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Ethynylation of Cysteines from Peptides to Proteins in Living Cells

Romain Tessier,^[a] Raj Kumar Nandi,^{[a]†} Brendan G. Dwyer,^{[b]†} Daniel Abegg,^{[b]†} Charlotte Sornay^{[c]†}, Javier Ceballos,^[a] Stéphane Erb,^[d] Sarah Cianferani,^[d] Alain Wagner,^[c] Guilhem Chaubet,^{*,[c]} Alexander Adibekian,^{**, [b]} and Jerome Waser^{***[a]}

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

- [a] Dr. R. Tessier, Prof. Dr. R. K. Nandi, Dr. J. Ceballos, Prof. Dr. J. Waser
Laboratory of Catalysis and Organic Synthesis
Ecole Polytechnique Fédérale de Lausanne
EPFL SB ISIC LCSO, BCH 4306, 1015 Lausanne, Switzerland
E-mail: jerome.waser@epfl.ch
- [b] B. Dwyer, Dr. D. Abegg, Prof. Dr. A. Adibekian
Department of Chemistry
The Scripps Research Institute
130 Scripps Way, Jupiter, Florida 33458, United-States
Email: aadibeki@scripps.edu
- [c] Charlotte Sornay, Dr Alain Wagner, Dr Guilhem Chaubet
Bio-Functional Chemistry (UMR 7199), LabEx Medalis, University of Strasbourg,
74 Route du Rhin, 67400 Illkirch-Graffenstaden, France
E-mail: chaubet@unistra.fr
- [d] Stéphane Erb, Dr Sarah Cianferani
Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO),
Université de Strasbourg, CNRS, IPHC UMR 7178, 67000
Strasbourg, France
- [†]These authors contributed equally.
Dr. R. Tessier
Present address: Department of Chemical Biology
Max Planck Institute of Molecular Physiology
Otto-Hahn-Strasse 11, 44227 Dortmund, Germany
Prof. Dr. R. K. Nandi
Present address: Department of Chemistry
Diamond Harbour Women's University
Sarisha, South 24 Parganas, West Bengal-743368, India

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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 alkylation of thiols such as dipeptide **1**, using triisopropylsilyl ethynylbenziodoxolone (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**).

A. Formation of terminal thioalkynes in two steps in organic solvents

B. Intracellular labeling of hyperreactive cysteine residues: Non-reactive internal alkyne

C. Labeling of peptide and surface cysteines: Addition reaction with loss of triple bond

D. This work: General one-pot cysteine ethynylation/CuAAC

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

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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,

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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 μ M (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 μ mol scale. ^[b]Reactions were carried out on a 0.50 μ mol scale. Yields were determined by relative integration based on HPLC-MS. See Supporting Information for details.

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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 Leu₅₅-His₆₃ 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 Trp₅₅₄-Ala₅₆₆ 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-N₃) was coupled to ethynylated glutathione (**5a**) and Human Serum Albumin Leu₅₅-His₆₃ 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-N₃. Cy7 = Cyanine7 dye. B. Alkyne hydration in acidic media. C. One-pot thiol alkylation 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

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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).

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

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-N₃, CuSO₄, THPTA, NaAsc, PBS (1X, pH 7.4), 25 °C, 24 h.

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 μ M 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 μ M 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

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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 μ M 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.

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 μ M 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).

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.

In comparison, we previously only found 2257 and 2184 peptides respectively enriched by 10 μ M 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

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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.

Acknowledgements

J. W. thanks ERC (European Research Council, Starting Grant iTools4MC, number 334840 and Consolidator Grant SeleCHEM, number 771170) and EPFL for financial support. C. S thanks Région Grand-Est and LabEx Medalis for financial support. Elija Grinhagena from Laboratory of Catalysis and Organic Synthesis at ISIC EPFL is thanked for finalizing the supporting information on peptide functionalization (adding HPLC spectra and mass data).

Conflict of interest

The authors declare no conflict of interest.

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

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RESEARCH ARTICLE

It always reaches its target! A direct ethynylation method for cysteines using hypervalent iodine reagents is reported. The reaction proceeds on peptides and antibodies, and can also be used for cysteine proteomic profiling in the living cell, displaying an improved coverage of the cysteinome compared to previously reported probes.

Romain Tessier, Raj Kumar Nandi, Brendan G. Dwyer, Daniel Abegg, Charlotte Sornay, Javier Ceballos, Stéphane Erb, Sarah Cianferani, Alain Wagner, Guilhem Chaubet, *Alexander Adibekian, ** and Jerome Waser***

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Ethynylation of Cysteines from Peptides to Proteins in Living Cells

Supporting Information

Author Contributions:

R. T. designed and synthesized the TMS-EBX reagents, discovered the reaction, optimized the process on glutathione, performed most of the scope of the reaction on peptides, applied the *in situ* CuAAC using Cy-7 and prepared the related part of the manuscript and experimental part.

R. K. N. synthesized the peptides, finished the scope of peptides, optimized the *in situ* CuAAC reaction with BnN₃ and Cy-7 and proofread the manuscript.

B. G. D. performed proteome labeling in lysates and in live cells, investigated the probe chemoselectivity by IAA pretreatment, prepared the MS-based proteomics samples, and prepared the related part of the manuscript and experimental part.

D. A. assisted in proteomics sample preparation, acquired the MS-based proteomics data, performed bioinformatics analysis of the proteomics data, and determined the proteome-wide chemoselectivity of probe 6d.

C. S. performed all trastuzumab conjugation experiments, optimized reactions with **6a** and **6c**, and prepared the experimental part.

J. C. performed the hydrolysis and Sonogashira reaction on the ethynylated glutathione and comparison experiments, prepared the related experimental part and proofread the manuscript.

S. E. ran all native mass spectrometry analyses on trastuzumab derivatives, collected the corresponding spectra, determined average degrees of conjugation and conversion values and prepared the experimental part.

S. C. supervised the mass spectrometry analyses on trastuzumab derivatives, validated the results and spectra quality, and proofread the experimental part.

A. W. helped with the design of bioconjugation reagents, liaised with S. C. to ensure smooth running of native mass spectrometry analyses and proofread the manuscript.

G. C. designed and supervised the antibody bioconjugation experiments, prepared the manuscript and proofread the experimental part.

A. A. designed the complex proteome labeling and MS-based proteomics experiments, supervised these experiments, and prepared the manuscript.

J. W. designed the overall research, supervised the labeling work on peptides and the functionalization of products, prepared the manuscript, proofread the experimental part and coordinated the overall project.

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1. Labeling of Peptides

1.1 General procedures

All reactions using anhydrous conditions were performed with oven-dried glassware, under an atmosphere of nitrogen, unless stated otherwise. Dichloromethane was dried by passage over activated alumina, under nitrogen atmosphere, on an Innovative Technology Solvent Delivery System (water content < 10 ppm, Karl-Fischer titration). DMSO was purchased from Sigma-Aldrich. All the Fmoc-protected amino acids, Rink Amide MBHA resin and 2-chlorotrityl chloride resin were purchased from GL Biochem. O-Benzotriazole-N,N,N',N'-tetramethyluronium-hexafluoro-phosphate (HBTU, GL Biotech), N,N-diisopropylethylamine (DIPEA, Iris Biotech GmbH) and hydroxybenzotriazole (HOBt, GL Biotech) were used as received. Oxytocin was purchased from Bachem AG and used without further purification. Cy7-azide was purchased from Jena Bioscience and used as such. All the other reagents were purchased from ABCR, Acros, Aldrich, AlfaAesar, Apollo Scientific, Fluorochem, Fluka, Roth and TCI and were used without additional purification. Melting points were measured on a Büchi B-540 melting point apparatus using open glass capillaries. The data is uncorrected. ¹H-NMR spectra were recorded on a Bruker DPX-400 400 MHz spectrometer in CDCl₃, DMSO-*d*₆ or D₂O. All signals are reported in ppm with the internal CHCl₃ signal at 7.26 ppm, the internal DMSO signal at 2.50 ppm or the internal H₂O signal at 4.79 ppm as standard. The data is being reported as: s = singlet, d = doublet, t = triplet, q = quadruplet, qi = quintet, m = multiplet or unresolved, br = broad signal, app = apparent, coupling constant(s) in Hz, integration, interpretation. ¹³C-NMR spectra were recorded with ¹H-decoupling on a Bruker DPX-400 100 MHz spectrometer in CDCl₃, DMSO-*d*₆ or D₂O. All signals are reported in ppm with the internal CHCl₃ signal at 77.0 ppm or the internal DMSO signal at 39.5 ppm as standard. Infrared spectra were recorded on a JASCO FT-IR B4100 spectrophotometer with an ATR PRO410-S and a ZnSe prisma and are reported as cm⁻¹ (w = weak, m = medium, s = strong, br = broad). High-resolution mass spectrometric measurements were performed by the mass spectrometry service of ISIC at the EPFL on a MICROMASS (ESI) Q-TOF Ultima API.

All reactions related to the ethynylation process were set up on the benchtop and carried out in an Eppendorf Thermomixer 5436, without oxygen exclusion. Buffers were not degassed and prepared with milliQ water. All the reactions were replicated three times and the reported yield is an average of these replicates. Phosphate buffers were prepared by mixing the adequate quantity of Na₂HPO₄ and NaH₂PO₄ in water.

1.2 HPLC-MS and preparative HPLC information

a. HPLC-MS analysis

HPLC-MS measurements were performed on an Agilent 1290 Infinity HPLC system with a G4226A 1290 Autosampler, a G4220A 1290 Bin Pump and a G4212A 1290 DAD detector, connected to a 6130 Quadrupole LC/MS, coupled with a Waters XBridge C18 column (250 x 4.6 mm, 5 μ m). Water:acetonitrile 95:5 + 0.1% formic acid (solvent A), water:acetonitrile 5:95 + 0.1% formic acid (solvent B) or water + 0.1% formic acid (solvent C) were used as the mobile phase, at a flow rate of 0.6 mL/min⁻¹. The column temperature was set up to 25 °C. Low-resolution mass spectrometric measurements were acquired using the following parameters: positive electrospray ionization (ESI), temperature of drying gas = 350 °C, flow rate of drying gas = 12 L. min⁻¹, pressure of nebulizer gas = 60 psi, capillary voltage = 2500 V and fragmentor voltage = 70 V. To obtain high-resolution mass spectrometric measurements, the desired fraction was recovered after separation on a Waters XBridge C18 column (250 x 4.6 mm, 5 μ m) and submitted to the mass spectrometry service of ISIC at the EPFL that uses a MICROMASS (ESI) Q-TOF Ultima API.

b. Preparative HPLC

Preparative RP-HPLC were performed on an Agilent 1260 HPLC system with a G2260A 1260 Prep ALS Autosampler, a G1361A 1260 Prep Pump, a G1365C 1260 MWD detector and a G1364B 1260 FC-PS collector, coupled with a Waters XBridge semi-preparative C18 column (19 x 150 mm, 5 μ m). Water + 0.1% TFA (solvent D), water:acetonitrile 5:95 + 0.1% TFA (solvent E), water (solvent F) or water:acetonitrile 5:95 (solvent G) were used as the mobile phase at a flow rate of 20 mL.min⁻¹. Following methods were used:

Method A: The gradient was programmed as follows: 100% D isocratic for 5 minutes followed by 100% D to 100% E in 20 minutes then isocratic for 5 minutes.

Method B: The gradient was programmed as follows: 100% F isocratic for 10 minutes followed by 100% F to 95% F in 5 minutes. Then, 95% F isocratic for 5 minutes. Finally, 95% F to 100% G in 5 minutes followed by isocratic for 5 minutes.

1.3 Peptide preparation

Solid-Phase Peptide Synthesis (SPPS):

Peptides were synthesized on an Advanced ChemTech 348-Ω parallel peptide synthesizer (AAPPTec) using standard Fmoc SPPS-chemistry and Rink Amide MBHA resin (0.26 mmol/g resin, 0.03 mmol scale) for C-terminal amide or 2-chlorotrityl chloride resin (1.00-1.60 mmol/g resin, 100-200 mesh, 0.03 mmol scale) for C-terminal carboxylic acid. The coupling was carried out by shaking the resin with a Fmoc-protected monomer (4.0 equiv.), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 4.0 equiv.), hydroxybenzotriazole (HOBt, 4.0 equiv.) and N,N-diisopropylethylamine (DIPEA, 6.0 equiv.), in dimethylformamide (1.3 mL), at 400 rpm, over 30 minutes. This step was accomplished twice. Capping was performed at the end of each coupling, followed by dimethylformamide wash (4 x 3 mL). Fmoc groups were then removed by shaking the resin with 20% v/v piperidine in dimethylformamide at 400 rpm, over 5 minutes. This step was carried out twice. Next, washing steps were achieved with dimethylformamide (5 x 3 mL). Finally, resin was dried with dichloromethane (5 x 3 mL).

Peptide cleavage and deprotection:

Peptides were deprotected and cleaved from the resin under reducing conditions, by treatment with 2.5% v/v water and 2.5% v/v thioanisole in neat trifluoroacetic acid (5 mL). The resulting mixture was shaken for 2 hours at 400 rpm, at room temperature. The resin was removed by filtration and peptides were precipitated in cold diethyl ether (50 mL), followed by a 2 hours incubation at -20 °C. Peptides were pelleted by centrifugation at 4000 rpm, at 4 °C, for 5 minutes. Finally, the mother liquors were carefully removed and crude peptides were dried under vacuum.

Peptide purification and analyses:

Peptides were dissolved in water with a minimum amount of organic co-solvent (acetonitrile, dimethylformamide or dimethyl sulfoxide). Peptides were then purified on preparative RP-HPLC using method A. Fractions containing the desired peptide were lyophilized. The purity was assessed by analyzing a 20 mM peptide solution by RP-HPLC (HPLC gradient: 100% A isocratic for 5 minutes followed by 100% A to 100% B in 15 minutes followed by 100% B isocratic for 3 minutes). At the same time, low-resolution mass spectrometric measurements were also acquired.

NH₂-Ala-Cys-Val-Ala-CONH₂ (**5b**):

LRMS (ESI) m/z : $[M + H]^+$ Calcd for C₁₄H₂₈N₅O₄S⁺ 362.186; Found 362.1.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Phe-Ala-CONH₂ (**5c**):

LRMS (ESI) m/z : $[M + H]^+$ Calcd for C₁₈H₂₈N₅O₄S⁺ 410.186; Found 410.1.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Pro-Ala-CONH₂ (5d):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₄H₂₆N₅O₄S⁺ 360.170; Found 360.1.

HPLC-MS chromatogram:

NH₂-Val-Cys-Phe-Ala-CONH₂ (5e):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₂₀H₃₂N₅O₄S⁺ 438.217; Found 438.2.

HPLC-MS chromatogram:

NH₂-Ile-Cys-Val-Ala-CONH₂ (5f):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₇H₃₄N₅O₄S⁺ 404.233; Found 404.3.

HPLC-MS chromatogram:

NH₂-Ala-Gln-Leu-Cys-CONH₂ (5g):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₇H₃₃N₆O₅S⁺ 433.223; Found 433.3.

HPLC-MS chromatogram:

NH₂-Cys-Ile-Glu-Ala-CONH₂ (5h):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₇H₃₂N₅O₆S⁺ 434.207; Found 434.2.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Asp-Ala-CONH₂ (5i):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₃H₂₄N₅O₆S⁺ 378.144; Found 378.1.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Asn-Ala-CONH₂ (5j):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₃H₂₅N₆O₅S⁺ 377.160; Found 377.1.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Met-Ala-CONH₂ (5k):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₄H₂₈N₅O₄S₂⁺ 394.158; Found 394.1.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Arg-Ala-CONH₂ (5l):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₅H₃₁N₈O₄S⁺ 419.218; Found 419.2.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Tyr-Ala-CONH₂ (5m):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₈H₂₈N₅O₅S⁺ 426.181; Found 426.1.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Ser-Ala-CONH₂ (5n):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₂H₂₄N₅O₅S⁺ 350.149; Found 350.1.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Trp-Ala-CONH₂ (5o):

LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{20}H_{29}N_6O_4S^+$ 449.197; Found 449.2.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Trp-Ala-CO₂H (5p):

LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{20}H_{28}N_5O_5S^+$ 450.181; Found 450.2.

HPLC-MS chromatogram:

NH₂-Ala-Cys-His-Ala-CONH₂ (5q):

LRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{15}H_{26}N_7O_4S^+$ 400.176; Found 400.2.

HPLC-MS chromatogram:

NH₂-Ala-Cys-Lys-Ala-CONH₂ (5r):

LRMS (ESI) m/z: [M + H]⁺Calcd for C₁₅H₃₁N₆O₄S⁺ 391.212; Found 391.2.

HPLC-MS chromatogram:

Human Serum Albumin Leu₅₅-His₆₃ sequence (5s):

LRMS (ESI) m/z: [M + 2H]²⁺Calcd for C₄₈H₇₂N₁₄O₁₅S²⁺ 558.251; Found 558.4.

HPLC-MS chromatogram:

GAP 26 (5t):

LRMS (ESI) m/z : $[M + 2H]^{2+}$ Calcd for $C_{70}H_{110}N_{20}O_{18}S^{2+}$ 775.401; Found 775.6.

HPLC-MS chromatogram:

HCV-1 e2 Trp₅₅₄-Ala₅₆₆ sequence (5u):

LRMS (ESI) m/z : $[M + 2H]^{2+}$ Calcd for $C_{61}H_{95}N_{17}O_{17}S_2^{2+}$ 700.826; Found 701.0.

HPLC-MS chromatogram:

1.4 Preparation of hypervalent iodine reagents (EBX)

a. Synthesis of TMS-EBX (6a)

2-Iodosylbenzoic acid (**19**):

Following a reported procedure,¹ sodium periodate (NaIO₄, 77.2 g, 361 mmol, 1.00 equiv.) and 2-iodobenzic acid (**18**) (89.5 g, 361 mmol, 1.00 equiv.) were suspended in 30% aqueous acetic acid solution (AcOH, 700 mL). The vigorously stirred mixture was heated and refluxed under air for 4 hours. The reaction mixture was then diluted with cold water (500 mL) and allowed to cool to room temperature. The mixture was stirred at room temperature for 45 minutes, and then poured into water (1.5 L). The crude product was collected by filtration, washed with a mixture of ice:water (3 x 300 mL) and cold acetone (3 x 300 mL). After air-drying overnight, 2-iodosylbenzoic acid (**19**) (74.3 g, 281 mmol, 78% yield) was recovered as a white solid.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.01 (dd, *J* = 7.5, 1.5 Hz, 1H, Ar*H*), 7.96 (ddd, *J* = 8.5, 7.2, 1.6 Hz, 1H, Ar*H*), 7.85 (dd, *J* = 8.2, 0.9 Hz, 1H, Ar*H*), 7.70 (td, *J* = 7.3, 1.0 Hz, 1H, Ar*H*).

¹³C NMR (101 MHz, DMSO-*d*₆) δ 167.7, 134.5, 131.5, 131.1, 130.4, 126.3, 120.4.

Spectroscopic data was consistent with the values reported in literature.¹

1-[(Trimethylsilyl)ethynyl]-1,2-benziodoxol-3(1*H*)-one (**6a**):

Following a reported procedure,² a solution of trimethylsilyl trifluoromethanesulfonate (TfOTMS, 7.35 mL, 40.7 mmol, 1.10 equiv.) was added dropwise to a stirred suspension of 2-iodosylbenzoic acid (**19**) (9.77 g, 37.0 mmol, 1.00 equiv.) in dichloromethane (112 mL) at room temperature. The mixture was then stirred for 60 minutes. Bis(trimethylsilyl)acetylene (9.22 mL, 40.7 mmol, 1.10 equiv.) was added dropwise to the reaction mixture. After 6 hours, a solution of saturated aqueous sodium bicarbonate was added (100 mL). The mixture was vigorously stirred for 30 minutes, then the two layers were separated and the organic layer was washed with additional portions of solution of saturated aqueous sodium bicarbonate (3 x 50 mL). The organic layer was dried over magnesium sulfate, filtered and concentrated under

¹ Kraszkiewicz, L.; Skulski, L. *Arkivoc* **2003**, 6, 120.

² Fernandez Gonzalez, D.; Brand, J. P.; Waser, J. *Chem. Eur. J.* **2010**, 16, 9457.

reduced pressure. Recrystallization from acetonitrile (120 mL) afforded TMS-EBX (**6a**) (8.86 g, 25.7 mmol, 70% yield) as a white solid.

¹H NMR (400 MHz, CDCl₃) δ 8.41 (dd, *J* = 6.8, 2.3 Hz, 1H, ArH), 8.19 (dd, *J* = 7.4, 1.7 Hz, 1H, ArH), 7.77 (m, 2H, 2 x ArH), 0.32 (s, 9H, TMS).

Spectroscopic data was consistent with the values reported in literature.²

b. Preparation of TMS-EBX derivatives

2-Iodosyl-5-nitrobenzoic acid (**20**):

Following a reported procedure,² 2-iodobenzoic acid (**18**) (10.0 g, 40.3 mmol, 1.00 equiv.) was suspended in a mixture of fuming nitric acid (6.6 mL) and concentrated sulfuric acid (13.4 mL). The reaction was equipped with a cooler, a vapor trap and was heated at 100 °C for 1 hour. The reaction mixture was then poured in a mixture of ice:water and the resulting precipitate was filtered. The resulting solid was refluxed in water (100 mL), filtered, washed with acetone (20 mL) and dried under vacuum to afford 2-iodosyl-5-nitrobenzoic acid (**20**) (4.10 g, 13.2 mmol, 33% yield).

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.69 (dd, *J* = 8.8, 2.5 Hz, 1H, ArH), 8.54 (d, *J* = 2.5 Hz, 1H, ArH), 8.08 (d, *J* = 8.8 Hz, 1H, ArH).

¹³C NMR (101 MHz, DMSO-*d*₆) δ 167.7, 148.3, 140.3, 136.0, 129.4, 127.2, 94.3.

Spectroscopic data was consistent with the values reported in literature.³

5-Nitro-1-[(trimethylsilyl)ethynyl]-1,2-benziodoxol-3(1H)-one (JW-RT-01, **6b**):

Following a slightly modified procedure,² a solution of trimethylsilyl trifluoromethanesulfonate (TfOTMS, 1.29 mL, 7.15 mmol, 1.10 equiv.) was added dropwise to a stirred suspension of 2-iodosyl-5-nitrobenzoic acid (**20**) (2.00 g, 6.50 mmol, 1.00 equiv.) in

³ Brand, J. P.; Chevalley, C.; Scopelliti, R.; Waser, J. *Chem. Eur. J.* **2012**, *18*, 5655.

dichloromethane (20 mL) at room temperature. The mixture was then stirred for 60 minutes. Bis(trimethylsilyl)acetylene (1.62 mL, 7.15 mmol, 1.10 equiv.) was added dropwise to the reaction mixture. After 6 hours, a solution of saturated aqueous sodium bicarbonate was added (20 mL). The mixture was vigorously stirred for 30 minutes, then the two layers were separated and the organic layer was washed with additional portions of solution of saturated aqueous sodium bicarbonate (3 x 10 mL). The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure. Recrystallization from acetonitrile (90 mL) afforded JW-RT-01 (**6b**) (1.05 g, 2.70 mmol, 42% yield) as a white solid.

m.p. (decomp.): 183 – 184 °C.

¹H NMR (400 MHz, CDCl₃) δ 9.14 (d, *J* = 2.5 Hz, 1H, *ArH*), 8.59 (dd, *J* = 8.9, 2.5 Hz, 1H, *ArH*), 8.46 (d, *J* = 8.9 Hz, 1H, *ArH*), 0.36 (s, 9H, TMS).

¹³C NMR (101 MHz, CDCl₃) δ 165.3, 151.2, 134.0, 128.8, 128.4, 126.9, 121.8, 118.9, 63.0, -0.3.

IR *v*_{max} 2942 (*w*), 2347 (*w*), 1632 (*s*), 1569 (*w*), 1532 (*m*), 1343 (*s*), 1252 (*w*), 1018 (*w*), 849 (*s*), 736 (*m*), 700 (*m*), 630 (*m*).

HRMS (ESI/QTOF) *m/z*: [M + H]⁺ Calcd for C₁₂H₁₃INO₄Si⁺ 389.9653; Found 389.9657.

5-Fluoro-2-iodosylbenzoic acid (**22**):

Following a reported procedure,³ sodium periodate (NaIO₄, 4.04 g, 18.9 mmol, 1.05 equiv.) and 2-iodo-5-fluorobenzoic acid (**21**) (4.79 g, 18.0 mmol, 1.00 equiv.) were suspended in 30% aqueous acetic acid solution (AcOH, 43 mL). The mixture was vigorously stirred and refluxed for 4 hours. The reaction mixture was then diluted with cold water (120 mL) and allowed to cool to room temperature, protecting it from light. After 45 minutes, the crude product was collected by filtration, washed on the filter with a mixture of ice:water (3 x 25 mL) and cold acetone (3 x 25 mL), and air-dried overnight in the dark to give 5-fluoro-2-iodosylbenzoic acid (**22**) (4.91 g, 17.4 mmol, 97% yield) as a colorless solid.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.21 (br, 1H, OH), 7.90 – 7.79 (m, 2H, 2 x *ArH*), 7.82 – 7.71 (m, 1H, *ArH*).

Spectroscopic data was consistent with the values reported in literature.³

5-Fluoro-1-[(trimethylsilyl)ethynyl]-1,2-benziodoxol-3(1H)-one (JW-RT-02, **6c**):

Following a slightly modified procedure,² a solution of trimethylsilyl trifluoromethanesulfonate (TfOTMS, 3.38 mL, 18.7 mmol, 1.10 equiv.) was added dropwise to a stirred suspension of 5-fluoro-2-iodosylbenzoic acid (**22**) (4.79 g, 17.0 mmol, 1.00 equiv.) in dichloromethane (52 mL) at room temperature. The mixture was then stirred for 60 minutes. Bis(trimethylsilyl)acetylene (4.24 mL, 18.7 mmol, 1.10 equiv.) was added dropwise to the reaction mixture. After 6 hours, a solution of saturated aqueous sodium bicarbonate was added (50 mL). The mixture was vigorously stirred for 30 minutes, then the two layers were separated and the organic layer was washed with additional portions of solution of saturated aqueous sodium bicarbonate (3 x 25 mL). The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure. Recrystallization from acetonitrile (100 mL) afforded JW-RT-02 (**6c**) (3.31 g, 9.13 mmol, 54% yield) as a crystalline white solid.

m.p. (decomp.): 173 – 174 °C.

¹H NMR (400 MHz, CDCl₃) δ 8.13 (dd, *J* = 9.0, 4.2 Hz, 1H, ArH), 8.09 (dd, *J* = 8.0, 2.9 Hz, 1H, ArH), 7.50 (ddd, *J* = 9.1, 7.5, 2.9 Hz, 1H, ArH), 0.32 (s, 9H, TMS).

¹³C NMR (101 MHz, CDCl₃) δ 165.7 (d, *J* = 253.9 Hz), 165.2 (d, *J* = 2.2 Hz), 134.2 (d, *J* = 7.6 Hz), 127.9 (d, *J* = 8.3 Hz), 122.4 (d, *J* = 24.5 Hz), 119.5 (d, *J* = 24.2 Hz), 117.9, 107.9 (d, *J* = 2.1 Hz), 63.6, -0.3.

IR ν_{max} 3075 (w), 2967 (w), 1617 (s), 1576 (m), 1454 (m), 1412 (m), 1308 (m), 1248 (m), 1210 (w), 1127 (w), 1082 (w), 1006 (w), 924 (w), 892 (w), 845 (s), 826 (m), 795 (m), 784 (m), 764 (m), 736 (w), 687 (s), 671 (m), 618 (w).

HRMS (ESI/QTOF) *m/z*: [M + H]⁺ Calcd for C₁₂H₁₃FIO₂Si⁺ 362.9708; Found 362.9704.

5-Methyl-2-iodosylbenzoic acid (**24**):

Following a reported procedure,³ sodium periodate (NaIO₄, 8.53 g, 39.9 mmol, 1.05 equiv.) and 2-iodo-5-methylbenzoic acid (**23**) (9.96 g, 38.0 mmol, 1.00 equiv.) were suspended in 30% aqueous acetic acid solution (AcOH, 102 mL). The mixture was vigorously stirred and refluxed for 4 hours. The reaction mixture was then diluted with cold water (270 mL) and allowed to cool to room temperature, protecting it from light. After 45 minutes, the crude product was

collected by filtration, washed on the filter with a mixture of ice:water (3 x 25 mL) and cold acetone (3 x 25 mL), and air-dried overnight in the dark to give 5-methyl-2-iodosylbenzoic acid (**24**) (9.70 g, 34.9 mmol, 92% yield) as a colorless solid.

¹H NMR (400 MHz, DMSO-*d*₆) δ 7.93 (br, 1H, OH), 7.84 (s, 1H, ArH), 7.78 (d, *J* = 8.4 Hz, 1H, ArH), 7.69 (d, *J* = 8.2 Hz, 1H, ArH), 2.47 (s, 3H, CH₃).

Spectroscopic data was consistent with the values reported in literature.³

5-Methyl-1-[(trimethylsilyl)ethynyl]-1,2-benziodoxol-3(1H)-one (JW-RT-03, **6d):**

Following a slightly modified procedure,² a solution of trimethylsilyl trifluoromethanesulfonate (TfOTMS, 6.86 mL, 38.0 mmol, 1.10 equiv.) was added dropwise to a stirred suspension of 5-methyl-2-iodosylbenzoic acid (**24**) (9.59 g, 34.5 mmol, 1.00 equiv.) in dichloromethane (105 mL) at room temperature. The mixture was then stirred for 60 minutes. Bis(trimethylsilyl)acetylene (8.60 mL, 38.0 mmol, 1.10 equiv.) was added dropwise to the reaction mixture. After 6 hours, a solution of saturated aqueous sodium bicarbonate was added (100 mL). The mixture was vigorously stirred for 30 minutes, then the two layers were separated and the organic layer was washed with additional portions of solution of saturated aqueous sodium bicarbonate (3 x 50 mL). The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure. Recrystallization from acetonitrile (40 mL) afforded JW-RT-03 (**6d**) (6.81 g, 19.0 mmol, 55% yield) as a pale brown solid.

m.p. (decomp.): 129 – 130 °C.

¹H NMR (400 MHz, CDCl₃) δ 8.22 (d, *J* = 2.2 Hz, 1H, ArH), 8.01 (d, *J* = 8.5 Hz, 1H, ArH), 7.59 (dd, *J* = 8.5, 2.2 Hz, 1H, ArH), 2.51 (s, 3H, CH₃), 0.31 (s, 9H, TMS).

¹³C NMR (101 MHz, CDCl₃) δ 166.8, 142.7, 135.9, 133.2, 131.3, 125.9, 116.8, 111.8, 64.2, 20.9, -0.3.

IR ν_{max} 3186 (w), 2960 (w), 2350 (w), 2026 (w), 1614 (s), 1571 (m), 1453 (m), 1403 (w), 1336 (m), 1254 (m), 1002 (w), 908 (w), 844 (s), 785 (s), 759 (w), 733 (m), 695 (s), 675 (s).

HRMS (ESI/QTOF) *m/z*: [M + H]⁺ Calcd for C₁₃H₁₆IO₂Si⁺ 358.9959; Found 358.9961.

4,5-Dimethoxy-2-iodosylbenzoic acid (**26**):

Following a reported procedure,² sodium periodate (NaIO_4 , 3.59 g, 16.8 mmol, 1.05 equiv.) and 2-iodo-4,5-dimethoxybenzoic acid (**25**) (4.93 g, 16.0 mmol, 1.00 equiv.) were suspended in 30% aqueous acetic acid solution (AcOH, 43 mL). The mixture was vigorously stirred and refluxed for 4 hours. The reaction mixture was then diluted with cold water (115 mL) and allowed to cool to room temperature, protecting it from light. After 45 minutes, the crude product was collected by filtration, washed on the filter with a mixture of ice:water (3 x 10 mL) and cold acetone (3 x 10 mL), and air-dried overnight in the dark to give 4,5-dimethoxy-2-iodosylbenzoic acid (**26**) (4.78 g, 14.8 mmol, 92% yield) as a colorless solid.

¹H NMR (400 MHz, $\text{DMSO}-d_6$) δ 7.45 (s, 1H, ArH), 7.23 (s, 1H, ArH), 3.88 (d, $J = 1.9$ Hz, 6H, 2 x OCH_3).

Spectroscopic data was consistent with the values reported in literature.³

4,5-Dimethoxy-1-[(trimethylsilyl)ethynyl]-1,2-benziodoxol-3(1H)-one (JW-RT-04, **6e**):

Following a slightly modified procedure,² a solution of trimethylsilyl trifluoromethanesulfonate (TfOTMS , 2.88 mL, 16.0 mmol, 1.10 equiv.) was added dropwise to a stirred suspension of 4,5-dimethoxy-2-iodosylbenzoic acid (**26**) (4.70 g, 14.5 mmol, 1.00 equiv.) in dichloromethane (44 mL) at room temperature. The mixture was then stirred for 60 minutes. Bis(trimethylsilyl)acetylene (3.61 mL, 16.0 mmol, 1.10 equiv.) was added dropwise to the reaction mixture. After 6 hours, a solution of saturated aqueous sodium bicarbonate was added (40 mL). The mixture was vigorously stirred for 30 minutes, then the two layers were separated and the organic layer was washed with additional portions of solution of saturated aqueous sodium bicarbonate (3 x 20 mL). The organic layer was dried over magnesium sulfate, filtered and concentrated under reduced pressure. Recrystallization from acetonitrile (120 mL) afforded JW-RT-04 (**6e**) (2.18 g, 5.39 mmol, 37% yield) as a white solid.

m.p. (decomp.): 176 – 177 °C.

¹H NMR (400 MHz, CDCl_3) δ 7.83 (s, 1H, ArH), 7.63 (s, 1H, ArH), 4.00 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 0.29 (s, 9H, TMS).

^{13}C NMR (101 MHz, CDCl_3) δ 166.9, 154.9, 152.3, 124.6, 116.4, 113.4, 107.9, 104.6, 65.5, 56.8, 56.5, -0.3.

IR ν_{max} 3055 (w), 2957 (w), 2849 (w), 1646 (m), 1625 (m), 1566 (w), 1498 (s), 1462 (w), 1443 (w), 1400 (m), 1315 (m), 1285 (m), 1267 (m), 1251 (w), 1216 (m), 1186 (w), 1126 (w), 1037 (w), 1018 (w), 846 (s), 780 (m), 761 (m), 730 (w), 687 (m), 653 (w).

HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{18}\text{IO}_4\text{Si}^+$ 405.0014; Found 405.0011.

c. Preparation of TIPS-EBX (**2**) and TES-EBX (**10**)

1-[(Triisopropylsilyl)ethynyl]-1,2-benziodoxol-3(1H)-one (**2**):

Following a reported procedure,² a cooled solution of trimethylsilyltriflate (TMSOTf, 19.9 mL, 110 mmol, 1.10 equiv.) was added dropwise to a stirred suspension of 2-iodosylbenzoic acid (**19**) (26.4 g, 100.0 mmol, 1.00 equiv.) in acetonitrile (350 mL) at 0 °C. The mixture was then allowed to warm to room temperature and was stirred for 15 minutes. Then trimethylsilyl(triisopropylsilyl)acetylene (28.0 g, 110 mmol, 1.10 equiv.) was added dropwise to the reaction mixture. After 30 minutes, pyridine (9.8 mL, 122 mmol, 1.10 equiv.) was added dropwise and, 15 minutes later, the reaction mixture was concentrated under reduced pressure. The collected solid was dissolved in dichloromethane (250 mL) and washed with a 1.0 N aqueous hydrochloric acid (150 mL). The aqueous layer was extracted with dichloromethane (250 mL), then the combined organic layers were washed with a saturated aqueous sodium bicarbonate (2 x 250 mL), dried over magnesium sulfate, filtered and concentrated *in vacuo*. The resulting solid was then recrystallized from acetonitrile and washed with hexanes (2 x 40 mL) to yield pure TIPS-EBX (**2**) (32.1 g, 74.9 mmol, 75% yield) as white crystals.

^1H NMR (400 MHz, CDCl_3) δ 8.43 (dd, J = 5.9, 3.3 Hz, 1H, ArH), 8.29 (dd, J = 6.0, 3.3 Hz, 1H, ArH), 7.76 (dd, J = 5.9, 3.3 Hz, 2H, 2 x ArH), 1.33 – 1.05 (m, 21H, TIPS).

^{13}C NMR (101 MHz, CDCl_3) δ 166.4, 134.5, 132.3, 131.4, 131.4, 126.1, 115.6, 113.9, 64.7, 18.4, 11.1.

Spectroscopic data was consistent with the values reported in literature.³

1-[(Triethylsilyl)ethynyl]-1,2-benziodoxol-3(1H)-one (**10**):

Following a reported procedure,² trimethylsilyltriflate (2.78 mL, 15.4 mmol, 1.1 equiv, freshly distilled over CaH₂) was added dropwise to a stirred solution of 2-iodosylbenzoic acid (**19**) (3.71 g, 14.0 mmol, 1.0 equiv) in acetonitrile (50 mL). After 15 min, (trimethylsilyl)(triethylsilyl)acetylene (3.26 g, 15.4 mmol, 1.1 equiv) was then added dropwise. After 30 min pyridine (1.25 mL, 15.4 mmol, 1.1 equiv) was added and the mixture was stirred for an additional 15 min. The solvent was then removed under reduced pressure and the yellow crude oil was dissolved in dichloromethane (50 mL). The organic layer was washed with 1 M HCl (50 mL), and the aqueous layer was extracted with CH₂Cl₂ (50 mL). The organic layers were washed twice with saturated NaHCO₃ (75 mL), dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. The resulting solid was recrystallized twice in CH₃CN. The solid was washed with cold acetonitrile, hexanes and dried under high vacuum to afford **10** (2.95 g, 7.64 mmol, 55% yield) as a slightly brown solid.

¹H NMR (400 MHz, CDCl₃) δ 8.40 (m, 1 H, ArH), 8.24 (m, 1 H, ArH), 7.75 (m, 2 H, ArH), 1.06 (t, *J* = 8.0 Hz, 9 H, SiCH₂CH₃), 0.73 (q, *J* = 8.0 Hz; 6H, SiCH₂CH₃).

¹³C NMR (101 MHz, CDCl₃) δ 166.5, 134.8, 132.5, 131.6, 131.3, 126.1, 115.5, 115.1, 64.6, 7.4, 4.1.

Spectroscopic data was consistent with the values reported in literature.²

1.5 Isolation of alkynylated glutathione 9a

a. Large scale reaction and characterization

A 500 mL round-bottom flask was charged with glutathione (**5a**) (154 mg, 500 μ mol, 1.00 equiv.) and milliQ water (250 mL). TMS-EBX (**6a**) (258 mg, 750 μ mol, 1.50 equiv.) was subsequently added, followed by 1,1,3,3-tetramethylguanidine (TMG) (88.0 μ L, 750 μ mol, 1.50 equiv.). No effort was made to exclude oxygen. The reaction mixture was stirred at 37 °C for 4 hours and then lyophilized. The resulting residue was partitioned between dichloromethane and water. The aqueous phase was washed with dichloromethane and subsequently purified by preparative RP-HPLC, employing method B (retention time: 7.5 – 9.5 minutes). Fractions containing the desired product were lyophilized to afford ethynylated glutathione **9a** TMG salt as a white solid (120 mg, 269 μ mol, 54% yield).

^1H NMR (400 MHz, D_2O) δ 4.81 (d, J = 4.7 Hz, 1H, CHCH_2S), 3.84 – 3.72 (m, 3H, CHCO_2H and CH_2CO_2^-), 3.32 (dd, J = 14.0, 4.6 Hz, 1H, CH_2S), 3.25 (s, 1H, SCCH), 3.05 (dd, J = 14.0, 8.9 Hz, 1H, CH_2S), 2.95 (s, 12H, TMG), 2.62 – 2.49 (m, 2H, $\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{N}$), 2.17 (q, J = 7.7 Hz, 2H, CHCH_2CH_2).

^{13}C NMR (101 MHz, D_2O) δ 176.1, 175.0, 173.9, 171.2, 161.3, 83.7, 73.2, 54.1, 53.1, 43.4, 38.8, 35.7, 31.5, 26.1.

HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{12}\text{H}_{18}\text{N}_3\text{O}_6\text{S}^+$ 332.0911; Found 332.0916.

b. Calibration

Calibration with alkynylated glutathione **9a** was achieved through the preparation of several samples of different concentrations and their analysis on RP HPLC. These analyses were repeated three times in order to obtain an average curve of calibration. The following linear regression was obtained: $Y = 0.00080296 \times X - 0.00898528$ and $R = 0.99987431$, where Y is the concentration in $\mu\text{mol/mL}$ of **9a** and X the absorbance area of the peak at 214 nm.

Figure S1: Calibration curve of **9a**.

1.6 Evaluation of reaction conditions

Table S1. Effect of EBX structure on the ethynylation of glutathione (5a)

<p>Reaction scheme showing the ethynylation of glutathione (5a) with an EBX reagent (2.0 equiv.) to form product 9a. Conditions: 200 mM PB pH 8.2, 2% v/v DMSO, 2 mM, 37 °C, 15 min.</p>			
Entry ^a	Reagent		Yield ^b
1		6a	87 % (95 %)
2		6d	74 % (82 %)
3		6e	81 % (90 %)
4		6c	87 % (90 %)
5		6b	99 % (99 %)
6		27	0 % (0 %)
7		10	60 % (76 %)

(a) Labeling conditions: 1.0 μ mol scale in 0.5 mL of non-degassed buffer (2% v/v DMSO). All the reactions were successfully replicated at least twice and the reported yield is an average of these replicates. (b) Calibrated HPLC yield based on absorbance at 214 nm. The yields in parentheses correspond to the yields after one hour of reaction.

Table S2. Effect of reaction conditions on the ethynylation of glutathione (5a)

Entry ^a	Buffer	pH	GSH conc.	Temperature	Reagent equiv.	Yield ^b
1	PB 200 mM	8.2	2 mM	37 °C	2.00	99 % (99 %)
2	Tris 200 mM	8.2	2 mM	37 °C	2.00	79 % (94 %)
3	HEPES 200 mM	8.2	2 mM	37 °C	2.00	42 % (75 %)
4	CHES 200 mM	8.2	2 mM	37 °C	2.00	59 % (85 %)
5	TAPS 200 mM	8.2	2 mM	37 °C	2.00	52 % (88 %)
6	PB 50 mM	8.2	2 mM	37 °C	2.00	95 % (99 %)
7	PB 100 mM	8.2	2 mM	37 °C	2.00	98 % (99 %)
8	PB 500 mM	8.2	2 mM	37 °C	2.00	95 % (95 %)
9	PB 200 mM	6.4	2 mM	37 °C	2.00	58 % (78 %)
10	PB 200 mM	7.2	2 mM	37 °C	2.00	90 % (93 %)
11	PB 200 mM	7.8	2 mM	37 °C	2.00	95 % (98 %)
12	PB 200 mM	8.8	2 mM	37 °C	2.00	99 % (99 %)
13	PB 200 mM	8.2	1 mM	37 °C	2.00	99 % (99 %)
14	PB 200 mM	8.2	200 μM	37 °C	2.00	97 % (97 %)
15	PB 200 mM	8.2	2 mM	r. t.	2.00	81 % (94 %)
16	PB 200 mM	8.2	2 mM	37 °C	1.20	93 % (97 %)
17	PB 200 mM	8.2	2 mM	37 °C	3.00	97 % (99 %)

(a) Labeling conditions: 1.0 μmol scale in 0.5 mL of non-degassed buffer (2% v/v DMSO). All the reactions were successfully replicated at least twice and the reported yield is an average of these replicates. (b) Calibrated HPLC yield based on absorbance at 214 nm. The yields in parentheses correspond to the yields after one hour of reaction.

1.7 Substrate scope of thiols

General procedure A:

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 200 mM solution of JW-RT-01 (**6b**) in DMSO (10.0 μ L, 2.00 μ mol, 2.00 equiv.) was diluted in a phosphate buffer (200 mM, pH 8.2, 390 μ L). The resulting mixture was shaken at room temperature at 420 rpm over 2 minutes and a 10.0 mM solution of the corresponding tetramer in 200 mM phosphate buffer pH 8.2 (100 μ L, 1.00 μ mol) was added in one portion. The solution was then vortexed few seconds and shaken at 37 °C at 700 rpm for 15 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

General procedure B:

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 100 mM solution of JW-RT-01 (**6b**) in DMSO (10.0 μ L, 1.00 μ mol, 2.00 equiv.) was diluted in a phosphate buffer (200 mM, pH 8.2, 390 μ L). The resulting mixture was shaken at room temperature at 420 rpm over 2 minutes and a 5.00 mM solution of the cysteine-containing peptide in 200 mM phosphate buffer pH 8.2 (100 μ L, 0.50 μ mol) was added in one portion. The solution was then vortexed few seconds and shaken at 37 °C at 700 rpm for 15 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

Yield calculation:

For the ethynylated glutathione **9a**, the yield was determined by comparing the average integration area of absorption peak at 214 nm of the product in the mixture to that of a standard curve.

For the other entries, the peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using slightly modified equation introduced by Li *et al.*:⁴ $\text{yield \%} = I_{\text{product}} / (I_{\text{starting}} + I_{\text{product}} + I_{\text{oxidation}} + I_{\text{side product}})$, where I_{starting} , I_{product} , $I_{\text{oxidation}}$ and $I_{\text{side product}}$ respectively represent the average ion counts of the remaining starting material, product, oxidized starting material and side product, if any.

⁴ Li, N.; Lim, R.; Edwardraja, S.; Lin, Q. *J. Am. Chem. Soc.* **2011**, *133*, 15316.

Ethynylated glutathione (**9a**):

Following general procedure A, glutathione (**5a**) afforded the title compound **9a** in 99% yield (retention time: 10.096 minutes).

HRMS (ESI/QTOF) m/z : $[M + H-1]^-$ Calcd for $C_{12}H_{16}N_3O_6S^-$ 330.0765; Found 330.0772.

HPLC gradient: 100% A isocratic for 10 minutes followed by 100% A to 100% B in 10 minutes.

HPLC-UV chromatogram at 214 nm:

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Val-Ala-CONH₂ (**9b**):

Following general procedure A, NH₂-Ala-Cys-Val-Ala-CONH₂ (**5b**) afforded the title compound **9b** in 93% yield (retention time: 7.426 minutes).

HRMS (ESI/QTOF) m/z : $[M + Na]^+$ Calcd for $C_{16}H_{27}N_5NaO_4S^+$ 408.1676; Found 408.1686.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Phe-Ala-CONH₂ (**9c**):

Following general procedure A, NH₂-Ala-Cys-Phe-Ala-CONH₂ (**5c**) afforded the title compound **9c** in 93% yield (retention time: 9.320 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₂₀H₂₇N₅NaO₄S⁺ 456.1676; Found 456.1685.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Pro-Ala-CONH₂ (**9d**):

Following general procedure A, NH₂-Ala-Cys-Pro-Ala-CONH₂ (**5d**) afforded the title compound **9d** in 91% yield (retention time: 14.152 minutes).

HRMS (ESI/QTOF) m/z : $[M + H]^+$ Calcd for $C_{16}H_{26}N_5O_4S^+$ 384.1700; Found 384.1695.

HPLC gradient: 100% A isocratic for 10 minutes followed by 100% A to 100% B in 10 minutes.

HPLC-MS chromatogram:

Ethynylated NH_2 -Val-Cys-Phe-Ala- $CONH_2$ (**9e**):

Following general procedure A, NH_2 -Val-Cys-Phe-Ala- $CONH_2$ (**5e**) afforded the title compound **9e** in 96% yield (retention time: 9.840 minutes).

HRMS (ESI/QTOF) m/z : $[M + H]^+$ Calcd for $C_{22}H_{32}N_5O_4S^+$ 462.2170; Found 462.2178.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ile-Cys-Val-Ala-CONH₂ (**9f**):

Following general procedure A, NH₂-Ile-Cys-Val-Ala-CONH₂ (**5f**) afforded the title compound **9f** in 94% yield (retention time: 8.639 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₁₉H₃₃N₅NaO₄S⁺ 450.2145; Found 450.2157.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Gln-Leu-Cys-CONH₂ (**9g**):

Following general procedure A, NH₂-Ala-Gln-Leu-Cys-CONH₂ (**5g**) afforded the title compound **9g** in 94% yield (retention time: 9.188 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₁₉H₃₂N₆NaO₅S⁺ 479.2047; Found 479.2055.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Cys-Ile-Glu-Ala-CONH₂ (9h):

Following general procedure A, NH₂-Cys-Ile-Glu-Ala-CONH₂ (**5h**) afforded the title compound **9h** in 87% yield (retention time: 8.831 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₁₉H₃₁N₅NaO₆S⁺ 480.1887; Found 480.1894.

HPLC gradient: 100% A to 100% B in 40 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Asp-Ala-CONH₂ (9i):

Following general procedure A, NH₂-Ala-Cys-Asp-Ala-CONH₂ (**5i**) afforded the title compound **9i** in 99% yield (retention time: 8.783 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₁₅H₂₃N₅NaO₆S⁺ 424.1261; Found 424.1262.

HPLC gradient: 100% A isocratic for 10 minutes followed by 100% A to 100% B in 10 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Asn-Ala-CONH₂ (**9j**):

Following general procedure A, NH₂-Ala-Cys-Asn-Ala-CONH₂ (**5j**) afforded the title compound **9j** in 99% yield (retention time: 7.607 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₁₅H₂₄N₆NaO₅S⁺ 423.1421; Found 423.1426.

HPLC gradient: 100% A isocratic for 10 minutes followed by 100% A to 100% B in 10 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Met-Ala-CONH₂ (**9k**):

Following general procedure A, NH₂-Ala-Cys-Met-Ala-CONH₂ (**5k**) afforded the title compound **9k** in 96% yield (retention time: 7.915 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₁₆H₂₇N₅NaO₄S₂⁺ 440.1397; Found 440.1404.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Arg-Ala-CONH₂ (**9l**):

Following general procedure A, NH₂-Ala-Cys-Arg-Ala-CONH₂ (**5l**) afforded the title compound **9l** in 59% yield (retention time: 5.737 minutes).

HRMS (ESI/QTOF) *m/z*: [M + H]⁺ Calcd for C₁₇H₃₁N₈O₄S⁺ 443.2183; Found 443.2183.

The reaction mixture was analyzed with two different gradients to resolve peak overlapping between desired product **9l**, the side-product, the disulfide, the buffer and the DMSO.

HPLC gradient: 96% C isocratic for 10 minutes followed by 96% C to 100% B in 10 minutes.

HPLC-MS chromatogram:

HPLC gradient: 93% C isocratic for 10 minutes followed by 93% C to 100% B in 10 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Tyr-Ala-CONH₂ (**9m**):

Following general procedure A, NH₂-Ala-Cys-Tyr-Ala-CONH₂ (**5m**) afforded the title compound **9m** in 84% yield (retention time: 7.763 minutes).

HRMS (ESI/QTOF) m/z : $[M + Na]^+$ Calcd for C₂₀H₂₇N₅NaO₅S⁺ 472.1625; Found 472.1640.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Ser-Ala-CONH₂ (**9n**):

Following general procedure A, NH₂-Ala-Cys-Ser-Ala-CONH₂ (**5n**) afforded the title compound **9n** in 97% yield (retention time: 10.145 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₁₄H₂₃N₅NaO₅S⁺ 396.1312; Found 396.1319.

HPLC gradient: 96% C isocratic for 10 minutes followed by 96% C to 100% B in 10 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Trp-Ala-CONH₂ (**9o**):

Following general procedure A, NH₂-Ala-Cys-Trp-Ala-CONH₂ (**5o**) afforded the title compound **9o** in 96% yield (retention time: 9.845 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₂₂H₂₈N₆NaO₄S⁺ 495.1785; Found 495.1786.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-Trp-Ala-CO₂H (**9p**):

Following general procedure A, NH₂-Ala-Cys-Trp-Ala-CO₂H (**5p**) afforded the title compound **9p** in 95% yield (retention time: 10.465 minutes).

HRMS (ESI/QTOF) *m/z*: [M + Na]⁺ Calcd for C₂₂H₂₇N₅NaO₅S⁺ 496.1625; Found 496.1631.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

Ethynylated NH₂-Ala-Cys-His-Ala-CONH₂ (**9q**):

Following general procedure A, NH₂-Ala-Cys-His-Ala-CONH₂ (**5q**) afforded the title compound **9q** in 93% yield (retention time: 7.476 minutes).

HRMS (ESI/QTOF) m/z : $[M + H]^+$ Calcd for $C_{17}H_{26}N_7O_4S^+$ 424.1761; Found 424.1766.

HPLC gradient: 97% C isocratic for 10 minutes followed by 97% C to 100% B in 10 minutes.

HPLC-MS chromatogram:

Ethynylated NH_2 -Ala-Cys-Lys-Ala- $CONH_2$ (**9r**):

Following general procedure A, NH_2 -Ala-Cys-Lys-Ala- $CONH_2$ (**5r**) afforded the title compound **9r** in 93% yield (retention time: 6.887 minutes).

HRMS (ESI/QTOF) m/z : $[M + H]^+$ Calcd for $C_{17}H_{31}N_6O_4S^+$ 415.2122; Found 415.2117.

HPLC gradient: 97% C isocratic for 10 minutes followed by 97% C to 100% B in 10 minutes.

HPLC-MS chromatogram:

Ethynylated Human Serum Albumin Leu₅₅-His₆₃ sequence (9s):

Following general procedure B, Human Serum Albumin Leu₅₅-His₆₃ sequence (**5s**) afforded the title compound **9s** in 97% yield (retention time: 17.278 minutes).

HRMS (ESI/QTOF) m/z : $[M + H]^+$ Calcd for $C_{50}H_{71}N_{14}O_{15}S^+$ 1139.4939; Found 1139.4883.

HPLC gradient: 100% A to 50% A in 35 minutes followed by 50% A to 100% B in 5 minutes.

HPLC-MS chromatogram:

Ethynylated GAP 26 (9t):

Following general procedure B, GAP 26 (**5t**) afforded the title compound **9t** in 68% yield (retention time: 15.205 minutes).

HRMS (ESI/QTOF) m/z : $[M + H]^+$ Calcd for $C_{72}H_{109}N_{20}O_{18}S^+$ 1573.7944; Found 1573.7970.

HPLC gradient: 100% A to 50% A in 35 minutes followed by 50% A to 100% B in 5 minutes.

HPLC-MS chromatogram:

Ethynylated HCV-1 e2 Trp₅₅₄-Ala₅₆₆ sequence (9u):

Following general procedure B, HCV-1 e2 Trp₅₅₄-Ala₅₆₆ sequence (5u) afforded the title compound 9u in 94% yield (retention time: 14.907 minutes).

HRMS (ESI/QTOF) m/z: [M + Na]⁺ Calcd for C₆₃H₉₃N₁₇NaO₁₇S₂⁺ 1446.6269; Found 1446.6274.

HPLC gradient: 100% A to 100% B in 40 minutes.

HPLC-MS chromatogram:

Attempt with a terminal cysteine and free carboxylic acid:

Ethynylated NH₂-Ala-Gln-Leu-Cys-CO₂H (9v):

Following general procedure A, $\text{NH}_2\text{-Ala-Gln-Leu-Cys-CO}_2\text{H}$ (**5v**) afforded a complex mixture. The low resolution mass of the compound with retention time of 9.576 minutes corresponded to **9h** (61% yield), but high resolution mass data could not be obtained.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-MS chromatogram:

1.8 Reaction of disulfide bond-containing molecules

a. Application on GSSG (**28**)

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a 2.00 mM solution of oxidized glutathione (**28**) in 200 mM phosphate buffer pH 8.2 (250 μ L, 0.50 μ mol) and a solution of tris(2-carboxyethyl)phosphine reagent (TCEP) in 200 mM phosphate buffer pH 8.2 (150 μ L, 0.75/2.50/5.00 μ mol, respectively 1.50/5.00/10.0 equiv.). The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at room temperature at 420 rpm over 5 or 60 minutes. Separately, a 200 mM solution of JW-RT-01 (**6b**) in DMSO (10.0 μ L, 2.00 μ mol, 4.00 equiv.) was diluted in a phosphate buffer (200 mM, pH 8.2, 90 μ L) and shaken at room temperature at 420 rpm over 2 minutes. Next, the solution of reduced glutathione was transferred to the mixture of hypervalent iodine reagent. The solution was then vortexed few seconds and shaken at 37 °C at 700 rpm for 15 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS and the yield was determined by comparing the integration area of absorption peak at 214 nm of the product in the mixture to that of a standard curve.

Entry	TCEP equiv.	Reduction time	Yield
1	1.50 equiv.	60 minutes	97%
2	5.00 equiv.	10 minutes	66%
3	10.0 equiv.	10 minutes	39%

b. Application on oxytocin (11)

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a 0.40 mM solution of oxytocin (**11**) in 200 mM phosphate buffer pH 8.2 (250 μ L, 0.10 μ mol) and a 1.00 mM solution of tris(2-carboxyethyl)phosphine reagent (TCEP) in 200 mM phosphate buffer pH 8.2 (150 μ L, 0.15 μ mol, 1.50 equiv.). The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at room temperature at 420 rpm over 60 minutes. Separately, in a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 40.0 mM solution of JW-RT-01 (**6b**) in DMSO (10.0 μ L, 0.40 μ mol, 4.00 equiv.) was diluted in a phosphate buffer (200 mM, pH 8.2, 90 μ L) and shaken at room temperature at 420 rpm over 2 minutes. Next, the solution of reduced oxytocin was transferred to the mixture of hypervalent iodine reagent. The solution was then vortexed few seconds and shaken at 37 °C at 700 rpm for 15 minutes to afford **12** in 86% yield (retention time: 15.657 minutes). No effort was made to exclude oxygen. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: $\text{yield \%} = I_{\text{product}} / (I_{\text{starting}} + I_{\text{product}} + I_{\text{side product}})$, where I_{starting} , I_{product} and $I_{\text{side product}}$ respectively represent the average ion counts of the remaining starting material, product and side product.

HRMS (ESI/QTOF) m/z : $[M + H]^+$ Calcd for $C_{47}H_{69}N_{12}O_{12}S_2^+$ 1057.4594; Found 1057.4587.

HPLC gradient: 100% A to 100% B in 40 minutes.

HPLC-MS chromatogram:

1.9 Functionalization of the thioalkyne product

One-pot ethynylation/CuAAC on glutathione (**5a**):

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 100 mM solution of JW-RT-01 (**6b**) in DMSO (2.0 μ L, 0.20 μ mol, 2.00 equiv.) was diluted in a phosphate buffer (200 mM, pH 8.2, 78.0 μ L). The resulting mixture was shaken at room temperature at 420 rpm over 2 minutes and a 5.00 mM solution of glutathione (**5a**) in 200 mM phosphate buffer pH 8.2 (20.0 μ L, 0.100 μ mol) was added in one portion. The solution was then vortexed few seconds and shaken at 37 °C at 700 rpm for 15 minutes to furnish **9a**. The reaction mixture was then allowed to cool to room temperature over 2 minutes.

Separately, a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a 50.0 mM solution of sodium ascorbate in water (2.4 μ L, 0.12 μ mol, 1.20 equiv.). Then, a 40.0 mM solution of THPTA in water (2.5 μ L, 0.10 μ mol, 1.00 equiv.) was added to the solution of sodium ascorbate and mixed through pipetting. Next, a 50.0 mM solution of copper sulfate in water (0.30 μ L, 15 nmol, 15 mol %) was added to the mixture of sodium ascorbate and THPTA. The resulting mixture was vortexed few seconds to ensure proper reagent mixing.

The solution of copper sulfate, THPTA and sodium ascorbate was then added to the reaction mixture containing **9a**. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 1 minute. Finally, a 50.0 mM solution of Cy7-N₃ in water (8.0 μ L, 0.40 μ mol, 4.00 equiv.) was added to the reaction mixture. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at room temperature at 420 rpm over 30 minutes to afford **13a** (retention time: 8.760 minutes). No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

HRMS (ESI/QTOF) m/z : $[M + H_2]^{-2}$ Calcd for C₆₀H₇₃N₉O₁₉S₅⁻² 691.6819; Found 691.6815.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-UV chromatogram at 214 nm:

One-pot ethynylation/CuAAC on Human Serum Albumin Leu₅₅-His₆₃ (**5t**):

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 100 mM solution of JW-RT-01 (**6b**) in DMSO (2.0 μ L, 0.20 μ mol, 2.0 equiv.) was diluted in a phosphate buffer (200 mM, pH 8.2, 78 μ L). The resulting mixture was shaken at room temperature at 420 rpm over 2 minutes and a 5.00 mM solution of Human Serum Albumin Leu₅₅-His₆₃ sequence (**5t**) in 200 mM phosphate buffer pH 8.2 (20 μ L, 0.10 μ mol) was added in one portion. The solution was then vortexed few seconds and shaken at 37 °C at 700 rpm for 15 minutes to furnish **9t**. The reaction mixture was then allowed to cool to room temperature over 2 minutes.

Separately, a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a 50.0 mM solution of sodium ascorbate in water (2.4 μ L, 0.12 μ mol, 1.20 equiv.). Then, a 40.0 mM solution of THPTA in water (2.5 μ L, 0.10 μ mol, 1.00 equiv.) was added to the solution of sodium ascorbate and mixed through pipetting. Next, a 50.0 mM solution of copper sulfate in water (0.30 μ L, 15 nmol, 15 mol %) was added to the mixture of sodium ascorbate and THPTA. The resulting mixture was vortexed few seconds to ensure proper reagent mixing.

The solution of copper sulfate, THPTA and sodium ascorbate was then added to the reaction mixture containing **9t**. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 1 minute. Finally, a 50.0 mM solution of Cy7-N₃ in water (8.0 μ L, 0.40 μ mol, 4.0 equiv.) was added to the reaction mixture. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at room temperature at 420 rpm over 30 minutes to afford **13b** (retention time: 13.273 minutes). No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

HRMS (ESI/QTOF) m/z: $[M + H_2]^{-2}$ Calcd for $C_{98}H_{126}N_{20}O_{28}S_5^{-2}$ 1095.3832; Found 1095.3859.

HPLC gradient: 100% A to 100% B in 40 minutes.

HPLC-UV chromatogram at 214 nm:

One-pot ethynylation/hydrolysis on glutathione (5a):

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 200 mM solution of JW-RT-01 (**6b**) in DMSO (30.0 μ L, 6.0 μ mol, 2.0 equiv.) was diluted in a phosphate buffer (200 mM, pH 8.2, 1170 μ L). The resulting mixture was shaken at room temperature over 2 minutes and a 10.0 mM solution of glutathione in 200 mM PB pH 8.2 (300 μ L, 3.00 μ mol) was added in one portion. The solution was then vortexed few seconds and shaken at 37 $^{\circ}$ C for 15 minutes. Then a 1M solution of citric acid in water (500 μ L) was added. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{12}H_{20}N_3O_7S^+$ 350.1016; Found 350.1014.

HPLC-UV Chromatogram (214 nm): t = 0 minute.

HPLC-UV Chromatogram (214 nm): t = 3 hours.

HPLC-UV Chromatogram (214 nm): t = 4 hours.

HPLC-UV Chromatogram (214 nm): t = 20 hours.

Time (h)	Abs. GS-ethynyl (9a)	Conversion (%)
0	398.9	0
3	34.0	92
4	21.3	95
20	0	100

One-pot ethynylation/Sonogashira coupling on glutathione (5a):

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 120 mM solution of JW-RT-01 (**6b**) in DMSO (10.0 μ L, 1.2 μ mol, 1.2 equiv.) was diluted in a phosphate buffer (200 mM, pH 8.2, 350 μ L). The resulting mixture was shaken at room temperature over 2 minutes and a 10.0 mM solution of glutathione in 200 mM PB pH 8.2 (100 μ L, 1.00 μ mol, 1.00 equiv) was added in one portion. The solution was then vortexed few seconds and shaken at 37 °C for 2 hours. Then in an eppendorf, Pd(OAc)₂ (0.1 mg, 0.4 μ mol, 0.45 equiv), copper iodide (0.5 mg, 2 μ mol, 2.6 equiv), 4-iodophenol (0.44 mg, 2.0 μ mol, 2.0 equiv) and TPPTS (1.0 mg, 1.8 μ mol, 1.8 equiv.) were placed and 40 μ L of DMF were added. To this suspension the reaction was added and the mixture was left to shake at 37 °C. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

HRMS 15 (ESI/QTOF) m/z : $[M + H]^+$ Calcd for $C_{18}H_{22}N_3O_7S^+$ 424.1173; Found 424.1168.

HRMS 30 (ESI/QTOF) m/z : $[M + H]^+$ Calcd for $C_{24}H_{33}N_6O_{12}S_2^+$ 661.1592; Found 661.1600.

HPLC-UV Chromatogram (214 nm): $t = 30$ minutes.

HPLC-MS Chromatogram: $t = 30$ minutes.

1.10 Competition experiments

2-Iodo-N-(prop-2-yn-1-yl)acetamide (**33**):

2-Iodo-N-(prop-2-yn-1-yl)acetamide (**33**) was synthesized according to literature procedure.⁵ 2-Iodoacetic acid (**31**) (200 mg, 1.08 mmol, 1 equiv), propargylamine (**32**) (68.9 μ L, 1.08 mmol, 1 equiv), EDCI (206 mg, 1.08 mmol, 1 equiv), and DMAP (13 mg, 0.11 mmol, 0.1 equiv) were dissolved in dichloromethane (3.6 mL, 0.3 M) and stirred at room temperature. The crude product was purified by flash column chromatography (40% ethyl acetate/pentane), yielding 2-Iodo-N-(prop-2-yn-1-yl)acetamide (**33**) (11 mg, 5%).

⁵ Z. V. Boskovic *et al.*, *ACS Chem. Biol.* **2016**, *11*, 1844-1851.

¹H NMR (CDCl₃, 400 MHz): δ 6.14 (s, 1H, NH), 4.08 (dd, *J* = 5.3, 2.6 Hz, 2H, CH₂N), 3.71 (s, 2H, CH₂I), 2.28 (t, *J* = 2.6 Hz, 1H, CH).

Spectroscopic data was consistent with the values reported in literature.⁵

Competition experiments between JR-RT-01 (**6b**) and iodoacetamide **33** for the functionalization of glutathione (**5a**) (reference 25):

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 1:1 solution of of JW-RT-01 (**6b**) (20 mM, 2.0 equiv) and iodoacetamide **33** (20 mM, 2.0 equiv.) in DMSO (10 μ L) was diluted in a phosphate buffer (200 mM, pH 8.2, 480 μ L). The resulting mixture was shaken at room temperature over 2 minutes and a 10.0 mM solution of the glutathione (**5a**) in 200 mM PB pH 8.2 (10 μ L, 0.10 μ mol) was added in one portion. The solution was then vortexed few seconds and shaken at 37 °C for 60 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

HPLC gradient: 100% A isocratic for 10 minutes followed by 100% A to 100% B in 15 minutes.

HPLC-UV Chromatogram (214 nm):

	N-propargyl IAA Product 34	GS-ethynyl (9a)
Abs. (mAU)	11.5	40.1

To approximate the product ratio and yield, the 214 nm absorbances of both products **9a** and **34** were considered the same. By this approximation, the iodoacetamide-derived product is formed in a 22% yield, while the ethynylated product was formed in a 78% yield, considering that no side product were formed and no starting materials remained. To further support this approximation, the reaction was run again with iodoacetamide **33** only as reagent (see below). In this case, full conversion of **9a** to **34** without side product was observed. The absorbance in this experiment was therefore assumed to correspond to a quantitative yield. Comparing the absorbance in both experiments shows that the iodoacetamide product **34** was formed in a 24% yield in the competitive experiment, further supporting our approximation.

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a solution of N-propargyl iodoacetamide **33** (20 mM, 2.0 equiv) equiv. in DMSO (10 μ L) was diluted in a phosphate buffer (200 mM, pH 8.2, 480 μ L). The resulting mixture was shaken at room temperature over 2 minutes and a 10.0 mM solution of **5a** in 200 mM PB pH 8.2 (10 μ L, 0.10 μ mol) was added in one portion. The solution was then vortexed few seconds and shaken at 37 $^{\circ}$ C for 60 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z : $[M + Na]^+$ Calcd for $C_{15}H_{22}N_4NaO_7S^+$ 425.1101; Found 425.1091.

HPLC-UV Chromatogram (214 nm):

(L-Arginyl glycyL-L- α -aspartyl-2-S-propyn-1-yl)L-cysteine (**35**):

(L-Arginyl glycyL-L- α -aspartyl-2-S-propyn-1-yl)L-cysteine (**35**) was synthesized according to the procedure described in the literature.⁶ To a solution of glutathione (0.75 mmol, 0.23 g) in water and MeOH (1:1, 5 mL), was added a 25% solution of NH₄OH (0.75 mmol, 4.5 mL, 1.0 equiv.) at 0 °C. A freshly distilled solution of propargyl bromide (8.9 μ L, 0.083 mmol, 1.1 equiv.) in MeOH (0.25 mL) was then introduced. The reaction was stirred during 3 hours at room temperature. The solution was concentrated under vacuum, water was then added (100 mL) and the solution was freeze-dried to provide product **35**.

Spectroscopic data was consistent with the values reported in literature.⁶

¹H NMR (CDCl₃, 400 MHz): δ 4.70 (dd, J = 8.9, 4.9 Hz, 1H, CHCH₂S), 3.80 (m, 3H, C_{alkyl}H), 3.39 (dd, J = 2.6, 1.2 Hz, 2H, CHCH₂S), 3.28 (dd, J = 14.3, 4.9 Hz, 1H, C_{alkyl}H), 3.04 (dd, J = 14.3, 8.9 Hz, 1H, C_{alkyl}H), 2.70 (t, J = 2.6 Hz, 1H, C_{alkyl}H), 2.6 (td, J = 7.4, 2.4 Hz, 2H, C_{alkyl}H), 2.18 (q, J = 7.4 Hz, 2H, C_{alkyl}H).

HRMS (ESI/QTOF) m/z : [M + H]⁺ Calcd for C₁₃H₂₀N₃O₆S⁺ 346.1067; Found 346.1066.

⁶ S. Lamandé-Langle, C. Collet, R. Hensienne, C. Vala, F. Chrétien, Y. Chapleur, A. Mohamadi, P. Lacolley, V. Regnault, *Bioorg. Med. Chem.* **2014**, 22, 6672-6683.

N⁵-((R)-3-(((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)thio)-1-((carboxymethyl)amino)-1-oxopropan-2-yl)-L-glutamine (36**):**

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a 50.0 mM solution of sodium ascorbate in water (2.4 μ L, 0.12 μ mol, 1.2 equiv.). Then, a 50.0 mM solution of THPTA in water (2.0 μ L, 0.10 μ mol, 1.0 equiv.) was added and mixed to the solution of sodium ascorbate through pipetting. Next, a 50.0 mM solution of copper sulfate in water (0.30 μ L, 15.0 nmol, 15 mol %) was added to the mixture of sodium ascorbate and THPTA. The resulting mixture was vortexed few seconds to ensure proper reagent mixing. A 1.07 mM solution of propargylated glutathione (**35**) (93 μ L, 0.10 μ mol, 1.0 equiv.) was added to the previous mixture and the mixture was vortexed. Finally, a 200 mM solution of benzyl azide in DMSO (2 μ L, 0.4 μ mol, 4.0 equiv) was added and the mixture was vortexed and left to react on the bench at room temperature. The reaction was followed by HPLC. After 30 min, an aliquote was taken and submitted to HPLC, showing complete conversion to cycloaddition product **36**.

HRMS (ESI/QTOF) m/z : [M + H]⁺ Calcd for C₂₀H₂₇N₆O₆S⁺ 479.1707; Found 479.1703.

HPLC gradient: 100% A to 100% B in 20 minutes.

HPLC-UV Chromatogram (214 nm):

N5-((R)-3-((1-benzyl-1H-1,2,3-triazol-4-yl)thio)-1-((carboxymethyl)amino)-1-oxopropan-2-yl)-L-glutamine (**37**):

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a 50.0 mM solution of sodium ascorbate in water (2.4 μL , 0.12 μmol , 1.2 equiv.). Then, a 50.0 mM solution of THPTA in water (2.0 μL , 0.10 μmol , 1.0 equiv.) was added and mixed to the solution of sodium ascorbate through pipetting. Next, a 50.0 mM solution of copper sulfate in water (0.30 μL , 15 nmol, 15 mol %) was added to the mixture of sodium ascorbate and THPTA. The resulting mixture was vortexed few seconds to ensure proper reagent mixing. A 1.07 mM solution of ethynylated glutathione (**9a**) (93 μL , 0.10 μmol , 1.0 equiv.) was added to the previous mixture and the mixture was vortexed. Finally, a 200 mM solution of benzyl azide in DMSO (2 μL , 0.4 μmol , 4.0 equiv) was added and the mixture was vortexed and left to react on the bench at room temperature. The reaction was followed by HPLC. After 30 min, an aliquote was taken and submitted to HPLC, showing full conversion to the cycloaddition product **37**.

HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{19}\text{H}_{24}\text{N}_6\text{O}_6\text{S}^+$ 465.1551; Found 465.1576

HPLC gradient: 100% A isocratic for 5 minutes followed by 100% A to 100% B in 15 minutes.

HPLC-UV Chromatogram (214 nm):

Retention time

Area (214 nm)

MS

12.71

666.8

465.2

Competition experiment for cycloaddition of propargylated glutathione (35**) and ethynylated glutathione (**9a**) with benzyl azide (reference 26):**

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a 50.0 mM solution of sodium ascorbate in water (2.4 μL , 0.12 μmol , 1.2 equiv.). Then, a 50.0 mM solution of THPTA in water (2.0 μL , 0.10 μmol , 1.0 equiv.) was added and mixed to the solution of sodium ascorbate through pipetting. Next, a 50.0 mM solution of copper sulfate in water (0.30 μL , 15.0 nmol, 15 mol %) was added to the mixture of sodium ascorbate and THPTA. The resulting mixture was vortexed few seconds to ensure proper reagent mixing. A 1:1 mixture of 1.07 mM solution of *S*-ethynylated glutathione and of 1.07 mM solution of *S*-propargylated glutathione (93 μL , 0.10 μmol , 1.0 combined equiv.) was added to the previous mixture and the mixture was vortexed. Finally, a 20 mM benzyl azide in DMSO (2 μL , 0.04 μmol , 0.4 equiv) was added and the mixture was vortexed and left to react on the bench at room temperature. The reaction was followed by HPLC. After 30 min, an aliquote was taken and submitted to HPLC.

HPLC gradient: 100% A isocratic for 10 minutes followed by 100% A to 100% B in 15 minutes.

No separation could be achieved for the triazole products **36** and **37**, nevertheless, mass analysis showed similar intensity for the molecule ion of the compounds. Therefore, similar reaction rates can be expected.

HPLC-UV Chromatogram (214 nm):

Retention time	Area (214 nm)	MS
7.79	54.2	330.3
9.00	68.5	346.4
17.05	546.4	465.2; 479.2

Accurate mass ESI QTOF spectrum:

1.11 Spectra of new compounds

2. Labeling of trastuzumab (16)

Material and methods:

All reagents and solvents were obtained from commercial sources and used without prior purifications. Antibody concentration was determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Illkirch, France). Antibody-conjugates were purified by gel filtration chromatography on Bio-Spin P-30 columns (Bio-Rad, Hercules, USA) pre-equilibrated with the buffer of interest. Vivaspin micro-concentrators (10 kDa cutoff, 500 μ L, Sartorius, Gottingen, Germany) were used for buffer exchange. Antibody deglycosylation was achieved by incubating Remove-iT[®] Endo S (New England Biolabs, Ipswich, USA).

Native mass spectrometry (MS) analyses of intact mAbs and conjugates were performed on a Synapt G2 HDMS (Waters, Manchester, UK) mass spectrometer coupled to an automated chip-based nanoESI infusion source (Triversa Nanomate, Advion, Ithaca, NY) operating in positive ion mode. Electrospray ionization was conducted at a capillary voltage of 1.70 kV and nitrogen nanoflow of 0.70 psi. Samples were infused at a concentration of 10 μ M. The mass spectrometer was operated in the positive mode. The extraction cone value was set to 5 V and the cone voltage was set to 120 V or 180 V for cysteine-conjugates and lysine-conjugates, respectively. The pressure in the interface region was fixed at 6 mbar. Acquisitions were performed in the m/z range 1,000–10,000 with a 4 s scan time. External calibration was performed using singly charged ions produced by a 2 g/L solution of cesium iodide in 2-propanol/water (50/50 v/v). MS data interpretations were performed using Mass Lynx V4.1 (Waters, Manchester, UK). Average DoC values were calculated using equation (1). These results were derived from the relative peak intensities measured from deconvoluted mass spectra.

$$DoC = \frac{(\sum_{k=0}^8 k \times \text{intensity DoCk})}{\sum_{k=0}^8 \text{intensity DoCk}} \quad (1)$$

General procedures:

Reduction of trastuzumab (16)

To a solution of trastuzumab (**16**) (5 mg/mL, 1 equiv., 100 μ L in PBS 1x with 1% EDTA, pH 7.5) was added TCEP (20 mM in H₂O, 10 equiv., 1.71 μ L). The reaction mixture was then incubated for 3 hours at 37 °C, before the excess of reagent was removed by gel filtration chromatography on Bio-Spin P-30 columns pre-equilibrated with PBS 1x (pH 7.5) to give a solution of reduced trastuzumab.

Conjugation step

To a solution of reduced trastuzumab (**16**) (5 mg/mL, 1 equiv., 100 μ L in PBS 1x, pH 7.5) was added the TMS-EBX reagent (10 mM solution in DMSO). The resulting solution was then incubated for a given time at either 4 °C, 25 °C or 37 °C, before the excess of reagent was removed by gel filtration chromatography on Bio-Spin P-30 columns pre-equilibrated with PBS 1x (pH 7.5) to give a solution of conjugated trastuzumab.

Functionalization step

To a solution of conjugated trastuzumab (1 equiv., 50 μ L in PBS 1x, pH 7.5) was added TAMRA- N_3 (100 mM solution in DMSO, 20 equiv.). A pre-mixed solution of $CuSO_4$ (10 mM solution in H_2O , 1 equiv.) and THPTA (10 mM solution in H_2O , 2 equiv.) was added to the reaction mixture, followed by sodium ascorbate (10 mM solution in H_2O , 3 equiv.). The resulting mixture was then incubated for 24 hours at 25 $^{\circ}C$ before the excess of reagent was removed by gel filtration chromatography on Bio-Spin P-30 columns pre-equilibrated with PBS 1/20x (pH 7.5). The resulting conjugate was then washed six times with PBS 1/20x (pH 7.5) containing 1% EDTA on Vivaspin centrifugal concentrators (500 μ L, 10 kDa) to remove chelated copper.

Sample preparation for native mass spectrometry

Prior to native MS experiments, antibody conjugates were deglycosylated at 37 $^{\circ}C$ for 2 hours with 0.4 units of Remove-iT[®] Endo S per microgram of antibody conjugates, before being desalted against 150 mM ammonium acetate solution buffered at $\sim$ pH 7.5 using ten cycles of concentration/dilution on Vivaspin centrifugal concentrators (500 μ L, 10 kDa). Protein concentration was then determined by UV absorbance using a NanoDrop spectrophotometer.

Table S3. Conjugation results with TMS-EBX (6a):

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

^a: reduced trastuzumab was utilized; ^b: as determined on non-reduced trastuzumab; ^c: see native mass spectrum S2; ^d: see native mass spectrum S5; ^e: see native mass spectrum S4

Table S4. Conjugation results with JW-RT-02 (6c):

Entry	Conditions ^a	Av. DoC	Conv. (%)	side reactivity ^b
1	6c (2 equiv.), PB buffer, pH 8.2, 37 °C, 5 min.	0.4	28	Y ^c
2	6c (8 equiv.), PBS buffer , pH 8.2, 37 °C, 5 min.	2.9	88	Y
3	6c (8 equiv.), PBS buffer, pH 6.5 , 37 °C, 5 min.	1.3	62	N
4	6c (8 equiv.), PBS buffer, pH 7.5 , 37 °C, 5 min.	2.7	88	Y
5	6c (8 equiv.), PBS buffer, pH 8.5 , 37 °C, 5 min.	3.0	90	Y
6	6c (8 equiv.), PBS buffer, pH 7.5, 25 °C , 5 min.	2.3	81	Y
7	6c (8 equiv.), PBS buffer, pH 7.5, 25 °C, 2 min.	1.1 ^d	55 ^d	N ^e
8	6c (8 equiv.) PBS buffer, pH 7.5, 4 °C , 5 min.	0	0	N

^a: reduced trastuzumab was utilized; ^b: as determined on non-reduced trastuzumab; ^c: see native mass spectrum S3; ^d: see native mass spectrum S7; ^e: see native mass spectrum S6

Native mass spectrometry analyses and spectra

Native mass spectra **S1**, **S2** and **S3** show the side reactivity (i.e. imperfect cysteine selectivity) observed when *non-reduced* trastuzumab (**16**) was reacted with the three TMS-EBX reagents – **6b** ($p\text{NO}_2$), **6a** ($p\text{H}$), and **6c** ($p\text{F}$), respectively – under the conditions optimized for peptides conjugation. While conjugation sites and mechanism are unknown, m/z values seem to suggest that this side reactivity is caused by the addition of a desilylated EBX reagent (see Figure S2).

Figure S2. Side-reactivity on non-reduced trastuzumab

Spectra **S4-S7** were obtained utilizing conditions for which no side reactivity was observed on non-reduced trastuzumab (see spectra **S4** and **S6**) with TMS-EBX reagents **6a** ($p\text{H}$) and **6c** ($p\text{F}$), respectively. The matching spectra obtained on *reduced* trastuzumab are also given (see spectra **S5** and **S7**), and correspond to conditions detailed in Table S1, entry 9, and in Table S2, entry 7, respectively.

Note: “DoC” has been shortened to “D” in all spectra.

Reaction conditions: non-reduced trastuzumab, JW-RT-01 **6b** (2 equiv.), PB buffer, pH 8.2, 37 °C, 5 min.

Native mass spectrum S1: charge states distribution of non-denaturing mass spectrum obtained under the conditions described hereinabove. The black box is a zoom on the 24+ charge state, showing side reactivity (D0 + 317 Da and D0 + 634 Da) and (de-)glycosylated adducts – the presence of hexose (+162 Da) is represented by a double asterisk, while the loss of fucose (-146 Da) is represented by a single asterisk.

Reaction conditions: non-reduced trastuzumab, TMS-EBX **6a** (2 equiv.), PB buffer, pH 8.2, 37 °C, 5 min.

Native mass spectrum S2: charge states distribution of non-denaturing mass spectrum obtained under the conditions described hereinabove. The black box is a zoom on the 24+ charge state, showing side reactivity (D0 + 271 Da and D0 + 541 Da) and (de-)glycosylated adducts – the presence of hexose (+162 Da) is represented by a double asterisk, while the loss of fucose (-146 Da) is represented by a single asterisk.

Reaction conditions: non-reduced trastuzumab, JW-RT-02 **6c** (2 equiv.), PB buffer, pH 8.2, 37 °C, 5 min.

Native mass spectrum S3: charge states distribution of non-denaturing mass spectrum obtained under the conditions described hereinabove. The black box is a zoom on the 24+ charge state, showing side reactivity (D0 + 287 Da and D0 + 580 Da) and (de-)glycosylated adducts – the presence of hexose (+162 Da) is represented by a double asterisk, while the loss of fucose (-146 Da) is represented by a single asterisk.

Reaction conditions: non-reduced trastuzumab, TMS-EBX **6a** (8 equiv.), PBS buffer, pH 7.5, 25 °C, 2 min.

Native mass spectrum S4: charge states distribution of non-denaturing mass spectrum obtained under the conditions described hereinabove. The black box is a zoom on the 24+ charge state, showing the absence of side reactivity.

Reaction conditions: reduced trastuzumab, TMS-EBX **6a** (8 equiv.), PBS buffer, pH 7.5, 25 °C, 2 min.

Native mass spectrum S5: charge states distribution of non-denaturing mass spectrum obtained under the conditions described hereinabove. The black box is a zoom on the 24+ charge state, showing conjugation up to D4.

Reaction conditions: non-reduced trastuzumab, JW-RT-02 **6c** (8 equiv.), PBS buffer, pH 7.5, 25 °C, 2 min.

Native mass spectrum S6: charge states distribution of non-denaturing mass spectrum obtained under the conditions described hereinabove. The black box is a zoom on the 24+ charge state, showing the absence of side reactivity.

Reaction conditions: reduced trastuzumab, JW-RT-02 **6c** (8 equiv.), PBS buffer, pH 7.5, 25 °C, 2 min.

Native mass spectrum S7: charge states distribution of non-denaturing mass spectrum obtained under the conditions described hereinabove. The black box is a zoom on the 24+ charge state, showing conjugation up to D4.

3. Proteomic experiments

Gel-based competition assay

HeLa lysate (50 µg in 25 µL) were treated with indicated compound (1 µL of 25X stock in DMSO) for 1 hour at 37 °C. Click chemistry was initiated by the addition of TAMRA azide (Sigma-Aldrich, 50 µM, 25x stock in DMSO), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Alfa Aesar, 1 mM, fresh 50x stock in water), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA, Sigma-Aldrich, 100 µM, 16x stock in DMSO:tBuOH 1:4), and copper(II) sulfate (1 mM, 50x stock in water) to the lysate and incubated in the dark for one hour at r.t. SDS-PAGE reducing loading buffer (4x) was added, and proteins were separated using a 11% SDS-PAGE gel. Gels were visualized using a Sapphire Biomolecular Imager (Azure Biosystems). Gels were stained with Coomassie Brilliant Blue dye for loading control.

Iodoacetamide competition assay

HeLa lysate (50 µg in 25 µL) were treated with iodoacetamide **33** (1 µL of 25X stock in DMSO) or 1 µL DMSO for 1 hour at 37 °C. The indicated amount of **6c** was added (1 µL of 25X stock in DMSO) and incubated for 1 hour at 37 °C. CuAAC click chemistry, SDS-PAGE, and in-gel fluorescence scanning were performed as described earlier.

Cell culture and lysate preparation

HeLa cells were maintained in DMEM media. All media were supplemented with 10% fetal calf serum (FCS), non-essential amino acids and penicillin/streptomycin. Cells were grown at 37 °C under 5% CO₂ atmosphere. Cells were allowed to grow to confluence and were harvested by scraping, centrifuged at 1'500 x g for five min. at 4 °C and resuspended in PBS. Cells were lysed by sonication to form cell lysates and protein concentration was determined using the Bradford assay.

In situ treatment of HeLa cells

HeLa cells (0.5e6 cells/mL) were seeded in a 6-well plate. During the following day, the media was changed for media containing indicated compound (1000x dilution of stock in DMSO). Cells were treated for 1.5 hours before washing (2x PBS), scraping, and collecting the cells (1.5k x g for 5 min at 4 °C). The cells were then sonicated. Protein concentration was measured via the Bradford assay. Samples (50 µg in 25 µL) were then prepared and subjected to CuAAC click chemistry, SDS-PAGE, and in-gel fluorescence scanning as described earlier.

Evaluation of compound cytotoxicity in HeLa cells

HeLa cells (20e3 cells/well) were seeded in a 96-well plate. During the following day, the media was changed for media containing indicated compound (1000x dilution of stock in DMSO) except for IAA (final 30 mM) which was dissolved directly in media. Cells were treated for 1.5 hours before imaging the cells using (Olympus IX51 inverted microscope). Media was then changed containing final 5% WST1 (Sigma Aldrich), incubated at 37 °C for 1 hour, and then absorbance at 420 nm and 630 nm was acquired using a BioTek Synergy H1 microplate reader.

Capture-and-release experiment for mass spectrometry

HeLa lysate (2 mg/mL, 5 mL) was treated with 10 µM **6d** (100x stock in DMSO) or DMSO for one hour at r.t. Photocleavable (PC) biotin azide **17** (Click Chemistry Tools, 60 µM, 50x stock

in DMSO), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50x fresh stock in water), tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (100 μ M, 16x stock in DMSO:tBuOH 1:4), and copper(II) sulfate (1 mM, 50x stock in water) were added to the proteome and left to react for one hour at r.t. Protein was precipitated by adding MeOH (4 vol.), CHCl₃ (1 vol.) and water (3 vol.) to the reaction mixture and the turbid mixture was centrifuged for 10 minutes at 20'000 x g at 4 °C yielding a protein layer between the aqueous and organic layers. The protein layer was isolated, dried and solubilized in 2 % SDS in PBS via sonication. Tube was centrifuged at 4'700 x g for five min. and soluble fraction was transferred to a new tube. PBS was added to give a final SDS concentration of 0.2 %. 500 μ L of streptavidin agarose beads were added and the mixture was rotated overnight at r.t. Beads were washed with 1 % SDS in PBS (2x 10 mL), PBS (3x 10 mL), and water (3x 10 mL). Beads were resuspended in 6 M urea in PBS (500 μ L), reduced with 10 mM neutralized TCEP (20x fresh stock in water) for 30 min. at r.t., and alkylated with 25 mM iodoacetamide (400 mM fresh stock in water) for 30 min. at r.t. in the dark. Beads were pelleted by centrifugation (1'400 x g, two min.) and resuspended in 300 μ L of 2 M urea in 50 mM NH₄HCO₃, 1 mM CaCl₂ (100x stock in water) and trypsin (Thermo Scientific, 2 μ L of 0.5 μ g/ μ L). The digestion was performed for 6 hours at 37 °C. Beads were washed with 1 % SDS in PBS (1x 10 mL), PBS (3x 10 mL), and water (3x 10 mL). Beads were resuspended in 2 mL PBS and photocleavage was performed with irradiation at 365 nm in a CL-1000 UV crosslinker (UVP) for one hour at 4 °C. Beads were centrifuged and supernatant was transferred to a new tube. Beads were resuspended in 1 mL PBS and incubated for one hour at 37 °C with shaking, centrifuged and supernatants were combined. Beads were resuspended in 1 mL PBS with 1 M NaCl and incubated overnight with shaking, centrifuged and supernatants were combined. Peptides were desalted over a self-packed C18 spin column and dried. Samples were analyzed by LC-MS/MS (see below) and the MS data was processed with Proteome Discoverer (see below).

LC-MS/MS analysis

Peptides were resuspended in water with 0.1 % formic acid (FA) and analyzed using EASY-nLC 1200 nano-UHPLC coupled to Q Exactive HF-X Quadrupole-Orbitrap mass spectrometer (Thermo Scientific). The chromatography column consisted of a 40 cm long, 75 μ m i.d. microcapillary capped by a 5 μ m tip and packed with ReproSil-Pur 120 C18-AQ 2.4 μ m beads (Dr. Maisch GmbH). LC solvents were 0.1 % FA in H₂O (Buffer A) and 0.1 % FA in 90 % MeCN: 10 % H₂O (Buffer B). Peptides were eluted into the mass spectrometer at a flow rate of 300 nL/min. over a 240 min. linear gradient (5-35 % Buffer B) at 65 °C. Data was acquired in data-dependent mode (top-20, NCE 28, R = 7'500) after full MS scan (R = 60'000, m/z 400-1'300). Dynamic exclusion was set to 10 s, peptide match to prefer and isotope exclusion was enabled.

Proteome Discoverer analysis

The MS data was processed with Proteome Discoverer (V2.1.1.21) using the Sequest HT algorithm⁷ and searched against the human proteome (Uniprot). Precursor mass tolerance

⁷ J. K. Eng, A. L. McCormack, J. R. Yates, *J. Am. Soc. Mass Spectrom.* **1994**, 5, 976-989.

was set to 10 ppm and 0.02 Da was used for the fragment mass tolerance. Minimum peptide length was set to 6 amino acids, PEP-value to $\leq 1\%$ and protein FDR to high. Methionine oxidation, carbamidomethylation of cysteine and **6d** PC cleaved adducts (+124.0749 Da) on cysteine, arginine, aspartic acid, glutamic acid, histidine, lysine, serine, threonine, tyrosine were searched as a dynamic modification. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (1) partner repository with the data set identifier PXD018165.⁸

Figure S3. TMS-EBXs are non-toxic in living cells. A) Images of HeLa cells following treatment with indicated compound for 1.5 h. 30 mM IAA was used as positive control. B) Quantification of HeLa cell viability following compound treatment (1.5 h) using WST1 absorbance assay.

⁸ Perez-Riverol et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res. 2019 Jan 8;47(D1):D442-D450.