol-bearing compounds in aqueous media. Our studies resulted in an efficient, chemoselective and clean labeling of cysteine-containing peptides and proteins without cleavage of the hypervalent bond (Scheme 2C).[20] Although the synthesized vinylbenziodoxolones (VBXs) opened the way for novel bioorthogonal transformations, the presence of a linker was mandatory and no alkyne bond remained in the product. From this point of view, the method was not fundamentally different from other reported alternatives. The fact that different products were obtained for hyperreactive or surface-exposed cysteines also limited the generality of the method. In particular, mixtures were obtained for cysteines with intermediate reactivity and/or surface localization.

Herein, we present the development and application of TMS-EBXs that selectively ethynylate cysteine residues, in a single step under physiological conditions (Scheme 2D). These reagents bear a labile trimethylsilyl group and can be fine-tuned to adjust the rate of product formation and the physical properties of the reagent in dependence of the desired application. The hypervalent iodine compounds were applied from simple peptides to complex proteins such as antibodies. One-pot CuAAC was possible with the obtained thioalkynes. The reagents could also be used for cysteine labeling in living cells. Proteomic profiling displayed an improved coverage of the cysteinome compared to our previous work using JW-RF-010 (**4**).

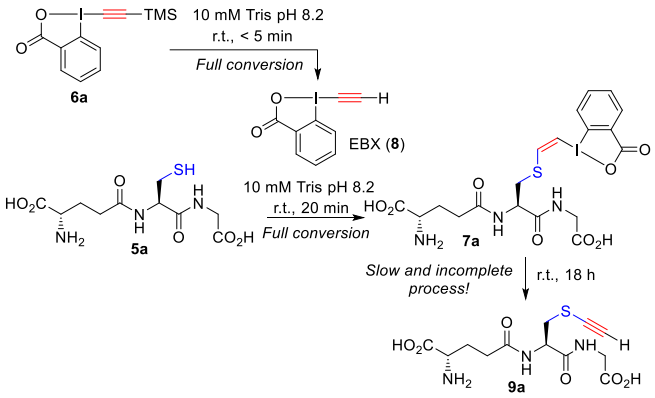


Scheme 2. Previous and current cysteine labeling studies using EBX reagents.

Results and Discussion

Our previous work with alkyl-substituted EBX reagents resulted in the exclusive formation of VBX products.[20] Nevertheless, our DFT calculations showed that the energy of the transition states leading to the alkyne product depended strongly on the structure of the substituent on the alkyne.[21] On one hand, alkyl-EBXs led to the formation of a relatively stable vinylic carbanion intermediate that can be easily protonated. On the other hand, a low energy pathway involving a 1,2-silicon shift was available for silyl-EBXs. Therefore, silyl-EBXs should give alkyne products, even in water. However, TIPS-EBX (**2**) cannot be used, as it is not soluble in aqueous media. To start our investigations, we selected the highly abundant glutathione (**5a**), a naturally occurring tripeptide that plays an essential role in primary metabolism as disulfide bond reductant,[22] and TMS-EBX (**6a**) in an aqueous buffer solution (Scheme 3). The use of the smaller trimethylsilyl group on the alkyne gave sufficient solubility in water. To our surprise, unsubstituted glutathione bound VBX **7a** was obtained instead of the expected silylated alkyne. This was due to a rapid and complete desilylation of TMS-EBX (**6a**) to give EBX (**8**) in a few minutes. EBX (**8**) is typically unstable, prepared *in situ* and employed at low temperature.[23] However, **8** displayed a remarkable stability in aqueous media. While no degradation was observed after 5 minutes, 86% of reagent **8** still remained after 2 hours in 10 mM Tris buffer at pH 8.2. This unusual stability might be explained by a coordinating effect from the buffer that stabilizes the reagent. Such effect was previously observed in organic solvent and allowed to crystallize an EBX-ACN complex.[24] In contrast to the previous substituted alkyl-VBX

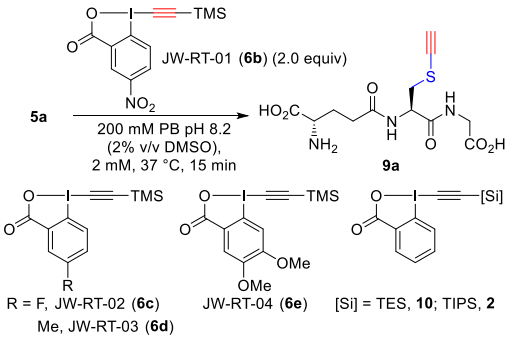
products,[20] **7a** underwent rearrangement into the ethynylated glutathione **9a**, giving a first access to the targeted product under physiological conditions. Nevertheless, the rearrangement was slow and incomplete after extended reaction times.



Scheme 3. Formation of EBX (**8**) and vinylbenziodoxolone **7a**, followed by its rearrangement into terminal thioalkyne **9a**.

We hypothesized that an electronic fine-tuning of the TMS-EBX aromatic core might have a critical impact on the rearrangement rate and efficiency. We therefore prepared several hypervalent iodine derivatives containing both electron withdrawing and donating groups (Table 1). These EBX compounds were prepared in two synthetic steps from commercially available compounds, without column chromatography purification and could be handled under air without any noticeable degradation (see Supporting Information). After optimization, a fast and practicable procedure could be developed for the thioethynylation based on the use of the more reactive nitro-substituted reagent JW-RT-01 (**6b**) (Table 1, entry 1). A stock solution of **6b** in DMSO was added to a non-degassed 200 mM phosphate buffer (PB) at pH 8.2 for desilylation. After 2 minutes, a solution of glutathione (**5a**) in the same buffer was transferred to the hypervalent iodine reagent. The reaction was then shaken at 37 °C for 15 minutes. The reaction afforded the ethynylated glutathione **9a** in 99% yield.[25] Compound **9a** is stable at neutral pH, but slowly hydrolyzes in acidic media. Under the optimized reaction conditions, a broad range of TMS-EBX reagents could be used. Nevertheless, lower yields were obtained with TMS-EBX (**6a**), JW-RT-02 (**6c**), JW-RT-03 (**6d**) and JW-RT-04 (**6e**) (entries 2-5). A complete desilylation of TES-EBX (**10**) was also observed, giving the desired product **9a** in a moderate yield after 15 minutes (entry 6). Finally, no conversion was observed in presence of TIPS-EBX (**2**) because of its lack of solubility in aqueous media (entry 7). We then examined the impact of the pH on the reactivity. At pH 7.2, the desired product **5a** was obtained in 90% yield within 15 minutes (entry 8). After only 15 minutes at pH 6.4, our labeling process produced 58% yield of the desired product **9a** (entry 9). An extended reaction time of 60 minutes increased the yield of **9a** to 78%.

Table 1. Evaluation of reaction conditions for the ethynylation of glutathione (**5a**).

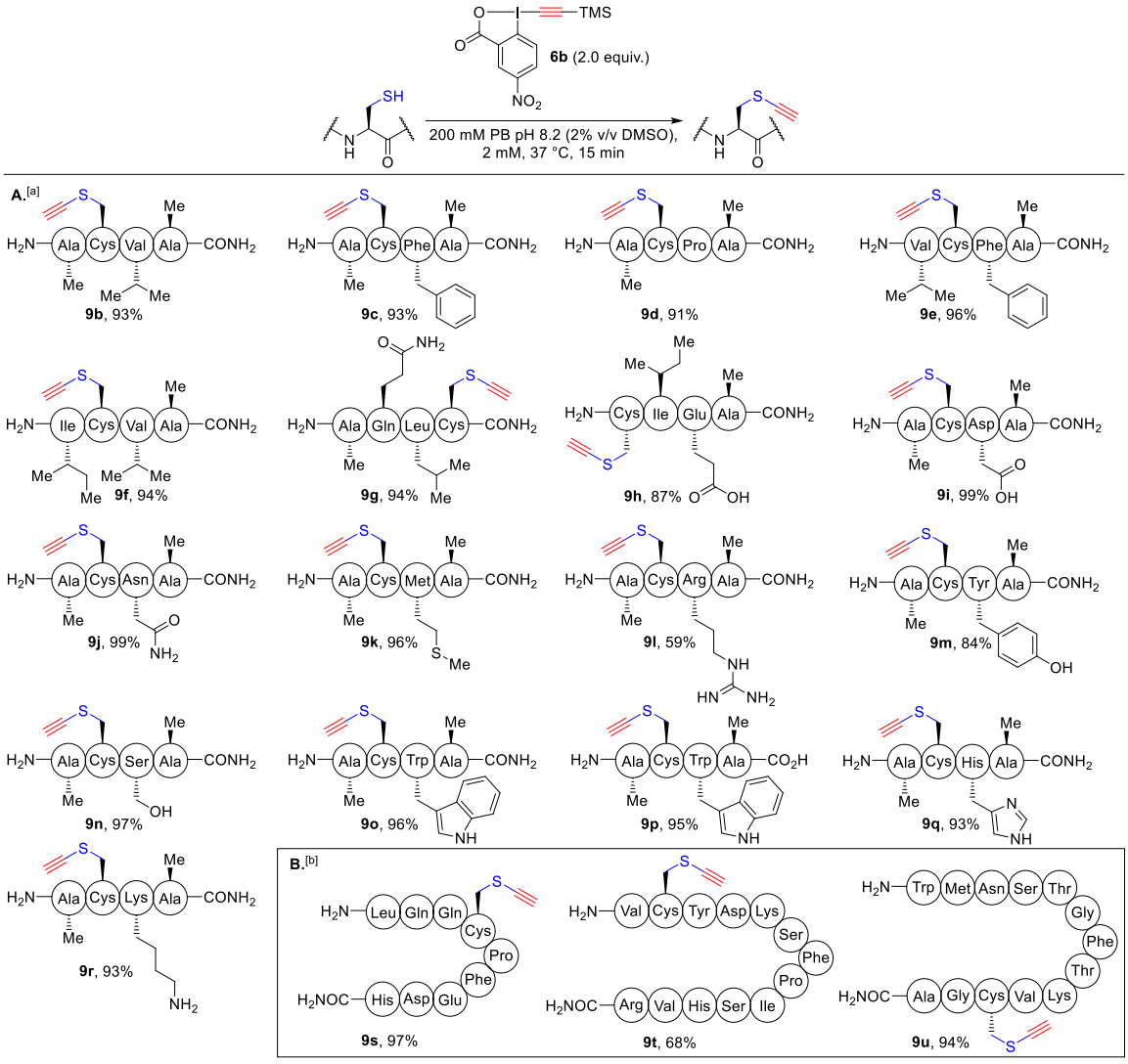


Entry	Variations from the above conditions	Yield
1	None	99% (99%)
2	TMS-EBX (**6a**)	87% (95%)
3	JW-RT-02 (**6c**)	87% (90%)
4	JW-RT-03 (**6d**)	74% (82%)
5	JW-RT-04 (**6e**)	81% (90%)
6	TES-EBX (**10**)	60% (76%)
7	TIPS-EBX (**2**)	0% (0%)
8	200 mM PB pH 7.2	90% (93%)
9	200 mM PB pH 6.4	58% (78%)
10	50 mM PB pH 8.2	95% (99%)
11	200 mM Tris pH 8.2	79% (94%)
12	200 mM HEPES pH 8.2	42% (75%)
13	200 mM TAPS pH 8.2	52% (88%)
14	Reaction at room temperature	81% (94%)
15	200 µM reaction molarity	97% (97%)
16	1.2 equiv. of **6b**	93% (97%)

Labeling conditions: 1.00 µmol glutathione (**5a**), JW-RT-01 (**6b**) (2.0 equiv.) in 0.5 mL of non-degassed 200 mM phosphate buffer pH 8.2 (2% v/v DMSO), 37 °C, 15 minutes. Calibrated HPLC yields based on absorbance at 214 nm (Figure S1). See Supporting Information for details. The yields in parentheses correspond to the yields after one hour of reaction. For complete robustness studies, see Supporting Information Tables S1 and S2.

We also investigated the tolerance of our ethynylation process to different buffers. Employing a 50 mM phosphate buffer did not alter significantly the yield and rate of the reaction (entry 10). Although the reaction rate significantly slowed down in Tris buffer, an extended reaction time of 60 minutes afforded 94% yield of the desired product (entry 11). Employing HEPES or TAPS buffers instead of a phosphate buffer resulted in a slowdown of the ethynylation process and a decrease of the yield (entries 12 and 13). Nevertheless, 75 and 88% yield were obtained after 60 minutes. Ethynylation of glutathione (**5a**) could be performed at room temperature, resulting in a 81% yield of the product **9a** after 15 minutes (entry 14). The reaction was still efficient when diluted to 200 µM (entry 15). Finally, a reduced amount of JW-RT-01 (**6b**) furnished the desired product **9a** in 93% yield (entry 16).

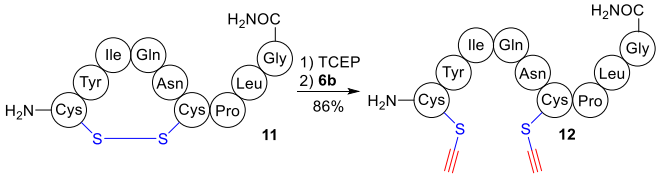
Once the robustness of our ethynylation process has been demonstrated, we investigated its scope first on tetrapeptides (Scheme 4A). Labeling of peptides containing valine (**9b**), phenylalanine (**9c**) and proline (**9d**) was obtained in 91-93% yield. Steric hindrance around the cysteine residue did not alter the reaction efficiency (**9e** and **9f**). We then introduced cysteine on the C-terminal position. When the C-terminal carbonyl of the peptide was an amide, the desired product **9g** was obtained in 94% yield. With a carboxylic acid in contrast, a mixture of products was obtained (See compound **9v** in Supporting Information). A *N*-terminal cysteine (**9h**) was well tolerated. Quantitative labeling was observed with cysteines in close proximity of aspartic acid (**9i**) and asparagine (**9j**). The presence of a methionine residue (**9k**) did not show any detrimental effect on the labeling. Undesired side reactivity was observed in presence of an arginine residue, resulting in the formation of the desired product **9l** in 59% yield. In the case of tyrosine, the desired product **9m** was obtained in 84% yield due to the formation of side-products.



Scheme 4. A. Scope of the ethynylation of tetrapeptides and **B.** Application to larger peptides. [a]Reactions were carried out on a 1.00 µmol scale. [b]Reactions were carried out on a 0.50 µmol scale. Yields were determined by relative integration based on HPLC-MS. See Supporting Information for details.

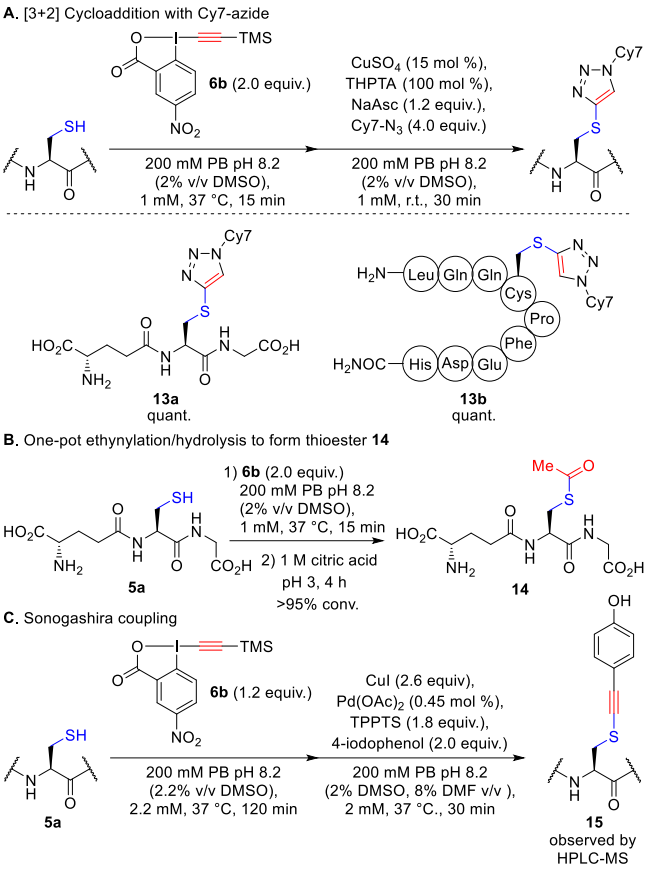
The reaction was also chemoselective in presence of serine (**9n**) and tryptophan (**9o**). When a carboxylic acid function was introduced instead of the amide in C-terminal position (**9p**), the product was obtained in 95% yield. This result confirms that a terminal carboxylic acid is an issue only in the case of C-terminal cysteine. Finally, no side reactivity occurred with tetramers containing histidine (**9q**) and lysine (**9r**). We then extended our ethynylation process to more complex peptides (Scheme 4B). On Human Serum Albumin Leu_{55}-His_{63} fragment, our optimized conditions afforded the desired product **9s** in 97% yield. After treatment with JW-RT-01 (**6b**), the peptidic gap junction blocker GAP26 furnished 68% yield of the alkynylated product **9t**. Finally, a Trp_{554}-Ala_{566} fragment of the hepatitis C virus envelope glycoprotein E2 was successfully converted in its corresponding thioalkyne product **9u** in 94% yield.

In nature, the vast majority of cysteines are engaged in disulfide bridges. It is therefore crucial to develop protocols to target these cysteine residues. The current most widespread procedure is an *in situ* cleavage of the disulfide bond, followed by labeling of the reduced cysteines. In our case, we envisioned that a one-pot protocol allowing disulfide bond reduction and subsequent cysteine ethynylation would be of high interest for the labeling of disulfide bound cysteines. Our investigations started with oxidized glutathione, as a model system, and tris(2-carboxyethyl)phosphine (TCEP), as reducing reagent (see Supporting Information). In presence of 1.5 equivalent of TCEP, oxidized glutathione was completely reduced within 60 minutes. Subsequent addition of 4.0 equivalents of JW-RT-01 (**6b**) (2.0 equivalents per reduced cysteine) afforded the desired product **9a** in 97% yield. Notably, the ethynylation process was also achieved in presence of 5.0 and 10 equivalents of the reducing reagent and without increasing the amount of **6b**. Although the hypervalent iodine reagent **6b** was significantly degraded in the presence of an excess of TCEP, these reactions respectively afforded the ethynylated glutathione **9a** in 66% and 39% yield. These promising results on oxidized glutathione prompted us to apply our one-pot procedure to the natural bioactive peptide oxytocin (**11**). To our delight, *in situ* reduction and subsequent ethynylation afforded peptide **12** in 86% yield (Scheme 5).



Scheme 5. Labeling of sulfur bridge-containing oxytocin (**11**). Conditions: 1.5 equiv. TCEP·HCl, 200 mM PB pH 8.2, r.t., 60 min, then 4.0 equiv. JW-RT-01 (**6b**), 200 mM PB pH 8.2 (2% v/v DMSO), 37 °C, 15 min. Reaction was carried out on a 0.10 µmol scale. Yield was determined by relative integration based on HPLC-MS. See Supporting Information for details.

Once the potential of our ethynylation process has been demonstrated, we investigated the reactivity of the terminal thioalkyne, starting with the well-known [3+2] cycloaddition between azides and triple bonds. Using tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) as ligand and sodium ascorbate (NaAsc) as reducing agent, the fluorescent probe Cy7-azide (Cy7-N_3) was coupled to ethynylated glutathione (**5a**) and Human Serum Albumin Leu_{55}-His_{63} fragment **5s** to give the corresponding products **13a** and **13b** (Scheme 6A).[26]



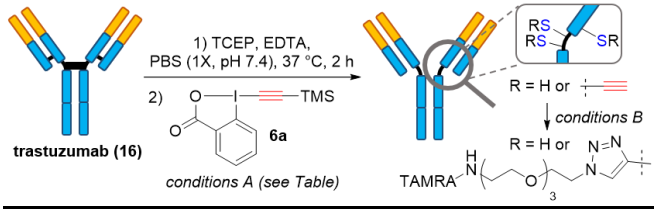
Scheme 6. A. Copper-catalyzed azide-alkyne cycloadditions between terminal thioalkynes and Cy7-N_3. Cy7 = Cyanine7 dye. **B**. Alkyne hydration in acidic media. **C**. One-pot thiol alkynylation and subsequent Sonogashira cross-coupling. TPPTS = Triphenylphosphine-3,3',3''-trisulfonic acid trisodium salt. See Supporting Information for further details about these procedures.

The ethynylation and cycloaddition steps could be conducted in a one-pot manner. Under mild acidic conditions (pH 3), thioalkyne **9a**, generated *in situ* from **5a**, was converted to the corresponding thioester **14** (Scheme 6B). Thioesters are key reactive intermediates in chemical biology, both in natural metabolism, for example for the production of fatty acids,[27a] and in *in vitro* methods to synthesize proteins.[27b] They can also be converted back to free cysteines.[27c] Finally, the thioalkyne moiety was engaged in a Sonogashira coupling (Scheme 6C).[28] The formation of the desired alkyne **15** was confirmed by HPLC-MS, together with a bisalkyne product resulting from a Glaser coupling and other side products (see Supporting Information for details).

These promising results prompted us to evaluate the reactivity and selectivity of JW-RT-01 (**6b**) on more complex substrates. We selected trastuzumab (**16**) (~150 kDa), an FDA-approved antibody used to fight breast cancer.[29] To our surprise, treatment of reduced trastuzumab (**16**) with reagent **6b** led to a complex mixture after the CuAAC step. This result suggested a partial decomposition of the antibody or side reactions occurring during the ethynylation or the cycloaddition steps. Control experiments conducted on the non-reduced antibody showed residual reactivity of JW-RT-01 (**6b**) even in absence of cysteine, leading to degradation of trastuzumab (**16**) as shown by native mass spectrometry (MS) (Figure S2). While arginine or tyrosine residues were also found to react with EBX reagents to some extent on tetrapeptides (Scheme 4), the reason and exact nature of this enhanced side-reactivity on antibody **16** are not clear at this stage. The formation of terminal alkynes is unlikely, as

highlighted by the antibody's lack of fluorescence when CuAAC reaction with a TAMRA-azide probe was attempted after treatment with JW-RT-01 (**6b**). Varying buffer composition and pH, or decreasing reaction time, temperature and number of equivalents, both on either reduced or non-reduced trastuzumab (**16**), did not allow to obtain better results, urging us to use the less reactive EBX reagents TMS-EBX (**6a**) and JW-RT-02 (**6c**) instead of JW-RT-01 (**6b**).

In presence of non-reduced trastuzumab, TMS-EBX (**6a**) showed still some side reactivity, but to a lower extent than JW-RT-01 (**6b**). Notably, under the previously optimized conditions (phosphate buffer pH 8.2, 37 °C), the reduced antibody afforded clean native mass spectra, allowing us to determine average degrees of conjugation (DoC) and conversion rates (Table 2, see Supporting Information Table S3 for detailed results). We observed that native MS profiles were identical after 5 or 15 minutes of incubation with 2 equivalents of **6a**. Increasing the number of equivalents of TMS-EBX (**6a**) from 2 to 8 and 16 resulted in increased DoC – from 0.8 to 3.7 and 4.4 – and conversion values – from 52% to 94% and 97% (Table 2, entries 1-3), highlighting a reactivity comparable to that of classical maleimides.[30] Switching from PB to PBS buffer had a negligible impact on the reactivity of **6a** (entry 4). In contrast, pH and temperature had a profound impact on the bioconjugation outcome (entries 5-8). Poor reactivity was found at pH 6.5, resulting in low average DoC and conversion values compared to pH ≥ 7.5, with the highest reactivity being found at pH 8.5, in line with the pKa of cysteine thiols (~8.0). Logically, reactivity decreased upon diminishing the reaction medium temperature (entry 8). While clean native mass spectra were obtained for all these experiments, side reactivity was still observed under these conditions on the non-reduced antibody. Therefore, we cannot definitely rule out side reactions on reduced trastuzumab (**16**), even though it could be outpaced by cysteine modification. In order to find out conditions at which **6a** would not react with the non-reduced antibody, different incubation times and temperatures were screened again. With 8 equivalents of **6a** in PBS buffer at pH 7.5, intact trastuzumab (**16**) could still be obtained after 2 minutes at 25 °C, and up to 5 minutes at 4 °C. Employing reduced trastuzumab, these conditions yielded an average DoC value of 1.2 with a conversion up to ~60% in just 2 minutes at 25 °C (entry 9). At 4 °C, only very low conversion was observed (Entry 10). Applied to the 4-F derivative JW-RF-02 (**6c**), similar results were obtained, in coherence with its reactivity comparable to the one of the unsubstituted parent compound **6a** (see Supporting Information Table S4 for detailed results).



Entry	Conditions A	Av. DoC	Conv. (%)
1	2 equiv., PB buffer, pH 8.2, 37 °C, 5 min.	0.8	52
2	***8 equiv.***, PB buffer, pH 8.2, 37 °C, 5 min.	3.7	94
3	***16 equiv.***, PB buffer, pH 8.2, 37 °C, 5 min.	4.4	97
4	8 equiv., ***PBS buffer***, pH 8.2, 37 °C, 5 min.	4.0	96
5	8 equiv., PBS buffer, ***pH 6.5***, 37 °C, 5 min.	0.9	50
6	8 equiv., PBS buffer, ***pH 7.5***, 37 °C, 5 min.	3.4	93
7	8 equiv., PBS buffer, ***pH 8.5***, 37 °C, 5 min.	4.4	97
8	8 equiv., PBS buffer, pH 7.5, ***25 °C***, 5 min.	1.8	74
9	8 equiv., PBS buffer, pH 7.5, 25 °C, ***2 min.***	1.2	60
10	8 equiv., PBS buffer, pH 7.5, ***4 °C***, 5 min.	0.1	13

Conditions B: TAMRA-N_3, $CuSO_4$, THPTA, NaAsc, PBS (1X, pH 7.4), 25 °C, 24 h.

Table 2. Evaluation of the reactivity of TMS-EBX reagent **6a** for the bioconjugation of the antibody trastuzumab (**16**)

We then evaluated the proteome-wide labeling of cysteines by the TMS-EBX reagents **6a-e**. HeLa cell lysates were treated for one hour at 37 °C with **6a-e** before CuAAC-mediated installation of a TAMRA dye and in-gel SDS-PAGE fluorescence scanning. Coomassie Brilliant Blue (CBB) staining was used as a loading control (Figure 1A). All five of the TMS-EBX reagents labeled HeLa lysates at 10 µM with JW-RT-02 (**6c**) showing the strongest labeling followed by TMS-EBX (**6a**) and JW-RT-03 (**6d**) (Figure 1B). To confirm that the compounds reacted with cysteines in a chemoselective manner, lysates were first treated with the cysteine blocking reagent iodoacetamide (IAA). To our delight, IAA pre-treatment abolished labeling of JW-RT-02 (**6c**), demonstrating that TMS-EBX reagents label proteomic cysteines in a highly chemoselective manner (Figure 1C). Having demonstrated the utility of TMS-EBX *in vitro*, we evaluated the *in situ* reactivity of the three best reagents in living HeLa cells. Cells were treated with the reagents at 3 or 10 µM concentration for 1.5 hours, without any noticeable toxicity – greater than 93 percent remaining viability was observed by imaging and by quantification with the WST1 absorbance assay (Figure S3). The cells were then collected, lysed, subjected to CuAAC-mediated installation of the TAMRA dye, and the probe-labeled proteins were visualized by fluorescence in-gel scanning. Interestingly, the probe JW-RT-03 (**6d**) exhibited the strongest labeling *in situ*, likely due to the stronger hydrophobicity of this probe enhancing the cellular permeability (Figure 1D). This result further demonstrated the importance of having a library of fine-tuned EBX reagents.

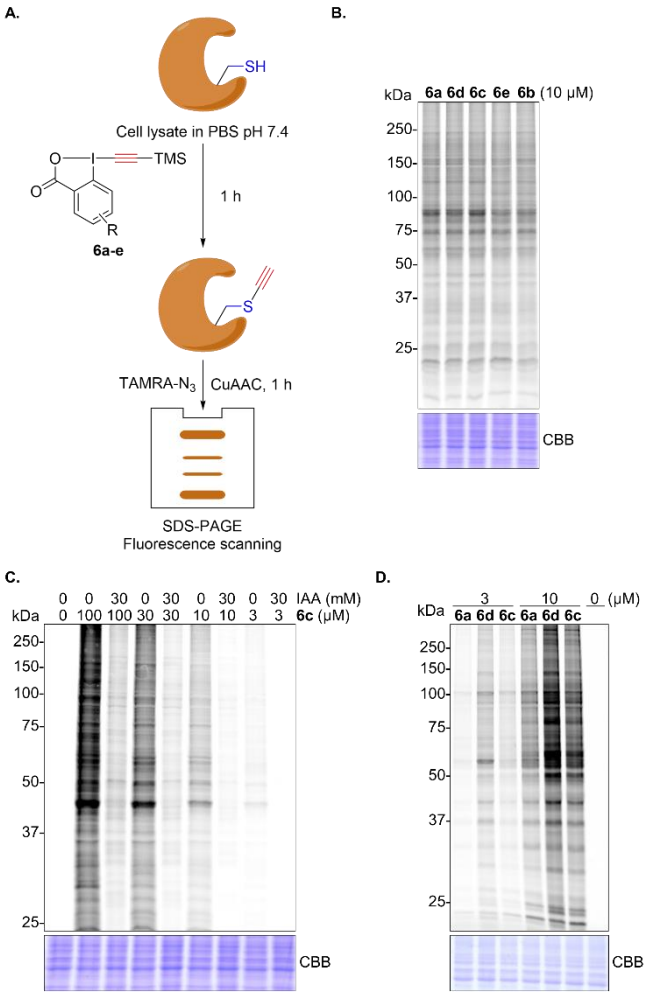


Figure 1. Gel-based proteomic evaluation of TMS-EBXs reactivity. **A.** HeLa cell lysate in PBS (pH 7.4) was treated with the TMS-EBX reagents before CuAAC installation of the TAMRA dye and in-gel SDS-PAGE fluorescence scanning. **B.** Fluorescence image of HeLa lysate treated with 10 μM TMS-EBX reagents. **C.** IAA (30 mM) competed the labeling of HeLa lysate by JW-RT-02(**6c**). **D.** JW-RT-03 (**6d**) exhibited the strongest *in situ* labeling using live HeLa cells after 1.5 h of treatment.

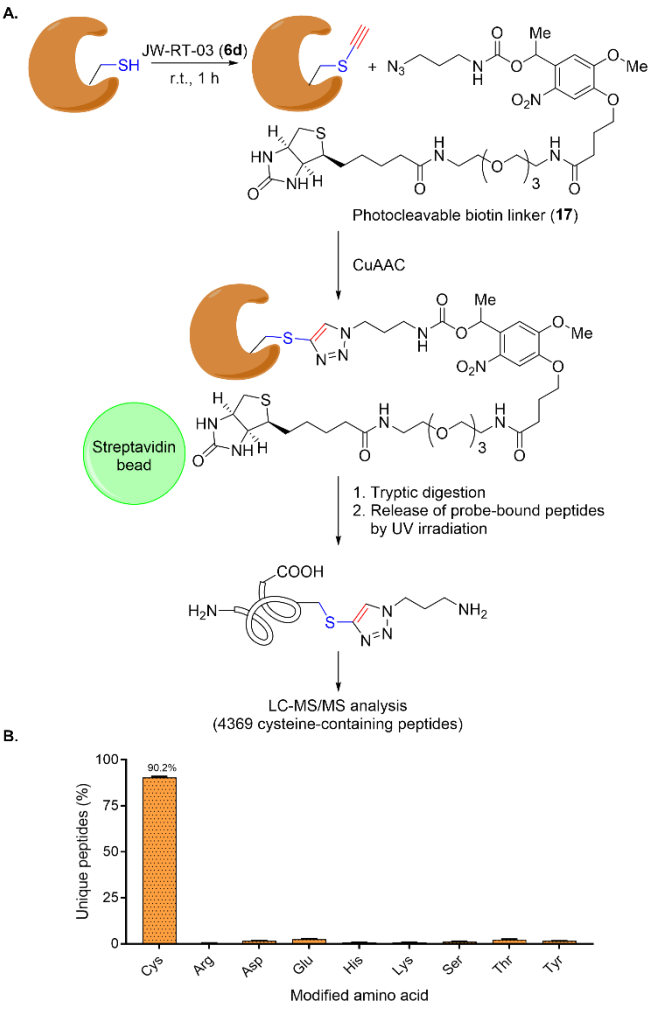


Figure 2. Proteomic chemoselectivity and reactivity of JW-RT-03 (**6d**) using LC-MS/MS analysis. **A.** MS-based capture-and-release strategy using a photocleavable biotin linker to identify peptides modified by reagent **6d**. **B.** Chemoselectivity of **6d** towards nucleophilic amino acids in HeLa lysate (n = 6). Error bars are S.E.M.

Having identified the most reactive probe *in situ* using gel-based proteomic techniques, we evaluated the chemoselectivity and cysteinome coverage of **6d** using mass spectrometry (MS)-based analysis. Using our established catch-and-release enrichment method,[19] HeLa lysates were treated with 10 μM of JW-RT-03 (**6d**) for one hour, followed by CuAAC-mediated conjugation to photocleavable biotin linker **17**, enrichment using streptavidin beads, tryptic digestion, release of probe-bound peptides, with UV irradiation, and LC-MS/MS analysis (Figure 2A). In total, 4325 cysteine-containing peptides were found to be labeled by JW-RT-03 (**6d**).

In comparison, we previously only found 2257 and 2184 peptides respectively enriched by 10 μM JW-RF-010 (**4**) and IAA alkyne from HeLa. Therefore, JW-RT-03 (**6d**) exhibits a greater coverage of reactive cysteines in the proteome than either of these probes. JW-RT-03 (**6d**) also exhibited good chemoselectivity towards cysteines (90.2%) relative to other nucleophilic amino acids (Asp, Glu, His, Lys, Ser, Thr, Tyr, Arg), as determined by searching for the corresponding mass adducts on all of these residues using the Sequest HT algorithm (Figure 2B).[31] Although arginine and tyrosine reacted with JW-RT-01 (**6b**) to some extent on tetrapeptides, minimal reactivity towards arginine (0.3%) and tyrosine (1.49%) was observed with JW-RT-03 (**6d**) throughout the proteome. Taken together, these results demonstrate that JW-RT-03 (**6d**) has greater coverage of reactive proteomic cysteines than JW-RF-010 or IAA alkyne, the current gold standard in the cysteinomics field, while maintaining high selectivity towards cysteines relative to other nucleophilic amino acids. Therefore, the TMS-EBX reagents offer tremendous potential as broad profile cysteine-reactive probes in chemoproteomics.

Conclusion

In summary, we described a procedure for cysteine ethynylation using TMS-ethynylbenziodoxolone (EBX) reagents. The reported labeling process displayed tolerance to various buffers, pH, temperatures and concentrations. Under native conditions, diverse cysteine-containing peptides formed Csp-S bonds, with the electron-deficient reagent JW-RT-01 (**6b**) performing best. Chemoselectivity was observed in presence of numerous nucleophilic amino acids. Although side reactivity was observed in presence of arginine and tyrosine residues, the corresponding thioalkynes were still generated in 59% and 84% yield respectively. With simple reducing pre-treatment, alkynylation of cysteines in disulfide bonds was performed on bioactive oxytocin (**11**). Finally, the resulting terminal thioalkynes were submitted to CuAAC, hydrolysis and Sonogashira cross-coupling in a one-pot manner. Cysteine alkynylation was also conducted on the more complex protein trastuzumab (**16**), a monoclonal antibody of ~150 kDa possessing 8 accessible cysteine residues. Using the less reactive TMS-EBX (**6a**) and JW-RT-02 (**6c**), good conversion and average DoC values were obtained, with an apparent high cysteine selectivity, as demonstrated by little to no conjugation on the non-reduced antibody under optimized conditions. Finally, the TMS-EBX compounds also performed labeling of HeLa lysates *in vitro* and in living HeLa cells. MS-based proteomics analysis showed that JW-RT-03 (**6d**) was the optimal reagent, especially for *in situ* labeling in living cells. Moreover, JW-RT-03 (**6d**) exhibited a greater coverage of the cysteinome than JW-RF-010 (**4**) and IAA-alkyne, while maintaining high chemoselectivity. Therefore, TMS-EBXs represent an excellent opportunity for broad cysteine-reactive probes in chemoproteomics and we anticipate broad application of these reagents in the near future.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: bioconjugation • cysteine ethynylation • hypervalent iodine • antibody functionalization • cysteine proteomic

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