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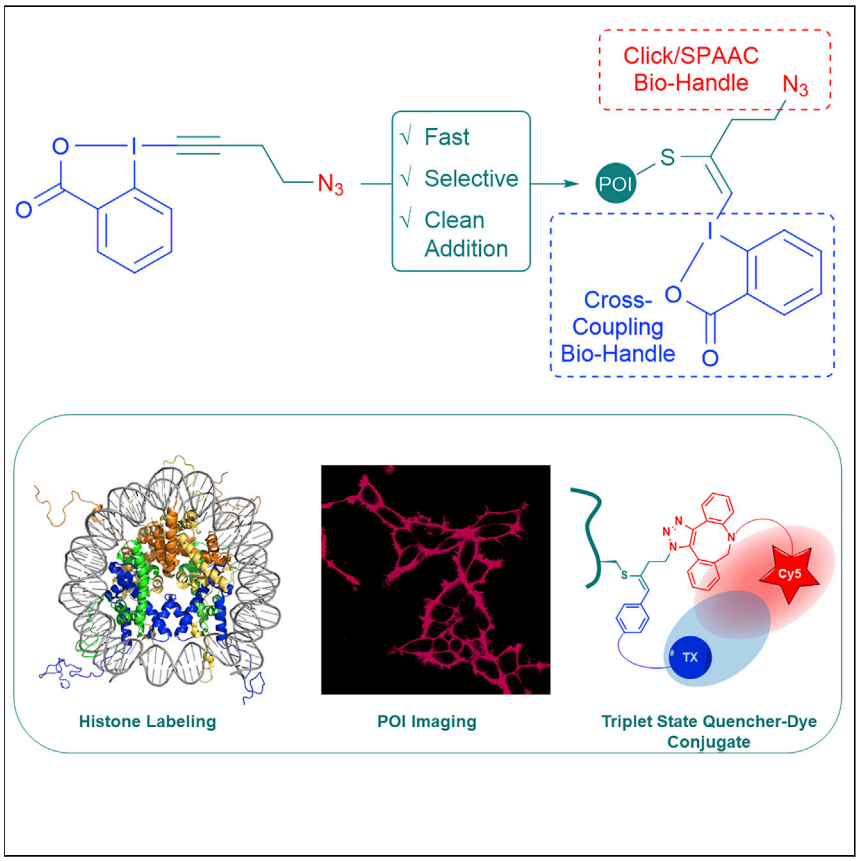
"Doubly Orthogonal" Labeling of Peptides and Proteins



Chemical biology develops molecular tools for studying biological processes, setting the basis for new diagnostics and therapeutics, and relies heavily on the ability to modify selectively biomolecules. In our work, we introduce hypervalent iodine bonds into peptides and proteins, via functionalization of cysteine, by using unique cyclic reagents developed in our group. The hypervalent bond can then be selectively modified in the presence of both natural and synthetic functional groups, opening new opportunities for applications in chemical biology.

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HIGHLIGHTS

Selective cysteine bioconjugation under physiological conditions

Formation of hypervalent iodine-bound peptides and proteins

Stable bioconjugates from simple amino acids to histones

Doubly orthogonal functionalization via azide-alkyne cycloaddition and cross-coupling

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"Doubly Orthogonal" Labeling of Peptides and Proteins

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SUMMARY

Herein, we report a cysteine bioconjugation methodology for the introduction of hypervalent iodine compounds onto biomolecules. Ethynylbenziodoxolones (EBXs) engage thiols in small organic molecules and cysteine-containing peptides and proteins in a fast and selective addition onto the alkynyl triple bond, resulting in stable vinylbenziodoxolone hypervalent iodine conjugates. The conjugation occurs at room temperature in an open flask under physiological conditions. The use of an azide-bearing EBX reagent enables a "doubly orthogonal" functionalization of the bioconjugate via strain-release-driven cycloaddition and Suzuki-Miyaura cross-coupling of the vinyl hypervalent iodine bond. We successfully applied the methodology on relevant and complex biomolecules, such as histone proteins. Through single-molecule experiments, we illustrated the potential of this doubly reactive bioconjugate by introducing a triplet-state quencher close to a fluorophore, which extended its lifetime by suppressing photobleaching. This work is therefore expected to find broad applications for peptide and protein functionalization.

INTRODUCTION

Efficient, selective, and flexible labeling techniques are essential for the study and manipulation of proteins.[1] Particularly in the last decade, the importance of site-selective modification has been highlighted in the quest for antibody-drug conjugates[2–6] and further emphasized by the development of stapled peptides for therapeutic interventions.[7–11] Novel bioconjugation techniques are still in high demand, but their development is complicated by the need for fast kinetics, high efficiency, and excellent selectivity under mild conditions. Nevertheless, several labeling methods exploiting both natural and unnatural amino acids have emerged.[12–17]

Because of its key role in the structure and catalytic activity of proteins, cysteine has been one of the most studied natural amino acids in chemical biology. Its low abundance in proteins and its intrinsic high nucleophilicity make it an ideal target for chemoselective modification techniques often based on site-directed mutagenesis (Scheme 1).[18–21] Accordingly, significant efforts have been made on the development of reagents enabling cysteine labeling with high selectivity and efficiency. Among those, alkylating halogenoalkanes, such as iodoacetamide, and maleimide derivatives are the most frequently employed. Although their reactivity has been extensively studied, significant limitations persist. For example, a lack of chemoselectivity is observed in the presence of nucleophilic amino acids, such as lysine, histidine, tyrosine, or tryptophan.[12,14,17] In the case of the widely used maleimides, a critical disadvantage is the well-known instability of their conjugates toward hydrolysis and external organosulfur attacks. This is mainly due to the reversibility of the

The Bigger Picture

Understanding the molecular basis of life is essential in the search for new medicines. Chemical biology develops molecular tools for studying biological processes, setting the basis for new diagnostics and therapeutics, and relies heavily on the ability to selectively modify biomolecules. Two approaches have been especially fruitful: (1) selective modification of natural biomolecules and (2) selective reaction between non-natural functionalities in the presence of biomolecules (the so-called orthogonal bioconjugation).

In our work, we contribute to both by transferring highly reactive hypervalent iodine reagents to cysteine residues in proteins and peptides. The obtained bioconjugates retain the reactive hypervalent bonds, which can be selectively functionalized via a metal-mediated reaction. Combined with a traditional azide tag, our approach allows a doubly orthogonal functionalization of biomolecules and is hence expected to be highly useful in chemical biology.

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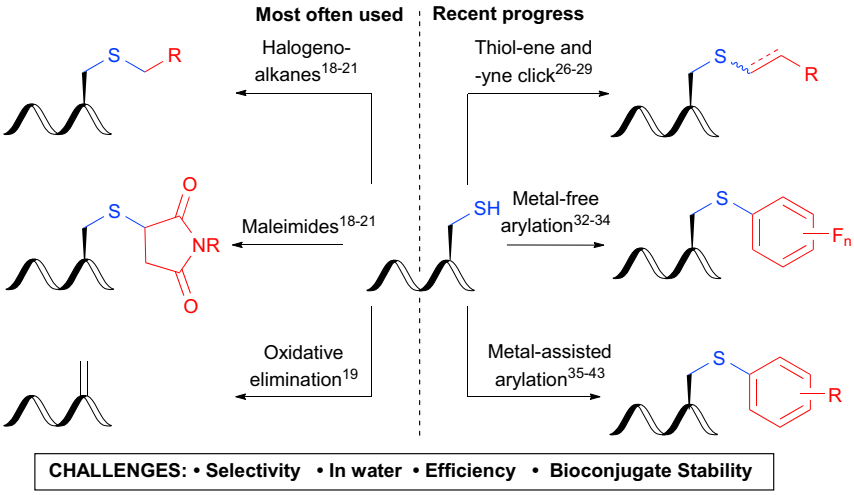

Scheme 1. State of the Art and Challenges in Cysteine Functionalization

thiol-maleimide reaction, which leads to loss of label.[22–25] Therefore, labeling methods leading to more stable adducts, including thiol-ene and -yne reactions,[26–29] oxidative elimination of cysteine to dehydroalanine followed by Michael addition,[19,30,31] metal-free arylations,[32–34] or metal-assisted functionalizations,[35–43] have been developed. Nevertheless, many of these methods suffer from low or uncontrolled reactivity, low chemoselectivity, or the need for organic co-solvents. Better performances can be found with the use of metal catalysts and reagents;[35–43] however, solubility and biocompatibility remain predominant challenges of metal-based methodologies.[44] Finally, many of these methods are based on chemical compounds requiring challenging preparations, careful storage, and/or stringent use of inert atmosphere.

In this context, hypervalent iodine reagents are interesting because they combine high reactivity with sufficient stability and low toxicity.[45] Nevertheless, applications for peptide and protein functionalization remain rare, probably as a result of concurring oxidation side reactions.[46–53] In 2018, Gaunt, Suero, and co-workers reported an elegant methionine functionalization using a hypervalent iodine reagent.[53] Our group previously achieved a highly efficient and chemoselective alkynylation of thiols by using hypervalent iodine-based ethynylbenziodoxolone[54–60] (EBX) reagents (**1**) in organic solvents, which was also successful for amino acids and dipeptides (Scheme 2A1).[61,62] In collaboration with the Adibekian group, we successfully applied JW-RF-010 (**1a**), an azide-bearing EBX, in the labeling of native cysteines *in vitro* and *in vivo* to afford alkynes as major products (Scheme 2A2).[63] **1a** displayed an exceptional stability with less than 3% decomposition after 14 days in D_2O, as well as an exceptional chemoselectivity, outperforming iodoacetamide, the gold standard in cysteine targeting. However, only hyper-reactive cysteines were efficiently functionalized in aqueous media, and the method is therefore not general. Common to all reported approaches for biomolecule functionalization with hypervalent iodine reagents is using the inherent reactivity of the hypervalent bond to perform the bioconjugation step.

In contrast, we present herein a highly efficient, fast, chemoselective, and clean labeling of cysteine-containing peptides and proteins to give stable adducts of EBX and thiols without cleaving the hypervalent bond (Scheme 2B). This methodology

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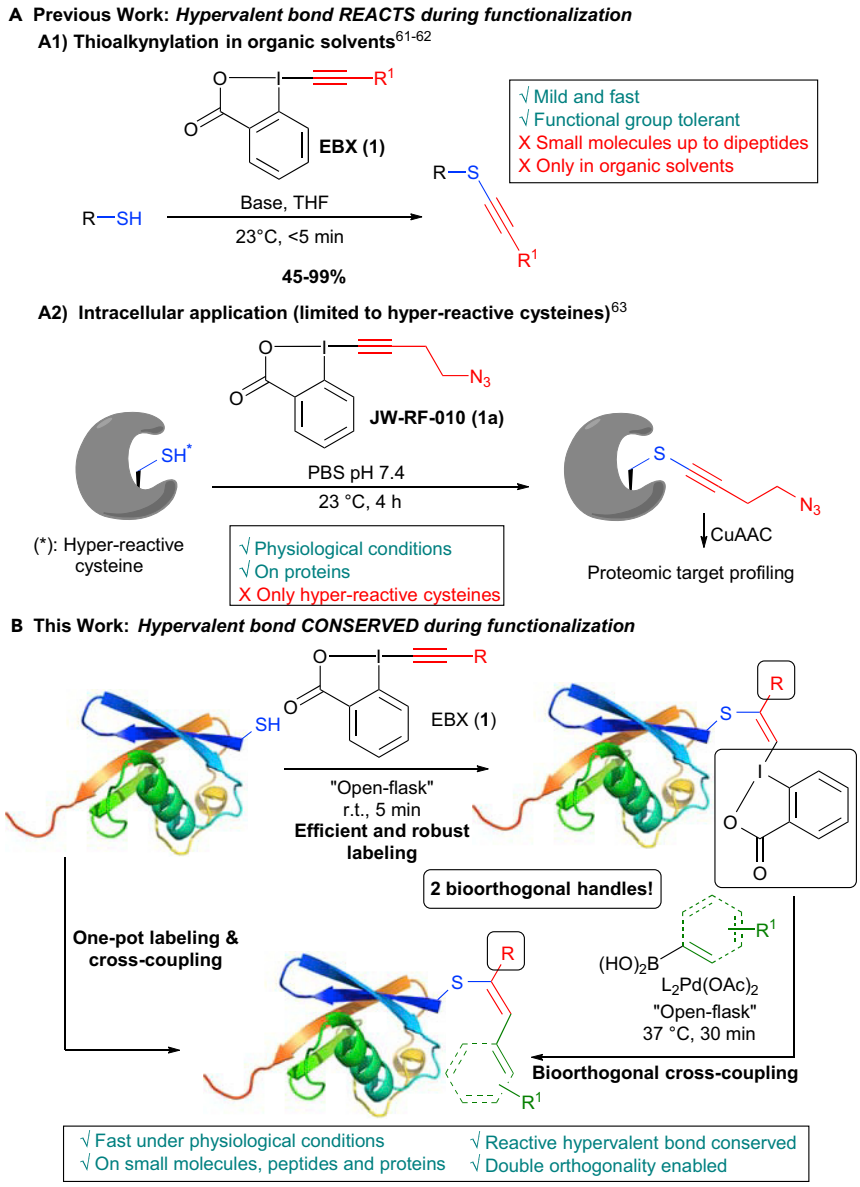
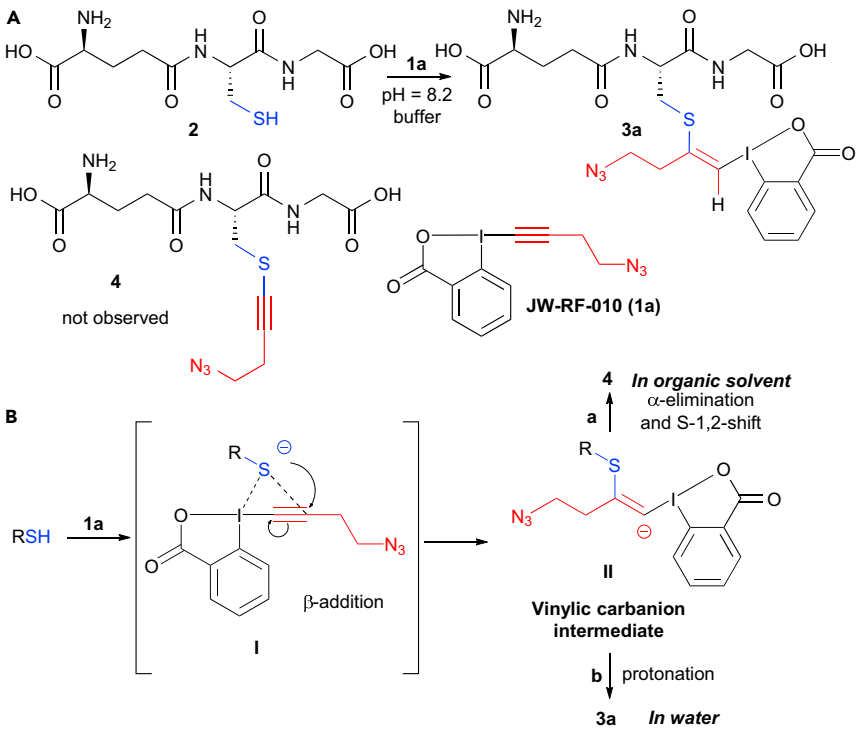


Scheme 2. Reaction of Cysteine with EBX Reagents

Previous work on thiol functionalization with reaction of the hypervalent bond (A) and approach conserving the hypervalent iodine bond presented in this work, enabling doubly orthogonal functionalization (B).

can be applied to all cysteine- and other thiol-bearing compounds, including modified ubiquitin and histone proteins. The peptide- and protein-bound vinyl benziodoxolone reagents are stable, yet their inherent reactivity[59,64–66] opens the door for bioconjugations orthogonal to natural functional groups existing in biomolecules. As a proof of concept, we report a palladium-catalyzed Suzuki-Miyaura cross-coupling reaction[67–71] selective at the vinyl hypervalent iodine bond (Scheme 2B).

Under physiological conditions, this efficient, robust, and fast methodology allows swift peptide and protein modifications without the need for incorporation of



Scheme 3. Reaction of EBX 1a with Glutathione (2)
Formation of vinylbenziodoxolone **3a** (A) and speculative reaction mechanism (B).

unnatural amino acids. In addition, we combined this powerful methodology with metal-free strain-promoted alkyne-azide cycloaddition (SPAAC),[72–74] which allowed functionalization of the adduct without cleaving the hypervalent bond, leading to "doubly orthogonal" modifications. We demonstrated this method's potential by introducing both a fluorophore and a triplet-state quencher (TSQ) on neuropeptide substance P, enabling single-molecule fluorescence experiments with increased sensitivity.

RESULTS AND DISCUSSION

Discovery and Optimization of the Hypervalent Iodine Transfer Reaction

Our study started with the functionalization of the thiol-containing glutathione (GSH, **2**) as a model substrate. **2** is a naturally occurring tripeptide that plays an essential role in primary metabolism as a disulfide-bond reductant and is present in high concentrations in cells. It was therefore surprising that alkynylation of **2** was not observed in cell lysate or living cells.[63] At that time, this was attributed to the lower acidity of **2** than of hyper-reactive cysteines. When **2** was treated with equimolar amounts of **1a** in a more basic buffer (10 mM Tris [pH 8.2]), vinylbenziodoxolone (VBX) **3a** was obtained instead of the expected alkynyl sulfide **4** (Scheme 3A). Our previous computational work on the mechanism of the thio-alkynylation reaction demonstrated that concerted β addition of sulfide via transition state **I** is favored for EBX reagents bearing an alkyl substituent (Scheme 3B).[75] With fully deprotonated sulfides in organic solvents, the formed vinylic carbanion intermediate **II** undergoes rapid α elimination and 1,2-sulfur shift to give alkynyl sulfides (Scheme 3B, route **a**). However, under the new conditions in water solution, direct protonation of **II** is favored to give VBX **3a** (Scheme 3B, route **b**).

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Indeed, we recently demonstrated that nitrogen and phenol nucleophiles can also be added to EBX reagents to give VBX compounds, and computational studies showed a very low barrier for protonation.[76] Solvent polarity also plays a key role, and in the case of thiol nucleophiles, increasing amounts of alkynylation were observed when DMSO was added to the reaction mixture both with a protected amino acid and with a tetrapeptide (see Figures S4 and S5). Mixtures of alkynes and VBXs were observed when water was added to organic solvents. Although our previous work on cell lysates and living cells were performed in aqueous buffer, the targeted cysteines were in protein active sites, whose properties are very different from those of the bulk solution. The lower dielectricity constant observed inside proteins[77] indicates that the local polarity is closer to that of organic solvents. 2D NMR spectroscopic studies established the *Z* configuration of the olefin, as well as the β addition regioselectivity, in agreement with our previous density functional theory calculations.[75] The developed transformation corresponds to the occurrence of a formal *trans*-addition of the thiol on the triple bond of the EBX reagent with perfect regio- and stereoselectivity, which is difficult to achieve for standard thiol-yne reactions.[26–28] Furthermore, the adduct is obtained without the need for further reagents, such as radical initiators, and without any byproduct formation.

To evaluate the potential of VBX for applications in chemical biology, it was first important to study the stability of adduct **3a**. No degradation of **3a** was observed up to 3 weeks at room temperature when it was kept as a solution in DMSO-d_6. Furthermore, **3a** exhibited a surprising resistance to high temperatures with no detectable degradation when being heated up to 100°C in pure water. Concerning pH stability, **3a** remained intact in an acetic acid buffer (10 mM [pH 4.0]) over 3 days. Only a slight degradation was observed in a basic CAPS buffer (10 mM [pH 11.0], 86% of remaining product after 3 days). Interestingly, no degradation, further addition, or exchange reaction was observed in the presence of 15 equiv of an external thiol nucleophile (30 mM tiopronin, 6× higher than the standard glutathione concentration in a cell) after 8 days. Finally, whereas the presence of a further 10 equiv of **1a** resulted in 14% degradation after 4 days, 5 equiv of **1a** did not lead to any decomposition.

Once the high stability of VBX **3a** had been confirmed, we started to evaluate the robustness of our labeling method (for a complete list of screened conditions, see Supplemental Experimental Procedures Section 6). The reaction is user friendly and easy to set up. **2** was simply dissolved in non-degassed Tris buffer (10 mM [pH 8.2]), and then **1a** was added from a DMSO stock solution. Then, the reaction was vortexed for a few seconds or homogenized with a simple pipette mixing and left to stand on the bench for 20 min. Unshaken and at room temperature, the reaction furnished a remarkable 81% yield of the desired product without the need for oxygen exclusion (Table 1, entry 1). 5 min of incubation already afforded the product in 55% yield (entry 2), and an extended labeling time of 60 min increased the yield to 87% (entry 3). The only side reactivity observed was oxidation of **2** into the dimeric disulfide. Interestingly, the absence of DMSO did not yield any significant loss of efficiency (entry 4), which could prove essential in the labeling of organic solvent-sensitive proteins. The reaction exhibited high tolerance toward buffer molarity (entry 5) and types of buffer (entries 6 and 7). The pH was found to be a crucial parameter for the reaction. At physiological pH (7.4), labeling was slowed down but still efficient (74% after 1 h, entry 8). At pH 6.5, only 29% of **3a** was obtained after 1 h (entry 9). Nevertheless, this low reactivity could be easily overcome with the use of 10 equiv of **1a** (entry 10). To our delight, the labeling remained efficient at either lower or higher temperatures (entries 11 and 12). Although diluting the reaction mixture

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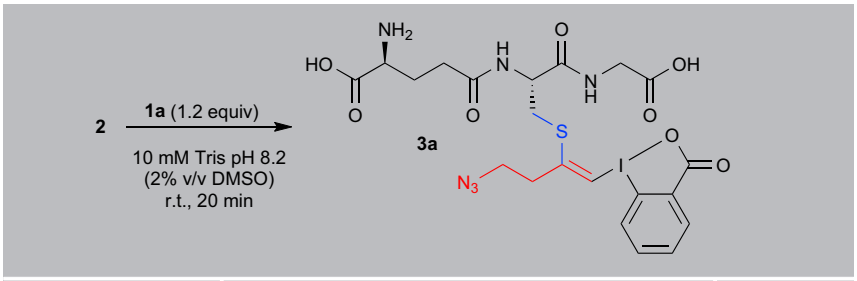
Table 1. Evaluation of Reaction Conditions for the Labeling of Glutathione (2)



Entry	Variations from the Above Conditions	Yield
1	none	81%
2	5 min reaction	55%
3	1 h reaction	87%
4	no DMSO	78% (86%)
5	100 mM Tris (pH 8.2)	78% (97%)
6	10 mM HEPES (pH 8.2)	77% (85%)
7	10 mM PBS (pH 8.2)	75% (91%)
8	10 mM PBS (pH 7.4)	54% (74%)
9	10 mM PBS (pH 6.5)	9% (29%)
10[a]	10 mM PBS (pH 6.5)	57% (88%)
11	4°C reaction temperature	67% (86%)
12	37°C reaction temperature	78% (87%)
13	200 μM reaction molarity	45% (76%)
14[b]	200 μM reaction molarity	76% (93%)
15[c]	3 equiv of **1a**	98%

Labeling conditions: 1.0 μmol glutathione (**2**) and 1.2 equiv of JW-RF-010 (**1a**) in 0.5 mL of non-degassed Tris buffer (pH 8.2; 2% v/v DMSO) at room temperature for 20 min. Calibrated HPLC yield is based on absorbance at 214 nm (see Figure S1). The yields in parentheses correspond to the yields after 1 h of reaction. For complete robustness studies, see Supplemental Experimental Procedures Section 6.

[a]10 equiv of **1a** was used.

[b]3 equiv of **1a** was used.

[c]The reaction was analyzed after 5 min.

from 2 mM to 200 μM increased the reaction time, it barely affected the labeling efficiency (entry 13). Furthermore, increasing the stoichiometry of **1a** to 3 equiv allowed for efficient labeling in 20 min and almost quantitative transformation in 1 h (entry 14) (for the complete list of screened conditions, see Table S1). Under these conditions, reactivity could be observed down to 2 μM reaction molarity (see Figures S2 and S3). A competition experiment between **1a** and iodoacetamide at pH 8.2 resulted in a 2:3 ratio of labeled products, showing that the two compounds reacted at similar rates. Finally, the use of 3 equiv of **1a** afforded quantitative functionalization of **2** within 5 min at 2 mM (entry 15). We selected these conditions to study the scope of the reaction.

Scope of the Bioconjugation Reaction

We therefore prepared a library of hypervalent iodine reagents, resulting in compounds bearing azide, alkyne, alcohol, and various other functional groups.[62,78] Most of these EBX compounds can be prepared in one synthetic step from commercial reagents and are stable toward air, light, and water. They can be stored on the bench under ambient atmosphere for weeks or at 4°C under nitrogen for years

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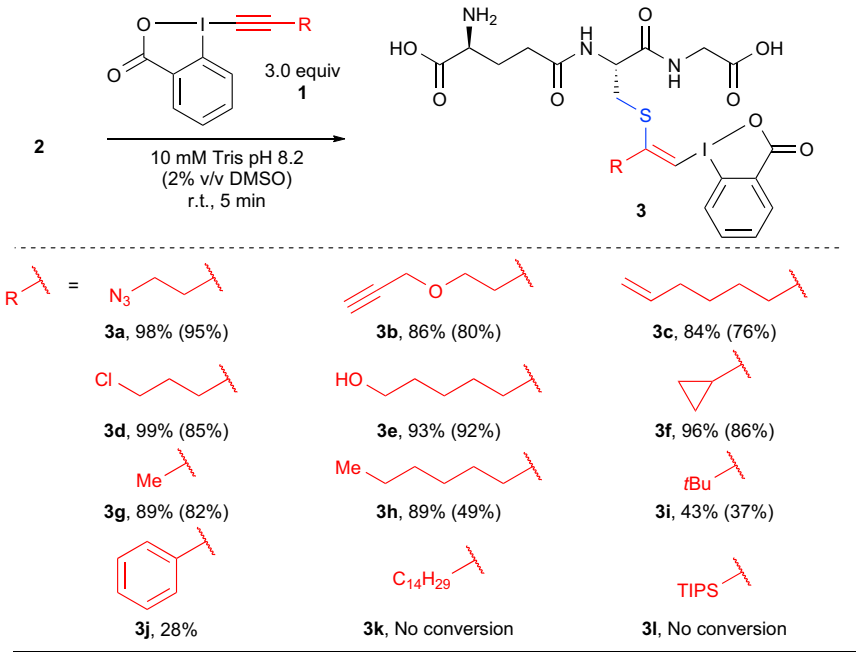


Scheme 4. Scope of Different EBX Reagents for Glutathione (2) Labeling

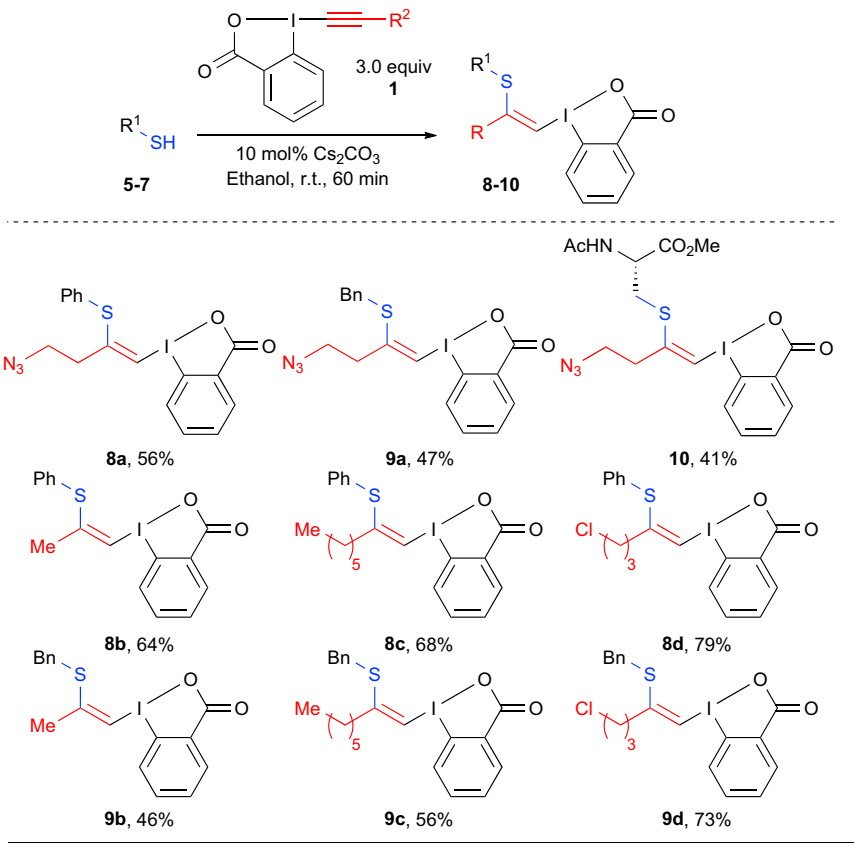
Reactions were carried out on a 1.00 μmol scale at a 2 mM concentration (see Supplemental Experimental Procedures Section 8). Yields were derived by comparison of the integration area of the product in its respective HPLC trace with that of the standard curve at 214 nm for **3a** (see Figure S1). Yields in parentheses represent those of the labeling reactions without DMSO as an organic co-solvent.

without exhibiting noticeable degradation. Our bioconjugation technique demonstrated a broad tolerance toward a large variety of functional groups (Scheme 4).

Indeed, azide (**3a**), terminal alkyne (**3b**) and alkene (**3c**), halogen (**3d**), alcohol (**3e**), and non-functionalized alkyl substituents (**3f–3h**) could be rapidly attached to **2** in excellent yields. To our delight, these reactions were also highly efficient without DMSO as an organic co-solvent. Because of their lipophilicity, solubility issues were observed with *t*Bu-EBX (**1i**), Ph-EBX (**1j**), $C_{14}H_{29}$-EBX (**1k**), and TIPS-EBX (**1l**), resulting in low yields or no conversion for products **3i–3l**.

The method could not be used with non-polar small organic molecules because of their low solubility in water. However, in such cases the reagents could be accessed in good yields under the conditions recently developed for N- and O-nucleophiles (catalytic cesium carbonate in ethanol; Scheme 5).[76] Phenyl and benzyl thiols (**5** and **6**) and protected cysteine (**7**) were added smoothly to reagent **1a** to give **8a**, **9a**, and **10**, respectively. X-ray analysis of reagents **8a**, **9a**, and **9b** confirmed the hypervalent structure, as well as the regio- and stereoselectivity of the thiol addition (see Data and Code Availability). The addition to other EBX reagents bearing a methyl group, a longer alkyl chain, or a chlorine functional group was also successful (products **8b–8d** and **9b–9d**).

We then investigated the scope of thiol reagents with **1a** under physiological conditions (Scheme 6). Starting with small molecules, we were pleased to observe quantitative labeling with tiopronin (**11**), 6-thioguanine (**12**), and thio-β-glucose (**13**) (Scheme 6A). We then extended the scope to the use of tetrapeptides containing



Scheme 5. Scope of Different EBX Reagents with Non-polar Small Organic Molecules in Ethanol
Reactions were carried out on a 0.50–11 mmol scale. Isolated yields after recrystallization or column chromatography are reported (see Supplemental Experimental Procedures Section 9a).

various natural amino acids (Scheme 6B). Cysteines in close proximity to phenylalanine (**14a**) or proline (**14b**) exhibited excellent reactivity. A more hindered environment around the cysteine residue with phenylalanine, valine, and isoleucine amino acids (**14c** and **14d**) did not show any detrimental effect on the labeling.

Whereas C-terminal cysteine was found to be more prone to oxidation, affording a lower efficiency (**14e**), N-terminal cysteine reacted efficiently (**14f**). Aspartic acid (**14 g**), asparagine (**14h**), and methionine (**14i**) were well tolerated. Only in the case of arginine (**14j**) was a lower yield obtained as a result of the formation of side products. High chemoselectivity was also observed in the presence of serine (**14k**), tyrosine (**14l**), and tryptophan (**14 m**). No side reactions occurred with the most nucleophilic amino acids histidine (**14n**) and lysine (**14o**). Finally, efficient labeling was still observed without DMSO for peptides **14b**, **14e**, **14h**, and **14l**.

These promising results on tetramers prompted us to apply our labeling technique to more complex biomolecules. After treatment with **1a**, human serum albumin Leu_{55}-His_{63} fragment (**15**) was converted to the corresponding hypervalent iodine bioconjugate **16** in 88% yield (Scheme 6C). We then decided to employ our method to modify cysteines in a protein context. As a model system, we chose the protein ubiquitin, which carries an N-terminal hexahistidine tag followed by a single cysteine residue (His6-Cys ubiquitin, **17**). To our delight, the treatment of **17** with only 2 equiv of **1a** under native conditions afforded a quantitative transformation into VBX **18** in

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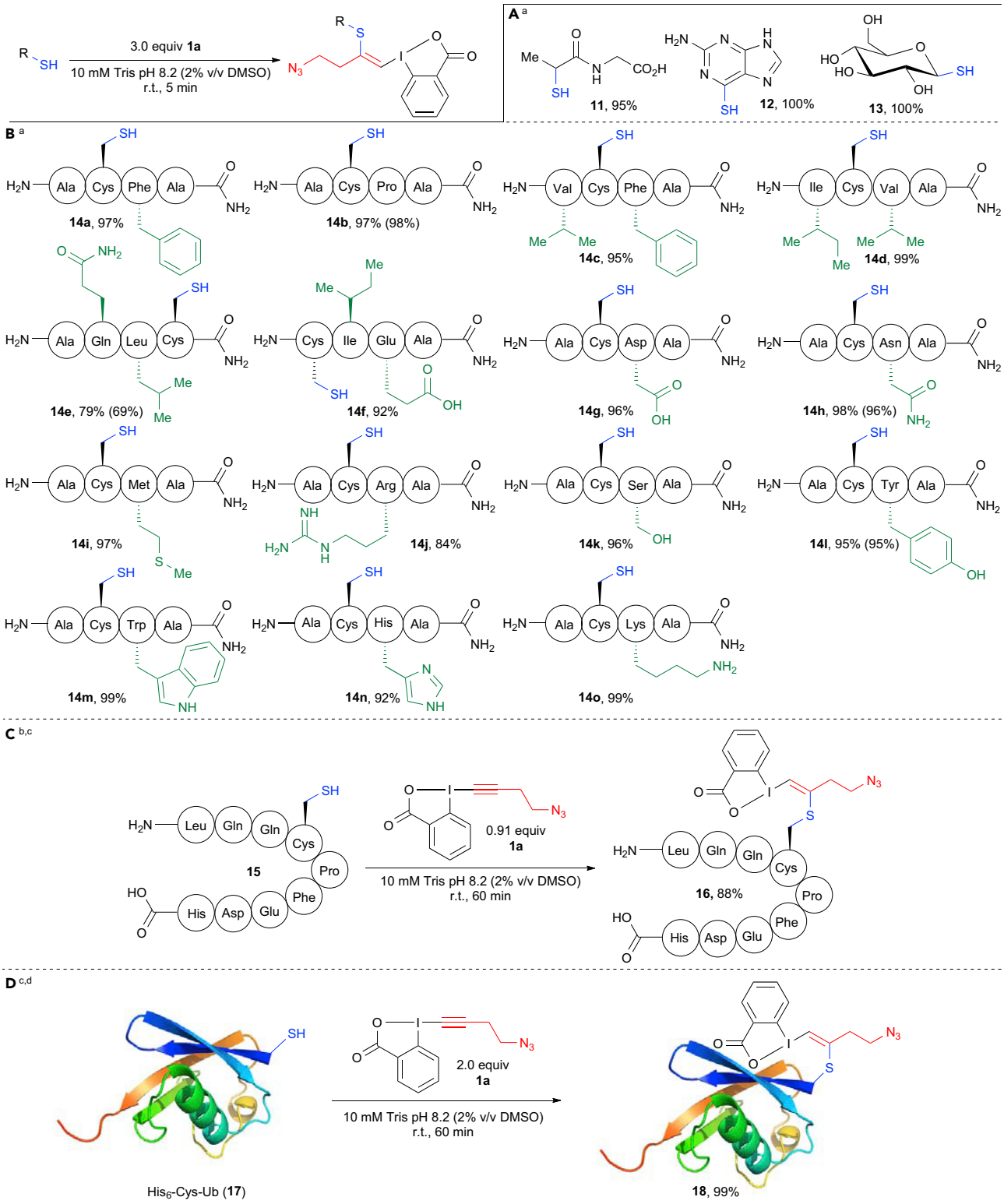

Scheme 6. Reaction with Peptides and Proteins

Scope of the functionalization of small molecules (A) and tetrapeptides (B) and applications on human serum albumin Leu_{55}-His_{63} sequence (**15**) (C) and His6-Cys-ubiquitin (**17**) (D).

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Scheme 6. *Continued*

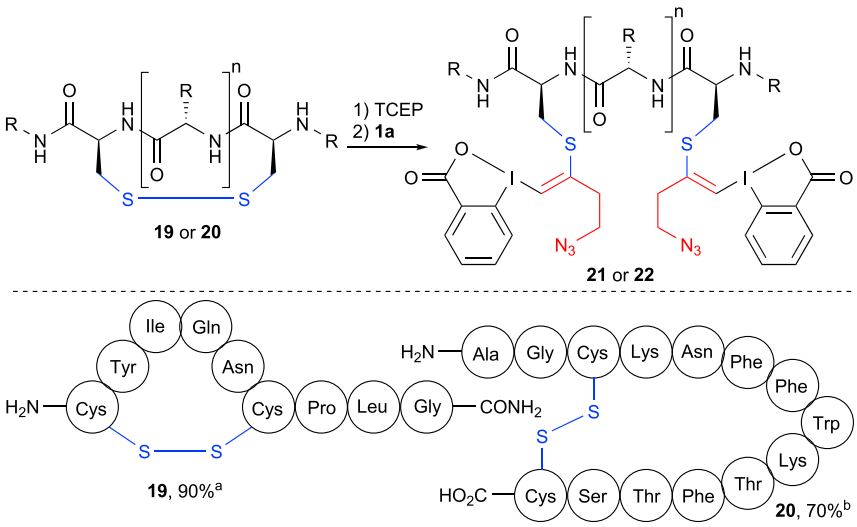
[a]Reactions were carried out on a 1.00 μmol scale at a 2 mM concentration. Yields were determined by relative integration based on HPLC-MS (see Supplemental Experimental Procedures Section 9b). Yields in parentheses represent the labeling reactions without DMSO.
[b]Reaction was carried out on a 1.30 μmol scale at 3 mM concentration. EBX (**1a**) was used substoichiometrically for ease of chromatogram analysis.
[c]Yields were determined by relative integration based on HPLC traces at 214 nm (see Supplemental Experimental Procedures Section 9b).
[d]Reaction was carried out on a 50.0 nmol scale at a 0.5 mM concentration.

less than 1 h (Scheme 6D). To the best of our knowledge, this constitutes the first example of a protein-bound hypervalent iodine reagent. This efficient conjugation was also compatible with a protein-denaturing buffer (see Supplemental Experimental Procedures Section 9e). Of note, side reactions due to arginine were not observed in ubiquitin, which contains five arginine residues. We thus conclude that such side reactions, as observed for **14j**, occur only when arginines are immediately next to the cysteine. Importantly, no reaction was observed with native (cysteine-free) ubiquitin (see Supplemental Experimental Procedures Section 9f), further demonstrating the cysteine specificity of the reaction in a protein context. Moreover, the correct modification site in ubiquitin was further confirmed by top-down mass spectrometry (see Supplemental Experimental Procedures Section 9e). It should be noted that because the reaction kinetics are dependent on the reagent concentrations, for larger proteins an increased concentration of EBX reagent might be required.

In nature, cysteine residues are known to form disulfide bonds, particularly in secreted, extracellular proteins. When these disulfides are targeted with cysteine labeling reagents, the standard protocol is an *in situ* disulfide cleavage with reducing reagents, such as tris(2-carboxyethyl)phosphine (TCEP). Therefore, a one-pot protocol allowing disulfide bond cleavage followed by reaction with EBX reagents would be highly useful for the labeling of disulfide-bound cysteines. As a proof of concept, we treated oxidized **2** with 1.5 equiv of TCEP followed by 6 equiv (3 equiv per reduced cysteine) of **1a**. To our delight, the product **3a** was obtained with 92% yield (see Supplemental Experimental Procedures Section 10a). Furthermore, even in the presence of 5 and 10 equiv of the reducing agent and no increase in the amount of **1a**, the product was still obtained in good yields (82% and 60%, respectively). Although a direct reaction between TCEP (reducing) and the EBX reagent (oxidizing) did take place, it was slower than the labeling reaction. Importantly, the hypervalent iodine conjugate **3a**, which still possesses reactive iodine and azide groups, did not show any degradation after 24 h in the presence of TCEP. With these exciting results in hand, we applied our one-pot reduction-labeling technique to larger peptides containing disulfide bridges. We were pleased to see excellent yields on challenging natural bioactive peptides, such as oxytocin (**19**) and somatostatin (**20**) (products **21** and **22**; Scheme 7) (see Supplemental Experimental Procedures Sections 10b and 10c).

Application for Fluorescent Protein Labeling

Next, we investigated the potential of the obtained bioconjugates for further functionalization. At first, we examined whether standard methods for azide modification could be used in the presence of the reactive hypervalent iodine center. In order to avoid the use of metal catalysts, we chose the well-known copper-free SPAAC.[72–74] In principle, two strategies could be followed: (1) labeling of the peptide or protein with **1a** followed by cycloaddition with a functionalized alkyne or (2) prior reaction of **1a** with the alkyne followed by reaction with the peptide or protein. The former appeared easier given that the reactivity of the hypervalent iodine of the VBX products is lower, whereas the latter is attractive for applications because it would allow preparation of multi-functionalized labeling reagents *in situ* starting from **1a** and commercially available alkynes. As a model system, we again chose **2** together



Scheme 7. Labeling of Sulfur-Bridge-Containing Peptides

Conditions: 1.5 equiv of TCEP·HCl and 100 mM Tris (pH 8.2) at room temperature for 60 min; then 6.0 equiv of **1a** and 100 mM Tris (pH 8.2; 2% v/v DMSO) at room temperature for 5 or 10 min. Yields were determined by relative integration based on HPLC traces at 214 nm (see Supplemental Experimental Procedures Sections 10b and 10c).

[a]Reaction was carried out on a 0.50 μmol scale.

[b]Reaction was carried out on a 0.10 μmol scale.

with broadly used dibenzocyclooctynes **23**[79] bearing a free acid (**23a**), a biotin (**23b**), or cyanine dyes (**23c** and **23d**) (Scheme 8A). Gratifyingly, both the cysteine addition followed by click reaction (**a**) and the click reaction followed by cysteine addition (**b**) approaches were successful with all cyclooctynes to give products **24a–24d**. This demonstrated that azide-cyclooctyne cycloaddition is fully orthogonal to hypervalent iodine reactivity. Both approaches of labeling also performed well on **15** and **17** to give **25** and **26** in quantitative yield (Scheme 8B).

To showcase the compatibility of our methodology with proteins more complex than ubiquitin and to determine whether the resulting conjugates are well tolerated, we generated fluorescently labeled nucleosomes. Nucleosomes consist of a central protein octamer formed from histones H2A, H2B, H3, and H4 and ~150 bp of DNA that is wrapped around the protein complex.[80] For labeling, we mutated glutamate 63 in H4 to cysteine (E63C) and expressed and purified the protein. We then assembled histone octamer complexes by using the remaining other human histone proteins (H2A, H2B, and H3C110A; see Supplemental Experimental Procedures Section 3d). The resulting protein complex **27** contained two cysteine residues, which efficiently reacted with **1a** within 60 min under aqueous conditions to yield **28** (Figure 1A). We then successfully labeled **28** with **23d** Cy5-DBCO via azide-cyclooctyne cycloaddition to afford product **29** (Figure 1A). SDS-PAGE analysis of product **29** revealed the high specificity of the labeling procedure given that only histone H4 exhibited a fluorescence signal (Figure 1B). The selectivity of the reaction was also corroborated by high-performance liquid chromatography (HPLC) and top-down tandem mass spectrometry (see Supplemental Experimental Procedures Section 9g).

To demonstrate that the protein functionality was not affected by the modification, we continued to reconstitute full nucleosomes, which will only form if the histone octamer structure is not disrupted. We thus combined **28** with a 170-bp-long segment

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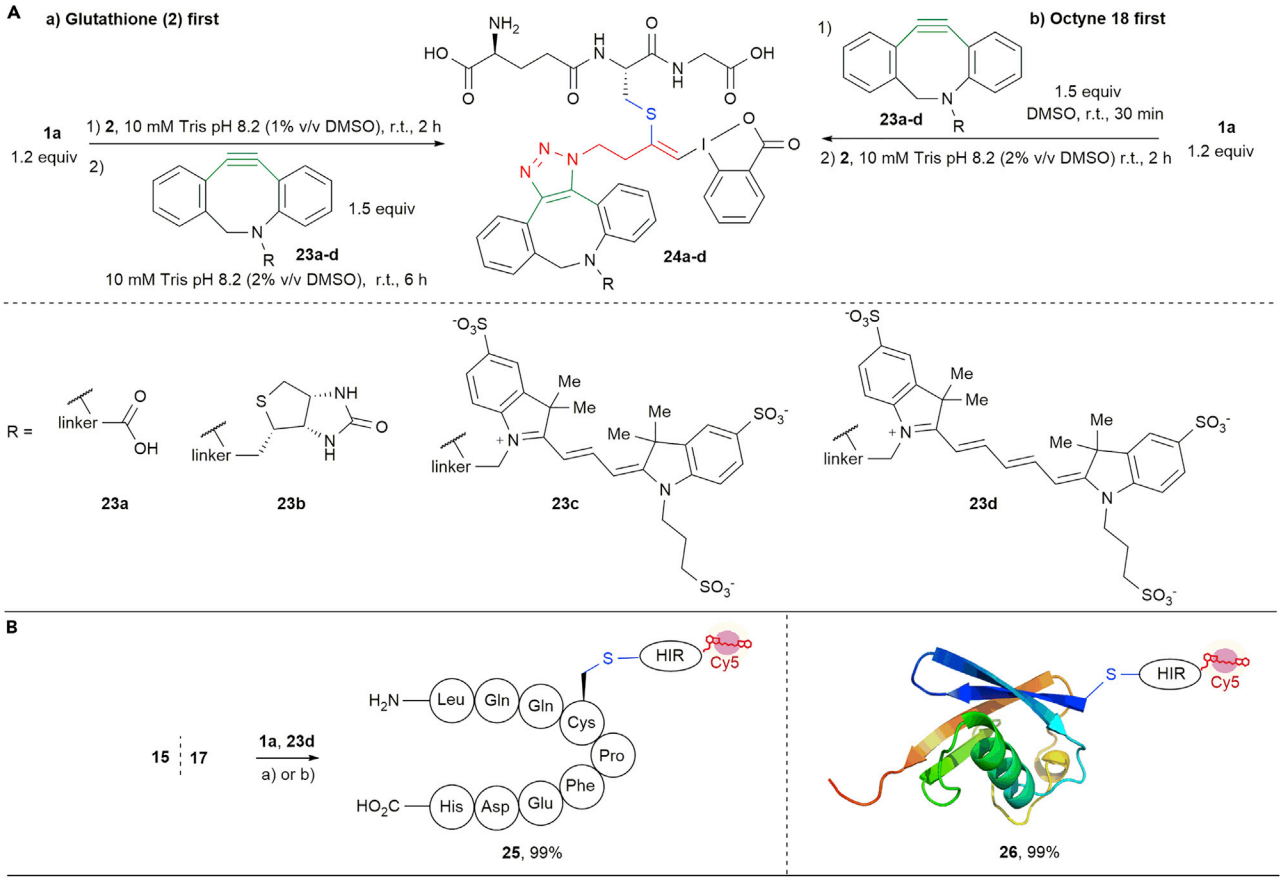

Scheme 8. Combination of Cysteine Labeling with Click Cycloaddition

(A) Labeling of glutathione (**2**) via cysteine addition followed by click reaction (**a**) and click reaction followed by cysteine addition (**b**) approaches. (B) Application to the labeling of **15** and **17**. All reactions were monitored by HPLC, and no side products were observed except <20% oxidation to disulfide during the thiol addition step. Reaction conditions were modified for **15** and **17** (See Supplemental Experimental Procedures Sections 11c and 11e–11g). HIR, hypervalent iodine reagent.

of the 601-nucleosome positioning sequence[81] and reconstituted nucleosomes via dialysis from high (2M NaCl) to low (10 mM KCl) salt (Figure 1C). To our delight, nucleosomes were readily formed, yielding both unmodified or EBX-modified (**28′**) nucleosomes with equal efficiency, as judged by native PAGE (Figure 1D). Finally, nucleosome assembly also proceeded smoothly with Cy5-labeled octamers to yield fluorescent nucleosomes **29′** (Figures 1E and 1F). Together, these experiments demonstrate that EBX modification at exposed cysteines is compatible with protein function and a viable strategy for highly efficient protein labeling.

Development of the "Doubly Orthogonal" Functionalization

After having demonstrated that selective reaction of the azide was possible, we investigated functionalization of the hypervalent bond. Encouraged by recent progress in the use of palladium catalysis for the functionalization of peptides and proteins,[67–71,82,83] we opted for a Suzuki-Miyaura cross-coupling. Although avoiding transition metals in biomolecule functionalization is preferred, it should be noted that the palladium content can easily be reduced down to 1.0 ppm through scavenging and size-exclusion chromatography.[70] Furthermore, palladium catalysis has even been used in living cells, in which it shows low toxicity.[69,82] After an extensive study on various palladium catalytic systems (see Tables S2–S8) with boronic

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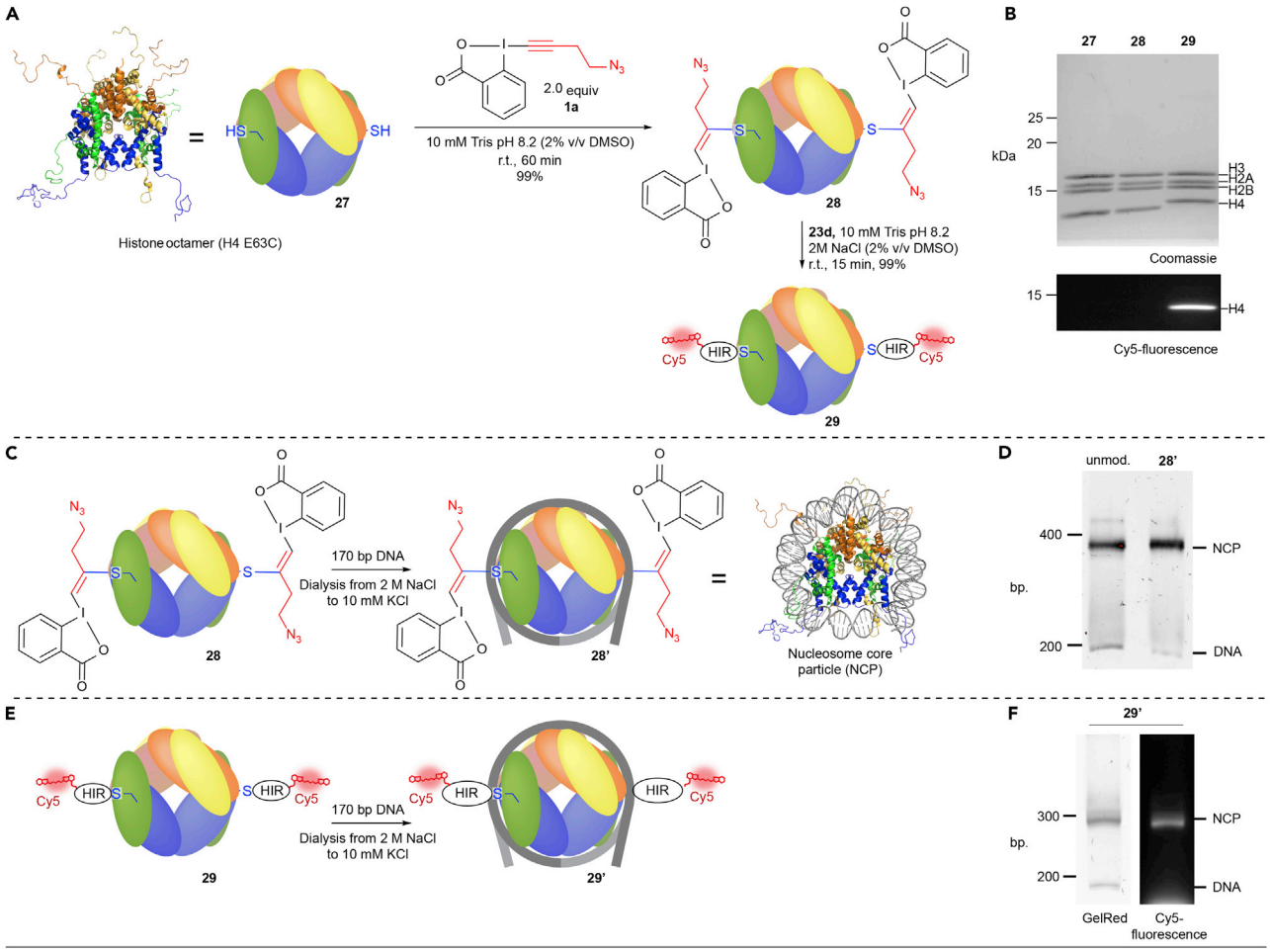

Figure 1. Labeling of Histones and Formation of Nucleosomes

(A) Functionalization of reconstituted histone octamers with EBX reagent **1a**. Reactions were carried out on a 2.4 nmol scale at a 40 μM concentration of histone octamers. Yields were determined by relative integration based on HPLC traces at 214 nm (see Supplemental Experimental Procedures Section 9h).

(B) Analysis of histone octamers by SDS-PAGE followed by staining with Coomassie blue or imaging of Cy5 fluorescence.

(C) Reconstitution of nucleosomes **28′** from EBX-labeled histone octamers (**28**) and the 170 bp 601 DNA sequence.

(D) Analysis of EBX-labeled nucleosomes **28′** and unmodified nucleosomes **27′** by native PAGE followed by GelRed staining.

(E) Reconstitution of nucleosomes **29′** from Cy5-labeled histone octamers (**29**) and the 170 bp 601 DNA sequence.

(F) Analysis of the Cy5-labeled nucleosome **29′** assembly by native PAGE followed by staining with GelRed or imaging of Cy5 fluorescence. NCP, nucleosome core particle. The gel migration is indicated in relation to a DNA ladder.

acid **30a**, air- and moisture-stable bis-lithium-2-(dimethylamino)-4,6-dihydroxylate-pyrimidine palladium diacetate complex **31a**[82] was found to be the most suitable catalyst-ligand system for our model reaction with VBX **3a**. Cross-coupling product **32a** was obtained in 70% yield (Table 2, entry 1). No degradation of the azide group was observed in the presence of the palladium catalyst. The reaction was run under air in a non-degassed phosphate buffer at 37°C for 30 min. Although DMSO helped to solubilize the boronic acid in aqueous media, it was non-essential for the reaction (entry 2). The reaction gave the desired product **32a** in satisfactory yields from pH 7.4 to 9.0 (entries 3 and 4). Using HEPES buffer instead of phosphate buffer resulted in a slightly less efficient coupling (entry 5). Tris buffer significantly slowed down the reaction rate, but a longer reaction time resulted in a satisfactory 51% yield (entry 6). A more concentrated buffer could also be used (entry 7). Cooling down to room

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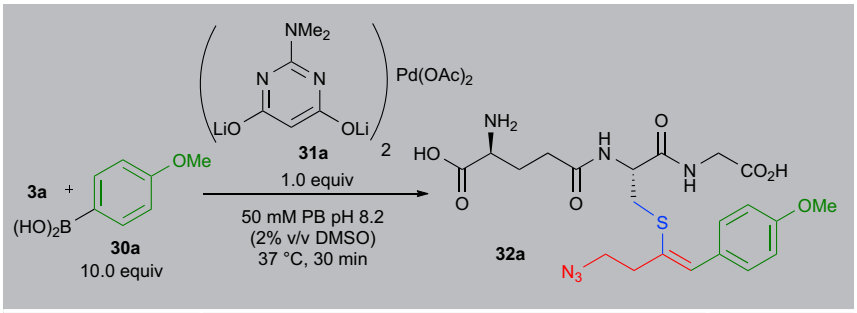
Table 2. Evaluation of the Reaction Conditions for the Cross-Coupling between 3a and 30a



Entry	Variations from the Optimized Conditions	Yield
1	none	70%
2	no DMSO	51%
3	50 mM PB (pH 7.4)	50%
4	50 mM PB (pH 9.0)	59%
5	50 mM HEPES (pH 8.2)	51%
6	50 mM Tris (pH 8.2)	19% (51%[a])
7	100 mM PB (pH 8.2)	58%
8	room temperature	53%
9	200 μM reaction molarity	63%

Reactions were carried out on a 1.00 μmol scale. Calibrated HPLC yield is based on absorbance at 214 nm (see Figure S6). For a complete list of examined catalysts, ligands, and conditions, see Tables S2–S9.
[a]The reaction was analyzed after 2 h.

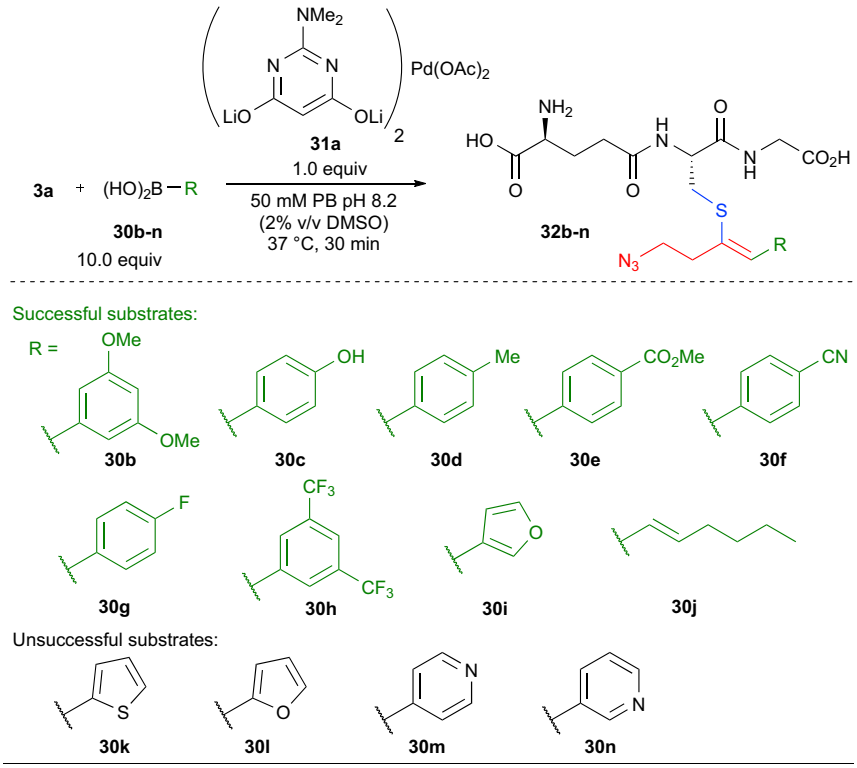
temperature still gave **32a** in 53% yield (entry 8). When the reaction was diluted from 2 mM to 200 μM, product **32a** could be obtained in 63% yield.

Once the robustness of the cross-coupling had been demonstrated, we examined the scope of boronic acids (Scheme 9). Different electron-rich phenyl boronic acid substrates, such as (3,5-dimethoxyphenyl)boronic acid (**30b**), (4-hydroxyphenyl) boronic acid (**30c**), or (4-methylphenyl)boronic acid (**30d**), could be coupled with **3a**. Electron-deficient substrates, such as (4-methoxycarbonylphenyl)boronic acid (**30e**) or (4-cyanophenyl)boronic acid (**30f**), as well as fluorinated arenes such as *para*-fluorobenzene (**30 g**) or 3,5-bis(trifluoromethyl)benzene (**30h**) were also successful. Cross-coupling of furyl (**30i**) and hexenyl (**30j**) boronic acids was possible as well. In contrast, heterocyclic boronic acids (**30k–30n**) could not be used.

We then applied our Suzuki-Miyaura cross-coupling to the more complex case of modified ubiquitin **17** (Scheme 10). To our delight, the cross-coupling worked in a one-pot labeling-cross-coupling approach to give **33**. Finally, both reactive handles were successfully employed in a one-pot labeling-SPAAC-Suzuki-Miyaura process, on a proof-of-principle scale, to afford doubly functionalized Cys-labeled ubiquitin **34**.

Application of "Doubly Orthogonal" Functionalization to Stabilize Fluorescent Dyes

We then exploited our reagents for fluorescent labeling of receptors on living cells. To this end, we synthesized substance P (**35**), a neuropeptide and a high-affinity ligand of the neurokinin 1 (NK1) receptor,[84] carrying an additional N-terminal

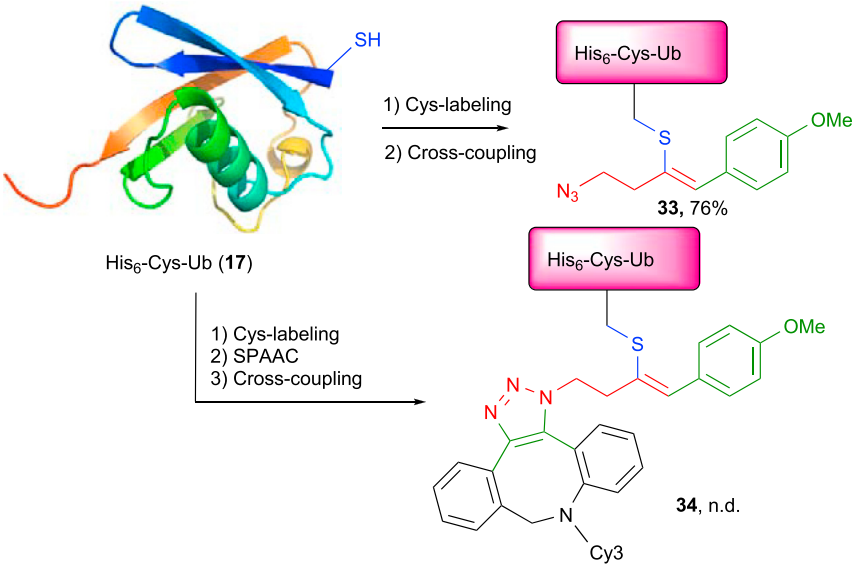


Scheme 9. Scope of Different Boronic Acid Reagents (30b–30d) for Cross-Coupling with 3a
Reactions were carried out on a 1.00 μmol scale. For all successful substrates, **32** was the major product observed by HPLC (see Supplemental Experimental Procedures Section 15).

cysteine residue (Figure 2A) (see Supplemental Experimental Procedures Section 18a). We functionalized **35** with EBX reagent **1a** and then labeling it with Cy5-DBCO (**23d**), producing the labeled peptide **36** (Figure 2B). We then tested the functionality of this construct by using a previously generated human embryonic kidney (HEK) cell line expressing green fluorescent protein (GFP)-tagged NK1 receptor at the cell surface[85,86] (Figure 2C). Substrate **36** readily labeled the cells and exhibited a distinct colocalization with the GFP-tagged NK1 receptor (Figure 2D), demonstrating the stability of our bioconjugate for experiments on live cells.

Fluorescent dyes often suffer from poor photophysics and photochemistry, resulting in dye bleaching. We envisioned that our dual-modification scheme might enable the attachment of photoprotection compounds that positively affect the photophysical and photochemical properties of nearby fluorophores.

We thus decided to exploit the doubly orthogonal functionalization scheme to increase the photostability of cyanine dye Cy5, which should increase dye brightness, decrease bleaching kinetics, and allow for longer tracks in single-particle tracking applications. To this end, we decided to place the TSQ 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in close proximity to the Cy5 fluorophore.[87] Such positioned TSQs reduce blinking rates, photobleaching rates, and dark-state lifetimes.[88] We therefore synthesized a boronic-acid adduct of Trolox (**37**). We then functionalized **35** with **1a** and coupled **37** via Suzuki-Miyaura cross-coupling (Figure 2E). The obtained product **38** was further labeled with DBCO-Cy5 as described above. Finally, a biotin moiety was attached to Lys5 for subsequent



Scheme 10. One-Pot Labeling-Suzuki-Miyaura and Labeling-SPAAC-Suzuki-Miyaura Functionalization of His6-Cys Ubiquitin (17)

n.d., yield not determined. Formation of **34** was confirmed by HPLC-MS.

surface immobilization using reagent **39** to peptides **36** and **38**, yielding the final peptides **40** (without Trolox) and **41** (with Trolox).

We then surface immobilized **40** and **41** (see Supplemental Experimental Procedures Section 19a) and observed the stability of individual Cy5 molecules by single-molecule total internal reflection (TIRF) microscopy (Figure 2F). Individual fluorescent molecules were imaged over time under conditions of oxygen exclusion. After 50 s of imaging, most Cy5 dyes were bleached in the absence of coupled Trolox in **40**, whereas for the Trolox-containing molecule **41**, a large portion of Cy5 was still fluorescent (Figure 2G). When measuring bleaching time constants for both conditions (Figures 2H and 2I), we detected a 3-fold increase in the dye's bleaching time constant as a result of the proximity to the Trolox moiety (from 17.9 $\pm$ 8.8 s for **40** to 55.9 $\pm$ 18.6 s for **41**). Together, these results demonstrate that a doubly orthogonal functionalization strategy can be exploited in a modular fashion to label proteins with fluorophores stabilized by a covalently coupled TSQ.

Conclusion

In summary, we have reported a general cysteine labeling protocol for the installation of a hypervalent iodine structure on both peptides and proteins. The obtained bioconjugate contains two reactive groups, an azide and a hypervalent iodine, which are orthogonal in reactivity to each other and to existing natural functional groups in peptides and proteins.

The cysteine labeling protocol proceeds with high efficiency, chemoselectivity, and functional-group tolerance under native conditions. The addition of thiols onto EBX reagents proceeds with perfect *Z* stereoselectivity on simple thiols and small peptides containing cysteine, but further studies will be needed to establish the stereoselectivity of the reaction on larger peptides and proteins. In contrast to previous methods based on the use of hypervalent iodine reagents, the reactive carbon-iodine bond does not react and is transferred intact to the biomolecule. A

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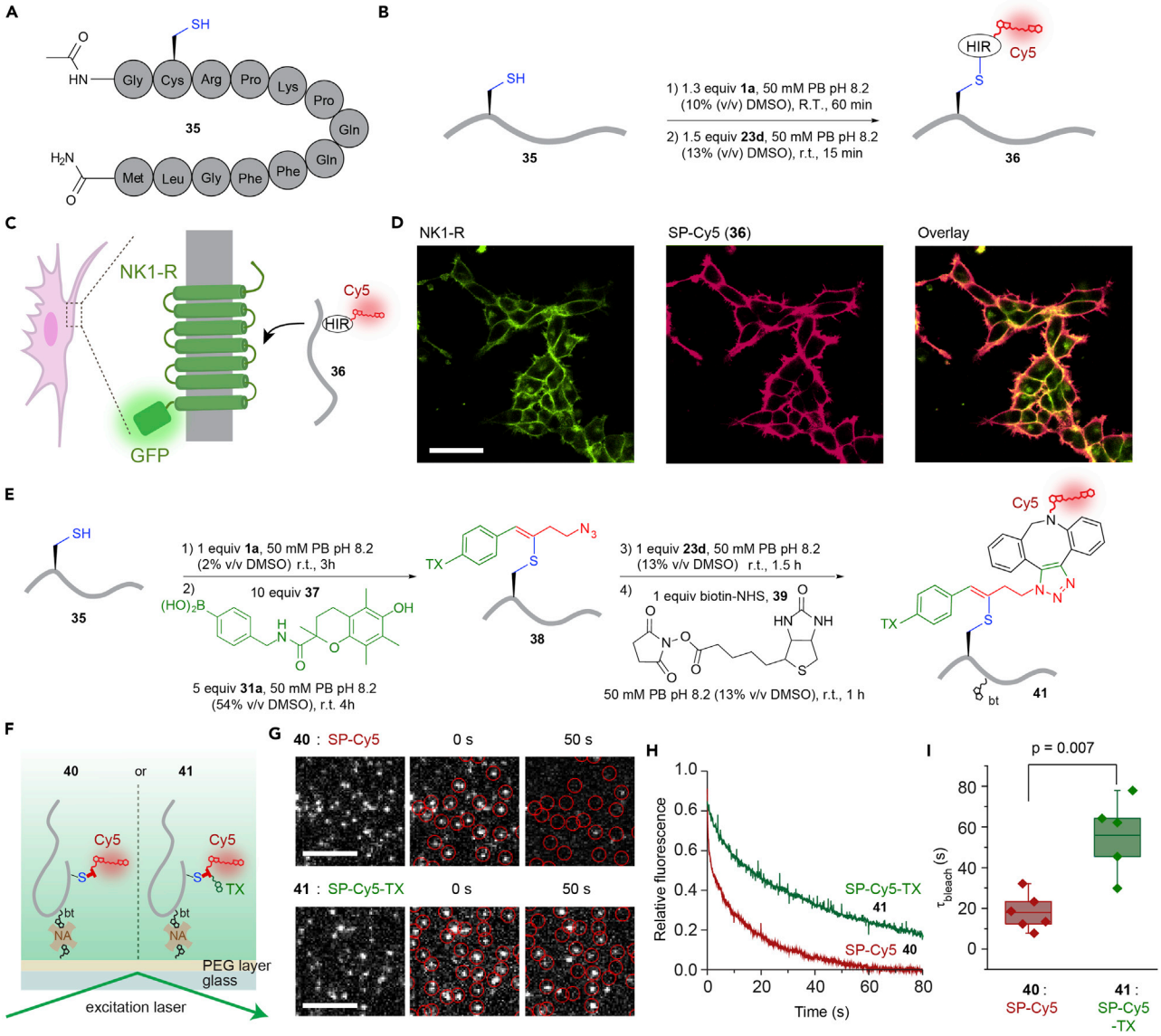

Figure 2. Application of the "Doubly Orthogonal" Functionalization to Stabilize Fluorescent Dyes

(A) Substance P (**35**) carrying N-acetylation and C-amidation with additional N-terminal Gly and Cys residues.

(B) Labeling and SPAAC of **35** (see Supplemental Experimental Procedures Section 11d).

(C) Scheme of the NK1-R-GFP-fusion-expressing cells and the cell-labeling experiment.

(D) Imaging of cells expressing GFP-NK1-receptor, which was labeled with **35**-Cy5 (SP-Cy5, **36**). Scale bar, 50 μm.

(E) Labeling-SPAAC-Suzuki-Miyaura functionalization with Trolox (TX) of **35** followed by biotin (bt) modification (see Supplemental Experimental Procedures Section 19b).

(F) Scheme of the single-molecule TIRF experiment.

(G) Single-molecule images of immobilized **40** and **41** at the indicated time points. Scale bar, 5 μm.

(H) Averaged fluorescence photobleaching kinetics for **40** and **41**.

(I) Fluorescence bleaching time constants from six experiments for **40** and five experiments for **41**. Error bars indicate the standard deviation (n = 5 or 6, p = 0.007, Student's t test).

wide range of peptidic-hypervalent iodine conjugates and a protein-bound hypervalent iodine compound were efficiently generated. Importantly, the obtained conjugates are compatible with protein structure and function, as demonstrated by the assembly of nucleosome particles.

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Additionally, the methodology could be extended to the functionalization of cysteines in disulfide bridges after a simple reductive pretreatment. The obtained bioconjugates allow "doubly orthogonal" functionalization: an azide group can be selectively functionalized by cycloaddition with cyclooctynes without affecting the hypervalent bond. Alternatively, the hypervalent iodine structure could be successfully engaged in an aqueous palladium-catalyzed cross-coupling with boronic acids without losing the azido group.

This provides a means for interesting and highly useful dual functionalization. Here, we demonstrated the usefulness of the approach by improving the photophysics of cyanine dyes by the attachment of a TSQ by using the dual-reactive handle. We thus provide a modular method for stabilizing organic dyes in biomolecules.

Altogether, our approach allows fast and selective peptide and protein modification. We are convinced that our work has just started to unravel the potential of hypervalent iodine reagents in biomolecule labeling, and the stage is now set for developing a day-to-day use in chemical biology and potential medicinal applications.

DATA AND CODE AVAILABILITY

Crystal structures for compounds **8a**, **9a**, and **9b** are available as Data S1, S2, and S3, respectively, and also at the Cambridge Crystallographic Data Centre under accession numbers CCDC: 1912427 (**8a**), CCDC: 1912430 (**9a**), and CCDC: 1862137 (**9b**). Single-molecule data are available at https://zenodo.org/ (https://doi.org/10.5281/zenodo.3246280).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.chempr.2019.06.022.

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AUTHOR CONTRIBUTIONS

R.T. discovered and designed the reaction; synthesized the reagents and substrates; performed the optimization studies, the scope of the reaction on peptides, and the modifications studies on peptides; and contributed to the redaction of the manuscript. J.C. synthesized the reagents and substrates, performed the modification studies on peptides, and contributed to the redaction of the manuscript. N.G. designed and performed the reactions on larger peptides; modified ubiquitin,

nucleosomes, and substance P; performed the cell experiments and single-molecule photobleaching studies; and contributed to the redaction of the manuscript. R.S.D. made several experiments important for the discovery and optimization of the reaction on glutathione. B.F. devised and supervised the experiments on larger peptides, ubiquitin, nucleosomes, cells, and single-molecule studies and participated in the redaction of the manuscript. J.W. managed the overall project, designed the reactions, planned the experiments on small molecules and peptides, and participated in the redaction of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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