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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. The methodology was successfully applied on relevant and complex biomolecules such as histone proteins. The potential of this doubly-reactive bioconjugate was illustrated with the introduction of a triplet-state quencher close to a fluorophore, extending its lifetime through suppression of photobleaching, as demonstrated on single molecule experiments. This work is therefore expected to find broad applications for peptide and protein functionalization.

bioconjugation, peptides, proteins, biomolecule functionalization, hypervalent iodine, chemical biology, selective reaction, cysteine, cross-coupling

The Bigger Picture

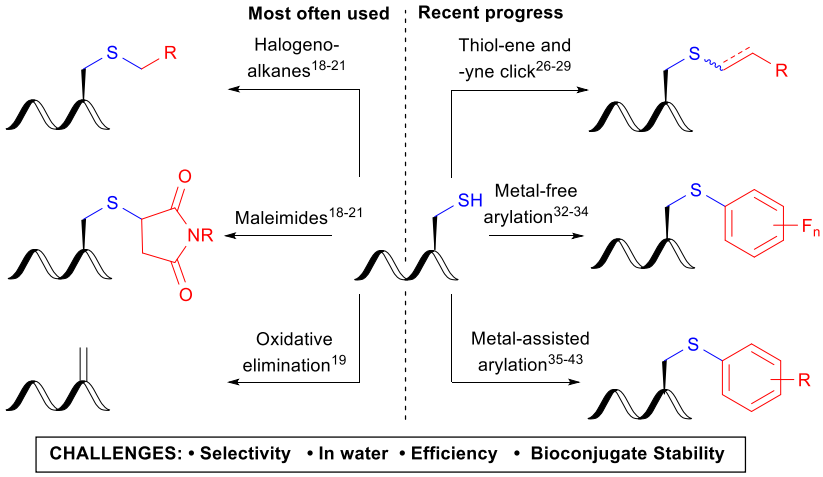
Understanding the molecular basis of life is essential in the search for new medicines. Chemical biology develops molecular tools to study biological processes, setting the basis for new diagnostics and therapeutics, and relies heavily on the ability to modify selectively biomolecules. Two approaches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

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 functionalized selectively via a palladium-catalyzed cross-coupling 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.

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 have emerged, exploiting both natural and unnatural amino acids.[12-17]

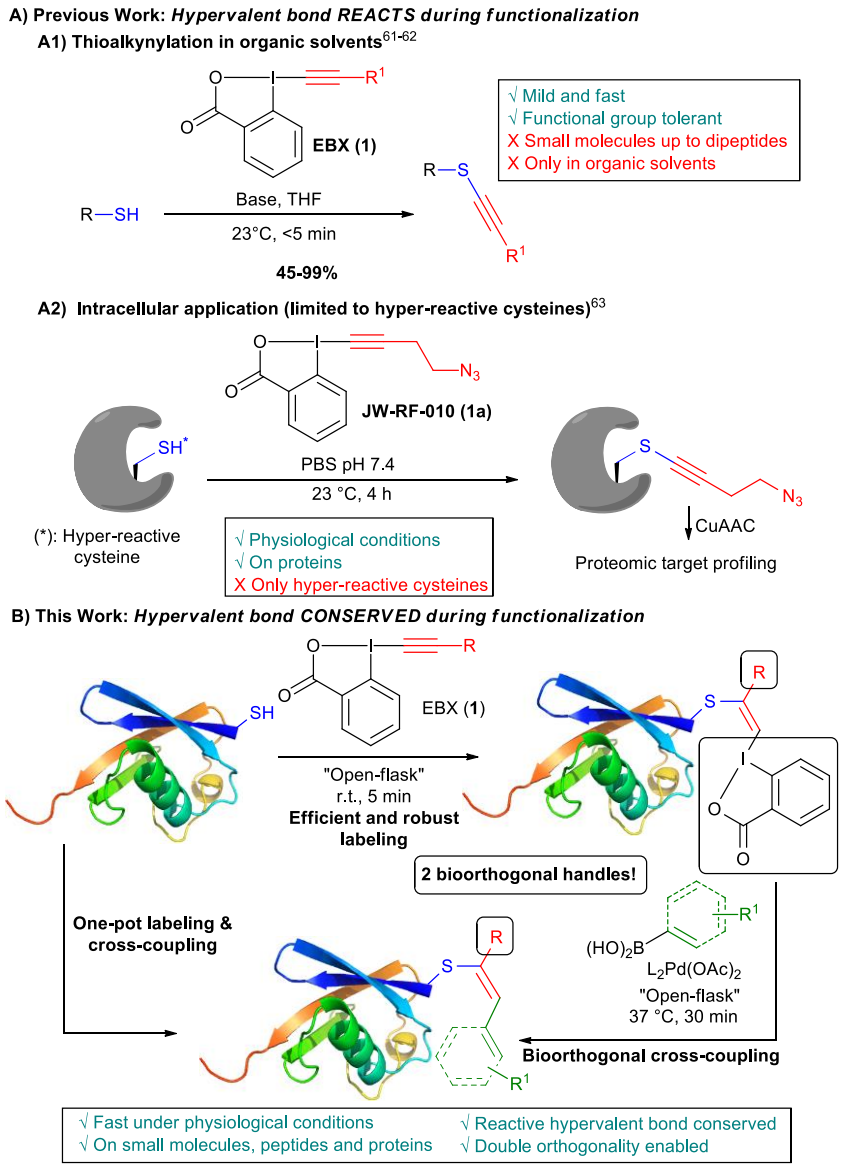
Because of its key role in the structure and catalytic activity of proteins, cysteine has been one of the most studied natural amino acids in chemical biology. Its low abundance in proteins and its intrinsic high nucleophilicity makes it an ideal target for chemoselective modification techniques often 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, lack of chemoselectivity is observed in 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 towards hydrolysis and external organosulfur attacks. This is mainly due to the reversibility of the thiol-maleimide reaction which leads to loss of label.[22-25] Therefore, new labeling methods leading to more stable adducts have been developed, 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] 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.



Scheme 1. State of the art and challenges in cysteine functionalization

In this context, hypervalent iodine reagents are interesting as they combine high reactivity with sufficient stability and low toxicity.[45] Nevertheless, applications for peptide/protein functionalization remain rare, probably due to concurring oxidation side reactions.[46-53] In 2018, Gaunt, Suero and co-workers reported an elegant functionalization of methionine using a hypervalent iodine reagent.[53] Our group previously reported a highly efficient and chemoselective alkynylation of thiols using hypervalent iodine-based EthynylBenziodoXolones[54-60] reagents (EBXs, **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] JW-RF-010 (**1a**) displayed an exceptional stability with less than 3% of 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 the use of 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 giving stable adducts of EBX and the thiols without cleavage of the hypervalent bond (Scheme 2B). This methodology can be applied to all cysteine- and other thiol-bearing compounds, including modified ubiquitin and histone proteins. The unprecedented peptide- and protein bound vinyl benziodoxolone reagents are stable, yet their inherent reactivity[59,64-66] opened the way for novel 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).



Scheme 2. 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).

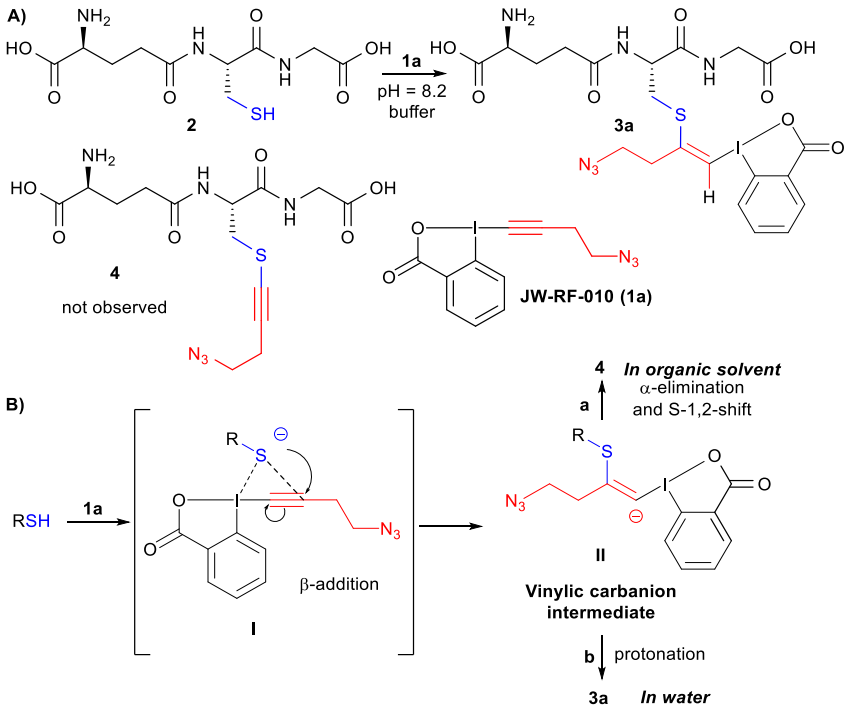
Efficient, robust, fast and under physiological conditions, this methodology allows swift peptide and protein modifications, without the need for incorporation of unnatural amino acids. In addition, we have combined this powerful methodology with metal-free Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC),[72-74] which allowed functionalization of the adduct without cleaving the hypervalent bond, leading to "doubly orthogonal"

modifications. The potential of the method was demonstrated by introducing both a fluorophore and a triplet state quencher 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 model substrate. GSH (**2**) is a naturally occurring tripeptide, which plays an essential role in primary metabolism as disulfide bond reductant and is present in high concentrations in cells. It was therefore surprising that alkynylation of **2** was not observed when working on cell lysate or living cells.[63] At that time, this was attributed to the low acidity of GSH (**2**) compared to hyper-reactive cysteines. But even when glutathione was treated with equimolar amounts of JW-RF-010 (**1a**) in a more basic buffer (10 mM Tris, pH 8.2), alkynyl sulfide **4** was not observed (Scheme 3A). Nevertheless, full conversion to a single product was obtained, which was identified as vinylbenziodoxolone (VBX) **3a**. Our previous computational work on the mechanism of the thio-alkynylation reaction had demonstrated that concerted β addition of sulfide via transition state **I** is favored for EBX 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 vinylbenziodoxolone (VBX) **3a** (Scheme 3B, route **b**).



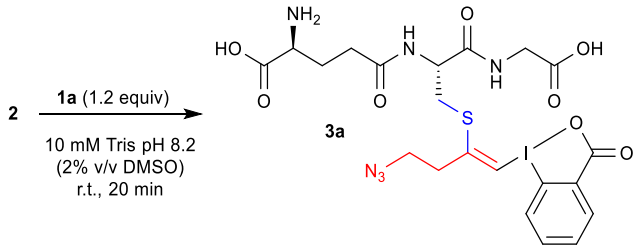
Scheme 3. Formation of vinylbenziodoxolone 3a (A) and speculative reaction mechanism (B).

Indeed, we recently demonstrated that also nitrogen and phenol nucleophiles can be added to EBX reagents to give VBX compounds, and computational studies showed a very low barrier for protonation.[76] Solvent polarity plays also a key role and in the case of thiol nucleophiles, increasing amounts of alkynylation were observed when adding DMSO to the reaction mixture, both with a protected amino acid and a tetrapeptide (see SI: Sections 7d and 7e). Mixtures of alkynes and VBXs were observed when water was added to organic solvents. Although our previous work on cell lysates and living cells had been performed in aqueous buffer, the targeted cysteines were in protein active sites, which have very different properties compared to the bulk solution. The lower dielectricity constant observed inside proteins[77] indicate that the local polarity is closer to the one of organic

solvents. 2D-NMR spectroscopic studies established the *Z* configuration of the olefin, as well as the β-addition regioselectivity, which are in agreement with our previous DFT calculations.[75] The developed transformation corresponds to a formal *trans*-addition of the thiol on the triple bond of the EBX reagent occurring 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 kept as a solution in DMSO-d_6. Furthermore, **3a** exhibited a surprising resistance to high temperatures, with no detectable degradation when heating 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, neither degradation, nor further addition, nor exchange reaction were observed in presence of 15 equivalents of an external thiol nucleophile (tiopronin, 30 mM, 6x higher than standard glutathione concentration in cell) after 8 days. Finally, while the presence of further 10 equivalents of JW-RF-010 (**1a**) resulted in 14% of degradation after 4 days, 5 equivalents of JW-RF-010 (**1a**) did not lead to any decomposition.

Table 1. Evaluation of reaction conditions for the labeling of glutathione (2).[a]



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[b]	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[c]	200 µM reaction molarity	76% (93%)
15[d]	3 equiv. of JW-RF-010 (**1a**)	98%

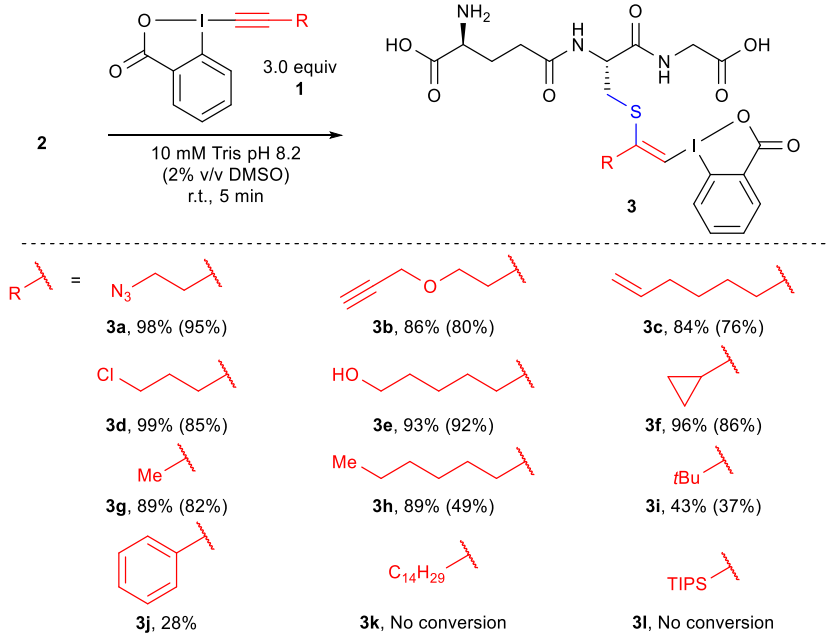
Labeling conditions: 1.0 µmol glutathione (**2**), JW-RF-010 (**1a**) (1.2 equiv) in 0.5 mL of non-degassed Tris buffer pH 8.2 (2% v/v DMSO), room temperature, 20 minutes. Calibrated HPLC yield based on absorbance at 214 nm (see SI: Section 5c, Figure S1). The yields in parentheses correspond to the yields after one hour of reaction. [a] For the complete robustness studies, see SI: Section 6. [b] 10 equiv of JW-RF-010 (**1a**) were used. [c] 3 equiv of JW-RF-010 (**1a**) were used. [d] The reaction was analyzed after 5 minutes.

Once the high stability of VBX **3a** had been confirmed, we started to evaluate the robustness of our labeling method. The reaction is user-friendly and easy to set up. Glutathione (**2**) was simply dissolved in non-degassed Tris buffer (10 mM, pH 8.2), followed by addition of JW-RF-010 (**1a**) from a DMSO stock solution. Then, the reaction was vortexed a few seconds, or homogenized with a simple pipette mixing, and left to stand on the bench for 20 minutes. 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 minutes incubation already afforded the product in 55% yield (entry 2), and an extended labeling time of 60 minutes increased the yield to 87% (entry 3). The only side-reactivity observed was glutathione oxidation into the dimeric disulfide. Interestingly, 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 towards 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 one hour, entry 8). At pH 6.5, only 29% of **3a** was obtained after one hour (entry 9). Nevertheless, this low reactivity could be easily overcome by using 10 equivalents of JW-RF-010 (**1a**) (entry 10). To our delight, the labeling remained efficient at either lower or higher temperatures (entries 11 and 12). While diluting the reaction mixture from 2 mM to 200 µM was found to increase the reaction time, it barely affected the labeling efficiency (entry 13). Furthermore, increasing the stoichiometry of JW-RF-010 (**1a**) to 3 equivalents allowed for efficient labeling in 20 minutes and almost quantitative transformation in one hour (entry 14). Using these conditions, reactivity could be observed down to 2 µM reaction molarity (See SI: Section 7c; Figures S2 and S3). A competition experiment between JW-RF-010 (**1a**) and iodoacetamide at pH 8.2, resulted in a 2:3 ratio of labeled products, showing that the two compounds react with similar rates. Finally, the use of 3 equivalents of JW-RF-010 (**1a**) afforded quantitative glutathione (**2**) functionalization within 5 minutes at 2 mM (entry 15). These conditions were selected to study the scope of the reaction.

Scope of the bioconjugation reaction

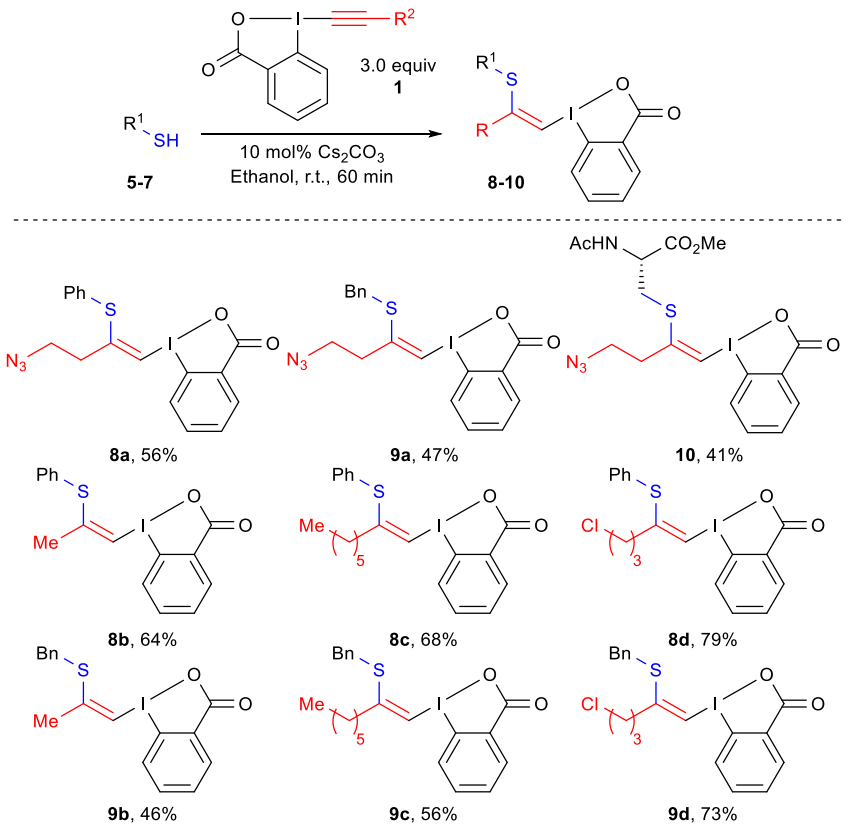
A library of hypervalent iodine reagents was therefore prepared, resulting in compounds bearing azide, alkyne, alcohol and various other functional groups.[62,78] Most of these EBX compounds can be prepared in one synthetic step from commercial reagents and are stable towards air, light and water. They can be stored on the bench under ambient atmosphere for weeks or at 4 °C under nitrogen for years without exhibiting noticeable degradation. Our bioconjugation technique demonstrated a broad tolerance towards a large variety of functional groups (Scheme 4).



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 SI: Section 8). Yields were derived by comparing the integration area of the product in their respective HPLC traces to that of the standard curve at 214 nm for 3a (see SI: Section 5c, Figure S1). Yields in parentheses represent that of the labeling reaction without DMSO as organic co-solvent.

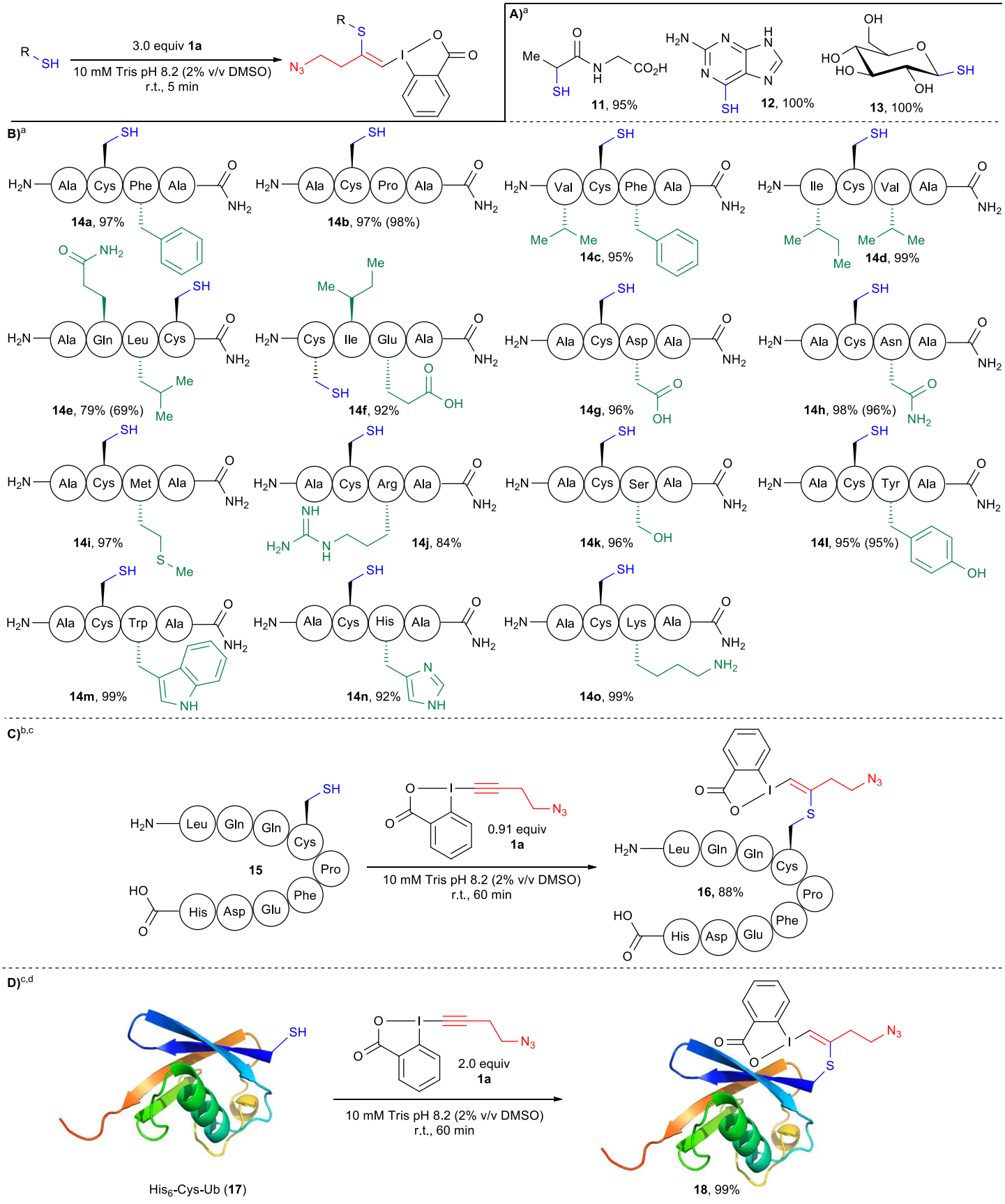
Indeed, azide (**3a**), terminal alkyne (**3b**) and alkene (**3c**), halogen (**3d**), alcohol (**3e**), as well as non-functionalized alkyl substituents (**3f-h**) could be rapidly attached to glutathione (**2**) in excellent yields. To our delight, these reactions were also highly efficient without DMSO as organic co-solvent. Due to 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-l**.

The method could not be used with non-polar small organic molecules, due to their low solubility in water. However, in such cases the reagents could be accessed in good yields using the conditions recently developed for N- and O- nucleophiles (catalytic cesium carbonate in ethanol, Scheme 5).[76] Phenyl and benzyl thiols (**5** and **6**) as well as protected cysteine (**7**) 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 stereo-selectivity of the thiol addition.[79] The addition to other EBX reagents bearing a methyl group, a longer alkyl chain or a chlorine functional group was also successful (products **8b-d** and **9b-d**).



Scheme 5. Scope of different EBX reagents with non-polar small organic molecules in ethanol. Reactions were carried out on a 0.50 to 11 mmol scale. Isolated yields after recrystallization or column chromatography are reported (see SI: Section 9a).

The scope of thiol reagents with JW-RF-010 (**1a**) under physiological conditions was then investigated (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 various natural amino acids (Scheme 6B). Cysteines in close proximity of phenylalanine (**14a**) or proline (**14b**) exhibited an 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.

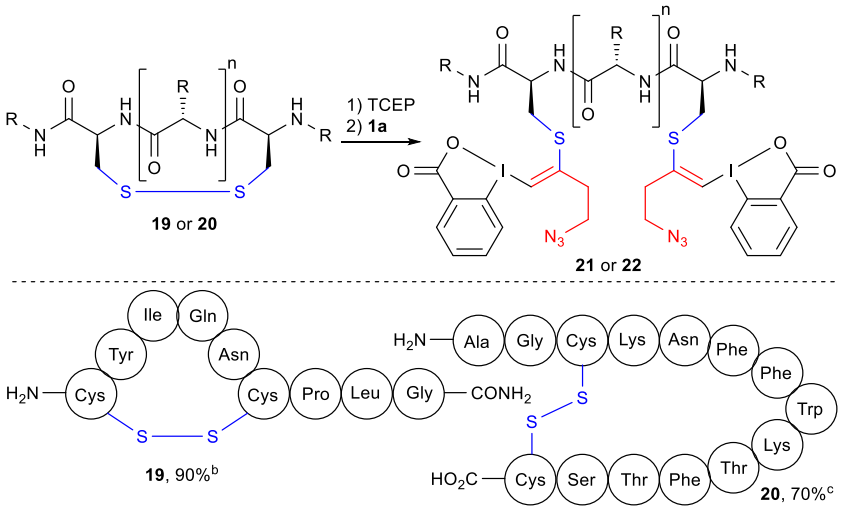

3.0 equiv 1a
10 mM Tris pH 8.2 (2% v/v DMSO)
r.t., 5 min
A)a
11, 95%
12, 100%
13, 100%
B)a
14a, 97%
14b, 97% (98%)
14c, 95%
14d, 99%
14e, 79% (69%)
14f, 92%
14g, 96%
14h, 98% (96%)
14i, 97%
14j, 84%
14k, 96%
14l, 95% (95%)
14m, 99%
14n, 92%
14o, 99%
C)b,c
15
0.91 equiv
1a
10 mM Tris pH 8.2 (2% v/v DMSO)
r.t., 60 min
16, 88%
D)c,d
His6-Cys-Ub (17)
2.0 equiv
1a
10 mM Tris pH 8.2 (2% v/v DMSO)
r.t., 60 min
18, 99%

Scheme 6. Scope of the functionalization of small molecules (A) and tetrapeptides (B); Applications on Human Serum Albumin Leu_{55}-His_{63} sequence (15) (C) and His6-Cys-ubiquitin (17) (D).[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 SI: Section 9b). Yields between brackets represent the labeling reaction without DMSO. [b] Reaction was carried out on a 1.30 µmol scale at 3 mM concentration. EBX (1a) was used substoichiometrically for the ease of chromatogram analysis. [c] Yields were determined by relative integration based on HPLC traces at 214 nm (See SI: Section 9b). [d] Reaction was carried out on a 50.0 nmol scale at a 0.5 mM concentration.

While *C*-terminal cysteine was found to be more prone to oxidation, affording a lower efficiency (**14e**), *N*-terminal cysteine reacted efficiently (**14f**). Aspartic acid (**14g**), asparagine (**14h**) and methionine (**14i**) were well tolerated. Only in case of arginine (**14j**) a lower yield was obtained due to the formation of side-products. High chemoselectivity was also observed in presence of serine (**14k**), tyrosine (**14l**) and tryptophan (**14m**). 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 JW-RF-010 (**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, carrying 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 equivalents of JW-RF-010 (**1a**) under native conditions afforded a quantitative transformation into VBX **18** in less than an hour (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 SI: 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**, only occur when arginines are immediately next to the cysteine. Importantly, no reaction was observed with native (cysteine-free) ubiquitin (see SI: 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 SI: Section 9e). It should be noted that, since the reaction kinetics are dependent on the reagent concentrations, for larger proteins an increase in EBX reagent concentration might be required.

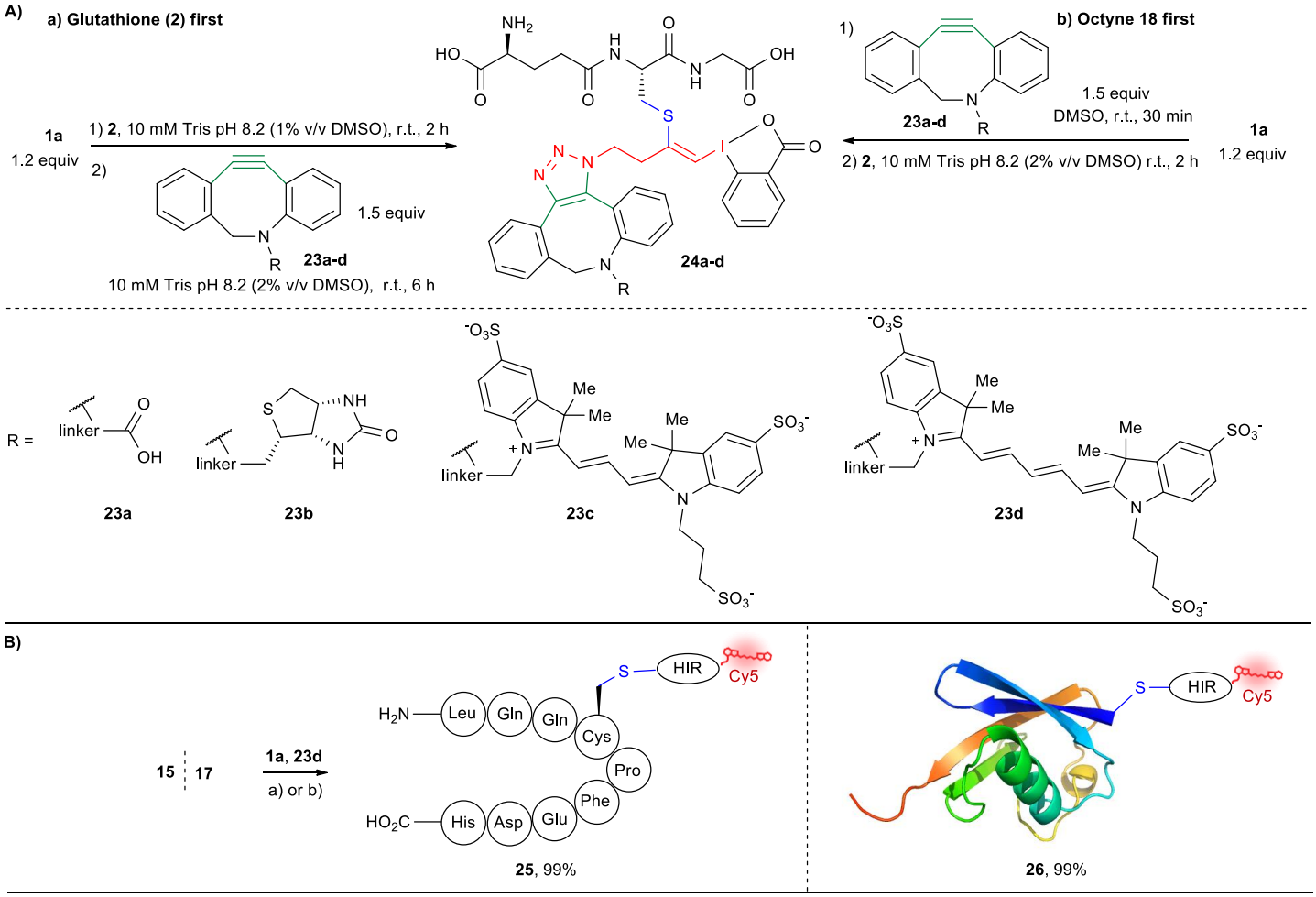
In nature, cysteine residues are known to form disulfide bonds, in particular in secreted, extracellular proteins. When targeting these disulfides with cysteine labeling reagents, the standard protocol is an *in situ* disulfide-cleavage with reducing reagents, such as tris(2-carboxyethyl)phosphine (TCEP). 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 glutathione with 1.5 equivalents of TCEP, followed by 6 equivalents (3 equivalents per reduced cysteine) of JW-RF-010 (**1a**). To our delight, the product **3a** was obtained with a yield 92% (see SI: Section 10a). Furthermore, even in presence of 5 and 10 equivalents of the reducing agent and without increasing the amount of **1a**, the product was still obtained in good yields (respectively 82% and 60%). While 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 hours in presence of TCEP. With these exciting results in hand, we applied our one-pot reduction/labeling technique on larger peptides containing disulfide bridges. We were pleased to see excellent yields on challenging natural bioactive peptides, such as oxytocin (**19**) and somatostatin (**20**) (Scheme 7) (see SI: Sections 10b and 10c).



Scheme 7. Labeling of sulfur bridge-containing peptides.[a] Conditions: 1.5 equiv. TCEP·HCl, 100 mM Tris pH 8.2, r.t., 60 min, then 6.0 equiv. 1a, 100 mM Tris pH 8.2 (2% v/v DMSO), r.t., 5 or 10 min. Yields were determined by relative integration based on HPLC traces at 214 nm (see SI: Sections 10b and 10c). [b] Reaction was carried out on a 0.50 µmol scale. [c] Reaction was carried out on a 0.10 µmol scale.

Application for fluorescent protein labeling

Next, we investigated the potential of the obtained bioconjugates for further functionalization. At first, we examined if standard methods for azide modification could be used in presence of the reactive hypervalent iodine center. In order to avoid the use of metal catalysts, we chose the well-known copper-free Strain-Promoted Alkyne-Azide Cycloaddition.[72-74] In principle, two strategies could be followed: (1) labeling of the peptide/protein with JW-RF-010 (**1a**), followed by cycloaddition with a functionalized alkyne or (2) prior reaction of JW-RF-010 (**1a**) with the alkyne, followed by reaction with the peptide/protein. The former appeared easier, as the reactivity of the hypervalent iodine of the VBX products is lower, whereas the latter is attractive for applications, as it would allow preparation of multi-functionalized labeling reagents in-situ starting from **1a** and commercially available alkynes. As a model system, we chose again glutathione (**2**), together with broadly used dibenzocyclooctynes **23**[80] bearing either a free acid (**23a**), a biotin (**23b**) or cyanine dyes (**23c-d**) (Scheme 8A). Gratifyingly, both the cysteine addition-click (**a**) and the click-cysteine addition (**b**) approaches were successful with all cyclooctynes. This demonstrated that azide-cyclooctyne cycloaddition is fully orthogonal to hypervalent iodine reactivity. Both approaches of labeling also performed well on Human Serum Albumin Leu_{55}-His_{63} sequence (**15**) and His-Cys-ubiquitin (**17**) (Scheme 8B).



Scheme 8. A) Labeling of glutathione (2) via the cysteine addition-click (a) and the click-cysteine addition approaches (b) and 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 SI: Sections 11c and 11e-g). HIR = Hypervalent Iodine Reagent

To showcase the compatibility of our methodology with more complex proteins than ubiquitin and determine if 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, as well as around 150 bp of DNA which iswrapped around the protein complex.[81] For labeling, we mutated glutamate 63 in H4 to cysteine (E63C) and expressed and purified the protein. We then assembled histone octamer complexes, using the remaining other human histone proteins (H2A, H2B and H3C110A, see SI: Section 3d). The resulting protein complex **27** contained two cysteine residues, which efficiently reacted with **1a** within 60 minutes 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). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the product **29** revealed the high specificity of the labeling procedure, as only histone H4 exhibited a fluorescence signal (Figure 1B). The selectivity of the reaction was also corroborated by HPLC and top-down MS-MS (see SI: Section 9g).

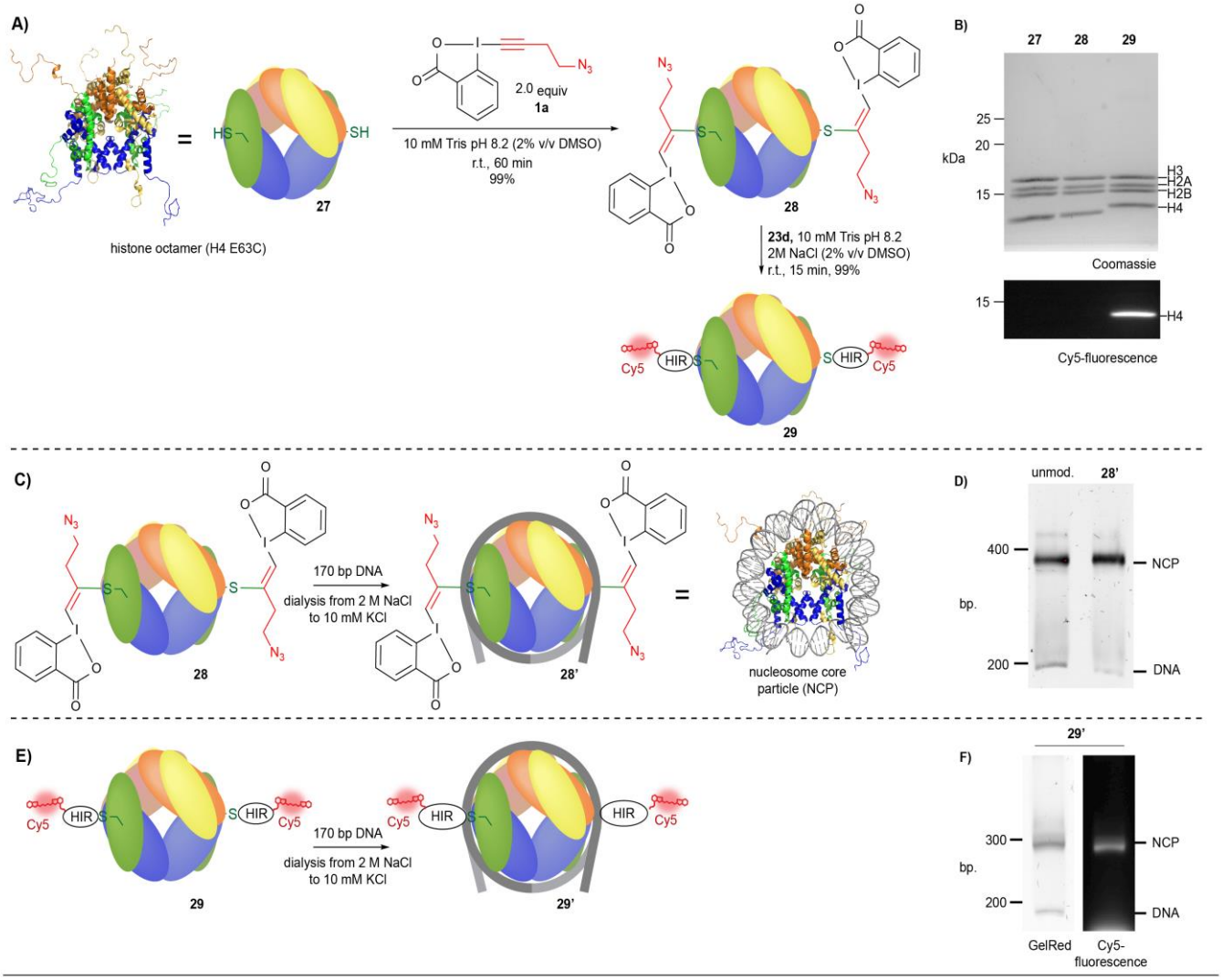


Figure 1. A) Functionalization of reconstituted histone octamers with EBX reagent 1a. Reactions were carried out on a 2.4 nmol scale at 40 µM concentration of histone octamers. Yields were determined by relative integration based on HPLC traces at 214 nm (See SI: Section 9h). B) Analysis of histone octamers 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 denotes nucleosome core particles. The gel migration is indicated relative to a DNA ladder.

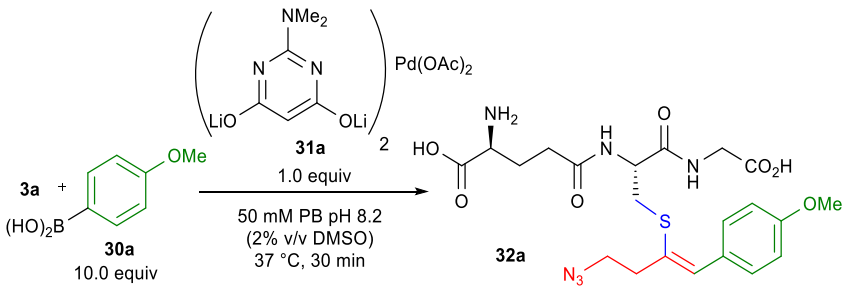
To demonstrate that the protein functionality was not affected by the modification we continued to reconstitute full nucleosomes, which will only form if the histone octamer structure is not disrupted. We thus combined **28** with a 170 bp-long segment of the 601 nucleosome positioning sequence[82] and reconstituted nucleosomes via dialysis from high (2M NaCl) to low (10 mM KCl) salt (Figure 1C). To our delight, nucleosomes were readily formed as judged by native PAGE (Figure 1D), yielding both unmodified or EBX-modified (**28'**) nucleosomes with equal efficiency. Finally, nucleosome assembly also proceeded smoothly with Cy5 labeled octamers to yield fluorescent nucleosomes **29'** (Figure 1E-F).

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,[67-71,83,84] we opted for a Suzuki-Miyaura cross coupling. While 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 been even used in living cells, showing low toxicity.[69,83] After an extensive study on various palladium catalysts (see SI: Section 14 Tables S5-11), air- and moisture-stable bis-lithium-2-(dimethylamino)-4,6-dihydroxylate-pyrimidine palladium diacetate complex **31a**[83] was found to be the most suitable catalyst for our model reaction between VBX **3a** and boronic acid **30a**. Cross coupling product **32a** was obtained in 70% yield (Table 2, entry 1). No degradation of the azide group was observed in presence of the palladium catalyst. The reaction was run under air in a non-degassed phosphate buffer at 37 ºC for 30 minutes. 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 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.

Table 2. Evaluation of the reaction conditions for the cross-coupling between 3a and 30a.[a]

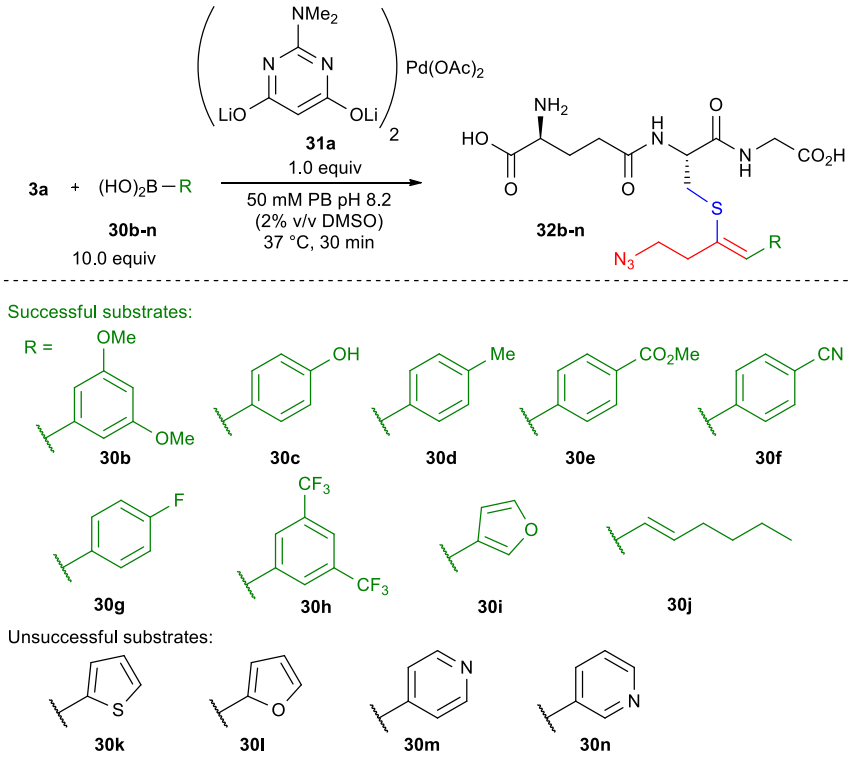


Entry	Variations from the optimized conditions	Yield
1	None	70%
2	No DMSO	51%
3	50 mM PB pH 7.4	50%
4	50 mM PB pH 9.0	59%
5	50 mM HEPES pH 8.2	51%
6	50 mM Tris pH 8.2	19% (51%[b])
7	100 mM PB pH 8.2	58%
8	Room temperature	53%
9	200 µM reaction molarity	63%

Reactions were carried out on a 1.00 µmol scale. Calibrated HPLC yield based on absorbance at 214 nm (see SI: Section 13c, Figure S9).[a] For a complete list of examined catalysts and conditions, see SI: Section 14. [b] The reaction was analyzed after 2 hours.

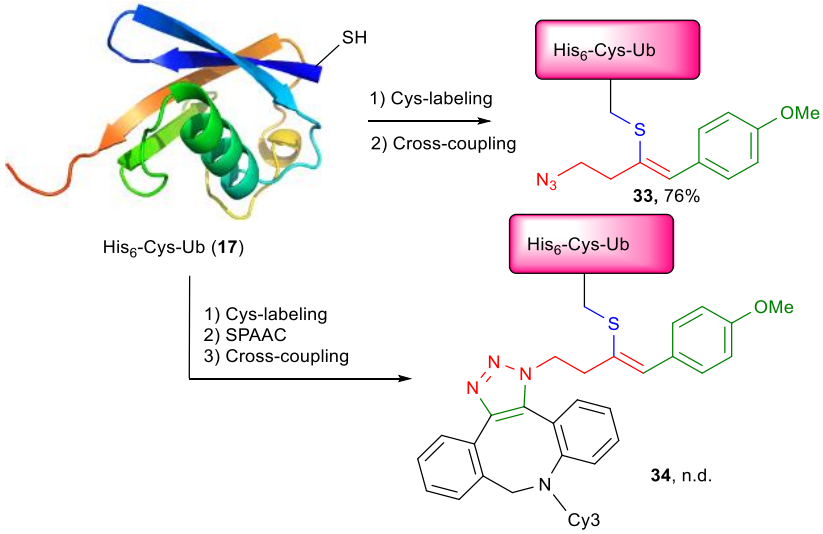
Once the robustness of the cross-coupling had been demonstrated, we examined the scope of boronic acids (Scheme 9). Different electron-rich phenyl boronic acid substrates, such as (3,5-dimethoxyphenyl)boronic acid (**30b**), (4-hydroxyphenyl)boronic acid (**30c**) or (4-methylphenyl)boronic acid (**30d**) could also 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 like *para*-fluorobenzene (**30g**)

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-n**) could not be used.



Scheme 9. Scope of different boronic acid reagents (30b-d) 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 SI: Section 16).

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 labeling/SPAAC/Suzuki-Miyaura one-pot process, on a proof-of-principle scale, to afford doubly-functionalized Cys-labeled Ubiquitin **34**.



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.

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, a neuropeptide and a high-affinity ligand of the neurokinin 1 receptor (NK1-receptor)[85], carrying an additional N-terminal cysteine residue (**35**, Figure 2A) (see SI: Section 18a). We functionalized **35** with EBX reagent **1a** followed by labeling with Cy5-DBCO (**23d**), producing the labeled peptide **36** (Figure 2B). We then tested the functionality of this construct using a previously generated human embryonic kidney (HEK) cell line expressing GFP-tagged NK1-receptor at the cell surface[86,87] (Figure 2C). Substrate **36** readily labeled the cells and exhibited a distinct colocalization with the GFP-tagged NK1-R (Figure 2D), demonstrating the applicability of our labeling approach for experiments on live cells.

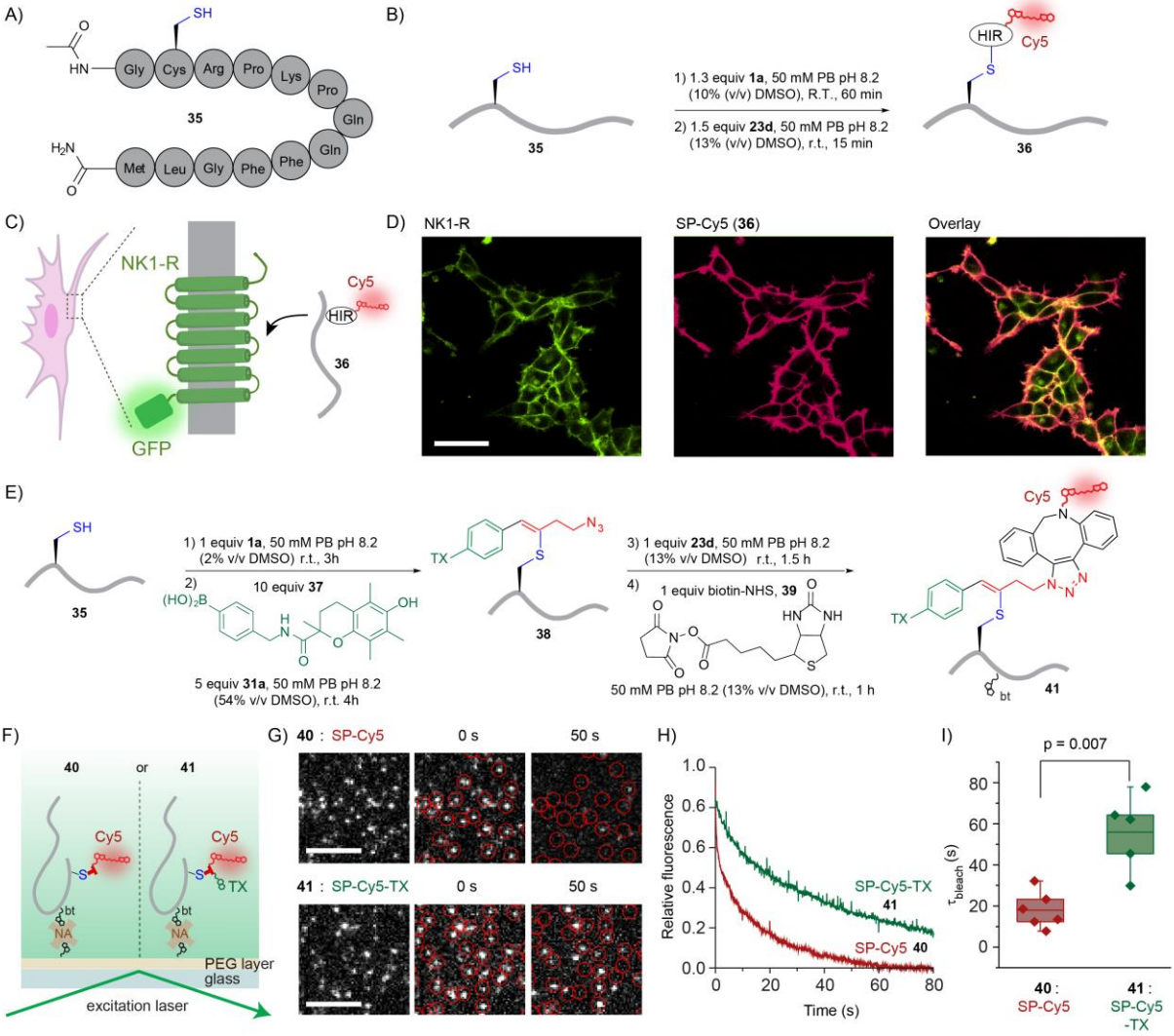


Figure 2. A) Substance P (35) carrying N-acetylation and C-amidation, with additional N-terminal Gly and Cys residues. B) Labeling/SPAAC of Substance P (see SI: Section 11d). C) Scheme of the

NK1-R - GFP fusion expressing cells and the cell-labeling experiment. D) Imaging of NK1-R expressing cells (GFP), labeled with Substance P-Cy5 (SP-Cy5, 36). The scale bar denotes 50 µm. E) Labeling/SPAAC/ Suzuki-Miyaura functionalization with Trolox (TX) of Substance P, followed by biotin (bt) modification (see SI: Section 19b. F) Scheme of the single-molecule TIRF experiment. G) Single-molecule images of immobilized 40 and 41 at indicated time points. The scale bar denotes 5 µm. H) Averaged fluorescence photobleaching kinetics for 40 and 41. I) Fluorescence bleaching time constants from n = 6 experiments for 40 and n = 5 experiments for 41.

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 which 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 triplet-state quencher (TSQ) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) in close proximity to the Cy5 fluorophore[88]. Such positioned TSQs reduce blinking rates, photobleaching rates and dark state lifetimes [89]. We therefore synthesized a boronic-acid adduct of Trolox (**37**). We then functionalized Substance P (**35**) with **1a** and coupled **37** via Suzuki-Miyaura cross coupling (Figure 2E). The obtained product **38** was further labeled using DBCO-Cy5 as described above. Finally, a biotin-moiety was attached to Lys5 for subsequent surface-immobilization, yielding the final peptide **41**.

We then surface-immobilized **41** (containing a Trolox moiety) and biotinylated Cy5-labeled Substance P without Trolox (**40**) (see SI: Section 19a), and observed the stability of individual Cy5 molecules using single-molecule total internal reflection (TIRF) microscopy (Figure 2F). Individual fluorescent molecules were imaged over time, under conditions of oxygen exclusion. After 50 seconds 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 were still fluorescent (Figure 2G). When measuring bleaching time constants for both conditions (Figure 2H, I) a 3-fold increase in the dye´s bleaching time constant was detected due to the proximity to the Trolox moiety (from 17.9 ± 8.8 s for **40** to 55.9 ± 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.

Conclusions

In summary, we have reported a general cysteine labeling protocol for the installation of an unprecedented 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. 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 wide range of peptidic-hypervalent iodine conjugates as well as the first 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.

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 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 triple-state quencher using the dual-reactive handle. We thus provide a modular method to stabilize organic dyes in biomolecules.

Taken together, our new 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 to develop a day-to-day use in chemical biology and potential medicinal applications.

SUPPLEMENTAL INFORMATION

Detailed experimental procedures and analytical data.

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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, 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 to the redaction of the manuscript. J. W. managed the overall project, designed the reactions, planned the experiments on small molecules and peptides and participated to the redaction of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Contents

Supplemental Data

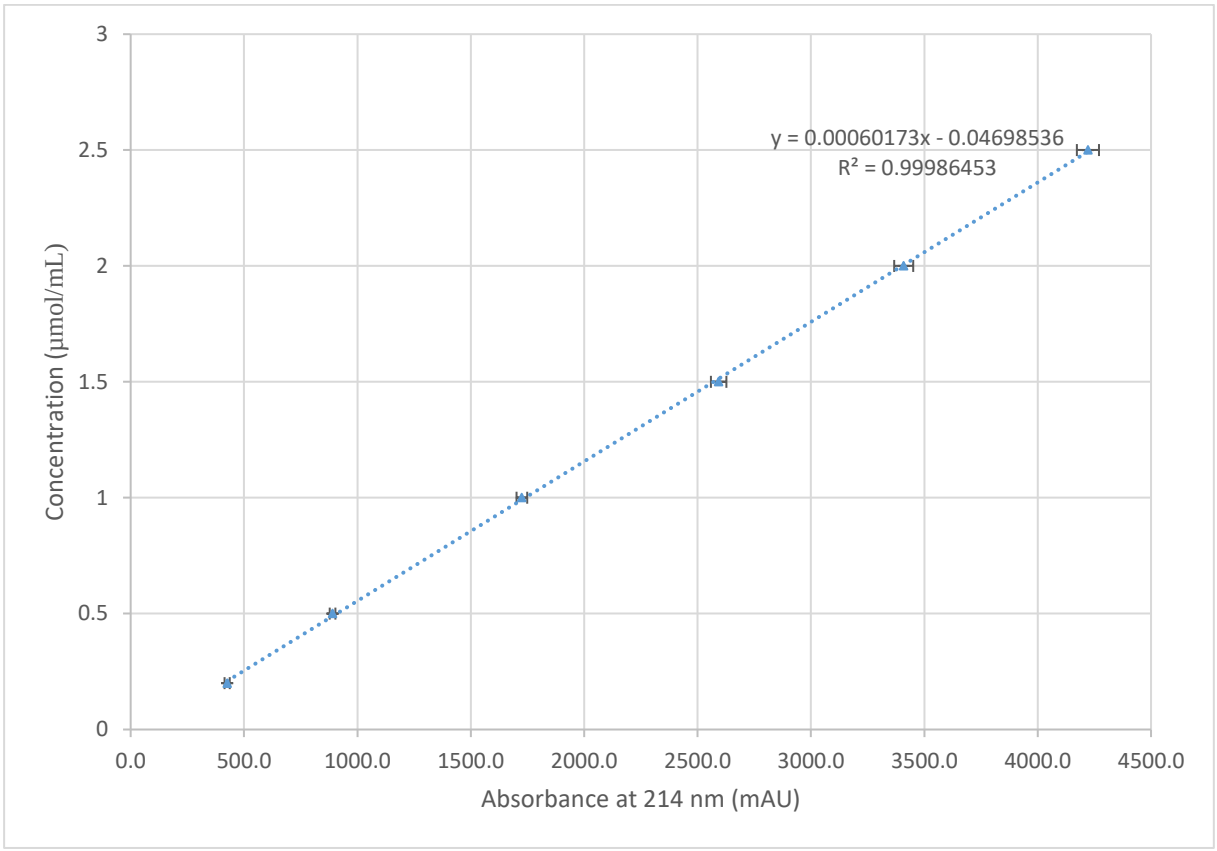


Figure S1: Calibration curve of **3a**.

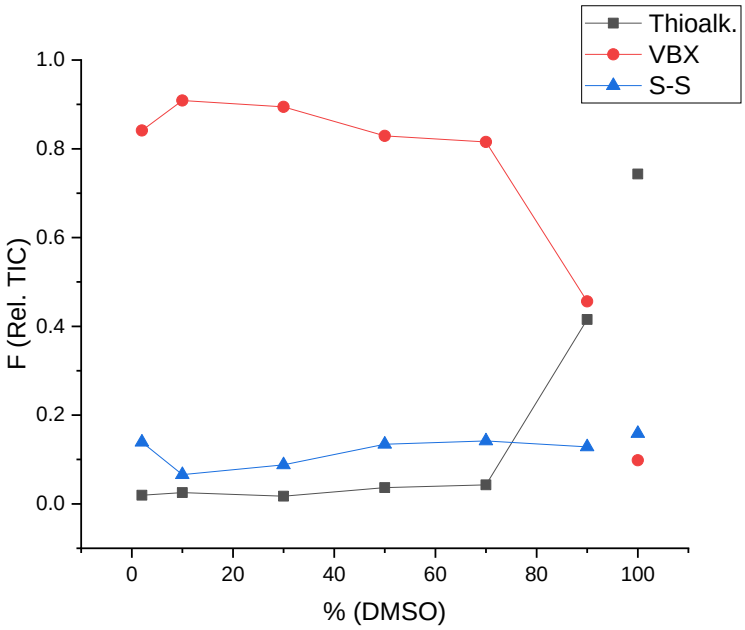


Figure S2: Normalized total ion count for Thioalkynyl (**74**), VBX (**10**) and disulfide (**75**) products vs the % of DMSO as solvent for the reaction of protected cysteine (**7**) with EBX reagent **1a**.

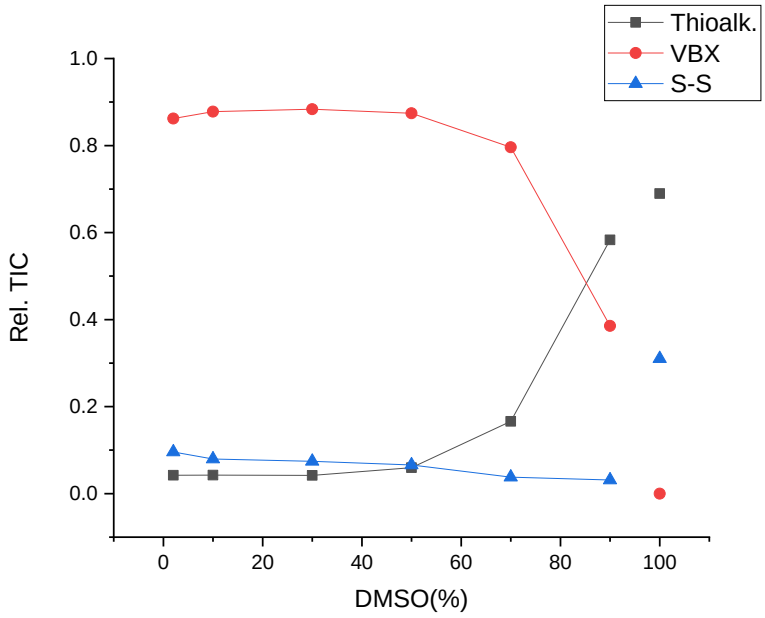


Figure S3: Normalized total ion count for Thioalkynyl (**73a**), VBX (**76**) and disulfide (**77**) products vs the % of DMSO as solvent for the reaction of ACYA (**14l**) with EBX reagent **1a**.

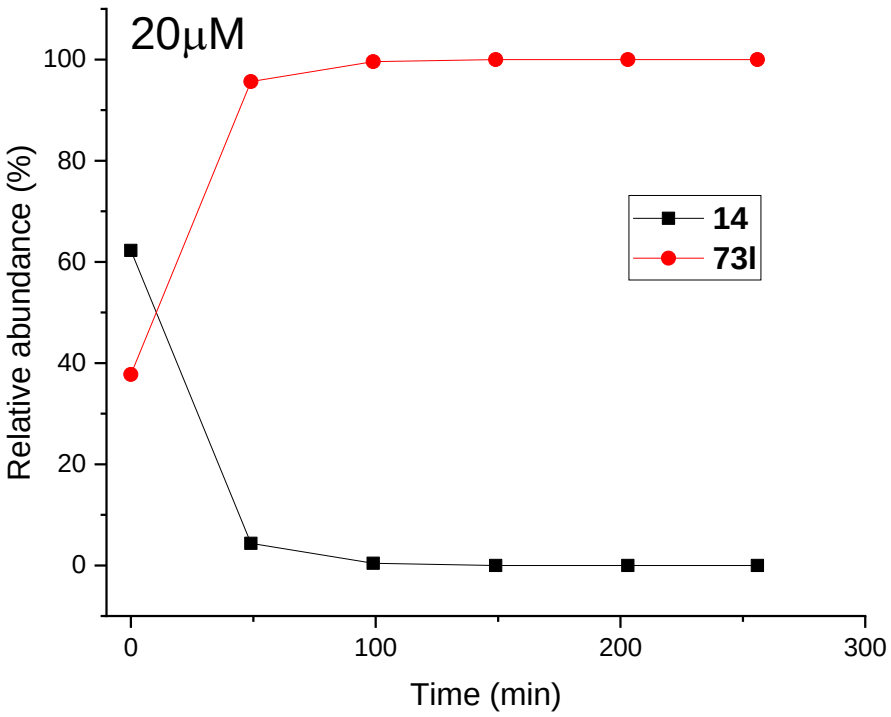


Figure S4: 20 µM reaction profile of **ACYA (14)** labeling under dilution conditions.

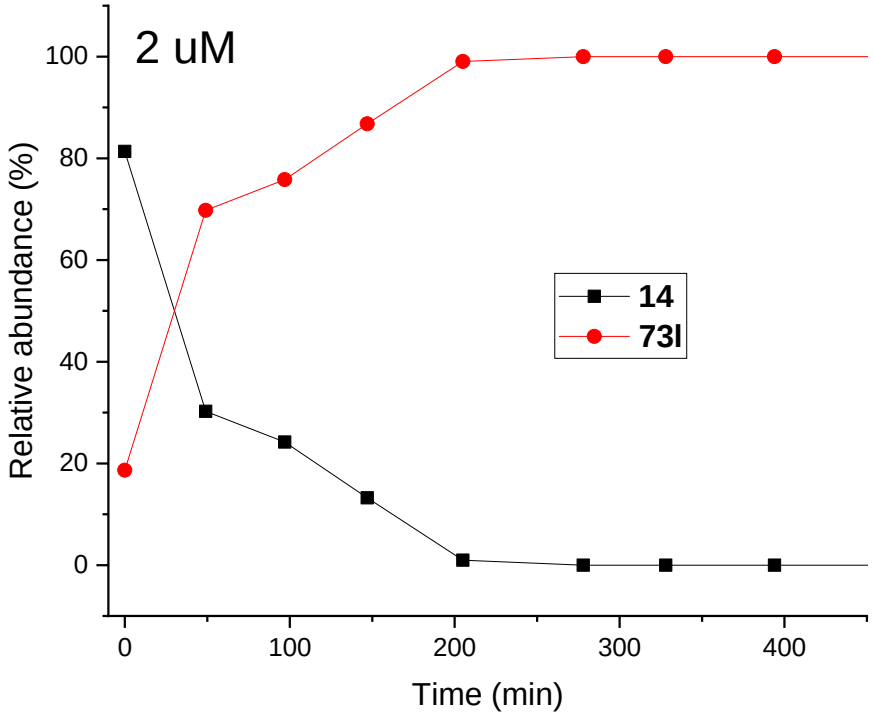


Figure S5: 2 µM reaction profile of **ACYA (14)** labeling under dilution conditions.

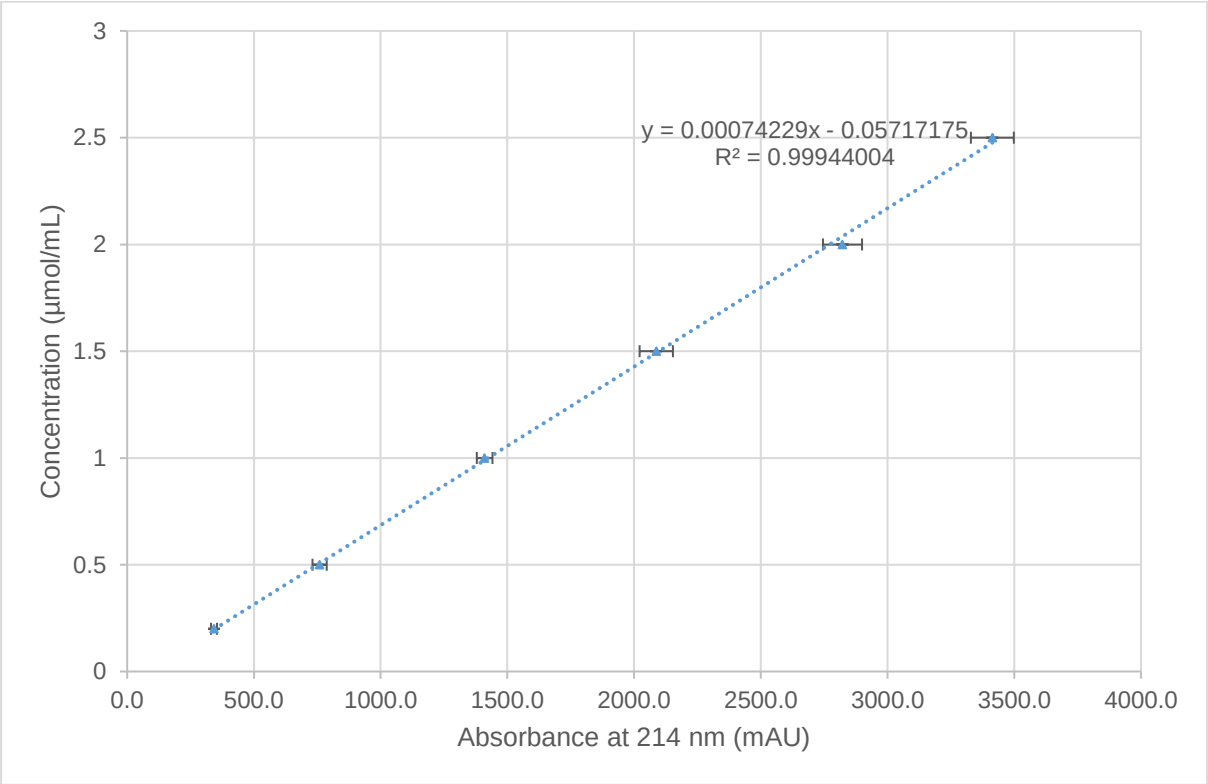
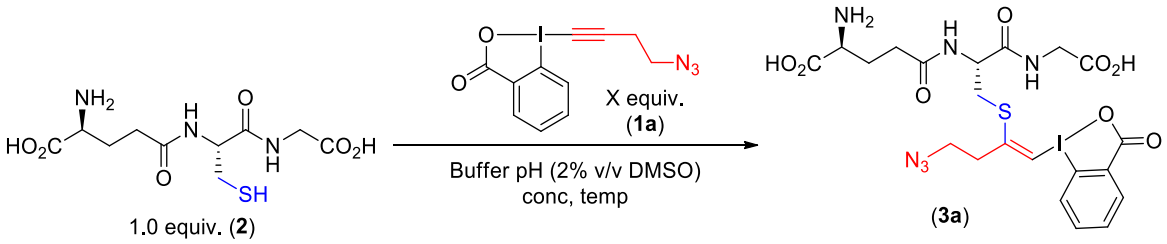


Figure S6: Calibration curve of **32a**.

Table S1: Reaction conditions evaluation on glutathione (**2**)



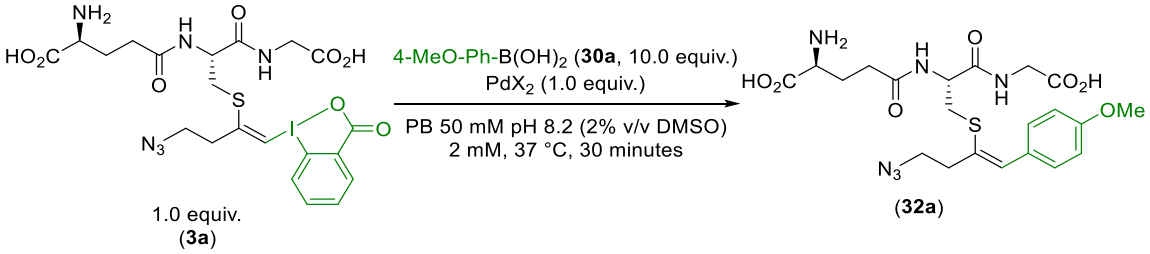
Entry	Buffer	pH	GSH conc.	Temperature	N_3-EBX equiv.	Time	Yield
1	10 mM PBS	6.5	2 mM	r.t.	3.00	20 min	25%
						60 min	54%
2	10 mM PBS	6.5	2 mM	r.t.	10.0	20 min	57%
						60 min	88%
3	10 mM PBS	7.4	2 mM	r.t.	1.20	20 min	54%
						60 min	74%
4	10 mM Tris	7.8	2 mM	r.t.	1.20	20 min	48%
						60 min	64%
5	10 mM Tris	8.0	2 mM	r.t.	1.20	20 min	71%
						60 min	89%
6	10 mM Tris	8.2	2 mM	r.t.	1.20	5 min	55%
						20 min	81%
						60 min	87%
7	10 mM PBS	8.2	2 mM	r.t.	1.20	20 min	75%
						60 min	91%
8	10 mM HEPES	8.2	2 mM	r.t.	1.20	20 min	77%
						60 min	85%

9	1 mM Tris	8.2	2 mM	r.t.	1.20	60 min	< 5%
10	100 mM Tris	8.2	2 mM	r.t.	1.20	20 min	78%
						60 min	97%
11	1 M Tris	8.2	2 mM	r.t.	1.20	20 min	66%
						60 min	66%
12	10 mM Tris	8.2	2 mM	4 °C	1.20	20 min	67%
						60 min	86%
13	10 mM Tris	8.2	2 mM	37 °C	1.20	20 min	78%
						60 min	87%
14	10 mM Tris	8.2	1 mM	r.t.	1.20	20 min	65%
						60 min	81%
15	10 mM Tris	8.2	200 µM	r.t.	1.20	20 min	45%
						60 min	76%
16	10 mM Tris	8.2	200 µM	r.t.	3.00	20 min	76%
						60 min	93%
17a	10 mM Tris	8.2	2 mM	r.t.	1.20	20 min	78%
						60 min	86%
18[b]	10 mM Tris	8.2	2 mM	r.t.	1.20	16 h	0%
19[c]	10 mM Tris	8.2	2 mM	r.t.	1.20	16 h	0%
20	10 mM Tris	8.2	2 mM	r.t.	2.00	5 min	82%
21	10 mM Tris	8.2	2 mM	r.t.	3.00	5 min	98%

a) The reaction was performed without DMSO. b) The reaction was performed with 1-(4-azidobut-1-ynyl)-3,3-bis(trifluoromethyl)-3(1H)-1,2-benziodoxole (**1m**) instead of (4-azidobut-1-ynyl)-1,2-benziodoxol-3(1H)-one (**1a**). c) (E)-1-(2-Cyclohexylvinyl)-1,2-benziodoxol-3(1*H*)-one (**1n**) or (E)-1-

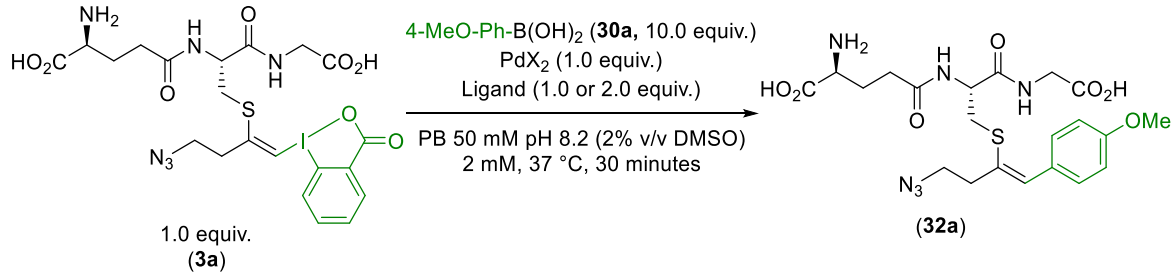
styryl-1,2-benziodoxol-3(1*H*)-one (**1o**) were used instead of (4-azidobut-1-ynyl)-1,2-benziodoxol-3(1H)-one (**1a**).

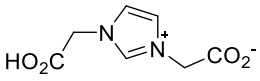
Table S2: Screening of ligandless palladium catalysts for the synthesis of cross-coupled product **32a**.



Entry	Palladium source	Yield	Unreacted intermediate
1	$Pd(OAc)_2$	0%	99%
2	$Pd(TFA)_2$	40%	0%
3	$Pd(NO_3)_2$	4%	96%
4	K_2PdCl_4	39%	0%
5	Na_2PdCl_4	36%	0%
6	Li_2PdCl_4	45%	0%

Table S3: Screening of non-preformed palladium plus ligand catalysts for the synthesis of cross-coupled product **32a**.





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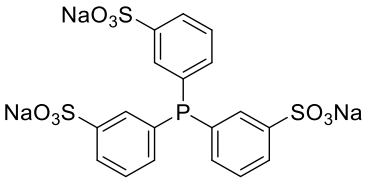

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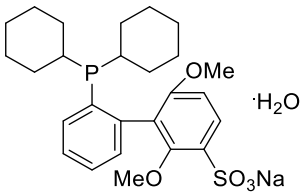

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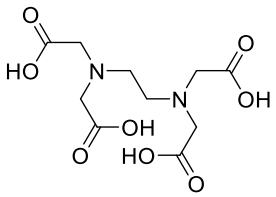

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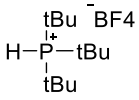
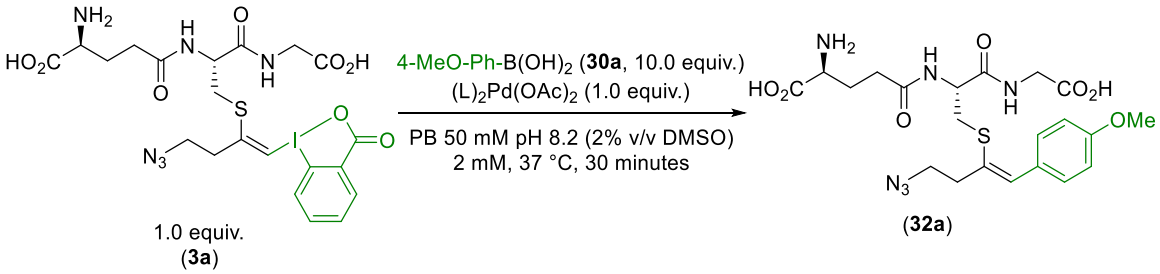


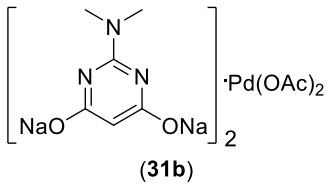
(**112**)

Entry	Palladium source	Ligand	L:Pd	Yield	Unreacted intermediate
1	Na_2PdCl_4	65	1:1	38%	0%
2	Na_2PdCl_4	65	2:1	42%	0%
3	Na_2PdCl_4	87	1:1	0%	0%
4	Na_2PdCl_4	87	2:1	0%	0%
5	Na_2PdCl_4	88	1:1	0%	12%
6	Na_2PdCl_4	88	2:1	0%	12%
7	Na_2PdCl_4	89	1:1	38%	0%
8	Na_2PdCl_4	89	2:1	30%	0%
9	Na_2PdCl_4	90	1:1	0%	88%

10	Na_2PdCl_4	90	2:1	0%	89%
11	$Pd(OAc)_2$	65	1:1	0%	92%
12	$Pd(OAc)_2$	65	2:1	0%	96%
13	$Pd(OAc)_2$	87	1:1	0%	46%
14	$Pd(OAc)_2$	87	2:1	0%	15%
15	$Pd(OAc)_2$	89	1:1	0%	93%
16	$Pd(OAc)_2$	89	2:1	0%	98%
17	$Pd(OAc)_2$	90	1:1	0%	93%
18	$Pd(OAc)_2$	90	2:1	0%	91%
19	$Pd(TFA)_2$	65	1:1	42%	0%
20	$Pd(TFA)_2$	65	2:1	41%	0%
21	$Pd(TFA)_2$	87	1:1	0%	0%
22	$Pd(TFA)_2$	87	2:1	0%	0%
23	$Pd(TFA)_2$	88	1:1	0%	29%
24	$Pd(TFA)_2$	88	2:1	0%	22%

Table S4: Screening of preformed palladium catalysts for the synthesis of cross-coupled product **32a**.

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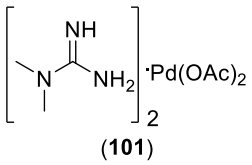
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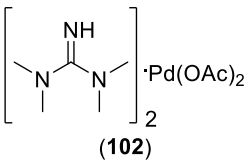
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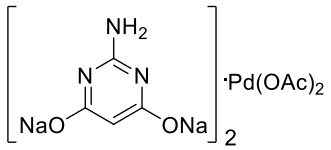
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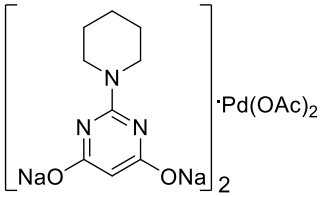
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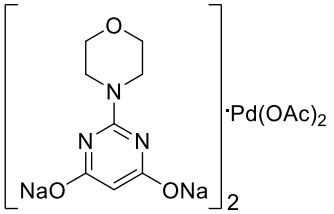
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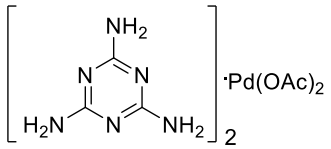
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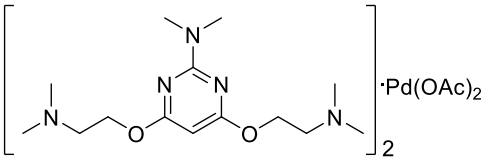
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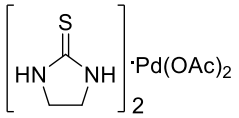
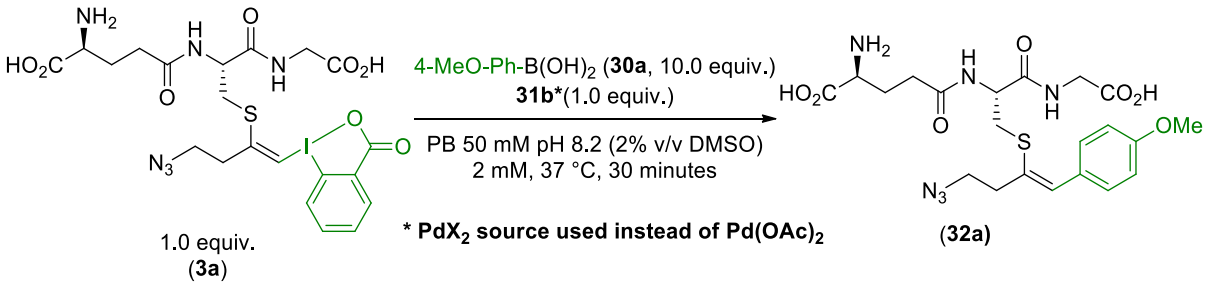

(**108**)

Entry	Palladium complex	Yield	Unreacted intermediate
1	22b	55%	0%
2	77	14%	0%
3	79	4%	61%
4	80	0%	99%
5	81	16%	12%
6	82	3%	58%
7	83	0%	99%
8	84	42%	0%

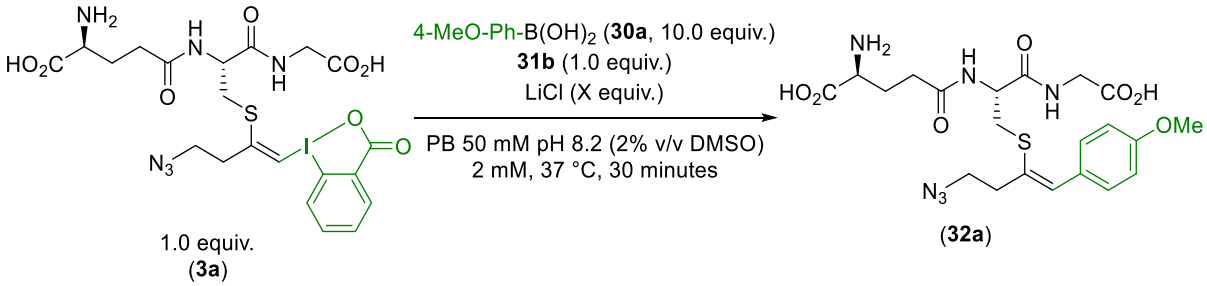
9	86	0%	99%

Table S5: Screening of palladium sources with ligand **92** for the synthesis of cross-coupled product **32a**.



Entry	Palladium source	Yield	Unreacted intermediate
1	$Pd(TFA)_2$	57%	0%
2	$Pd(NO_3)_2$	52%	0%
3	K_2PdCl_4	47%	0%
4	Na_2PdCl_4	50%	0%

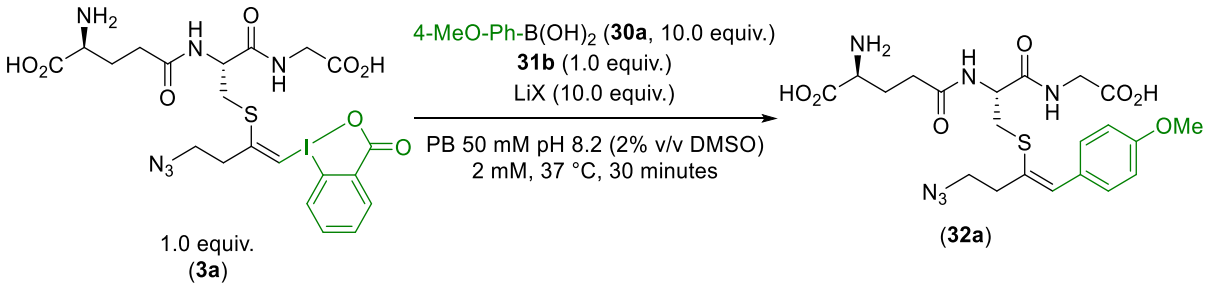
Table S6: Screening of LiCl addition for the synthesis of cross-coupled product **32a**.



Entry	LiCl equivalents	Yield	Unreacted intermediate
1	1.00 equiv.	61%	0%
2	2.00 equiv.	62%	0%
3	5.00 equiv.	61%	0%

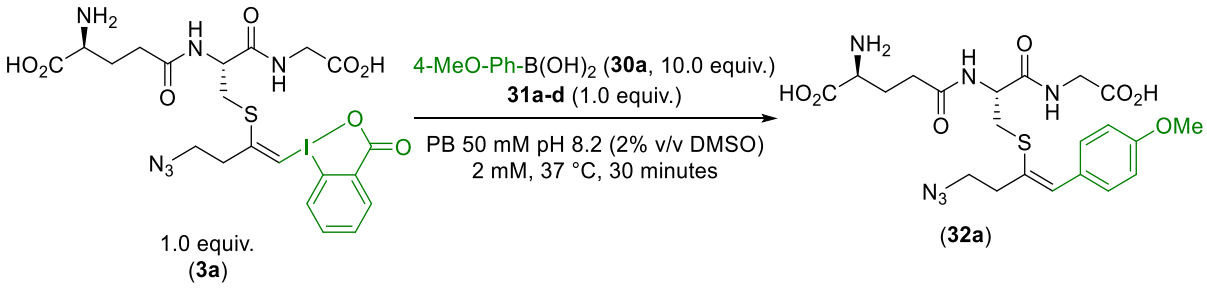
4	10.0 equiv.	61%	0%
5	20.0 equiv.	61%	0%

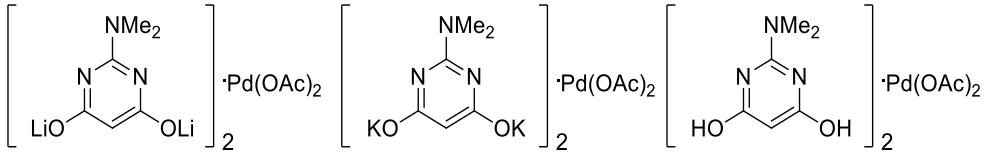
Table S7: Screening of Lithium source for the synthesis of cross-coupled product **32a**



Entry	Lithium source	Yield	Unreacted intermediate
1	LiF	57%	0%
2	LiBr	66%	0%
3	LiI	26%	0%
4	$LiNO_3$	60%	0%
5	LiTFA	58%	0%

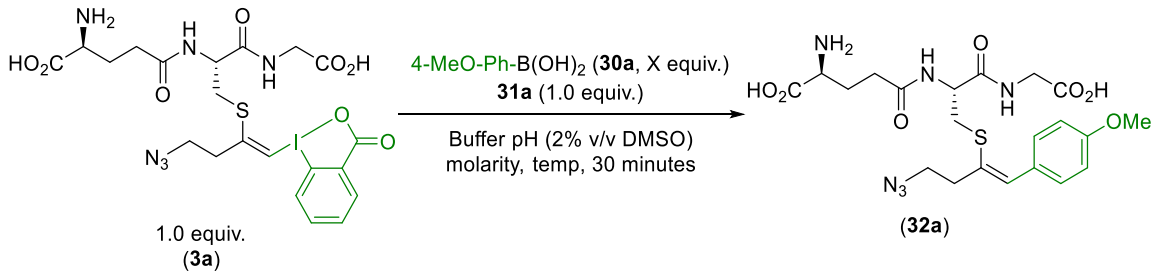
Table S8: Screening of ligand **92**-based catalysts for the synthesis of cross-coupled product **32a**





Entry	Palladium complex	Yield	Unreacted intermediate
1	22a	70%	0%
2	22b	51%	0%
3	22d	15%	0%

Table S9: Screening of buffer and temperature conditions for the synthesis of cross-coupled product **32a**



Entry	Buffer	pH	GSH conc.	Temperature	X equiv.	Yield
1	50 mM PB	8.2	2 mM	37 °C	10.0	70%
2	50 mM PB	8.2	2 mM	r.t.	10.0	53%
3a	50 mM PB	8.2	2 mM	37 °C	10.0	51%
4	50 mM HEPES	8.2	2 mM	37 °C	10.0	51%
5	50 mM Tris	8.2	2 mM	37 °C	10.0	19% (51%[b])
6	10 mM PB	8.2	2 mM	37 °C	10.0	41%
7	100 mM PB	8.2	2 mM	37 °C	10.0	58%
8	Water		2 mM	37 °C	10.0	15%
9	50 mM PB	7.4	2 mM	37 °C	10.0	50%
10	50 mM PB	9.0	2 mM	37 °C	10.0	59%
11	50 mM PB	8.2	1 mM	37 °C	10.0	59%
12	50 mM PB	8.2	200 µM	37 °C	10.0	63%
13	50 mM PB	8.2	2 mM	37 °C	5.00	32%
14	50 mM PB	8.2	2 mM	37 °C	2.00	9%
15	50 mM PB	8.2	2 mM	37 °C	1.00	< 5%

a) The reaction was performed without DMSO. b) The reaction was analyzed after 2 hours.

Supplemental Experimental Procedures

1. General procedures

All reactions using anhydrous conditions were performed with oven-dried glassware, under an atmosphere of nitrogen, unless stated otherwise. Tetrahydrofuran, acetonitrile, diethyl ether and dichloromethane (DCM) were dried by passage over activated alumina, under nitrogen atmosphere, on an Innovative Technology Solvent Delivery System (water content < 10 ppm, Karl-Fischer titration). Dichloroethane and ethanol were purchased from Acros and trifluoroethanol was purchased from Fluorochem. DMSO was purchased from Sigma-Aldrich. All the Fmoc-protected amino acids and Rink Amide MBHA resin were purchased from GL Biochem. O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU, GL Biotech), N,N-di*iso*propylethylamine (DIPEA, Iris Biotech GmbH) and hydroxybenzotriazole (HOBt, GL Biotech) were used as received. Oxytocin and somatostatin were purchased from Bachem AG and used without further purification. All the other reagents were purchased from ABCR, Acros, AlfaAesar, Apollo Scientific, Fluorochem, Fluka, Roth, Sigma-Aldrich and TCI and were used as such. For flash chromatography, distilled technical grade solvents were used. Chromatographic purification was performed as flash chromatography using Macherey-Nagel silica 40-63, 60 Å, using the solvents indicated as eluent with 0.1 – 0.5 bar pressure. TLC was performed on Merck silica gel 60 F_{254} TLC aluminum plates and visualized with UV light or permanganate stain. Melting points were measured on a Büchi B-540 melting point apparatus using open glass capillaries. The data is uncorrected. ^{1}H-NMR spectra were recorded on a Brucker DPX-400 400 MHz spectrometer in $CDCl_3$, DMSO-d_6 or D_2O. All signals are reported in ppm with the internal $CHCl_3$ signal at 7.26 ppm, the internal DMSO signal at 2.50 ppm or the internal H_2O 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.^{13}C-NMR spectra were recorded with ^{1}H-decoupling on a Brucker DPX-400 100 MHz spectrometer in $CDCl_3$, DMSO-d_6 or D_2O. All signals are reported in ppm with the internal $CHCl_3$ signal at 77.0 ppm or the internal DMSO signal at 39.5 ppm as standard. Spectra were fully assigned using COSY, HSQC, HMBC and ROESY. 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 labeling and Suzuki-Miyaura cross-coupling reactions were set up on the benchtop and carried out under ambient conditions (without oxygen exclusion). Buffers were not degassed and prepared with milliQ water. For Suzuki-Miyaura cross-coupling reactions, the experiments were heated up using an Eppendorf Thermomixer 5436. All the reactions were replicated at least twice and the furnished yield is an average of these replicates.

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 µm). Water:acetonitrile 95:5 (solvent A) and water:acetonitrile 5:95 (solvent B), each containing 0.1% formic acid, were used as the mobile phase, at a flow rate of 0.6 mL/min^{-1}. The gradient was programmed as follows: 100% A to 100% B in 20 minutes. 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. In few cases, the gradient was programmed as follows: 100% A to 100% B in 40 minutes, in order to resolve peak overlapping. The other parameters were untouched. 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 µm) and submitted to the mass spectrometry service of ISIC at the EPFL that uses a MICROMASS (ESI) Q-TOF Ultima API. Recurrent HPLC peaks: Tris/HEPES/Phosphate buffers (4.0-4.2 minutes), DMSO (4.8-5.0 minutes) and formic acid contaminant (5.5-6.5 minutes, wide peak).

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 µm). Water (solvent A) and water:acetonitrile 5:95 (solvent B), each containing 0.1% TFA, 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% A isocratic for 5 minutes followed by 100% A to 100% B in 20 minutes then isocratic for 5 minutes.

Method B: The gradient was programmed as follows: 95% A to 78% A in 20 minutes then 78% A to 100% B in 10 minutes then isocratic for 5 minutes.

Method C: The gradient was programmed as follows: 100% A isocratic for 10 minutes followed by 100% A to 50% A in 10 minutes. Then, 50% A to 100% B in 5 minutes followed by isocratic for 5 minutes.

Method D: The gradient was programmed as follows: 100% A to 100% B in 30 minutes.

c. Methods for ubiquitin, nonamer peptide, Substance P analogue and histone octamers

HPLC analysis:

Analytical RP-HPLC analysis was performed on an Agilent 1260 series instrument using an analytical Agilent Zorbax C18 column (150 × 4.6 mm, 5 µm particle size) at a flow rate of 1.0 mL/min. All RP-HPLC analyses were done with 0.1% (v/v) trifluoroacetic acid (TFA) in water (RP-HPLC solvent A) and 90% acetonitrile with 0.1% (v/v) TFA in water (RP-HPLC solvent B) as mobile phases. Typically, a gradient from 0-70% solvent A to solvent B over 30 minutes was used for analytical RP-HPLC analyses unless otherwise stated.

Prep HPLC analysis:

Purification of proteins on a semi-preparative scale was performed on an Agilent 1260 series instrument using an semi-preparative Agilent Zorbax C18 column (250 × 9.4 mm, 5 µm particle size) at a flow rate of 4 mL/min. Preparative RP-HPLC purifications were done on an Agilent 1260 preparative HPLC system with a preparative Agilent Zorbax C18 column (250 × 21.2 mm, 7 µm particle size) at a flow rate of 20 mL/min.

ESI-MS analysis:

Electrospray ionization mass spectrometric (ESI-MS) analysis was conducted on a Shimadzu MS2020 single quadrupole instrument connected to a Nexera UHPLC system. Mass spectra were acquired by electrospray ionization in positive ion mode in the mass range of 200-2000m/z.

Top-down LC-HCD-MS/MS analysis of ubiquitin and H4 histone protein:

For LC-MS and LC-MS/MS analysis, samples were separated on an Acquity UPLC Protein BEH C4 column (300 Å, 1.7 µm, 1 x 150 mm, at 60°C) using a gradient from 15% to 25% solvent B in 1 min, then from 25 to 32 % solvent B in 11 min with flow rate of 90 µl/min (Solvents: solvent A (H_2O with 0.1 % formic acid (FA)) and solvent B (acetonitrile with 0.1 % FA)). Eluting proteoforms were analysed on a QExactive HF Orbitrap-FT-MS (Thermo Fisher Scientific, Bremen, Germany). MS1 and MS2 scans were performed in protein mode, with 120'000 and 240'000 resolution, respectively, averaging 10 µscans. HCD (higher-energy collisional dissociation) fragmentation was performed at 2 different NCE (normalized collision energy) values for each protein using PRM mode with wide isolation window.

MS1 data were analysed with Protein Deconvolution 4.0 software (Thermo Fisher Scientific) using Xtract algorithm, 95% fit factor. MS2 data were deconvoluted with Mash Suite software (GE research group, University of Wisconsin) with 70% fit factor and S/N 0.01. For each protein 2 different NCE conditions for HCD-MS/MS analysis were used to create a fragmentation map with assigned b- and y-fragments using ProSight Lite software (Kelleher research group) with 15 ppm mass accuracy. Key fragments were validated manually. All displayed masses are monoisotopic ones.

3. Peptides, ubiquitin, histone proteins and histone octamers preparation

a. 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). The coupling was carried out by shaking Rink Amide MBHA 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-di*iso*propylethylamine (DIPEA, 6.0 equiv.), in dimethylformamide (DMF, 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).

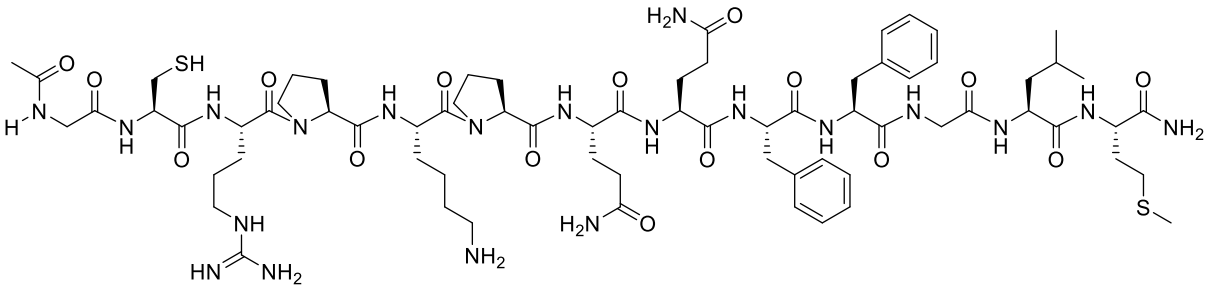
Peptide cleavage and deprotection:

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 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. At the same time, low-resolution mass spectrometric measurements were also acquired. In order 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 µm) and submitted to the mass spectrometry service of ISIC at the EPFL that uses a MICROMASS (ESI) Q-TOF Ultima API.

b. Preparation of Substance P analogue (**35**)



(**35**)

The peptide (**35**) was synthesised by the Tribute peptide synthesiser (PTI) on Rink-Amide resin. The synthesis was performed on 0.1 mmol scale using Fmoc chemistry. Fmoc N-protected amino acids with

standard acid labile protecting groups were employed. To maximise synthesis yield, amino acids were double coupled where necessary. Briefly, the N-terminal Fmoc-group was deprotected by incubating the resin with 20% (v/v) piperidine in DMF. Activation of amino acid (0.50 mmol, 5.00 eq.) was achieved by addition of O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU, 0.48 mmol, 4.80 eq.) and DIPEA (1.00 mmol, 10.0 eq.). The coupling step was performed by adding the activated amino acid to the resin, followed by 30 min incubation at room temperature. When the full-length peptide was assembled, the peptidyl-resin was washed with DMF, and transferred to a reaction vessel for manual SPPS. Acetylation of the N-terminus was achieved by incubation of the peptidyl-resin with 15 mL of 10% acetic anhydride, 10% DIPEA in DMF for 30 min under N_2 bubbling. To ensure maximal yields, the acetylation reaction was repeated. The peptidyl-resin was washed with DMF, DCM and methanol and dried under vacuum. The peptide was cleaved from the resin using a cleavage solution of 2.5% TIS, 2.5% H_2O in TFA. The crude peptide was precipitated by addition of ice-cold diethyl ether, recovered by centrifugation, dissolved in 50% (v/v) acetonitrile in H_2O, flash-frozen and lyophilised. Purification of the crude peptide was performed on a preparative RP-HPLC employing a linear gradient from 20 to 60% solvent B over 30 min. Fractions were analysed, pooled accordingly and lyophilised (yield: 28%). Purified peptide **35** was analysed and characterised by analytical RP-HPLC and ESI-MS (retention time:19.380 minutes).

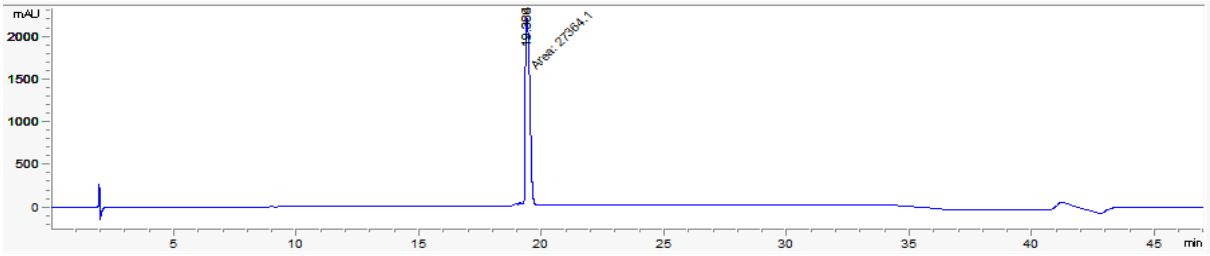


Figure S7: HPLC-UV chromatogram at 214 nm of **35**.

ESI-MS: Calcd. mass 1549.9 Da; Found 1549.3 Da.

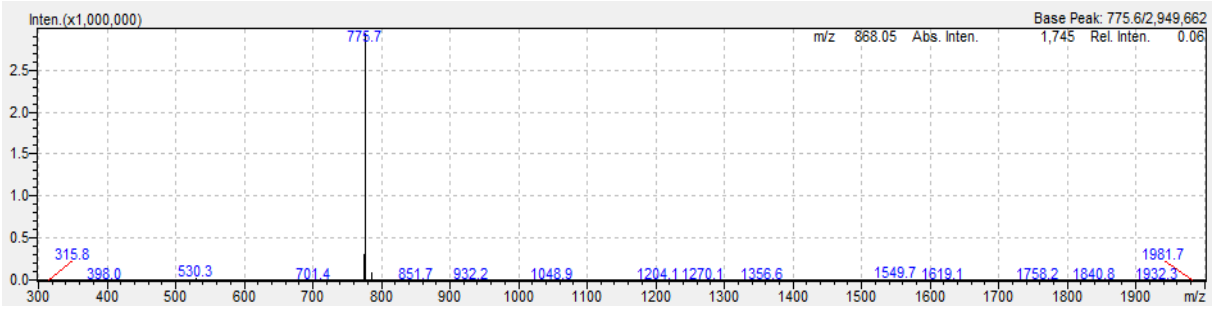


Figure S8: ESI-MS spectrum of **35.**

c. Preparation of cys-ubiquitin (**17**), H4E63C histone protein (**42**) and human wild-type core histones (**43**)

Instrumentation:

Bacterial cells for recombinant protein expression were grown in an HT infors AG incubator. E. coli cells were lysed by sonication using a Vibra-cell VCX 750 Sonics & Materials sonicator. Sedimentations were accomplished in an Avanti J20 XPI centrifuges and rotors (JA-12 and JA-8.1000) from Beckman Coulter. The Mini-Protean II system for SDS-PAGE was from BioRad. Gels were imaged using a ChemiDoc MP imaging system from BioRad.

Expression and purification of cys-ubiquitin (**17**):

Cys-ubiquitin (**17**) was recombinantly expressed as N-terminal fusion to a his6-tag in E. coli BL21(DE3)pLysS cells. Cells were grown in LB medium (supplemented with 100 µg/mL ampicillin as selection marker) at 37 °C until reaching an OD_{600} of 0.6. Protein expression was induced with 0.2 mM (isopropylthio-β-galactoside) IPTG and cells were further incubated at 37 °C for 4 hours. Subsequently, cells were harvested by centrifugation (4000 g, 4 °C, 10 minutes) and the cell pellet was resuspended in lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8). Cells were lysed by sonication and cell lysate was centrifuged (15000 g, 4 °C, 15 minutes). The lysate supernatant was applied to Ni- NTA resin previously equilibrated with lysis buffer. The protein was bound to the resin by gentle nutating for 1 hour at 4 °C. The flowthrough was collected and the resin was washed with 2 x column volumes (CV) wash buffer I (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, 1 mM 2-mercaptoethanol, pH 8) followed by 2 x CV wash buffer II (50 mM sodium phosphate, 300 mM sodium chloride, 50 mM imidazole, 1 mM 2-mercaptoethanol, pH 8). Finally, the protein was eluted with 6 x 500 µL elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole, 1 mM 2-mercaptoethanol, pH 8). Fractions were analyzed by SDS-PAGE, protein-containing fractions were pooled. The protein was incubated at 4°C for 30 minutes with 10 mM TCEP and submitted to preparative RP-HPLC purification (30 - 70% HPLC solvent B over 30 minutes). Collected fractions were analyzed by analytical RP-HPLC and ESI-MS (retention time: 21.268 minutes). Pure fractions were pooled, lyophilized and stored at - 20 °C. Typical yields were 5 mg of cys-ubiquitin (**17**) per liter bacterial culture.

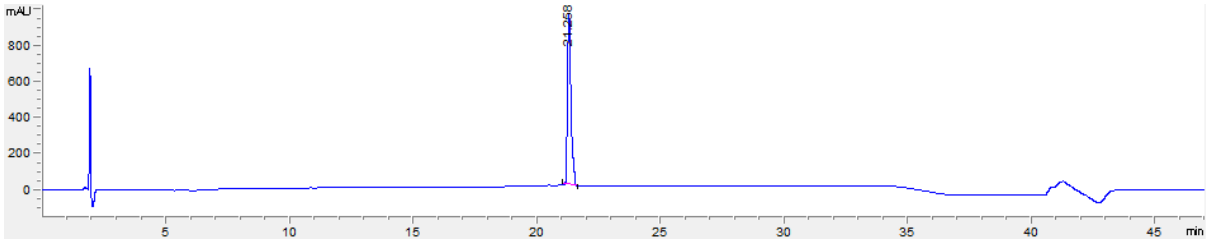


Figure S9: HPLC-UV chromatogram at 214 nm of Cys-Ubiquitin (**17**).

ESI-MS: Calcd mass 10700.1 Da; Found 10699.6 Da.

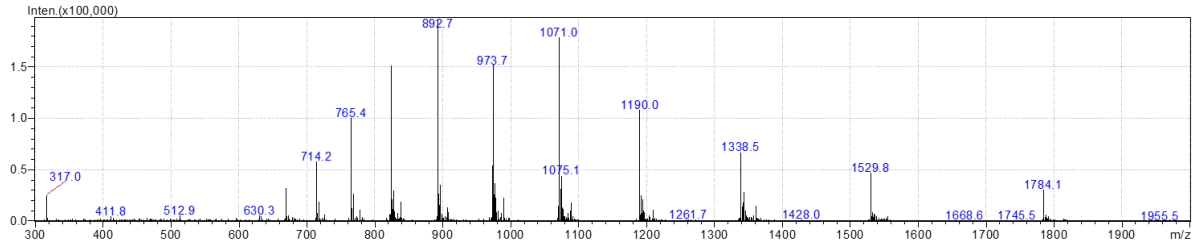


Figure S10: ESI-MS spectrum of Cys-Ubiquitin (**17**).

Expression and purification of H4E63C (**42**):

H4E63C was recombinantly expressed in E. coli BL21(DE3)pLysS cells. Cells were grown in LB medium (supplemented with 100 µg/mL ampicillin as selection marker) at 37 °C until reaching an OD_{600} of 0.6. Protein expression was induced with 0.4 mM IPTG and cells were further incubated at 37 °C for 3 hours. Subsequently, cells were harvested by centrifugation (4000 g, 4 °C, 10 minutes) and the cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, 200 mM sodium chloride, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM Phenylmethanesulfonylfluoride (PMSF), 1x Protease inhibitors (Roche, 1 tablet per 50 mL), pH 7.5). Cells were lysed by sonication and cell lysate was centrifuged (15000 g, 4 °C, 20 minutes). The pelleted insoluble inclusion bodies were washed twice with lysis buffer containing 1% Triton X-100 and once with lysis buffer without detergent. Inclusion bodies were soaked in DMSO for 30 min at room temperature and solubilized in resolubilization buffer (7 M guanidinium-hydrochloride (GdmHCl), 20 mM Tris-HCl, 10mM (1,4-dithiothreitol) DTT, pH 7.5) for 1 h at room temperature while stirring, centrifuged (15000xg, 4°C, 15min) to remove insoluble material and the supernatant dialyzed

overnight against buffer A (6 M GdmHCl, 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 10mM DTT, 0.2 mM PMSF, pH 7.5). The dialyzed inclusion bodies were subjected to Sepharose ion exchange chromatography (HiTrap SP FF 5 mL) using buffer A and a gradient from 10% to 100% buffer B (6 M GdmHCl, 10 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 10mM DTT, 0.2 mM PMSF, pH 7.5) over 15 column volumes (CV). Elution fractions were analyzed by SDS-PAGE (15% polyacrylamide gel), histone containing fractions were pooled and dialyzed overnight against 1% acetic acid at 4°C. The purified histone H4E63C were analyzed by analytical RP-HPLC and ESI-MS, flash frozen with liquid N_2, lyophilized and stored at -20°C as a dry powder (retention time: 21.076 minutes). Typical yields were 50 mg of H4E63C (**42**) per liter bacterial culture.

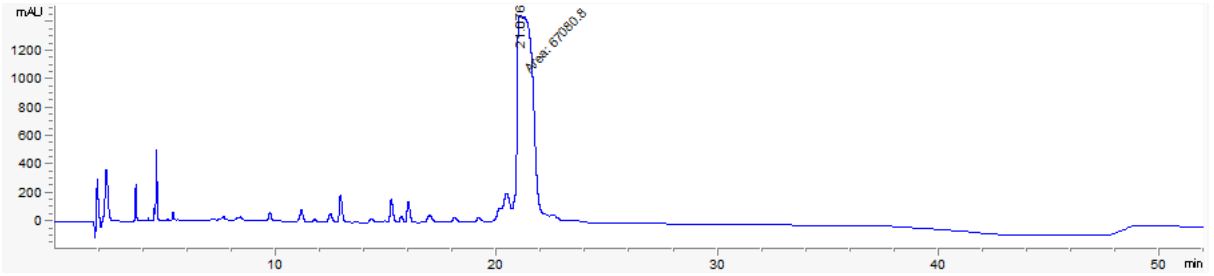


Figure S11: HPLC-UV chromatogram at 214 nm of **42.**

ESI-MS Calcd mass 11210 Da; Found 11209 Da.

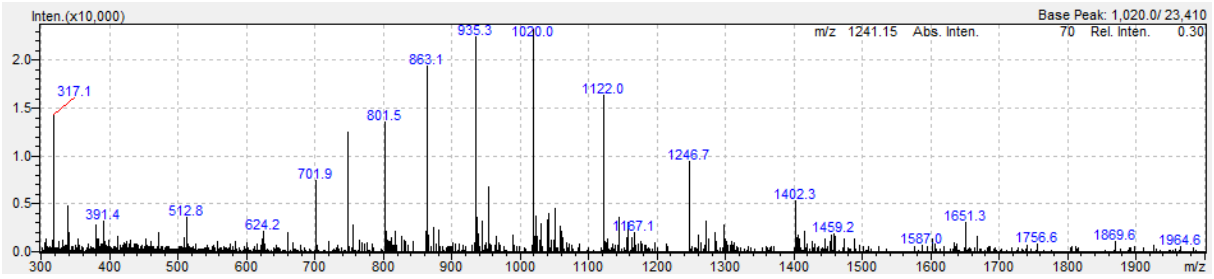


Figure S12: ESI-MS spectrum of **42.**

Expression and purification of human wild-type core histones (**43**):

Human wild-type core histones H2A, H2B, H3_C110A and H4 were expressed and purified as described previously.[1]

d. Refolding and purification of H4E63C (**27**) and unmodified histone octamers (**43**)

Lyophilised recombinant modified histone proteins were dissolved in unfolding buffer (6 M GdmHCl, 20 mM Tris-HCl, 1 mM EDTA, 5 mM DTT, pH 7.5 at 4°C). The concentration of each histone was determined by UV-absorbance measurements, and calculated using the extinction coefficients and molar weights listed below:

Table S10: Extinction coefficients and molecular weights used in the determination of histone concentration.

Histone	**Extinction coefficient (280nm)**	**Molecular weight**
hH2A	4470 $M^{-1}cm^{-1}$	13964 g/mol

hH2B	7450 $M^{-1}cm^{-1}$	13759 g/mol
hH3_C110A	4470 $M^{-1}cm^{-1}$	15209 g/mol
hH4	5970 $M^{-1}cm^{-1}$	11236 g/mol
H4E63C	5970 $M^{-1}cm^{-1}$	11210 g/mol

1 equiv. of H3 was mixed with 1 equiv. H4, 1.1 equiv. hH2A and 1.1 equiv. hH2B to a final protein concentration of 1 mg/mL in unfolding buffer. The solution was transferred in a Slide-A-Lyzer™ dialysis cassette (7K molecular weight cut-off (MWCO)) and dialysed at 4° C overnight against refolding buffer (2 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 8.3 at 4°C). The refolded octamers (**27**) or unmodified (**43**) were removed from the dialysis cassette and concentrated with a centrifugal concentrator Vivaspin500 (10K MWCO) to a final concentration of 40 μM. Subsequently, they were purified by size exclusion chromatography (SEC) on a Superdex S200 10/300GL column in refolding buffer (w/o DTT). Octamer containing fractions were pooled and the octamers concentration was quantified by UV-absorbance measurements. 2 equiv. DTT were added to the solution, the octamers were concentrated to a final concentration of 40 μM and stored at 4°C. Octamers purity was confirmed by SDS-PAGE (17% or 15% polyacrylamide gel).

SEC chromatograms:

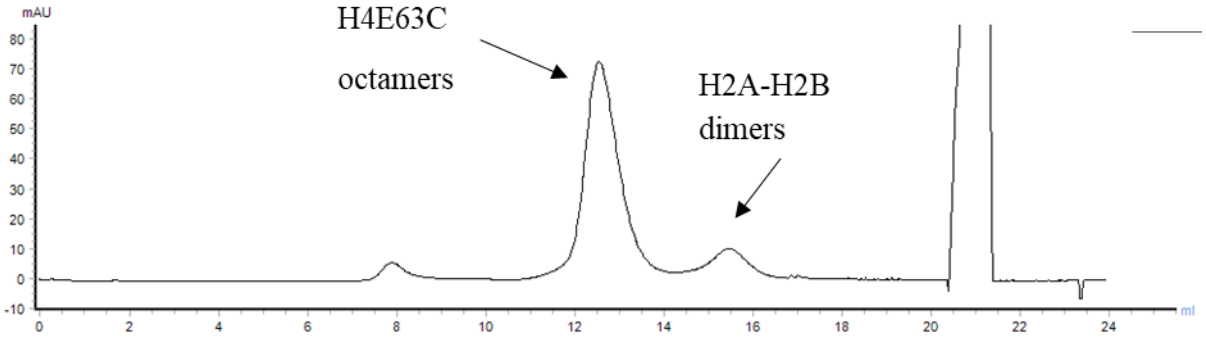


Figure S13: Size exclusion chromatogram of H4E63C octamers (**27**).

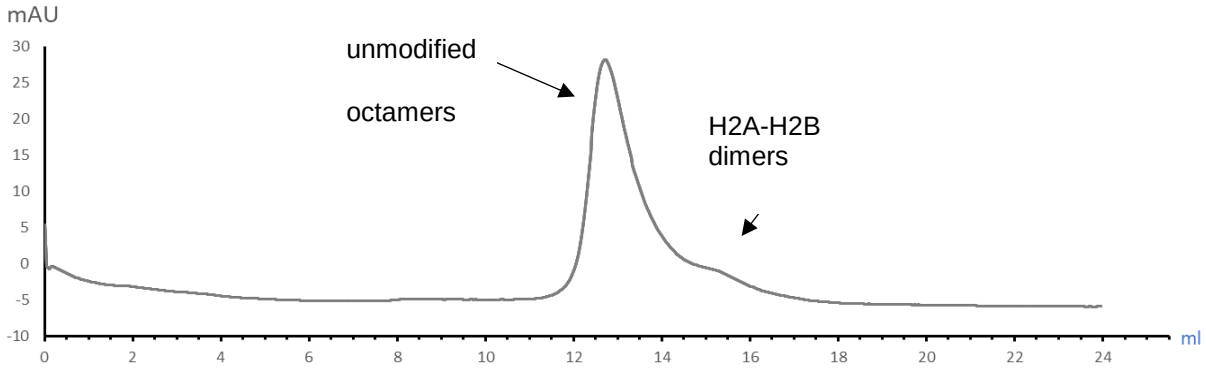


Figure S14: Size exclusion chromatogram of unmodified octamers (**43**).

SDS-PAGE:

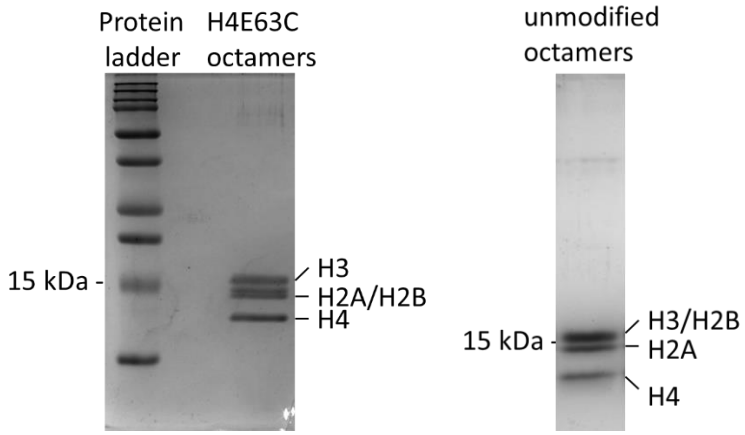
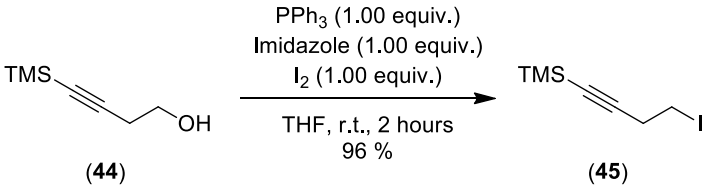


Figure S15: SDS-PAGE gel of H4E63C octamers (**27**) and unmodified octamers (**43**).

4. Preparation of hypervalent iodine reagents (EBX)

a. Syntheses of alkyne substrates

(4-Iodo-but-1-yn-1-yl)trimethylsilane (**45**)



(**44**) (**45**)

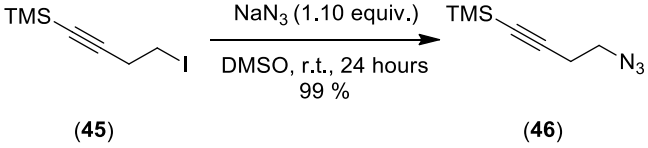
Following a slightly modified procedure,[2] triphenylphosphine (PPh_3, 37.9 g, 145 mmol, 1.00 equiv.) was added to a cooled solution of 4-(trimethylsilyl)but-3-yn-1-ol (**44**) (20.6 g, 145 mmol, 1.00 equiv.) in tetrahydrofuran (545 mL) at 0 °C. Upon dissolution, imidazole (9.84 g, 145 mmol, 1.00 equiv.) was added, followed by iodine (I_2, 36.7 g, 145 mmol, 1.00 equiv.). The resulting mixture was then allowed to warm to room temperature and was stirred for 2 hours. It was then diluted with diethyl ether (400 mL) and washed with 10% aqueous sodium thiosulfate (400 mL). The aqueous layer was extracted with additional portions of diethyl ether (2 x 150 mL) and the combined organic layers were washed with brine (400 mL), dried over magnesium sulfate, filtered and concentrated *in vacuo*. The resulting white suspension was filtered through a plug of silica, eluting with pentane, to afford pure (4-iodo-but-1-yn-1-yl)trimethylsilane (**45**) (34.9 g, 138 mmol, 96% yield) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.20 (t, *J* = 7.5 Hz, 2H, CH_2CH_2I), 2.78 (t, *J* = 7.5 Hz, 2H, CH_2CH_2I), 0.14 (s, 9H, TMS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 105.1, 86.9, 25.3, 1.1, 0.1.

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

(4-Azidobut-1-yn-1-yl)trimethylsilane (46)



(**45**) (**46**)

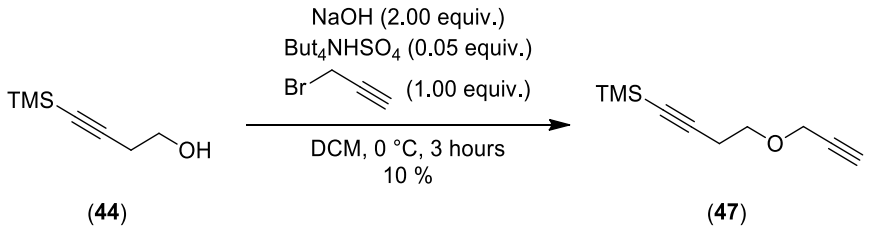
Following a slightly modified procedure,[4] (4-iodobut-1-yn-1-yl)trimethylsilane (**45**) (34.9 g, 138 mmol, 1.00 equiv.) was added to a 0.5 M solution of sodium azide in dimethyl sulfoxide (NaN_3, 304 mL, 152 mmol, 1.10 equiv.). The reaction mixture was stirred for 24 hours at room temperature, then slowly poured into a mixture of ice:water (800 mL). The aqueous layer was extracted with diethyl ether (3 x 300 mL) and the combined organic layers were washed with water (2 x 200 mL), brine (200 mL), dried over magnesium sulfate, filtered and concentrated under reduced pressure. The light yellow crude liquid was purified through a plug of silica, eluting with pentane, to afford pure (4-azidobut-1-yn-1-yl)trimethylsilane (**46**) (22.8 g, 136 mmol, 99% yield) as a colorless liquid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.37 (t, *J* = 6.8 Hz, 2H, $CH_2CH_2N_3$), 2.52 (t, *J* = 6.8 Hz, 2H, $CH_2CH_2N_3$), 0.15 (s, 9H, TMS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 102.8, 87.3, 49.8, 21.1, 0.0.

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

Trimethyl(4-(prop-2-yn-1-yloxy)but-1-yn-1-yl)silane (**47**)



(**44**) (**47**)

Following a reported procedure,[4] 4-(trimethylsilyl)but-3-yn-1-ol (**44**) (4.00 g, 28.1 mol, 1.00 equiv.) was dissolved in dichloromethane (60 mL) and the solution was cooled down at 0 °C. Then, tetrabutylammonium hydrogensulfate (But_4NHSO_4, 0.477 g, 1.41 mmol, 0.05 equiv.) was added, followed by sodium hydroxide (NaOH, 2.25 g, 56.2 mmol, 2.00 equiv.). The reaction mixture was stirred at this temperature for 5 minutes and then propargyl bromide (3.03 mL, 28.1 mmol, 1.00 equiv.) was added. The resulting yellow reaction mixture was stirred for 4 hours at 0 °C and quenched with water (60 mL) keeping the internal temperature at 0 °C. The aqueous layer was extracted with dichloromethane (60 mL), then the combined organic layers were dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude yellow oil was purified by column chromatography (SiO_2, Pentane:Ethyl acetate 99:1) affording pure trimethyl(4-(prop-2-yn-1-yloxy)but-1-yn-1-yl)silane (**47**) (1.44 g, 8.00 mmol, 10% yield) as a colorless liquid.

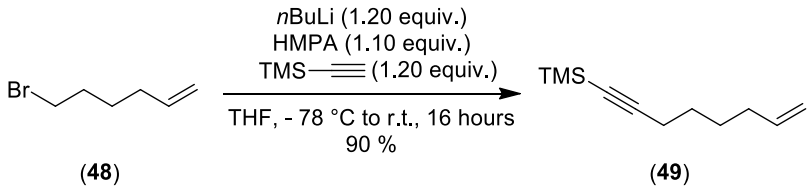
R_f 0.22 (Pentane:Ethyl acetate 99:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.19 (dd, *J* = 6.9, 2.4 Hz, 2H, CCC*H*$_2$O), 3.66 (q, *J* = 7.1 Hz, 2H, OC*H*$_2$), 2.53 (t, *J* = 7.2, 2H, OCH_2CH_2), 2.44 (dt, *J* = 3.2, 2.4 Hz, 1H, CC*H*), 0.14 (s, 9H, TMS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 103.3, 86.1, 79.6, 74.7, 68.3, 58.3, 21.3, 0.2.

Spectroscopic data was consistent with the values reported in literature.[4]

Trimethyl(oct-7-en-1-yn-1-yl)silane (**49**)



(**48**) (**49**)

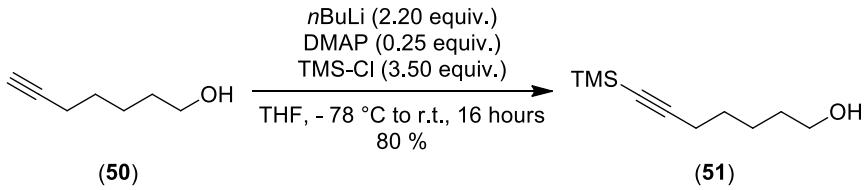
Following a reported procedure,[4] a cooled 2.5 M solution of *n*-butyllithium in hexanes (*n*BuLi, 6.99 mL, 17.5 mmol, 1.20 equiv.) was added dropwise to a stirred solution of trimethylsilylacetylene (2.49 mL, 17.5 mmol, 1.20 equiv.) in tetrahydrofuran (15 mL) at - 78 °C. After stirring for 1 hour at this temperature, a solution of 6-bromohex-1-ene (**48**) (2.05 mL, 14.6 mmol, 1.00 equiv.) and hexamethylphosphoramide (HMPA, 2.79 mL, 16.0 mmol, 1.10 equiv.) in THF (7.5 mL) was added dropwise. The mixture was stirred for 1 hour at - 78 °C, followed by 24 hours of stirring at room temperature. The solution was then cooled to 0 °C, quenched by a saturated aqueous solution of ammonium chloride (40 mL) and diluted with water (7.5 mL) and ethyl acetate (37.5 mL). The layers were separated and the aqueous layer extracted with ethyl acetate (3 x 40 mL). The combined organic layers were washed with water (2 x 75 mL), brine (75 mL), dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude liquid was purified through a plug of silica, eluting with pentane, to afford pure trimethyl(oct-7-en-1-yn-1-yl)silane (**49**) (2.35 g, 13.0 mmol, 90% yield) as a colorless liquid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (ddt, *J* = 16.9, 10.2, 6.7 Hz, 1H, CH_2CHCH_2), 5.04-4.93 (m, 2H, CH_2CHCH_2), 2.22 (t, *J* = 7.0 Hz, 2H, CH_2), 2.09-2.04 (m, 2H, CH_2), 1.55-1.46 (m, 4H, CH_2), 0.14 (s, 9H, TMS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 138.9, 114.7, 107.4, 84.5, 33.4, 28.2, 28.1, 20.1, 0.3.

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

7-(Trimethylsilyl)hept-6-yn-1-ol (**51**)



(**50**) (**51**)

Following a reported procedure,[9] hept-6-yn-1-ol (**50**) (5.00 g, 44.6 mmol, 1.00 equiv.) was dissolved in tetrahydrofuran (150 mL) and the solution was cooled down at - 78 °C. A cooled 2.5 M solution of *n*-butyllithium in hexanes (*n*BuLi, 39.2 mL, 98.0 mmol, 2.20 equiv.) was added dropwise, followed by 4-(dimethylamino)pyridine (DMAP, 1.36 g, 11.1 mmol, 0.25 equiv.). After stirring for 1 hour at this temperature, trimethylsilyl chloride (TMS-Cl, 20.4 mL, 156 mmol, 3.50 equiv.) was added dropwise. The mixture was then allowed to warm to room temperature. After 2 hours of stirring, the reaction was quenched with a 1.0 N aqueous hydrochloric acid (50 mL) and vigorously stirred at room temperature over 30 minutes. The mixture was then diluted with ethyl acetate (200 mL) and the layers were separated. The aqueous phase was extracted with additional portions of ethyl acetate (3 x 50 mL). The combined organic layers were collected, washed with a solution of saturated aqueous sodium bicarbonate (100 mL), brine (50 mL), dried over magnesium sulfate, filtered and concentrated *in vacuo*. After purification by column chromatography (SiO_2, Pentane:Ethyl acetate 4:1), 7-(trimethylsilyl)hept-6-yn-1-ol (**51**) (6.58 g, 35.7 mmol, 80% yield) was obtained as a colorless oil.

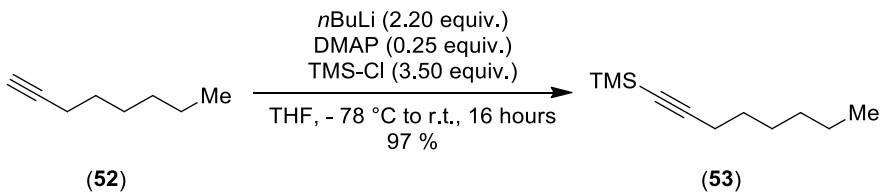
R_f 0.32 (Pentane:Ethyl acetate 4:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.65 (td, *J* = 6.5, 1.0 Hz, 2H, CH_2OH), 2.24 (td, *J* = 7.0, 1.0 Hz, 2H, $CCCH_2$), 1.63-1.51 (m, 4H, CH_2), 1.51-1.41 (m, 2H, CH_2), 0.14 (s, 9H, TMS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 107.4, 84.6, 62.9, 32.3, 28.6, 25.1, 19.9, 0.3.

Spectroscopic data was consistent with the values reported in literature.[7]

Trimethyl(oct-1-yn-1-yl)silane (**53**)



(**52**) (**53**)

Following a reported procedure,[9] oct-1-yne (**52**) (1.35 mL, 8.98 mmol, 1.00 equiv.) was dissolved in tetrahydrofuran (30 mL) and the solution was cooled down at - 78 °C. A cooled 2.5 M solution of *n*-butyllithium in hexanes (*n*BuLi, 7.91 mL, 19.8 mmol, 2.20 equiv.) was added dropwise, followed by 4-

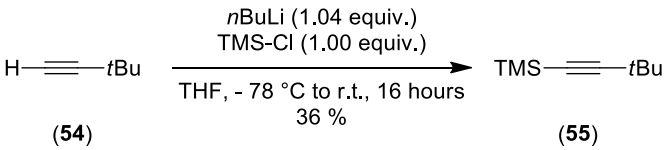
(dimethylamino)pyridine (DMAP, 274 mg, 2.25 mmol, 0.25 equiv.). After stirring for 1 hour at this temperature, trimethylsilyl chloride (TMS-Cl, 4.02 mL, 31.4 mmol, 3.50 equiv.) was added dropwise. The mixture was then allowed to warm to room temperature. After stirring overnight, the reaction was quenched with a 1.0 N aqueous hydrochloric acid (10 mL) and vigorously stirred at room temperature over 30 minutes. The mixture was then diluted with ethyl acetate (40 mL) and the layers were separated. The aqueous phase was extracted with additional portions of ethyl acetate (3 x 20 mL). The combined organic layers were collected, washed with a solution of saturated aqueous sodium bicarbonate (20 mL), brine (10 mL), dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude product was passed through a short plug of silica, eluting with pentane to give trimethyl(oct-1-yn-1-yl)silane (**53**) (1.60 g, 8.75 mmol, 97% yield) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.21 (t, *J* = 7.0 Hz, 2H, CH_2CC), 1.54-1.47 (m, 2H, CH_2), 1.39-1.23 (m, 6H, CH_2), 0.88 (t, *J* = 6.9 Hz, 3H, CH_3), 0.14 (s, 9H, TMS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 107.8, 84.3, 31.3, 28.6, 28.5, 22.6, 19.9, 14.1, 0.2.

Spectroscopic data was consistent with the values reported in literature.[8]

(3,3-Dimethylbut-1-yn-1-yl)trimethylsilane (**55**)



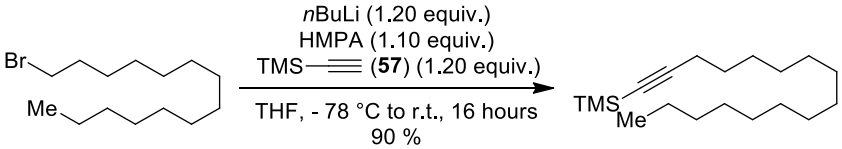
(**54**) (**55**)

Following a reported procedure,[9] a cooled 2.5 M solution of *n*-butyllithium in hexanes (*n*BuLi, 3.28 mL, 8.19 mmol, 1.04 equiv.) was added dropwise to a stirred solution of 3,3-dimethylbut-1-yne (**54**) (1.00 mL, 8.03 mmol, 1.02 equiv.) in tetrahydrofuran (26.5 mL) at - 78 °C. After stirring for 2 hours at this temperature, trimethylsilyl chloride (TMS-Cl, 1.00 mL, 7.88 mmol, 1.00 equiv.) in tetrahydrofuran (5.0 mL) was added dropwise. The mixture was then allowed to warm to room temperature. After stirring overnight, the reaction was treated with a saturated aqueous solution of ammonium chloride (40 mL) and was extracted with dichloromethane (2 x 40 mL). The combined organic phase were washed with brine, dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude product was passed through a short plug of silica, eluting with pentane to give (3,3-dimethylbut-1-yn-1-yl)trimethylsilane (**55**) (435 mg, 2.82 mmol, 36% yield) as a colorless liquid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.21 (s, 9H, *t*Bu), 0.12 (s, 9H, TMS).

Spectroscopic data was consistent with the values reported in literature.[9]

Hexadec-1-yn-1-yltrimethylsilane (**58**)



(**56**) (**58**)

Following a reported procedure,[4] a cooled 2.5 M solution of *n*-butyllithium in hexanes (*n*BuLi, 10.2 mL, 25.4 mmol, 1.20 equiv.) was added dropwise to a stirred solution of trimethylsilylacetylene (**57**) (3.62

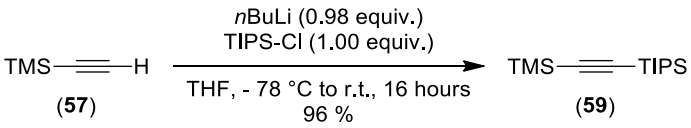
mL, 25.4 mmol, 1.20 equiv.) in tetrahydrofuran (4.5 mL) at - 78 °C. After stirring for 1 hour at this temperature, a solution of 1-bromotetradecane (**56**) (5.89 mL, 21.2 mmol, 1.00 equiv.) and hexamethylphosphoramide (HMPA, 4.06 mL, 23.3 mmol, 1.10 equiv.) in THF (2.5 mL) was added dropwise. The mixture was stirred for 1 hour at - 78 °C, followed by 24 hours of stirring at room temperature. The solution was then cooled to 0 °C, quenched by a saturated aqueous solution of ammonium chloride (15 mL) and diluted with water (3 mL) and ethyl acetate (15 mL). The layers were separated and the aqueous layer extracted with ethyl acetate (3 x 15 mL). The combined organic layers were washed with water (2 x 30 mL), brine (30 mL), dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude liquid was purified through a plug of silica, eluting with pentane, to afford pure hexadec-1-yn-1-yltrimethylsilane (**58**) (5.65 g, 19.2 mmol, 90% yield) as a colorless liquid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.20 (t, *J* = 7.1 Hz, 2H, CCC*H*$_2$), 1.54-1.47 (m, 2H, C*H*$_2$), 1.38-1.22 (m, 22H, C*H*$_2$), 0.88 (t, *J* = 6.7 Hz, 3H, CH_2C*H*$_3$), 0.14 (s, 9H, TMS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 107.7, 84.3, 32.4, 29.9, 29.8, 29.7, 29.5, 29.3, 29.0, 28.9, 23.0, 20.0, 14.3, 0.3.

Spectroscopic data was consistent with the values reported in literature.[4]

Trimethylsilyl(triisopropylsilyl)acetylene (**59**)



Following a reported procedure,[10] a cooled 2.5 M solution of *n*-butyllithium in hexanes (*n*BuLi, 86.0 mL, 209 mmol, 0.98 equiv.) was added dropwise to a stirred solution of trimethylsilylacetylene (**57**) (30.3 mL, 213 mmol, 1.00 equiv.) in tetrahydrofuran (330 mL) at - 78 °C. After stirring for 2 hours at this temperature, tri*iso*propyl chloride (TIPS-Cl, 45.6 mL, 213 mmol, 1.00 equiv.) was added dropwise. The mixture was then allowed to warm to room temperature. After stirring overnight, the reaction was treated with a saturated aqueous solution of ammonium chloride (300 mL) and was extracted with diethyl ether (2 x 300 mL). The combined organic phase were dried over magnesium sulfate, filtered and concentrated under reduced pressure. The crude product was passed through a short plug of silica, eluting with pentane to afford trimethylsilyl(tri*iso*propylsilyl)acetylene (52.5 g, 206 mmol, 96% yield) as a colorless liquid (**59**).

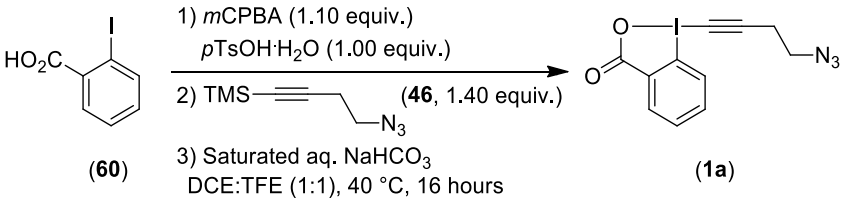
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.08 (m, 21H, TIPS), 0.18 (s, 9H, TMS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 116.3, 110.3, 18.7, 11.2, 0.2.

Spectroscopic data was consistent with the values reported in literature.[10]

b. Syntheses of ethynylbenziodoxolone reagents

(4-Azidobut-1-ynyl)-1,2-benziodoxol-3(1H)-one (**1a**)



Following a reported procedure,[4] 2-iodobenzoic acid (**60**) (24.1 g, 97.0 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 18.5 g, 97.0 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 23.9 g, 107 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (81 mL) and 2,2,2-trifluoroethanol (81 mL). After 1 hour stirring at 40 °C, (4-azidobut-1-yn-1-yl)trimethylsilane (**46**) (22.7 g, 136 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (1000 mL) and treated with a solution of saturated aqueous sodium bicarbonate (1000 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 500 mL). The organic layers were combined, dried over magnesium sulfate; filtered and concentrated under reduced pressure. Purification by column chromatography (SiO_2, Ethyl acetate) afforded (4-azidobut-1-ynyl)-1,2-benziodoxol-3(1H)-one (**1a**) (5.23 g, 15.3 mmol, 16% yield) as a white solid.

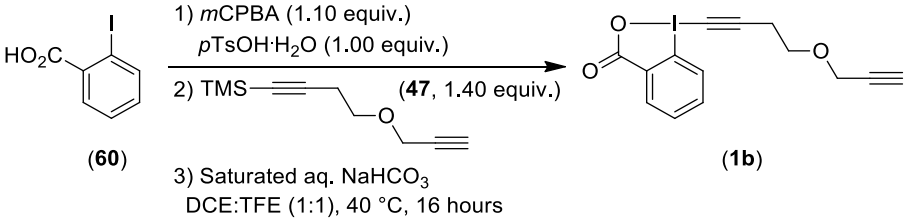
R_f 0.47 (Ethyl acetate:Methanol 9:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.37 (d, *J* = 7.5 Hz, 1H, Ar*H*), 8.21 (d, *J* = 7.5 Hz, 1H, Ar*H*), 7.80-7.70 (m, 2H, Ar*H*), 3.56 (t, *J* = 6.5 Hz, 2H, $CH_2CH_2N_3$), 2.86 (t, *J* = 6.5 Hz, 2H, $CH_2CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.2, 134.9, 132.3, 131.6, 131.4, 126.8, 115.8, 104.5, 49.4, 42.7, 21.5.

Spectroscopic data was consistent with the values reported in literature.[11]

4-(Prop-2-yn-1-yloxy- but-1-yn-1-yl)-1,2-benziodoxol-3(1H)-one (**1b**)



Following a reported procedure,[4] 2-iodobenzoic acid (**60**) (1.75 g, 7.06 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 1.34 g, 7.06 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 1.74 g, 7.77 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (5.9 mL) and 2,2,2-trifluoroethanol (5.9 mL). After 1 hour stirring at 40 °C, trimethyl(4-(prop-2-yn-1-yloxy)but-1-yn-1-yl)silane (**47**) (1.78 g, 9.88 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (25 mL) and treated with a solution of saturated aqueous sodium bicarbonate (25 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 20 mL). The organic layers were combined, dried over magnesium sulfate; filtered and concentrated under reduced pressure. Purification

by column chromatography (SiO_2, Ethyl acetate) afforded 4-(prop-2-yn-1-yloxy- but-1-yn-1-yl)-1,2-benziodoxol-3(1H)-one (**1b**) (177 mg, 0.500 mmol, 60% yield) as a white solid.

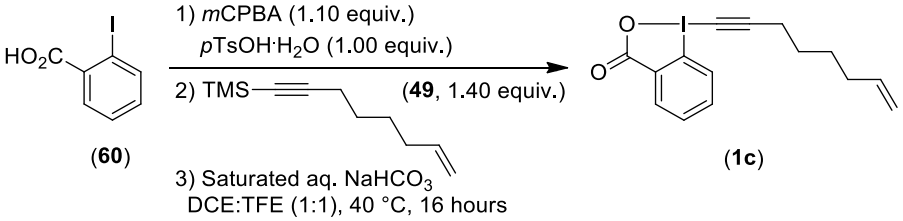
Rf 0.10 (Ethyl acetate).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (dd, *J* = 7.0, 2.2 Hz, 1H, Ar*H*), 8.26 (dd, *J* = 8.1, 1.1 Hz, 1H, Ar*H*), 7.83-7.70 (m, 2H, Ar*H*), 4.25 (d, *J* = 2.4 Hz, 2H, OCH_2CC), 3.78 (t, *J* = 6.3 Hz, 2H, OCH_2CH_2), 2.90 (t, *J* = 6.3 Hz, 2H, OCH_2CH_2), 2.49 (t, *J* = 2.4 Hz, 1H, CC*H*).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.6, 135.0, 132.5, 131.7, 131.5, 126.5, 115.8, 105.9, 79.2, 75.3, 67.4, 58.6, 41.5, 21.9.

Spectroscopic data was consistent with the values reported in literature.[4]

(Oct-6-en-1-ynyl)-1,2-benziodoxol-3(1*H*)-one (**1c**)



Following a reported procedure,[4] 2-iodobenzoic acid (**60**) (1.47 g, 5.94 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 1.13 g, 5.94 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 1.46 g, 6.53 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (5.0 mL) and 2,2,2-trifluoroethanol (5.0 mL). After 1 hour stirring at 40 °C, trimethyl(oct-7-en-1-yn-1-yl)silane (**49**) (1.50 g, 8.32 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (90 mL) and treated with a solution of saturated aqueous sodium bicarbonate (90 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 60 mL). The organic layers were combined, dried over magnesium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (SiO_2, Ethyl acetate) afforded (oct-6-en-1-ynyl)-1,2-benziodoxol-3(1*H*)-one (**1c**) (457 mg, 1.29 mmol, 22% yield) as a white solid.

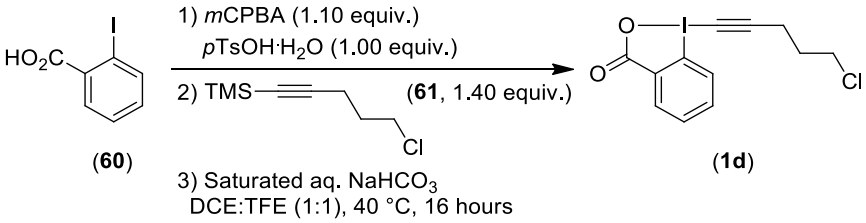
R_f 0.34 (Ethyl acetate).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42-8.39 (m, 1H, Ar*H*), 8.19-8.15 (m, 1H, Ar*H*), 7.78-7.72 (m, 2H, Ar*H*), 5.81 (ddt, *J* = 17.0, 10.2, 6.7 Hz, 1H, CH_2CHCH_2), 5.07-4.98 (m, 2H, CH_2CHCH_2), 2.61 (t, *J* = 7.0 Hz, 2H, CH_2), 2.15-2.09 (m, 2H, CH_2), 1.72-1.51 (m, 4H, CH_2).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 138.0, 134.6, 132.1, 131.6, 131.3, 126.4, 115.7, 115.0, 109.3, 39.1, 33.0, 27.9, 27.5, 20.3.

Spectroscopic data was consistent with the values reported in literature.[4]

(5-Chloropent-1-ynyl)-1,2-benziodoxol-3(1*H*)-one (**1d**)



(**60**) (**1d**)

Following a reported procedure,[4] 2-iodobenzoic acid (**60**) (1.18 g, 4.76 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 0.905 g, 4.76 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 1.17 g, 5.24 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (4.0 mL) and 2,2,2-trifluoroethanol (4.0 mL). After 1 hour stirring at 40 °C, (5-chloropent-1-yn-1-yl)trimethylsilane (**61**) (1.16 g, 6.66 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (40 mL) and treated with a solution of saturated aqueous sodium bicarbonate (40 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 20 mL). The organic layers were combined, dried over magnesium sulfate; filtered and concentrated under reduced pressure. Purification by column chromatography (SiO_2, Ethyl acetate) afforded (5-chloropent-1-ynyl)-1,2-benziodoxol-3(1*H*)-one (**1d**) (481 mg, 1.38 mmol, 29% yield) as a white solid.

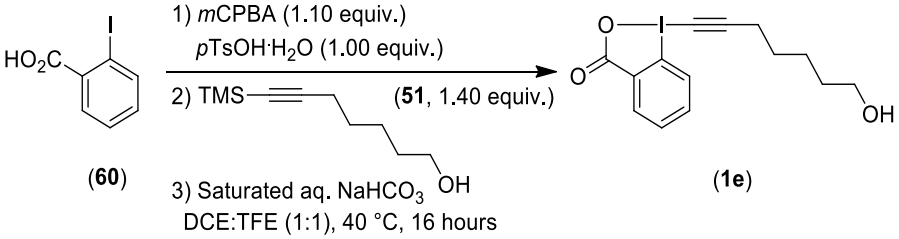
R_f 0.15 (Ethyl acetate).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43-8.39 (m, 1H, Ar*H*), 8.20-8.15 (m, 1H, Ar*H*), 7.80-7.73 (m, 2H, Ar*H*), 3.71 (t, *J* = 6.1 Hz, 2H, ClC$H_2$$CH_2$), 2.83 (t, *J* = 6.9 Hz, 2H, CCC$H_2$$CH_2$), 2.17-2.08 (m, 2H, $ClCH_2CH_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 134.7, 132.8, 131.9, 131.6, 126.6, 115.8, 106.9, 43.7, 41.0, 30.9, 18.1.

Spectroscopic data was consistent with the values reported in literature.[4]

5-Pentanolethynyl-1,2-benziodoxol-3(1H)-one (**1e**)



(**60**) (**1e**)

Following a reported procedure,[4] 2-iodobenzoic acid (**60**) (7.69 g, 31.0 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 5.90 g, 31.0 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 7.64 g, 34.1 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (25.8 mL) and 2,2,2-trifluoroethanol (25.8 mL). After 1 hour stirring at 40 °C, 7-(trimethylsilyl)hept-6-yn-1-ol (**51**) (8.00 g, 43.4 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (500 mL) and treated with a solution of saturated aqueous sodium bicarbonate (500 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 150 mL). The organic layers were combined, dried over magnesium sulfate; filtered and concentrated under reduced pressure. Purification by column

chromatography (SiO_2, Ethyl acetate:Methanol 95:5) afforded 5-pentanolethynyl-1,2-benziodoxol-3(1H)-one (**1e**) (1.60 g, 4.30 mmol, 14% yield) as a white solid.

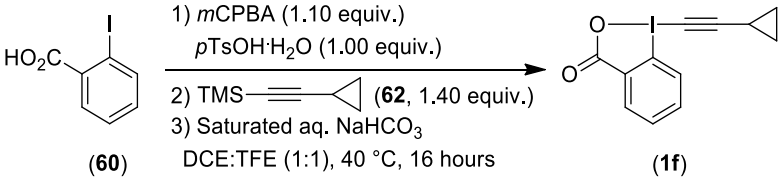
R_f 0.24 (Ethyl acetate:Methanol 9:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 (dd, *J* = 7.2, 2.0 Hz, 1H, Ar*H*), 8.15 (d, *J* = 8.0 Hz, 1H, Ar*H*), 7.79-7.64 (m, 2H, Ar*H*), 3.66 (t, *J* = 5.9 Hz, 2H, CH_2OH), 2.59 (t, *J* = 6.9 Hz, 2H, CCCH_2), 1.73-1.49 (m, 7H, CH_2 and O*H*).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.2, 134.8, 132.2, 131.6, 131.4, 126.6, 115.6, 109.8, 62.2, 38.7, 32.0, 27.9, 25.2, 20.5.

Spectroscopic data was consistent with the values reported in literature.[4]

(2-Cyclopropylethynyl)-1,2-benziodoxol-3(1*H*)-one (**1f**)



(**60**) (**1f**)

Following a reported procedure,[12] 2-iodobenzoic acid (**60**) (6.41 g, 25.8 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 4.91 g, 25.8 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 6.36 g, 28.4 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (21.5 mL) and 2,2,2-trifluoroethanol (21.5 mL). After 1 hour stirring at 40 °C, trimethyl(2-cyclopropylethynyl)silane (**62**) (4.99 g, 36.1 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (400 mL) and treated with a solution of saturated aqueous sodium bicarbonate (400 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 100 mL). The organic layers were combined, dried over magnesium sulfate, filtered and concentrated under reduced pressure. Purification by column chromatography (SiO_2, Ethyl acetate) afforded (2-cyclopropylethynyl)-1,2-benziodoxol-3(1H)-one (**1f**) (2.11 g, 6.76 mmol, 26% yield) as a white solid.

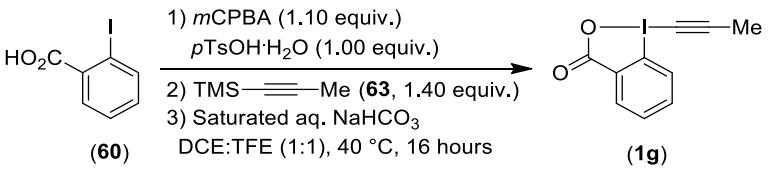
R_f 0.46 (Ethyl acetate:Methanol 9:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (dd, J = 7.0, 2.1 Hz, 1H, Ar*H*), 8.18-8.09 (m, 1H, Ar*H*), 7.81-7.63 (m, 2H, Ar*H*), 1.59 (tt, J = 8.2, 5.0 Hz, 1H, C*H*), 1.07-0.85 (m, 4H, CH_2CH_2).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 134.7, 132.3, 131.7, 131.4, 126.2, 115.9, 113.3, 35.0, 9.8, 1.1.

Spectroscopic data was consistent with the values reported in literature.[12]

Propynyl-1,2-benziodoxol-3(1*H*)-one (**1g**)



(**60**) (**1g**)

Following a reported procedure,[4] 2-iodobenzoic acid (**60**) (1.42 g, 5.73 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 1.09 g, 5.73 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 1.41 g, 6.30 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (4.8 mL) and 2,2,2-trifluoroethanol (4.8 mL). After 1 hour stirring at 40 °C, trimethyl(prop-1-yn-1-yl)silane (**63**) (0.900 g, 8.02 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (40 mL) and treated with a solution of saturated aqueous sodium bicarbonate (40 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 30 mL). The organic layers were combined, dried over magnesium sulfate; filtered and concentrated under reduced pressure. Purification by column chromatography (SiO_2, Ethyl acetate) afforded propynyl-1,2-benziodoxol-3(1*H*)-one (**1g**) (410 mg, 1.43 mmol, 25% yield) as a white solid.

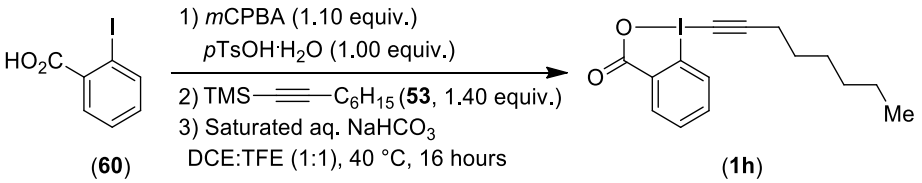
R_f 0.10 (Ethyl acetate).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42-8.39 (m, 1H, Ar*H*), 8.22-8.14 (m, 1H, Ar*H*), 7.78-7.73 (m, 2H, Ar*H*), 2.27 (s, 3H, CCC*H*$_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.1, 134.7, 132.2, 131.6, 131.4, 126.6, 115.7, 104.9, 38.5, 5.7.

Spectroscopic data was consistent with the values reported in literature.[4]

Octynyl-1,2-benziodoxol-3(1*H*)-one (**1h**)



(**60**) (**1h**)

Following a reported procedure,[4] 2-iodobenzoic acid (**60**) (856 mg, 3.45 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 656 mg, 3.45 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 851 mg, 3.80 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (2.9 mL) and 2,2,2-trifluoroethanol (2.9 mL). After 1 hour stirring at 40 °C, trimethyl(oct-1-yn-1-yl)silane (**53**) (881 mg, 4.83 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (40 mL) and treated with a solution of saturated aqueous sodium bicarbonate (40 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 20 mL). The organic layers were combined, dried over magnesium sulfate; filtered and concentrated under reduced pressure. Purification by column chromatography (SiO_2, Ethyl acetate) afforded octynyl-1,2-benziodoxol-3(1*H*)-one (**1h**) (548 mg, 1.54 mmol, 45% yield) as a white solid.

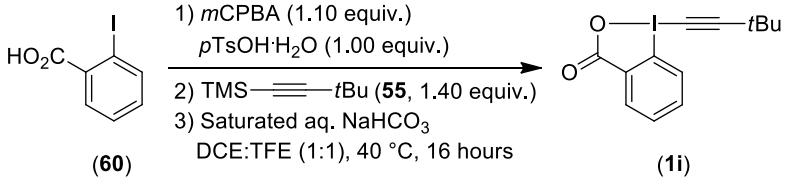
R_f 0.25 (Ethyl acetate).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42-8.39 (m, 1H, Ar*H*), 8.19-8.16 (m, 1H, Ar*H*), 7.77-7.72 (m, 2H, Ar*H*), 2.59 (t, *J* = 7.1 Hz, 2H, CCC*H*$_2$), 1.69-1.59 (m, 2H), 1.50-1.43 (m, 2H), 1.37-1.31 (m, 4H), 0.94-0.89 (m, 3H, CH_2CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 134.7, 132.5, 131.7, 131.6, 126.3, 115.7, 109.9, 39.4, 31.3, 28.7, 28.3, 22.6, 20.6, 14.1.

Spectroscopic data was consistent with the values reported in literature.[4]

3,3-Dimethylbutynyl-1,2-benziodoxol-3(1*H*)-one (**1i**)



(**60**) (**1i**)

Following a reported procedure,[4] 2-iodobenzoic acid (**60**) (689 mg, 2.78 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 529 mg, 2.78 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 685 mg, 3.06 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (2.3 mL) and 2,2,2-trifluoroethanol (2.3 mL). After 1 hour stirring at 40 °C, (3,3-dimethylbut-1-yn-1-yl)trimethylsilane (**55**) (601 mg, 3.89 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (50 mL) and treated with a solution of saturated aqueous sodium bicarbonate (50 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 30 mL). The organic layers were combined, dried over magnesium sulfate; filtered and concentrated under reduced pressure. Purification by column chromatography (SiO_2, Ethyl acetate) afforded 3,3-dimethylbutynyl-1,2-benziodoxol-3(1*H*)-one (**1i**) (673 mg, 2.05 mmol, 74% yield) as a white solid.

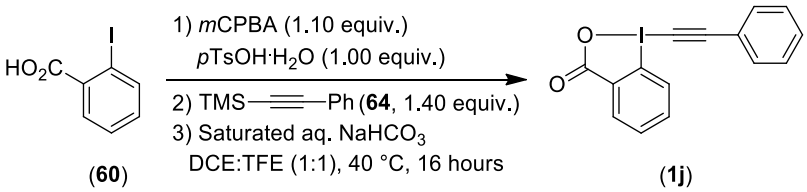
R_f 0.36 (Ethyl acetate).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43-8.39 (m, 1H, Ar*H*), 8.15-8.10 (m, 1H, Ar*H*), 7.78-7.73 (m, 2H, Ar*H*), 1.38 (s, 9H, *t*Bu).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 134.8, 132.5, 131.6, 131.6, 126.0, 117.6, 115.7, 38.3, 30.7, 29.7.

Spectroscopic data was consistent with the values reported in literature.[4]

1-Phenylethynyl-1,2-benziodoxol-3(1*H*)-one (**1j**)



(**60**) (**1j**)

Following a modified procedure,[13] 2-iodobenzoic acid (**60**) (1.21 g, 4.87 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 0.926 g, 4.87 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 1.20 g, 5.36 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (4.0 mL) and 2,2,2-trifluoroethanol (4.0 mL). After 1 hour stirring at 40 °C, trimethyl(phenylethynyl)silane (**64**) (1.19 g, 6.82 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (40 mL) and treated with a solution of saturated aqueous sodium bicarbonate (40 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous phase was extracted with additional portions of dichloromethane (3 x 20 mL). The

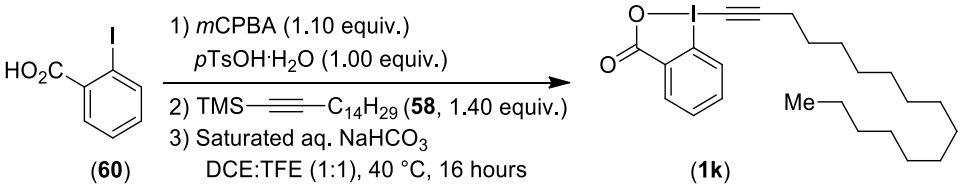
organic phases were combined, dried over magnesium sulfate; filtered and concentrated under reduced pressure. The resulting solid was then recrystallized from acetonitrile and washed with acetonitrile (2 x 20 mL) to yield pure 1-phenylethynyl-1,2-benziodoxol-3(1*H*)-one (**1j**) (648 mg, 1.86 mmol, 38% yield) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45-8.41 (m, 1H, Ar*H*), 8.28-8.23 (m, 1H, Ar*H*), 7.81-7.75 (m, 2H, Ar*H*), 7.62-7.59 (m, 2H, Ar*H*), 7.51-7.41 (m, 3H, Ar*H*).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 135.0, 133.0, 132.6, 131.7, 131.5, 130.9, 128.9, 126.4, 120.7, 116.3, 106.7, 50.3.

Spectroscopic data was consistent with the values reported in literature.[13]

Hexadecynyl-1,2-benziodoxol-3(1*H*)-one (**1k**)



(**60**) (**1k**)

Following a reported procedure,[4] 2-iodobenzoic acid (**60**) (552 mg, 2.18 mmol, 1.00 equiv.), *para*-toluene sulfonic acid monohydrate (*p*TsOH·H_2O, 419 mg, 2.18 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 538 mg, 2.40 mmol, 1.10 equiv.) were dissolved in a mixture of dichloroethane (1.8 mL) and 2,2,2-trifluoroethanol (1.8 mL). After 1 hour stirring at 40 °C, hexadec-1-yn-1-yltrimethylsilane (**58**) (900 mg, 3.06 mmol, 1.40 equiv.) was added in one portion. The reaction mixture was stirred for an additional 14 hours at the same temperature, then the resulting suspension was filtered and the volatiles were removed under reduced pressure. The resultant residue was dissolved in dichloromethane (30 mL) and treated with a solution of saturated aqueous sodium bicarbonate (30 mL). The mixture was vigorously stirred for 1 hour, then the two layers were separated and the aqueous layer was extracted with additional portions of dichloromethane (3 x 20 mL). The organic layers were combined, dried over magnesium sulfate; filtered and concentrated under reduced pressure. Purification by column chromatography (SiO_2, Ethyl acetate) afforded hexadecynyl-1,2-benziodoxol-3(1*H*)-one (**1k**) (221 mg, 0.472 mmol, 22% yield) as a white solid.

R_f 0.36 (Ethyl acetate).

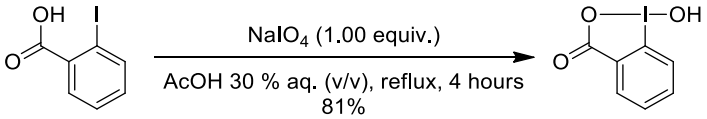
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43-8.39 (m, 1H, Ar*H*), 8.19-8.15 (m, 1H, Ar*H*), 7.78-7.72 (m, 2H, Ar*H*), 2.59 (t, *J* = 7.1 Hz, 2H, CCCH_2), 1.65 (p, *J* = 7.1 Hz, 2H, CCCH_2CH_2), 1.49-1.42 (m, 2H, CH_2), 1.36-1.20 (m, 20H, CH_2), 0.87 (t, *J* = 6.7 Hz, 3H, CH_2CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 108.0, 84.3, 32.2, 30.1, 30.0, 29.8, 29.7, 29.3, 29.0, 28.9, 22.9, 20.0, 14.5, 0.3.

Spectroscopic data was consistent with the values reported in literature.[4]

Synthesis of 1-[(tri*iso*propylsilyl)ethynyl]-1,2-benziodoxol-3(1*H*)-one (**1l**)

2-iodosylbenzoic acid (**65**)



(**60**) (**65**)

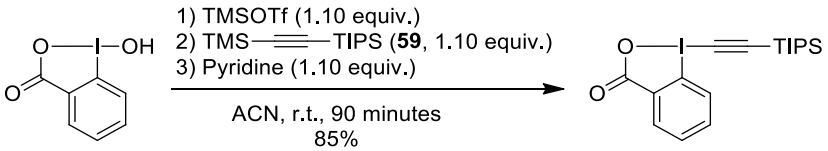
Following a reported procedure,[14] sodium periodate ($NaIO_4$, 77.2 g, 361 mmol, 1.00 equiv.) and 2-iodobenzoic acid (**60**) (89.5 g, 361 mmol, 1.00 equiv.) were suspended in 30% aqueous acetic acid solution (700 mL). The vigorously stirred mixture was heated and refluxed under air for 4 hours. The 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, 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 (**65**) (74.3 g, 281 mmol, 78% yield) was recovered as a white solid.

^{1}H 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*).

^{13}C 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.[14]

1-[(Tri*iso*propylsilyl)ethynyl]-1,2-benziodoxol-3(1H)-one (**1l**)



(**65**) (**1l**)

Following a reported procedure,[15] a cooled solution of trimethylsilyl trifluoromethanesulfonate (TMSOTf, 19.9 mL, 110 mmol, 1.10 equiv.) was added dropwise to a stirred suspension of 2-iodosylbenzoic acid (**65**) (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(tri*iso*propylsilyl)acetylene (**59**) (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 (**1l**) (32.1 g, 74.9 mmol, 75% yield) as white crystals.

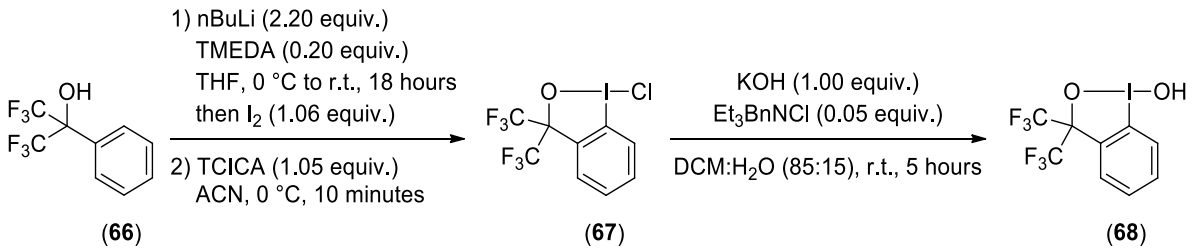
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (dd, *J* = 5.9, 3.3 Hz, 1H, Ar*H*), 8.29 (dd, *J* = 6.0, 3.3 Hz, 1H, Ar*H*), 7.76 (dd, *J* = 5.9, 3.3 Hz, 2H, Ar*H*), 1.33-1.05 (m, 21H, TIPS).

^{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.[15]

Synthesis of 1-(4-azidobut-1-yn-1-yl)-3,3-bis(trifluoromethyl)-1,3-dihydro-1λ^3-benzo[*d*][1,2]iodaoxole (**1m**)

1-Hydroxy-3,3-bis(trifluoromethyl)-3-(1H)-1,2-benziodoxole (**68**)



(**66**) (**67**) (**68**)

Following a reported procedure,[16] tetramethylethylenediamine, distilled over KOH, (TMEDA, 1.26 mL, 8.32 mmol, 0.20 equiv.) was added to a solution of *n*-butyllithium in hexanes (*n*BuLi, 36.6 mL, 91.0 mmol, 2.20 equiv.). After 15 minutes, the solution was cooled to 0 °C and 1,1,1,3,3,3-hexafluoro-2-phenylpropan-2-ol (**66**) (7.00 mL, 41.6 mmol, 1.00 equiv.), in tetrahydrofuran (36 mL), was added dropwise. The reaction was stirred 30 minutes at 0 °C, followed by 18 hours at room temperature. Iodine (I_2, 11.2 g, 44.1 mmol, 1.06 equiv.) was added in small portions at 0 °C. The mixture was stirred at 0 °C for 30 minutes and then 4 hours at room temperature. The reaction was quenched with a solution of saturated aqueous ammonium chloride (100 mL) and extracted with diethyl ether (100 mL). The aqueous layer was then extracted twice with diethyl ether (3 x 50 mL). The organic layers were combined, washed twice with a solution of saturated aqueous sodium thiosulfate (2 x 50 mL), dried over magnesium sulfate, filtered and reduced to afford 1,1,1,3,3,3-hexafluoro-2-(2-iodophenyl)propan-2-ol (**69**) (15.2 g, 41.2 mmol, 99%) as an orange oil which was used without further purification. The crude oil was dissolved in acetonitrile (190 mL) under air and a solution of trichloro*iso*cyanuric acid (TCICA, 3.25 g, 14.0 mmol, 1.05 equiv.) in acetonitrile (20 mL) was then added dropwise at 0 °C. After 10 minutes, diethyl ether (100 mL) was added, the resulting suspension was removed and the filtrate was concentrated *in vacuo*. The resulting solid was dissolved into diethyl ether (50 mL), filtered, dried and washed with small amounts of dichloromethane to afford 1-chloro-3,3-bis(trifluoromethyl)-3-(1H)-1,2-benziodoxole (**67**) (9.33 g, 23.1 mmol, 56%) as a yellow solid.

^{1}H NMR ($CDCl_3$, 101 MHz) δ 8.09 (d, *J* = 8.4 Hz, 1H, Ar*H*), 7.87-7.82 (m, 1H, Ar*H*), 7.73 (d, *J* = 4.8 Hz, 2H, Ar*H*).

^{13}C NMR ($CDCl_3$, 101 MHz) δ 134.0, 132.2, 131.8, 129.9, 128.7, 122.6 (q, 290 Hz), 113.6, 84.9.

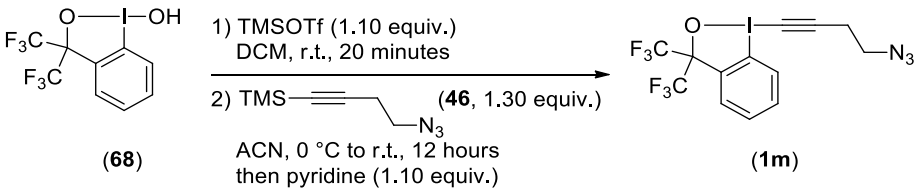
Spectroscopic data was consistent with the values reported in literature.[17]

Following a reported procedure,[18] benzyltriethylammonium chloride ($Et_{3b}nNCl$, 260 mg, 1.15 mmol, 0.05 equiv.) was added to a stirring solution of 1-chloro-1,3-dihydro-3,3-bis(trifluoromethyl)-1,2-benziodoxole (**67**) (9.33 g, 23.1 mmol, 1.00 equiv.) in dichloromethane (130 mL) and potassium hydroxide (KOH, 1.29 g, 23.1 mmol, 1.00 equiv.) in water (24 mL). The reaction was stirred for 5 hours under air. The organic layer was separated, dried over magnesium sulfate and concentrated *in vacuo*. The resulting solid was purified over a silica plug with ethyl acetate, then recrystallized in ethyl acetate and washed with pentane to afford 1-hydroxy-3,3-bis(trifluoromethyl)-3-(1H)-1,2-benziodoxole (**68**) (3.40 g, 8.81 mmol, 38%) as a colorless solid.

^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.01-7.90 (m, 2H, Ar*H*), 7.79-7.67 (m, 2H, Ar*H*).

Spectroscopic data was consistent with the values reported in literature.[13]

1-(4-Azidobut-1-ynyl)-3,3-bis(trifluoromethyl)-3(1H)-1,2-benziodoxole (**1m**)



(**68**) (**1m**)

Following a slightly modified procedure,[13] trimethylsilyl trifluoromethanesulfonate (TMSOTf, 398 µL, 2.20 mmol, 1.10 equiv.) was added dropwise to a stirred solution of 1-hydroxy-3,3-bis(trifluoromethyl)-3-(1H)-1,2-benziodoxole (**68**) (772 mg, 2.00 mmol, 1.00 equiv.) in dichloromethane (3.8 mL). After 20 minutes, the solution was concentrated *in vacuo* in an ice bath, at 0 °C, and the resulting oil was dissolved in acetonitrile (7.5 mL) under argon. (4-azidobut-1-ynyl)trimethylsilane (**46**) (435 mg, 2.60 mmol, 1.30 equiv.) was added dropwise at 0 °C and after 12 hours, pyridine (178 µL, 2.20 mmol, 1.10 equiv.) was added at 0 °C. The mixture was stirred for 3 hours and the solvent was removed *in vacuo*. The resulting oil was filtered over a silica plug with diethyl ether. Purification by column chromatography (SiO_2, Pentane:Ethyl acetate 3:1) afforded 1-(4-azidobut-1-ynyl)-3,3-bis(trifluoromethyl)-3(1H)-1,2-benziodoxole (**1m**) (408 mg, 0.881 mmol, 44% yield) as a white solid.

R_f 0.41 (Pentane:Ethyl acetate 3:1).

m.p.: 119-120 °C.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (dt, *J* = 7.4, 2.7 Hz, 1H, Ar*H*), 7.83 (br, 1H, Ar*H*), 7.74 – 7.65 (m, 2H, Ar*H*), 3.53 (t, *J* = 6.6 Hz, 2H, $CH_2CH_2N_3$), 2.80 (t, *J* = 6.6 Hz, 2H, $CH_2CH_2N_3$).

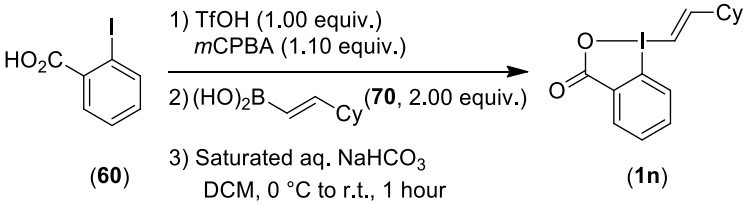
^{13}C NMR (101 MHz, $CDCl_3$) δ 133.1, 131.4, 130.1, 130.0, 130.0, 123.7 (q, *J* = 290.4 Hz), 111.0, 103.0, 81.70 (p, *J* = 29.7 Hz), 49.7, 47.2, 21.4.

IR (vmax, cm-1) 2162 (w), 2104 (m), 1559 (w), 1512 (w), 1464 (w), 1392 (w), 1340 (w), 1263 (w), 1216 (m), 1192 (m), 1153 (m), 1082 (w), 1006 (w), 957 (w), 910 (m), 857 (m).

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + H]^+$ Calcd for $C_{13}H_9F_6IN_3O^+$ 463.9689; Found 463.9684.

c. Syntheses of vinylbenziodoxolone reagents

(E)-1-(2-Cyclohexylvinyl)-1,2-benziodoxol-3(1*H*)-one (**1n**)



(**60**) (**1n**)

Following a reported procedure,[19] trifluoromethanesulfonic acid (TfOH, 0.533 mL, 6.00 mmol, 1.00 equiv.) was added dropwise to a stirred solution of 2-iodobenzoic acid (**60**) (1.49 g, 6.00 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 1.48 g, 6.60 mmol, 1.10 equiv.) in dichloromethane (37.5 mL) at 0 °C. After 15 minutes stirring at room temperature, (E)-(2-cyclohexylvinyl)boronic acid (**70**) (1.85 g, 12.0 mmol, 2.00 equiv.) was added in one portion at 0 °C. The reaction mixture was stirred for an additional 1 hour at room temperature, then the resulting mixture was concentrated *in vacuo*. Diethyl ether (300 mL) was added and the resulting mixture was vigorously stirred over 30 minutes. The solid was filtered off and washed with portions of diethyl ether (5 x 50 mL). The resulting solid was dissolved in dichloromethane (50 mL) and treated with a solution of saturated aqueous sodium bicarbonate (50 mL). The mixture was vigorously stirred for 1 hour, then the organic layer was diluted with dichloromethane (100 mL) and the two layers were separated. The aqueous layer was extracted with additional portions of dichloromethane (3 x 50 mL) and the combined organic layers were washed with water (50 mL) and brine (50 mL), then dried over magnesium sulfate and filtered. The volatiles were removed to afford pure (E)-1-(2-cyclohexylvinyl)-1,2-benziodoxol-3(1*H*)-one (**1n**) (0.654 g, 1.84 mmol, 31% yield) as a beige solid.

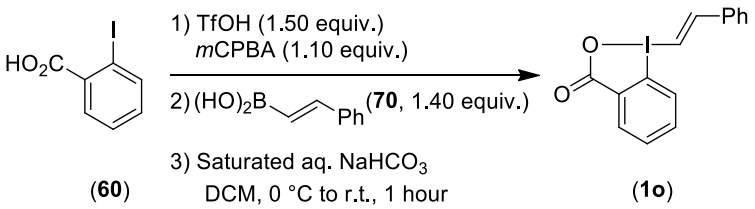
R_f 0.46 (Dichloromethane:Methanol 95:5).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 – 8.33 (m, 1H, Ar*H*), 7.55 (tt, *J* = 7.2, 5.3 Hz, 2H, Ar*H*), 7.50 – 7.39 (m, 1H, Ar*H*), 6.98 (dd, *J* = 15.4, 6.8 Hz, 1H, IC*H*CH), 6.55 (dd, *J* = 15.3, 1.2 Hz, 1H, ICHC*H*CH), 2.45 – 2.30 (m, 1H, CHC*H*$(CH_2)_2$), 1.96 – 1.75 (m, 4H, CH_2), 1.75 – 1.65 (m, 1H, CH_2), 1.45 – 1.09 (m, 5H, CH_2).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 164.0, 133.7, 133.4, 132.9, 130.7, 125.8, 114.6, 98.9, 44.6, 31.8, 25.8, 25.6.

Spectroscopic data was consistent with the values reported in literature.[19]

(E)-1-Styryl-1,2-benziodoxol-3(1*H*)-one (1o)



(**60**) (**1o**)

Following a reported procedure,[19] trifluoromethanesulfonic acid (TfOH, 0.666 mL, 7.50 mmol, 1.50 equiv.) was added dropwise to a stirred solution of 2-iodobenzoic acid (**60**) (1.24 g, 5.00 mmol, 1.00 equiv.) and *meta*-chloroperoxybenzoic acid (*m*CPBA-77%, 1.23 g, 5.50 mmol, 1.10 equiv.) in dichloromethane (31.3 mL) at 0 °C. After 15 minutes stirring at room temperature, (E)-styrylboronic acid (**70**) (1.04 g, 7.00 mmol, 1.40 equiv.) was added in one portion at 0 °C. The reaction mixture was stirred for an additional 1 hour at room temperature, then the resulting mixture was treated with a solution of saturated aqueous sodium bicarbonate (50 mL). The mixture was vigorously stirred for 1 hour, then the

organic layer was diluted with dichloromethane (100 mL) and the two layers were separated. The aqueous layer was extracted with additional portions of dichloromethane (3 x 50 mL) and the combined organic layers were washed with water (50 mL) and brine (50 mL), then dried over magnesium sulfate, filtered and concentrated *in vacuo*. Diethyl ether (300 mL) was added and the resulting mixture was vigorously stirred over 30 minutes. The solid was filtered off and washed with portions of diethyl ether (5 x 50 mL) to afford (E)-1-styryl-1,2-benziodoxol-3(1*H*)-one (**1o**) (0.602 g, 1.72 mmol, 34% yield) as a pale brown solid.

R_f 0.30 (Dichloromethane:Methanol 95:5).

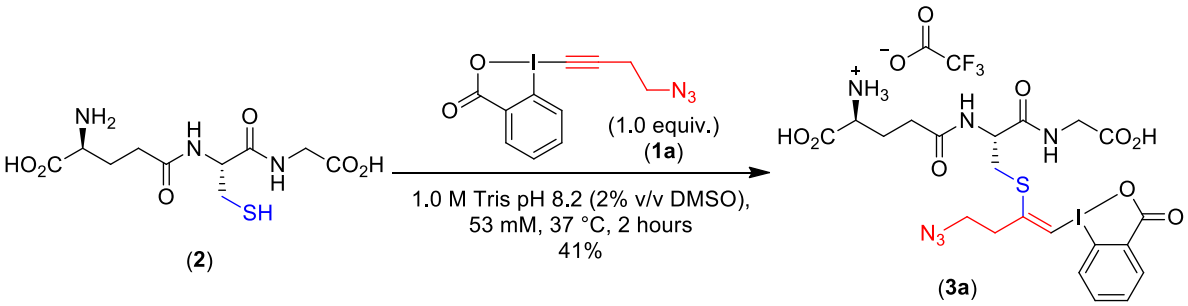
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (dt, *J* = 7.1, 1.2 Hz, 1H, Ar*H*), 7.80 (d, *J* = 15.7 Hz, 1H, Ar*H*), 7.61 – 7.40 (m, 8H, Ar*H*), 7.33 (d, *J* = 15.7 Hz, 1H, Ar*H*).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.2, 154.3, 135.1, 133.7, 133.4, 132.9, 131.3, 130.8, 129.3, 127.9, 126.5, 115.6, 99.8.

Spectroscopic data was consistent with the values reported in literature.[19]

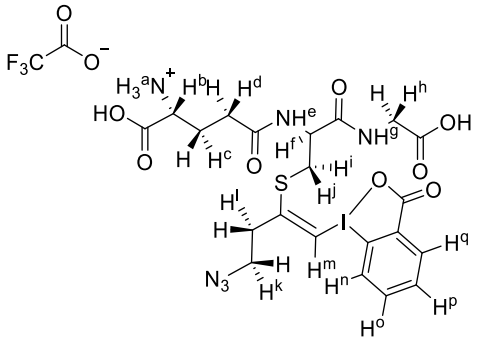
5. Isolation of hypervalent iodine glutathione 3a

a. Large scale reaction

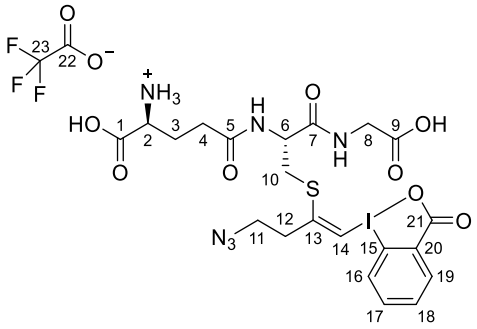


A 1.5 mL Eppendorf Safe-Lock microcentrifuge was charged with glutathione (**2**) (24.6 mg, 80.0 µmol, 1.00 equiv.) and (4-azidobut-1-ynyl)-1,2-benziodoxol-3(1H)-one (**1a**) (27.3 mg, 80.0 µmol, 1.00 equiv.). The reagents were dissolved in a mixture of Tris buffer (1.0 M, pH 8.2, 1470 µL) and DMSO (30 µL). The resulting mixture was vigorously shaken for 2 hours at 37 °C. No effort was made to exclude oxygen. The reaction mixture was filtered and purified by preparative RP-HPLC using method B (retention time: 14 – 16.5 minutes). Fractions containing the desired product were lyophilized to afford S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) trifluoroacetic acid salt (25.0 mg, 32.8 µmol, 41% yield) as a white solid.

b. Characterization and structure analysis



^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (t, J = 5.9 Hz, 1H, H^g), 8.40 (d, J = 8.3 Hz, 1H, H^e), 8.36 – 8.23 (d, J = 5.4 Hz, 3H, H^a), 8.18 – 8.11 (m, 1H, H^q), 7.69 – 7.62 (m, 2H, H^o and H^p), 7.62 – 7.56 (m, 1H, H^n), 7.21 (s, 1H, H^m), 4.43 (td, J = 8.7, 4.7 Hz, 1H, H^f), 3.95 (d, J = 5.4 Hz, 1 H, H^b), 3.82 – 3.61 (m, 4H, H^h and H^k), 3.23 (dd, J = 13.8, 4.7 Hz, 1H, H^i), 3.12 – 3.00 (m, 2H, H^l), 2.97 (dd, J = 13.8, 9.0 Hz, 1H, H^j), 2.37 – 2.21 (m, 2H, H^d), 2.07 – 1.89 (m, 2H, H^c).



^{13}C NMR (101 MHz, DMSO-d_6) δ 171.7 (C^5), 171.3 (C^1 or C^9), 171.2 (C^1 or C^9), 170.1 (C^7), 166.6 (C^{21}), 158.4 (q, J = 32.4 Hz, C^{22}), 156.9 (C^{13}), 134.4 (C^{20}), 134.1 (C^{17}), 132.3(C^{19}), 130.9 (C^{18}), 127.9 (C^{16}), 117.3 (q, J = 298 Hz, C^{23}), 114.1 (C^{15}), 106.5 (C^{14}), 52.9 (C^6), 52.1 (C^2), 49.7 (C^{11}), 41.2 (C^8), 35.8 (C^{12}), 34.2 (C^{10}), 30.9 (C^4), 26.2 (C^3).

m.p.: 117-118 °C.

IR (vmax, cm-1) 3435 (m), 3060 (w), 2940 (w), 2522 (w), 2106 (s), 1736 (m), 1657 (s), 1532 (s), 1430 (w), 1369 (m), 1189 (s), 1133 (s), 1078 (w), 1022 (m), 1004 (m).

HRMS (ESI) calculated for $C_{21}H_{26}IN_6O_8S^+$ $[M+H]^+$ 649.0572; found 649.0584.

^{1}H NMR COSY, HSQC, and ROESY (DMSO-d6, 400 MHz) were consistent with this attribution.

(Z)-configuration attribution: H^m could be assigned due to its unique vinylic shift and its HSQC correlation with the only vinylic carbon of the molecule C^{14}. Meanwhile, H^l could be assigned based on COSY and HMBC correlations with H^k and C^{11}. Finally, ROESY correlation between H^m and H^l confirmed the (Z) configuration of the double bond. No ROESY correlation could be observed with H^j nor H^i.

c. Calibration

Calibration with S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) was achieved through the preparation of several samples of different concentrations and their analysis on RP HPLC. These analyses were repeated five times in order to obtain an average curve of calibration. The following linear regression was obtained: Y = 0.00060173 x X - 0.04698536 and R = 0.99986453, where Y is the concentration in µmol/mL of **3a** and X the absorbance area of the peak at 214 nm.

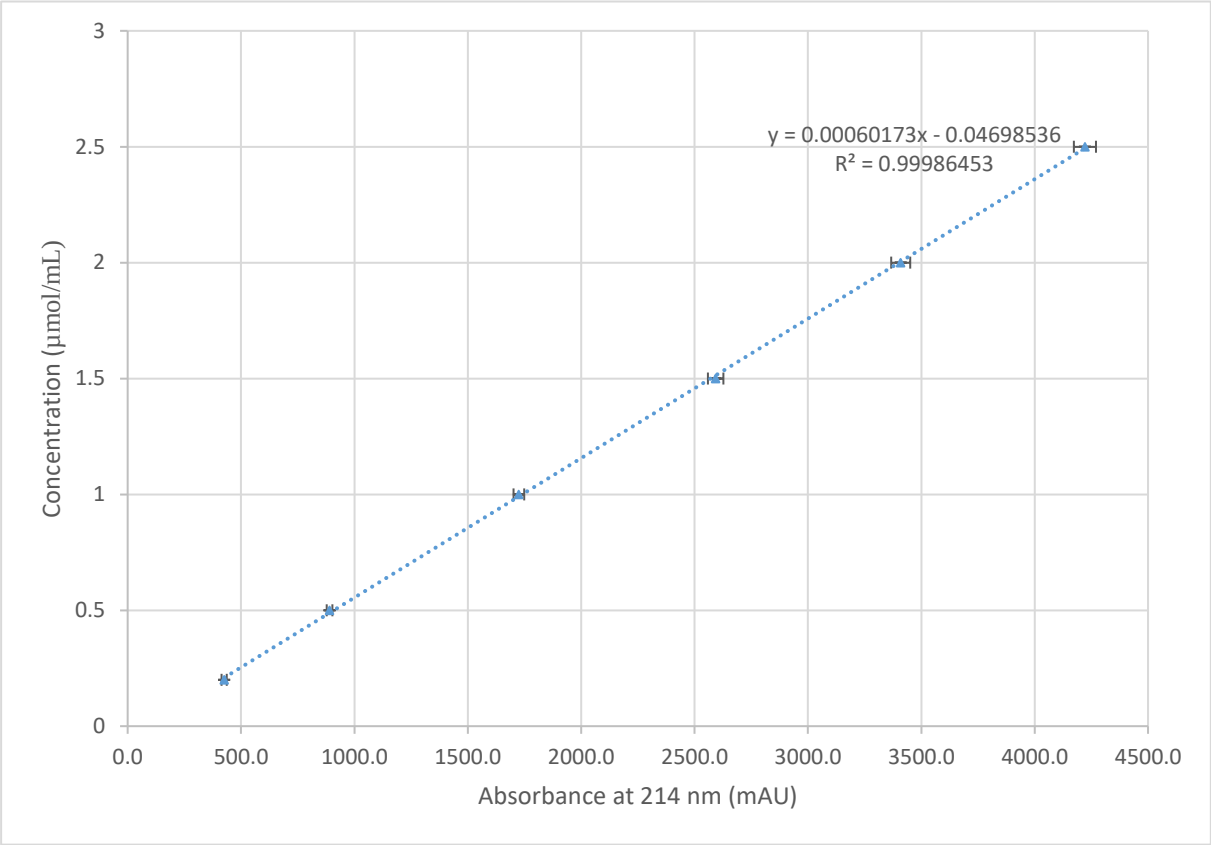


Figure S1: Calibration curve of **3a**.

6. Stability evaluation of the hypervalent iodine-glutathione structure 3a

This structure being reported for the first time, some stability evaluation was performed. The degradation evolution was followed by HPLC-MS.

a. Stability evaluation at low pH

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) (1.62 mg, 2.50 µmol) was dissolved in an acetic acid buffer (1.0 M, pH 4.0, 1250 µL) and shaken at room temperature. After 3 days, S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) remained intact.

b. Stability evaluation at high pH

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) (1.62 mg, 2.50 µmol) was dissolved in a CAPS buffer (10.0 mM, pH 11.0, 1250 µL) and shaken at room temperature. After 3 hours, only minor degradation could be observed (96% of the S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone available). After 3 days, 86% of the S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) remained intact.

c. Stability evaluation towards temperature

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) (1.62 mg, 2.50 µmol) was dissolved in milliQ water (1250 µL). The solution was sampled after 10 minutes shaking at 37 °C, 50 °C, 60 °C, 80 °C and 100 °C. Only a minor degradation was noticed at 100 °C.

d. Stability evaluation in presence of tiopronin (**11**) as an external thiol nucleophile

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) (0.65 mg, 1.00 µmol), Tris buffer (100 mM, pH 8.2, 490 µL) and DMSO (10 µL). Then, tiopronin (**11**) (2.45 mg, 15.0 µmol, 15.0 equiv.) was added in one portion and the solution was shaken at room temperature. After 8 days, S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) remained intact.

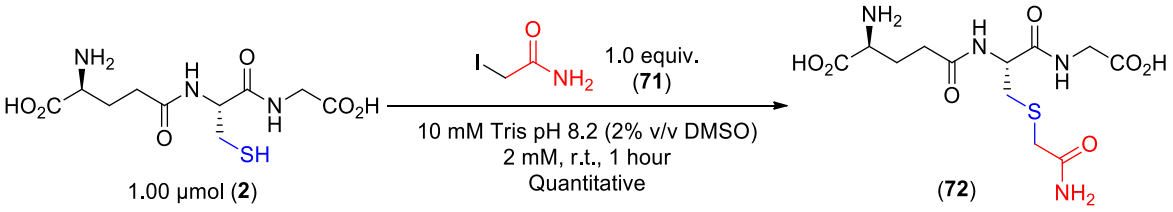
e. Stability evaluation in presence of N_3-EBX (**1a**)

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) (0.65 mg, 1.00 µmol) and Tris buffer (100 mM, pH 8.2, 490 µL). Then, a 1.00 M solution of N_3-EBX reagent (**1a**) in DMSO (10.0 µL, 10.0 µmol, 10.0 equiv.) was added and the resulting mixture was shaken at room temperature. After 4 days, 86% of the S-glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**) remained intact while 72% was still available after 8 days. When only 5.00 equiv. of N_3-EBX reagent (**1a**) was used, no degradation was observed after 4 days.

7. Evaluation of reaction conditions

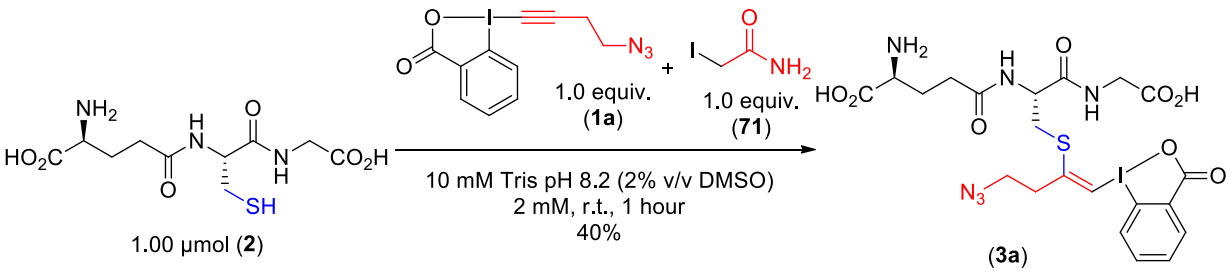
a. Competition experiment with iodoacetamide (**71**)

Control experiment with iodoacetamide (**71**):



In a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 100 mM solution of glutathione (**2**) in 10 mM Tris buffer (10.0 µL, 1.00 µmol) was diluted with Tris buffer (10 mM, pH 8.2, 480 µL). Then, a 100 mM solution of iodoacetamide (**71**) in DMSO (10.0 µL, 1.00 µmol, 1.00 equiv.) was added. The resulting solution was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 60 minutes. After this time, the reaction was analyzed by HPLC-MS, revealing quantitative conversion to **72**.

Competition experiment using stoichiometric amount of iodoacetamide (**71**) and N_3-EBX (**1a**):



In a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 100 mM solution of glutathione **(2)** in 10 mM Tris buffer (10.0 µL, 1.00 µmol) was diluted with Tris buffer (10 mM, pH 8.2, 480 µL). Separately, a 200 mM solution of iodoacetamide (**71**) in DMSO (5.00 µL, 1.00 µmol, 1.00 equiv.) and a 200 mM solution of N_3-EBX reagent (**1a**) in DMSO (5.00 µL, 1.00 µmol, 1.00 equiv.) were mixed. The resulting mixture was added to the solution of glutathione. The reaction mixture was then vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 60 minutes. HPLC-MS analysis of the mixture exhibited a yield of 40% in favor of **3a**. The rest of the mixture was composed of glutathione-acetamide conjugate **72** and oxidized glutathione.

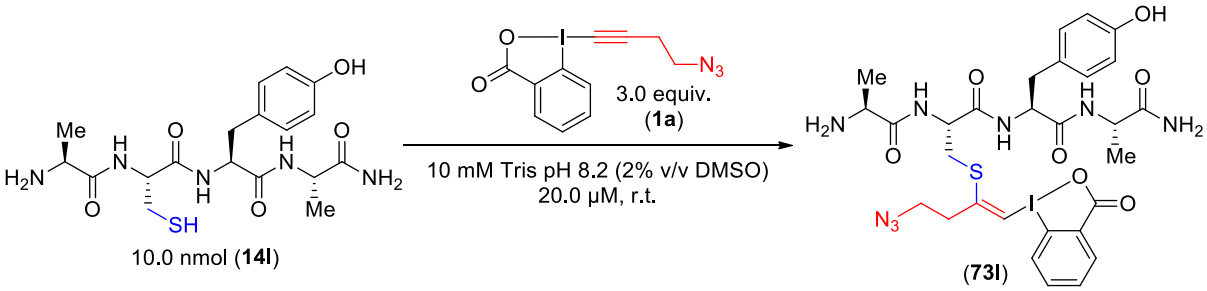
b. Concentration evaluation on Ala-Cys-Tyr-Ala (**14l**)

MS analysis conditions:

Quantitative analyses were conducted on a Xevo G2-S QTOF mass spectrometer coupled to the Acquity UPLC Class Binary Solvent manager and BTN sample manager (Waters, Corporation, Milford, MA). The sample manager system temperature was maintained at 10 °C and the injection volume was 2.0 µL and 5.0 µL for respectively 20 µM and 2 µM reactions. Mass spectrometer detection was operated in positive ionization using the ZSpray™ dual-orthogonal multimode ESI/APCI/ESCi® source. The TOF mass spectra were acquired in the sensitive mode over the range of m/z 50-800 at an acquisition rate of 0.036 sec/spectra. The instrument was calibrated using a solution of sodium formate (0.01 mg/L in *iso*propanol:water 90:10). A mass accuracy better than 5 ppm was achieved using a Leucine Enkephalin solution as lock-mass (200 pg/uL in acetonitrile:water (50:50)) infused continuously using the LockSpray

source. Source settings were as follows: cone = 25 V; capillary = 3 kV, source temperature = 150 °C; desolvation temperature = 500 °C, cone gas = 10 L/h, desolvation gas = 500 L/h. The separation was achieved using an ACQUITY UPLC® BEH C18 1.7 µm column, 2.1 mm x 50 mm (Waters) heated at 30°C. Mobile phase consisted of 0.1% formic acid in water as eluent A and 0.1% formic acid in acetonitrile as eluent B. The separation was carried out at 0.4 mL/min over a 6.0 min total run time using the following program: 0-2 min, 1% B; 2-5 min, 1-95% B; 5-5.1 min, 1% B; 5.1-6 min, 1% B to reequilibrate the system in initial conditions.

20 µM reaction:



A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a 1.00 mM solution of Ala-Cys-Tyr-Ala (**14l**) in 10 mM Tris buffer (10.0 µL, 10.0 nmol) and Tris buffer (10 mM, pH 8.2, 480 µL). Then, a 3.00 mM solution of N_3-EBX reagent (**1a**) in DMSO (10.0 µL, 30.0 nmol, 3.00 equiv.) was added. The resulting solution was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature. The reaction mixture was sampled and analyzed by LC-MS. The following graphic represents the relative presence of Ala-Cys-Tyr-Ala (**14l**) and final product **73l** ions. The absolute ion presence values of Ala-Cys-Tyr-Ala (**14l**) and final product **73l** were added and normalized to 100%.

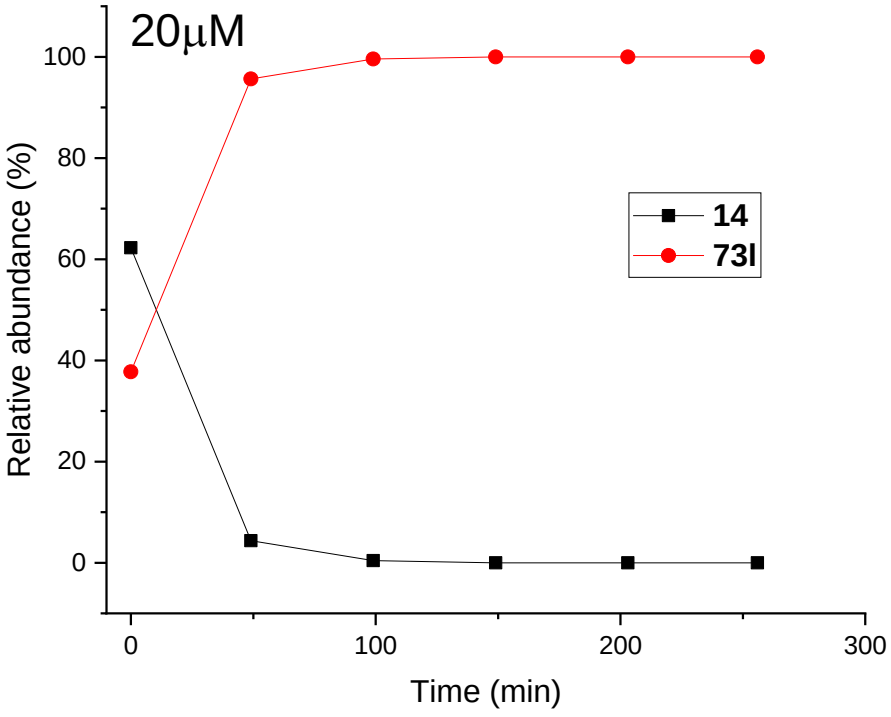
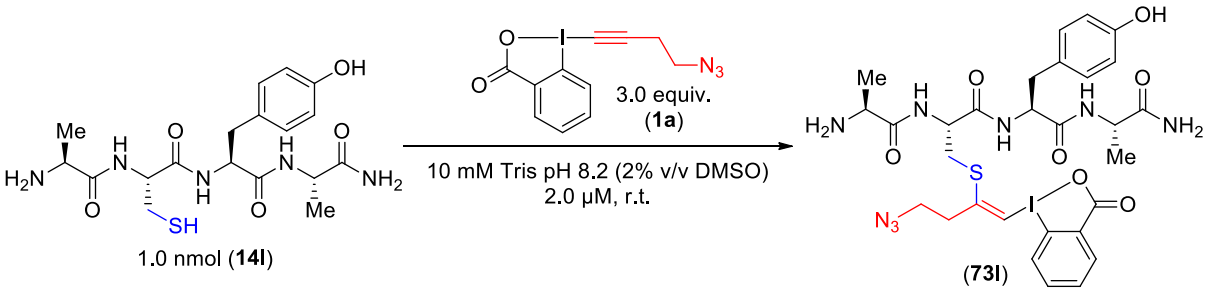


Figure S4: 20 µM reaction profile of **ACYA (14)** labeling under dilution conditions.

2 µM reaction:



A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a 100 µM solution of Ala-Cys-Tyr-Ala (**14l**) in 10 mM Tris buffer (10.0 µL, 1.00 nmol) and Tris buffer (10 mM, pH 8.2, 480 µL). Then, a 300 µM solution of N_3-EBX reagent (**1a**) in DMSO (10.0 µL, 3.00 nmol, 3.00 equiv.) was added. The resulting solution was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature. The reaction mixture was sampled and analyzed by LC-MS. The following graphic represents the relative presence of Ala-Cys-Tyr-Ala (**14l**) and final product **73l** ions. The absolute ion presence values of Ala-Cys-Tyr-Ala (**14l**) and final product **73l** were added and normalized to 100%.

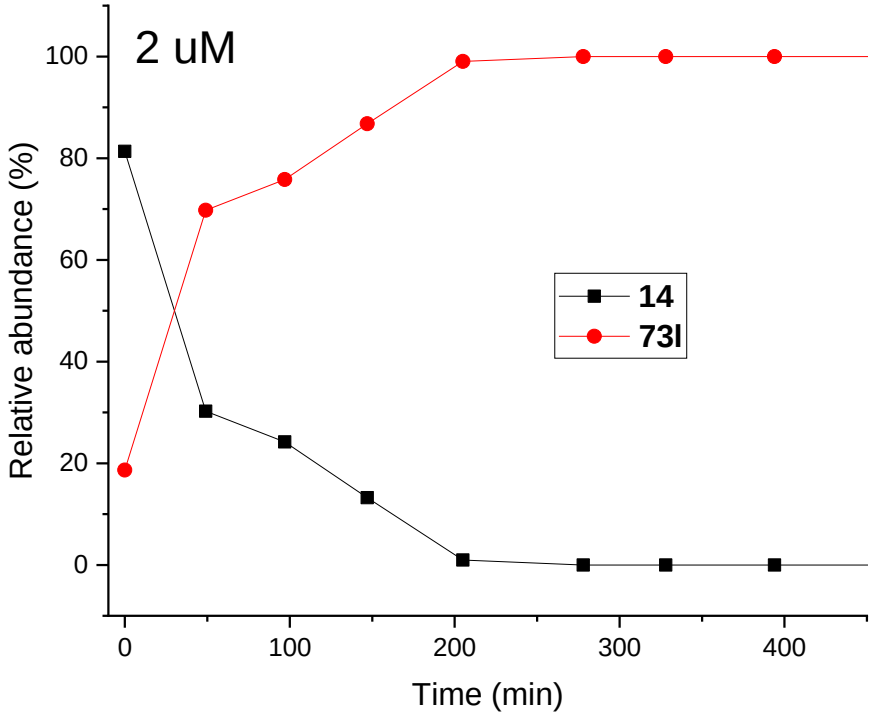
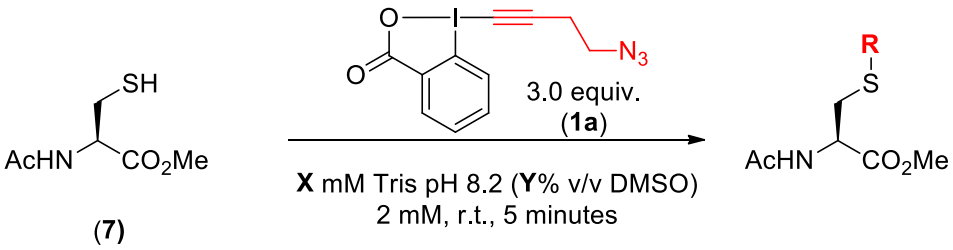
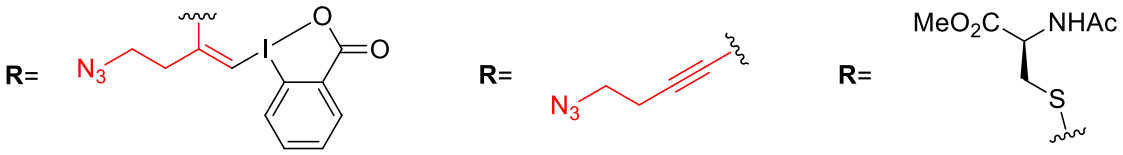


Figure S5: 2 µM reaction profile of **ACYA (14)** labeling under dilution conditions.

c. Tris Buffer/DMSO ratio evaluation on protected cysteine (**7**)





(**10**) **VBX** (**74**) **Thioalk.** (**75**) **S-S**

Conditions Screened

X(mM)	10	10.9	12.9	19.6	32.7	98	-
Y(%)	2	10	30	50	70	90	100

General procedure for Tris Buffer/DMSO ratio evaluation:

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a solution of protected cysteine (**7**) (0.050 mL, 20 mM, 1.0 equiv.) in 98 mM Tris pH 8.2 buffer, the mixture was then diluted with water ($V_{2\rightarrow 90\%dmso}$(µL)= 440, 400, 300, 200, 100, 0) to reach **X** mM concentration of Tris buffer. Then, from a stock solution 300 mM of N_3-EBX reagent (**1a**) in DMSO, 10 µL (3.00 µmol, 3.00 equiv.) were taken and diluted further with DMSO ($V_{2\rightarrow 90\%dmso}$(µL)= 0, 40, 140, 240,340, 440) and the mixture was added to the previous buffer solution. The resulting solution was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes. The reaction mixture was then sampled and analyzed by LC-MS. The absolute ion presence values of **VBX** (**10**), **Thioalk.** (**74**) and **S-S** (**75**) were added and normalized to 100%.

Procedure for the 100% DMSO reaction:

A small cylindrical vial was charged with (R)-methyl 2-acetamido-3-mercaptopropanoate (**7**) (1.0 mg, 5.64 µmol, 1.0 equiv.) in DMSO (2.0 mM), 1,1,3,3-tetramethylguanidine in DMSO (0.78 µL, 6.21 µmol, 1.1 equiv.) was then added and the mixture was then vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes before adding a N_3-EBX reagent (**1a**) (5.8 mg, 17.0 µmol, 3.0 equiv.). After 15 minutes the solution was sampled and analyzed by LC-MS. The absolute ion presence values of VBX (**10**), Thioalk**.** (**74**) and S-S (**75**) were added and normalized to 100%.

Table S11: Tris Buffer/DMSO ratio evaluation for protected cysteine (**7**).

DMSO(%)	Thioalk.(%)	VBX(%)	S-S(%)
2	2	82	16

10	3	91	7
30	2	89	9
50	4	83	13
70	4	82	14
90	42	46	13
100[a]	74	10	16

Values obtained as normalized ion count (Rel. TIC).
a) The values obtained for DMSO 100% were obatined in a different experiment.

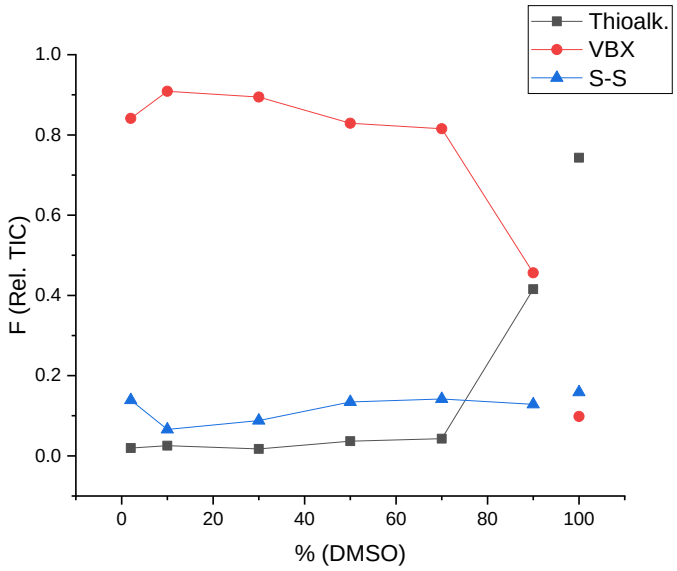


Figure S2: Normalized total ion count for Thioalkynyl (**74**), VBX (**10**) and disulfide (**75**) products vs the % of DMSO as solvent for the reaction of protected cysteine (**7**) with EBX reagent **1a**.

2% DMSO

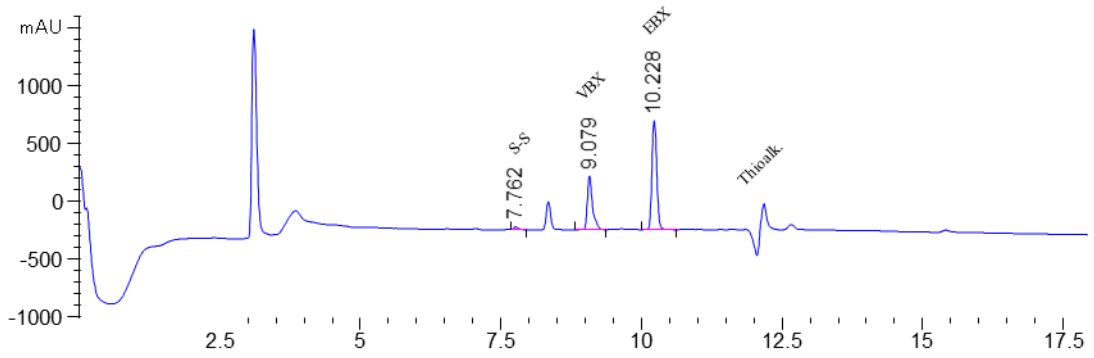


Figure S16: HPLC-UV chromatogram of the 2% DMSO reaction at 214 nm.

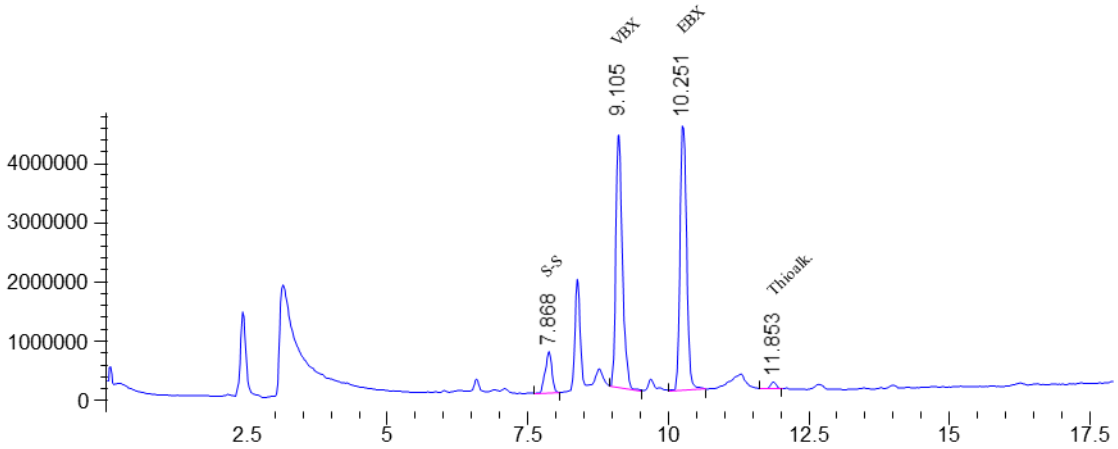


Figure S17: HPLC-MS of the 2% DMSO reaction chromatogram

10% DMSO

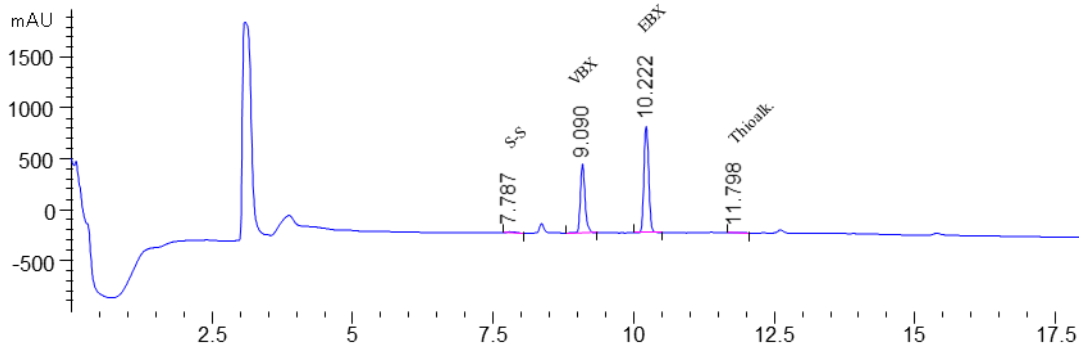


Figure S18: HPLC-UV chromatogram of the 10% DMSO reaction at 214 nm.

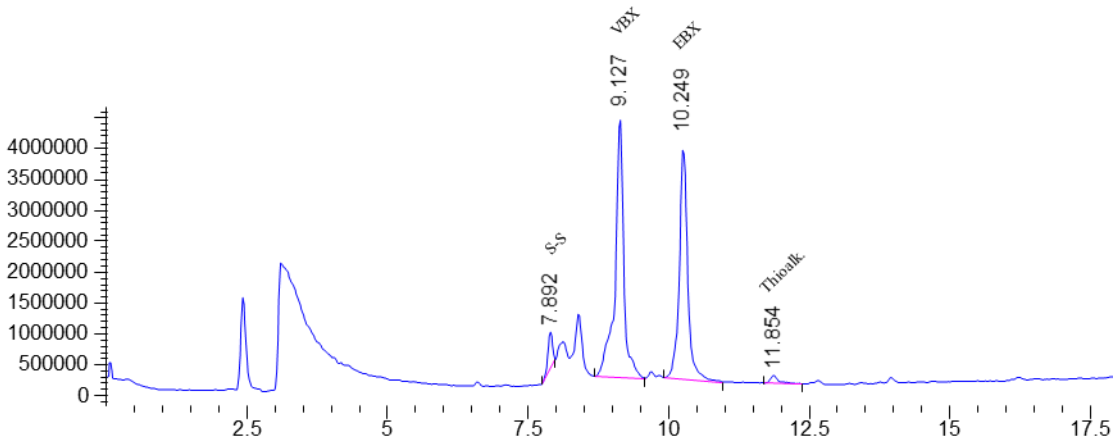


Figure S19: HPLC-MS chromatogram of the 10% DMSO reaction.

30% DMSO

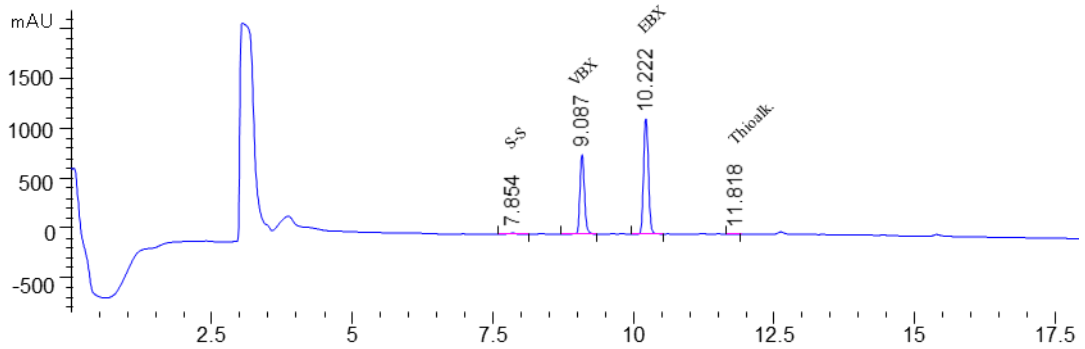


Figure S20: HPLC-UV chromatogram of the 30% DMSO reaction at 214 nm.

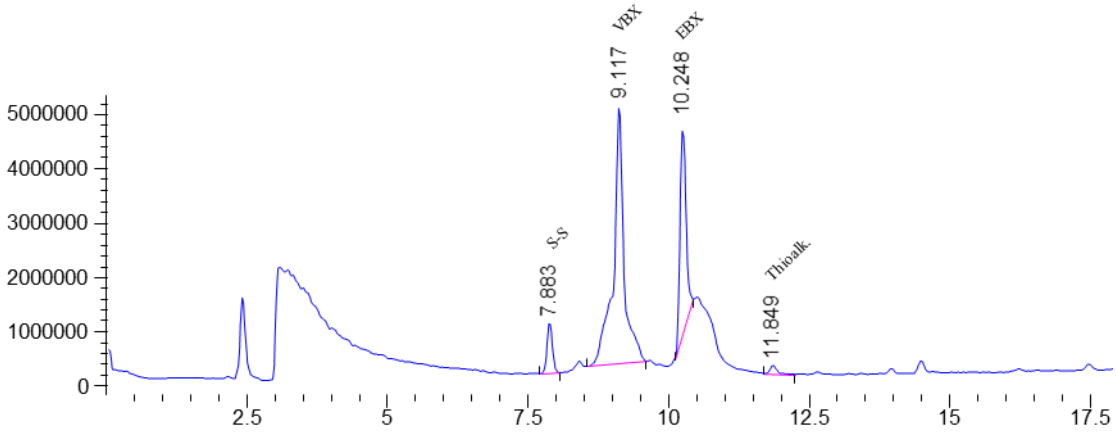


Figure S21: HPLC-MS chromatogram of the 30% DMSO reaction.

50% DMSO

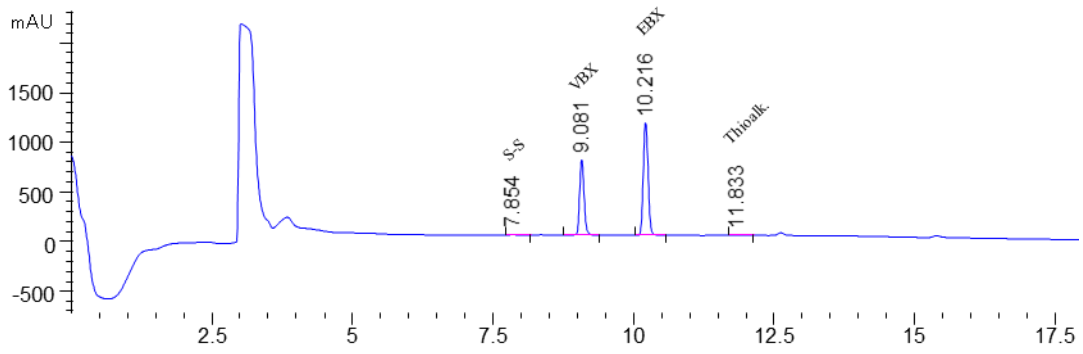


Figure S22: HPLC-UV chromatogram of the 50% DMSO reaction.at 214 nm

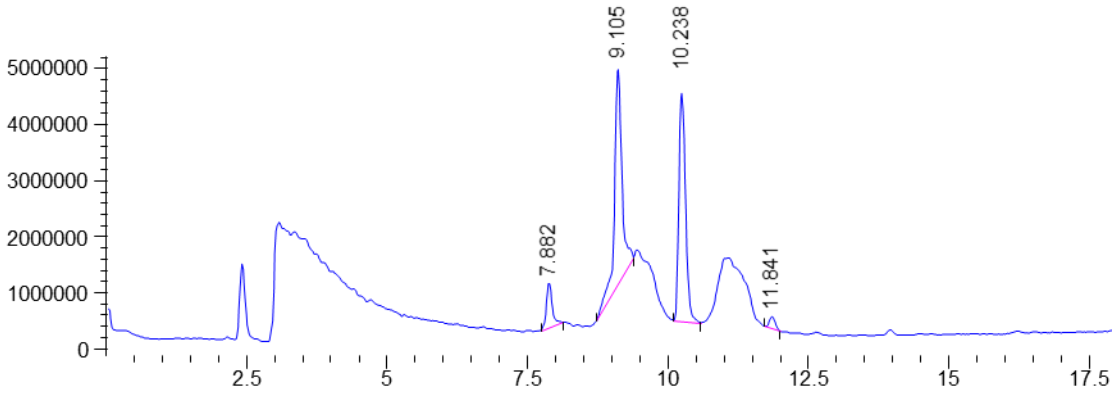


Figure S23: HPLC-MS chromatogram of the 50% DMSO reaction.

70% DMSO

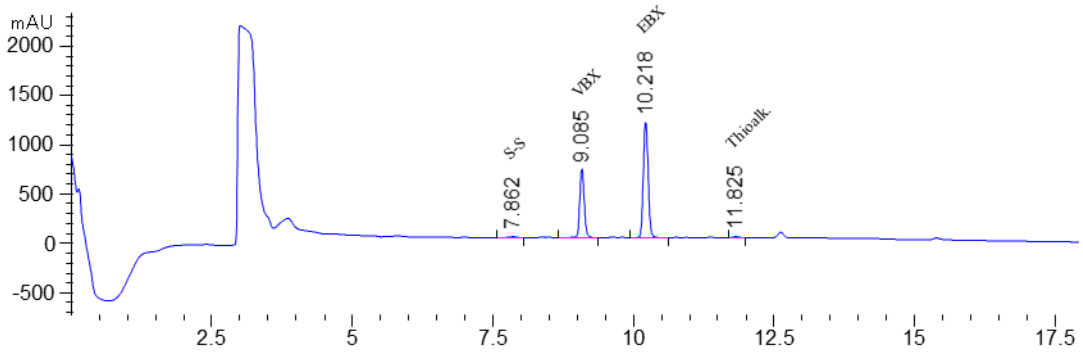


Figure S24: HPLC-UV chromatogram of the 70% DMSO reaction at 214 nm.

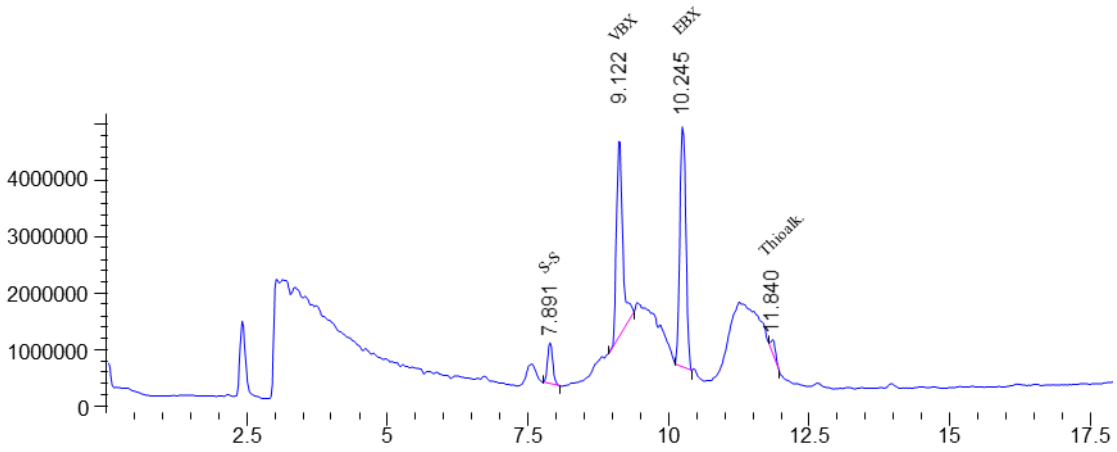


Figure S25: HPLC-MS chromatogram of the 70% DMSO reaction.

90% DMSO

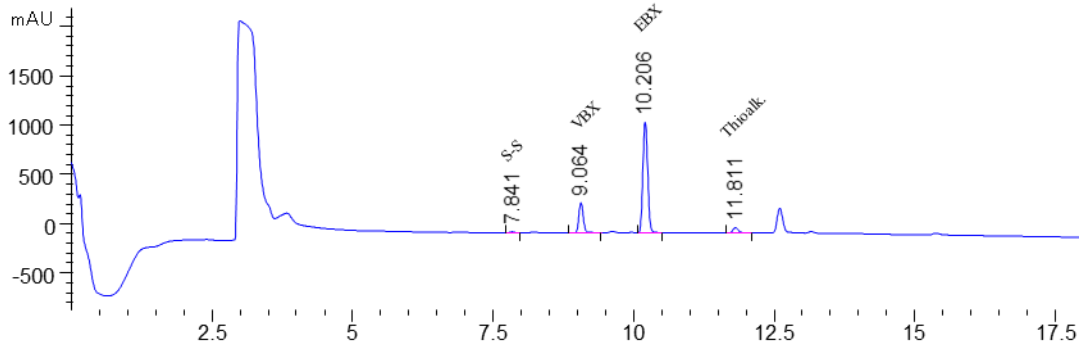


Figure S26: HPLC-UV chromatogram of the 90% DMSO reaction. at 214 nm.

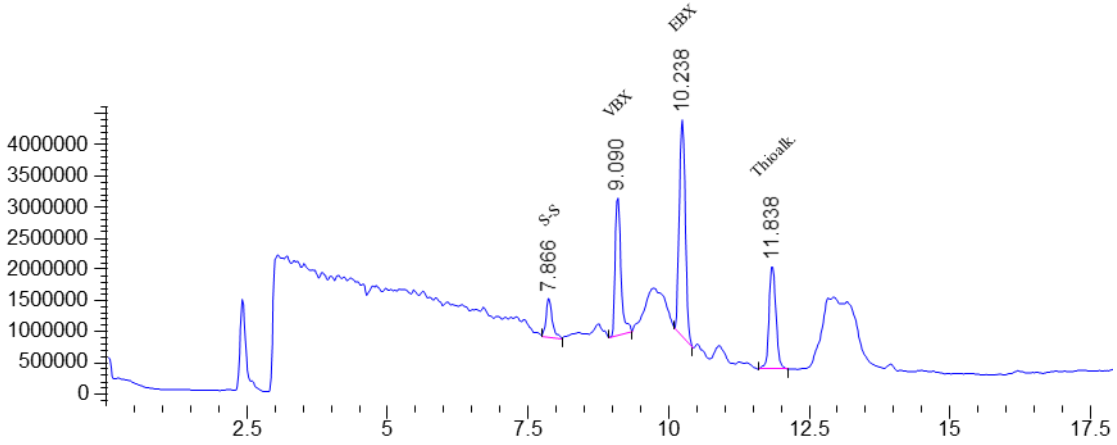


Figure S27: HPLC-MS chromatogram of the 90% DMSO reaction.

100% DMSO

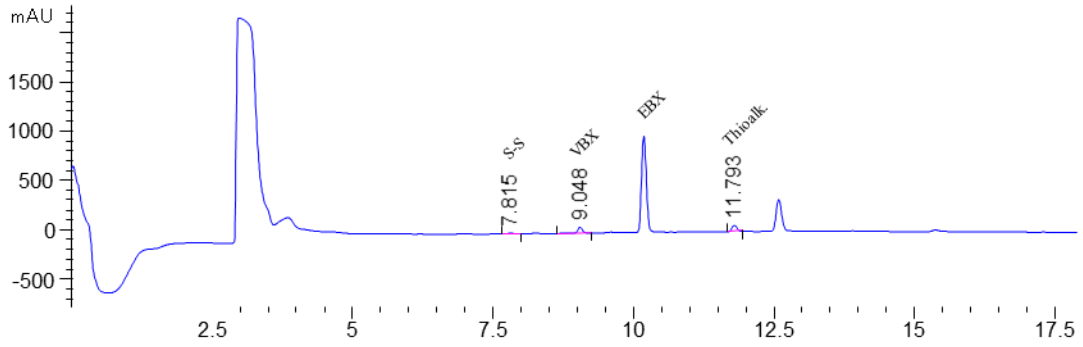


Figure S28: HPLC-UV chromatogram of the 100% DMSO reaction at 214 nm.

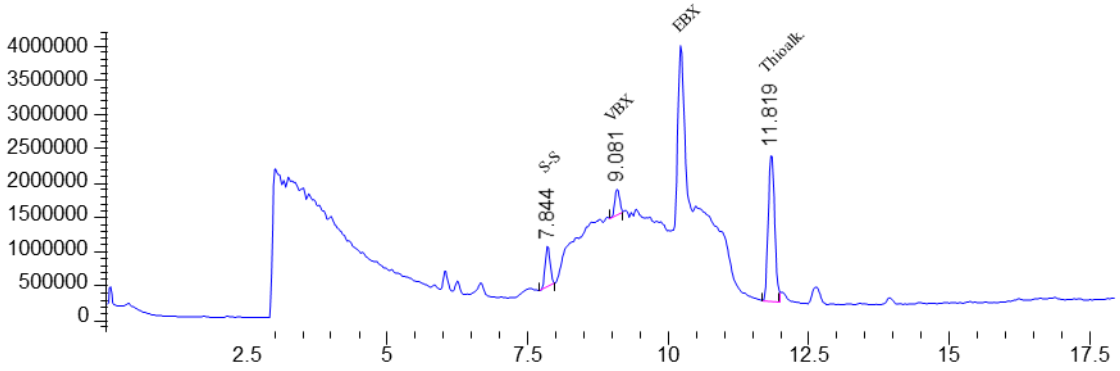
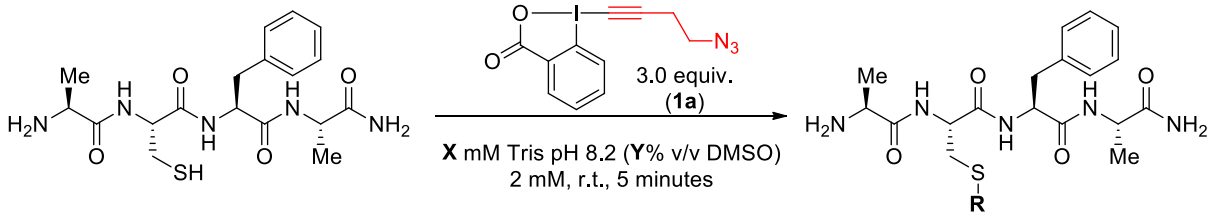
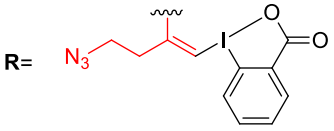


Figure S29: HPLC-MS chromatogram of the 100% DMSO reaction.

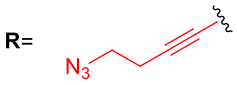
d. Tris Buffer/DMSO ratio evaluation on Ala-Cys-Phe-Ala (**14a**)



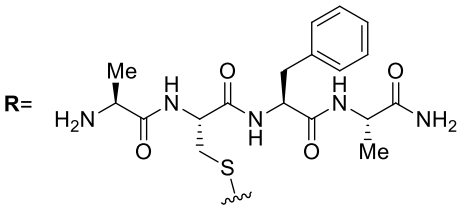
(**14a**)



(**73a**) **VBX**



(**76**) **Thioalk.**



(**77**) **S-S**

Conditions Screened

X(mM)	10	10.9	12.9	19.6	32.7	98	-
Y(%)	2	10	30	50	70	90	100

General procedure for the Tris Buffer/DMSO ratio evaluation on Ala-Cys-Phe-Ala:

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with a solution of Ala-Cys-Tyr-Ala (**14l**) (0.042 mL, 19.9 mM, 1.0 equiv.) in 98 mM Tris pH 8.2 buffer, the mixture was then diluted with water ($V_{2\rightarrow 90\%dmso}$(µL)= 370, 336, 252, 168, 84, 0) to reach **X** mM concentration of Tris buffer. Then, from a stock solution 300 mM of N_3-EBX reagent (**1a**) in DMSO, 8.34 µL (2.502 µmol, 3.00 equiv.) were taken and diluted further with DMSO ($V_{2\rightarrow 90\%dmso}$(µL)= 0, 33, 117, 200, 284, 367)) and the mixture was added to the previous buffer solution. The resulting solution was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes. The reaction mixture was then sampled and analyzed by LC-MS. The absolute ion presence values of VBX (**73a**), Thioalk**.** (**76**) and S-S (**77**) were added and normalized to 100%.

Procedure for the 100% DMSO reaction:

A small cylindrical vial was charged with a solution of Ala-Cys-Phe-Ala (**14a**) (2.25 mL, 2.0 mM, 1.0 equiv.) in DMSO, a solution of TMG in DMSO (49.4 µL, 0.1M, 1.1 equiv.) was then added and the mixture was then vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes before adding a final solution of N_3-EBX reagent (**1a**) in DMSO (135 µL, 0.1M, 3.0 equiv.). After 15 minutes the solution was sampled and analyzed by LC-MS. The absolute ion presence values of VBX (**73a**), Thioalk**.** (**76**) and S-S (**77**) were added and normalized to 100%.

Table S12: Tris Buffer/DMSO ratio evaluation for tetramer ACFA (**14a**).

DMSO(%)	Thioalk.(%)	VBX(%)	S-S(%)
2	4	86	10
10	4	88	8
30	4	88	7
50	6	87	7
70	17	80	4
90	58	39	3
100[a]	69	0	31

Values obtained as normalized ion count (Rel. TIC).
A) The values obtained for DMSO 100% were 57btained in a different experiment following procedure for the 100% DMSO reaction.

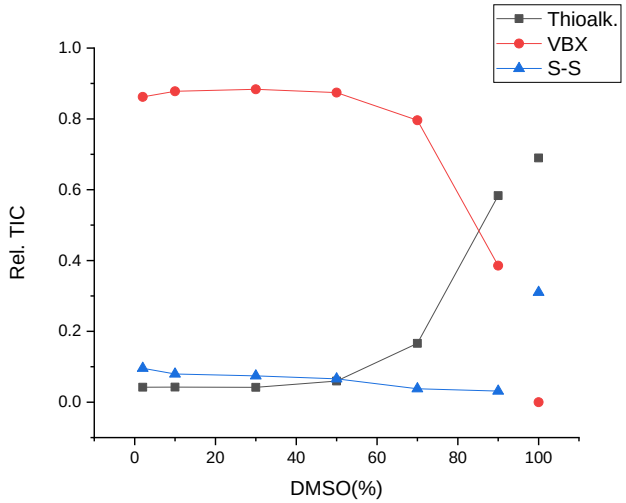


Figure S3: Normalized total ion count for Thioalkynyl (**73a**), VBX (**76**) and disulfide (**77**) products vs the % of DMSO as solvent for the reaction of ACYA (**14l**) with EBX reagent **1a**.

2% DMSO

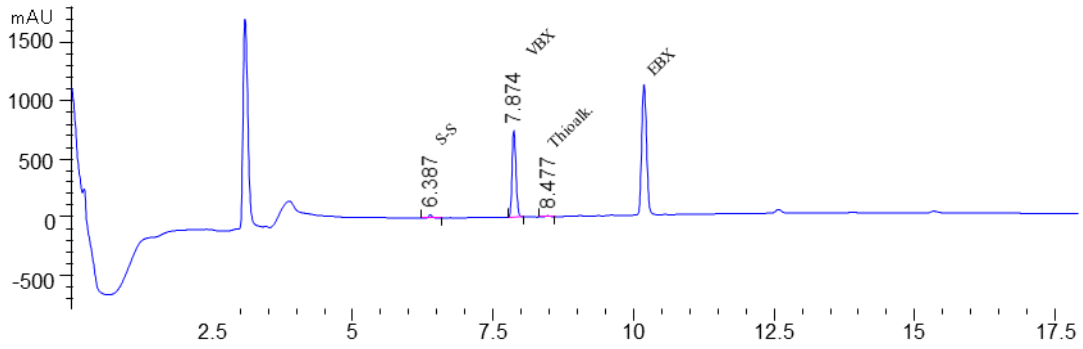


Figure S30: HPLC-UV chromatogram of the 2% DMSO reaction at 214 nm.

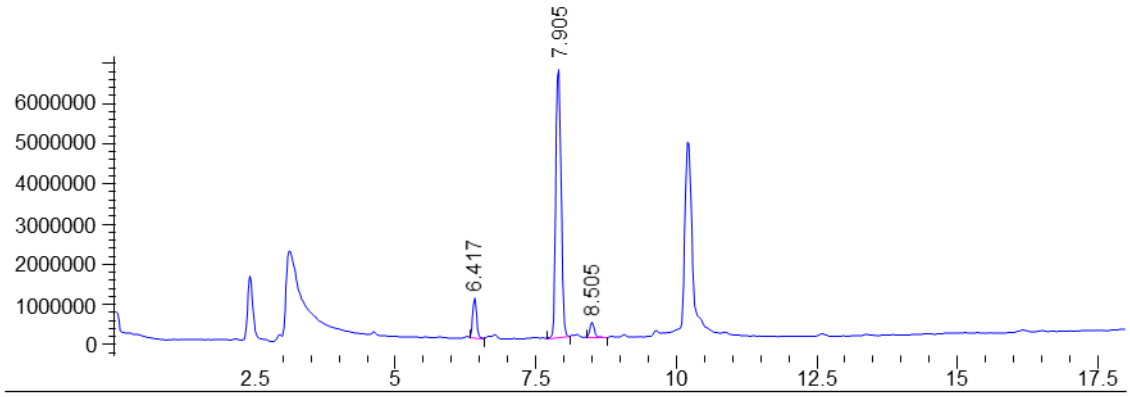


Figure S31: HPLC-MS chromatogram of the 2% DMSO reaction.

10% DMSO

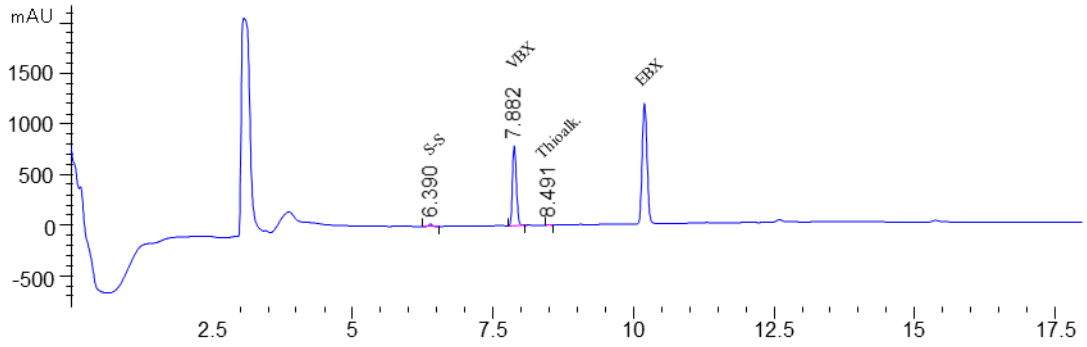


Figure S32: HPLC-UV chromatogram of the 10% DMSO reaction at 214 nm.

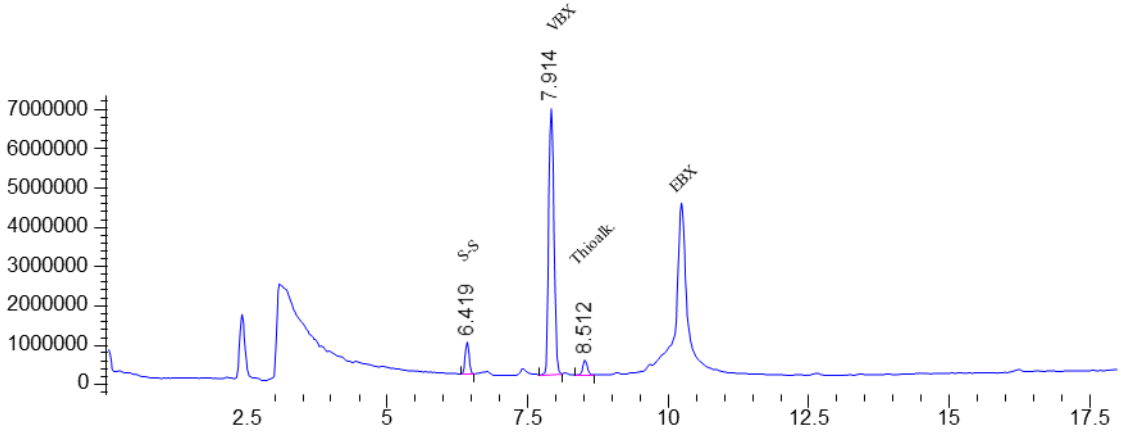


Figure S33: HPLC-MS chromatogram of the 10% DMSO reaction.

30% DMSO

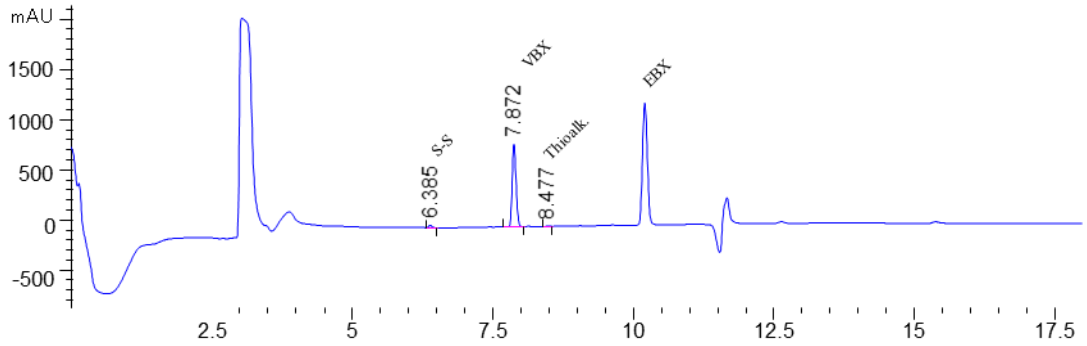


Figure S34: HPLC-UV chromatogram of the 30% DMSO reaction at 214 nm.

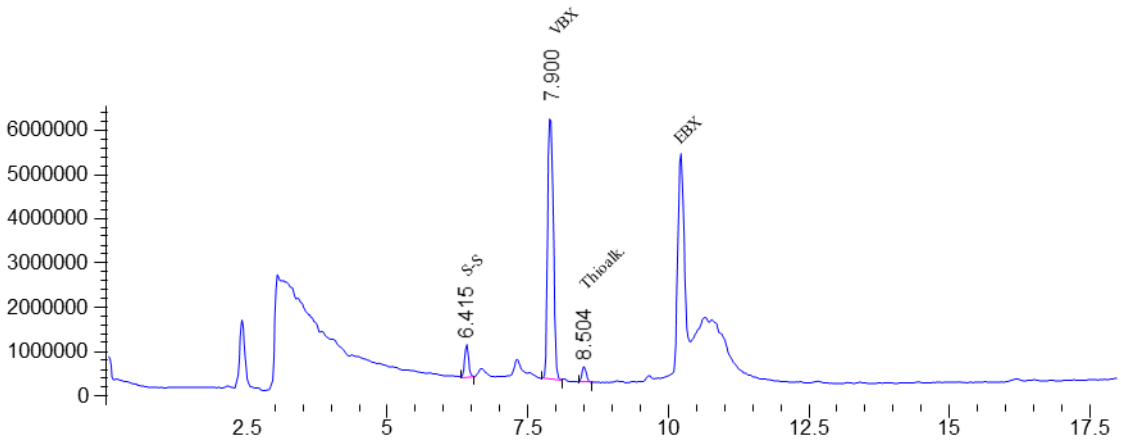


Figure S35: HPLC-MS chromatogram of the 30% DMSO reaction.

50% DMSO

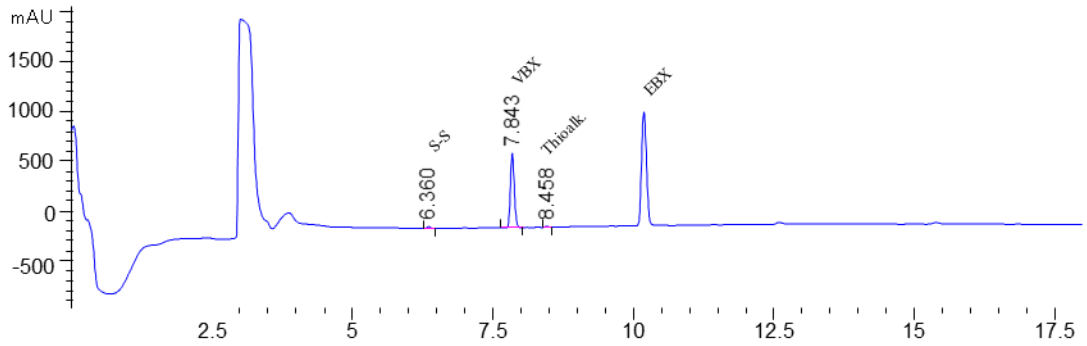


Figure S36: HPLC-UV chromatogram of the 50% DMSO reaction at 214 nm.

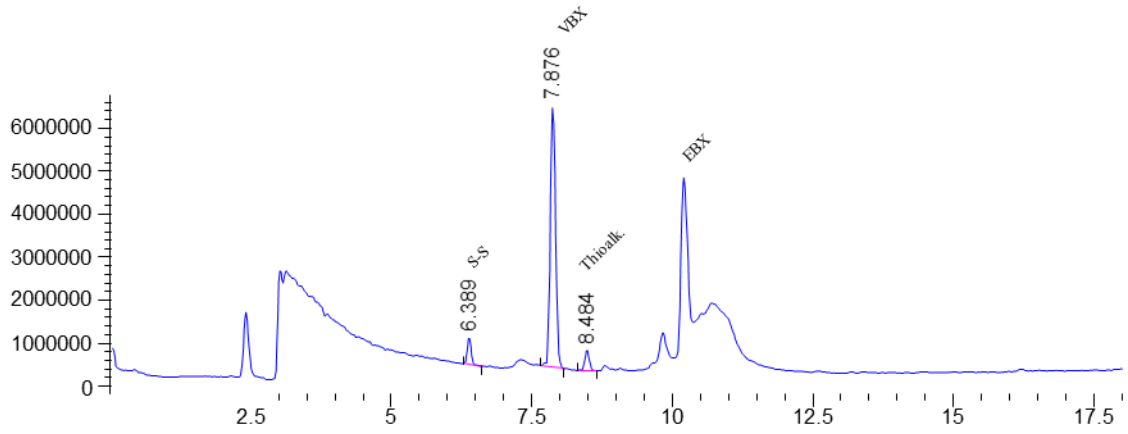


Figure S37: HPLC-MS chromatogram of the 50% DMSO reaction.

70% DMSO

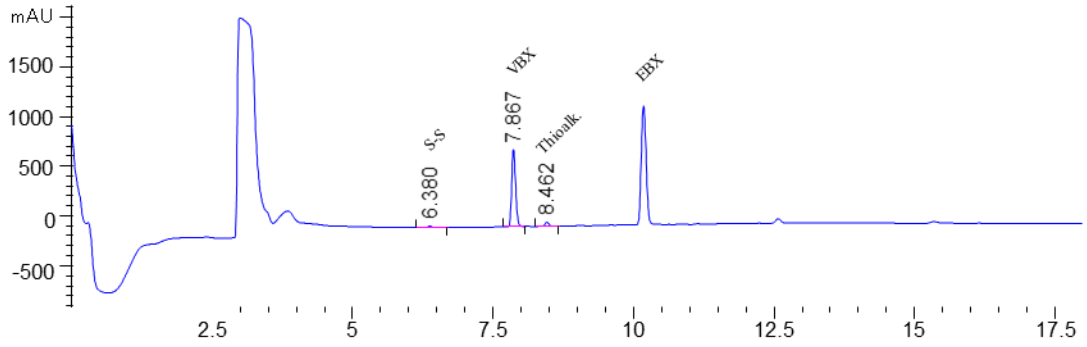


Figure S38: HPLC-UV chromatogram of the 70% DMSO reaction at 214 nm.

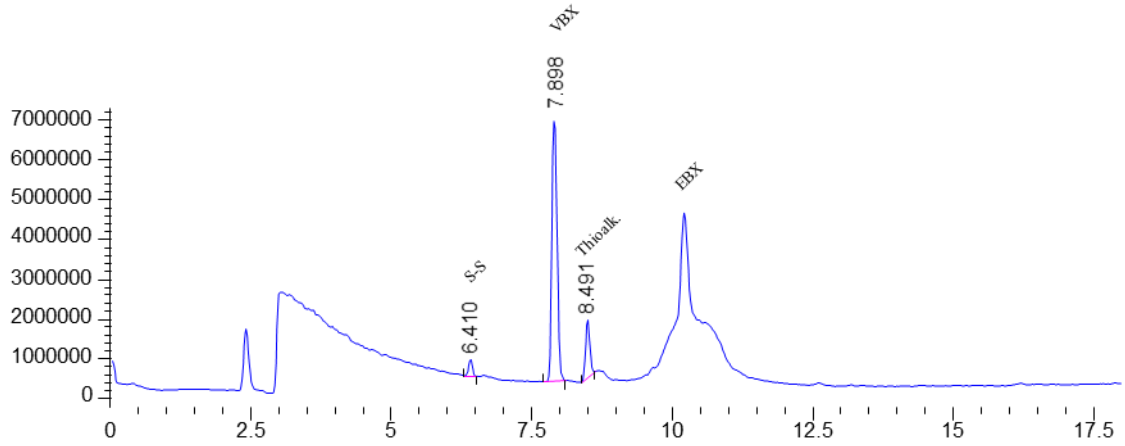


Figure S39: HPLC-MS chromatogram of the 70% DMSO reaction.

90% DMSO

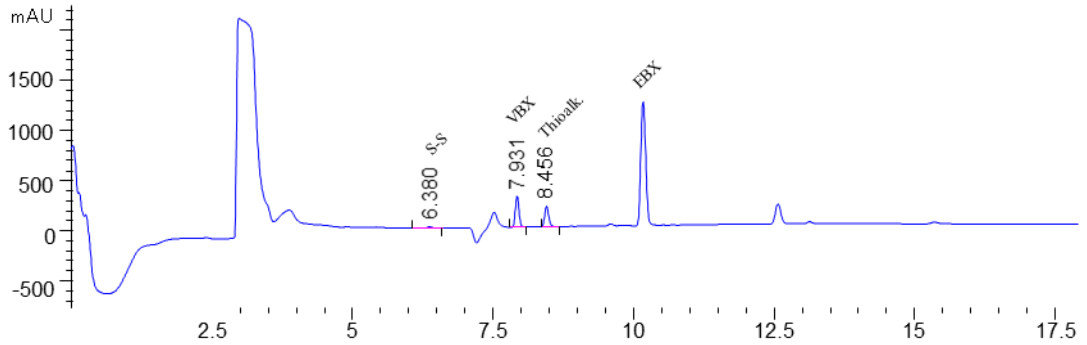


Figure S40: HPLC-UV chromatogram of the 90% DMSO reaction at 214 nm.

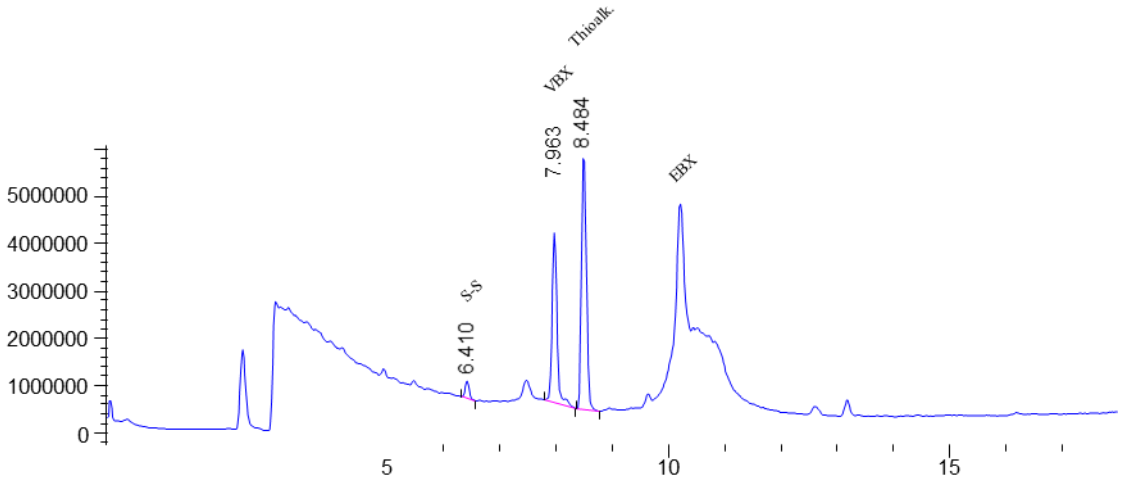


Figure S41: HPLC-MS chromatogram of the 90% DMSO reaction.

100% DMSO

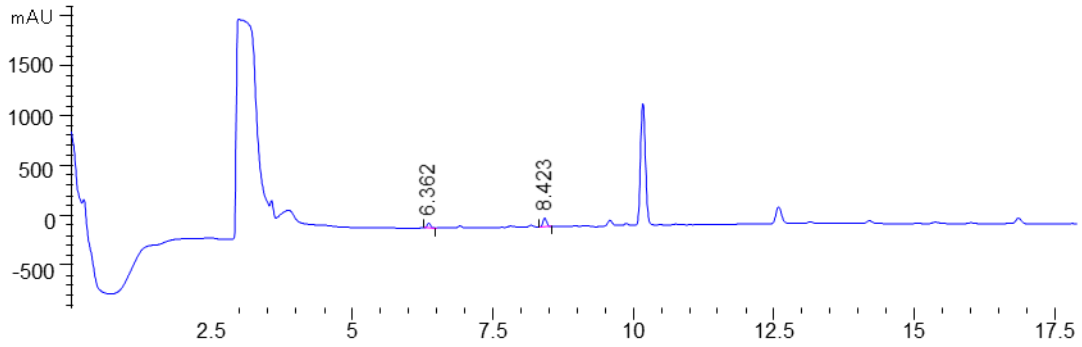


Figure S42: HPLC-UV chromatogram of the 100% DMSO reaction at 214 nm.

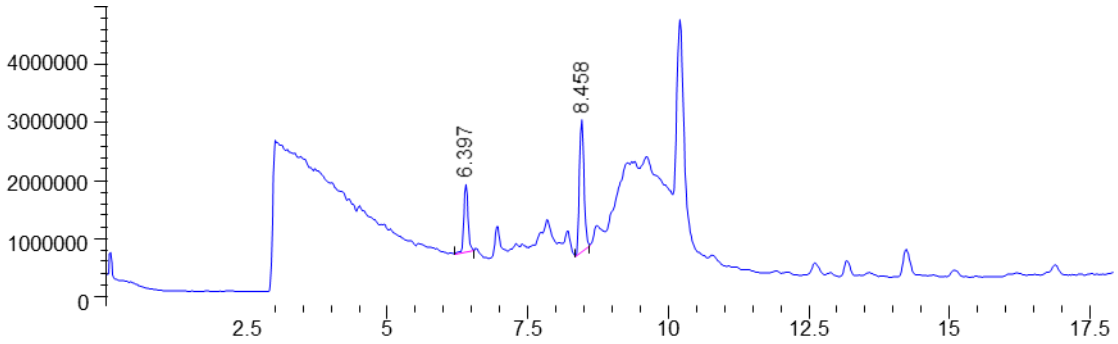
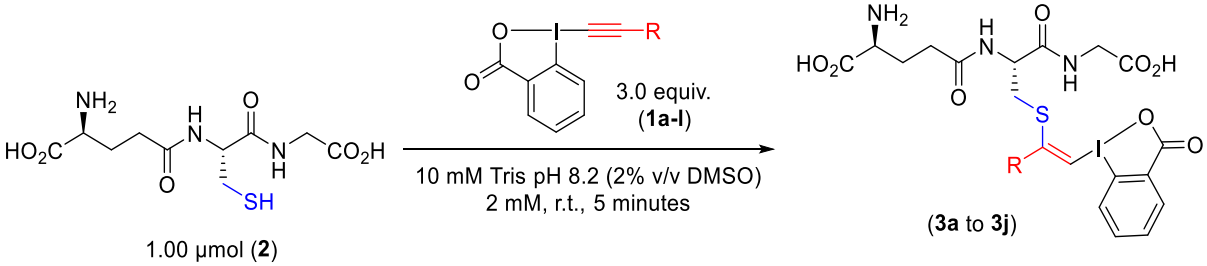


Figure S43: HPLC-MS chromatogram of the 100% DMSO reaction.

8. Substrate scope of EBX reagents



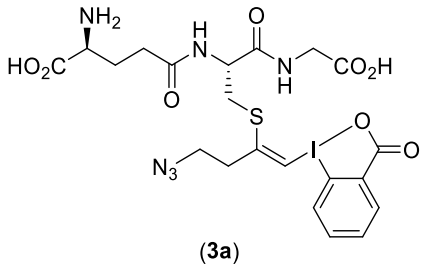
General procedure A:

In a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 100 mM solution of glutathione (**2**) in 10 mM Tris buffer pH 8.2 (10.0 µL, 1.00 µmol) was diluted with Tris buffer (10 mM, pH 8.2, 480 µL). The resulting solution was vortexed few seconds and a 300 mM solution of EBX reagent (**1a-l**) in DMSO (10.0 µL, 3.00 µmol, 3.00 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS and 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. The different glutathione-EBX conjugates (**3b to 3j**) were considered to have similar absorbance than the calibrated glutathione-N_3-EBX product **3a**.

General procedure B:

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with the corresponding EBX reagent (**1a-l**) (3.00 µmol, 3.00 equiv.) and Tris buffer (10 mM, pH 8.2, 490 µL). The resulting solution was vigorously shaken over 5 minutes and a 100 mM solution of glutathione (**2**) in 10 mM Tris buffer pH 8.2 (10.0 µL, 1.00 µmol) was added in one portion. The resulting mixture was vortexed few seconds and slowly shaken at room temperature for 5 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. The different glutathione-EBX conjugates (**3b to 3j**) were considered to have similar absorbance than the calibrated glutathione-N_3-EBX product **3a**.

S-Glutathione-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**3a**)



(**3a**)

Following general procedure A, (4-azidobut-1-ynyl)-1,2-benziodoxol-3(1H)-one (**1a**) afforded the title compound **3a** in 98% yield (retention time: 9.285 minutes).

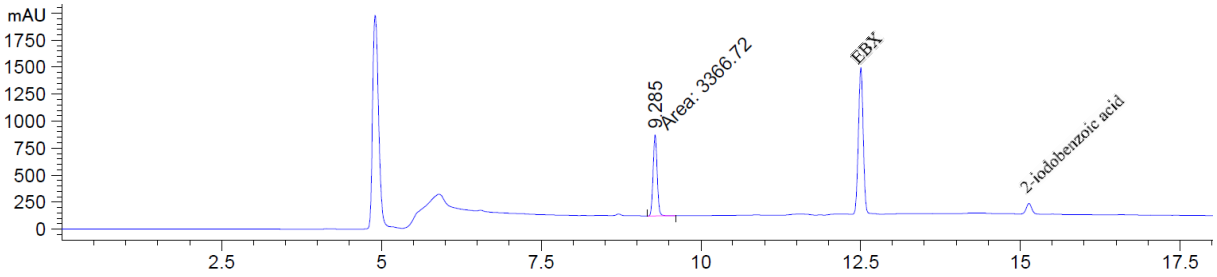


Figure S44: HPLC-UV chromatogram at 214 nm of **3a**.

Following general procedure B, (4-azidobut-1-ynyl)-1,2-benziodoxol-3(1H)-one (**1a**) afforded the title compound **3a** in 95% yield (retention time: 9.310 minutes).

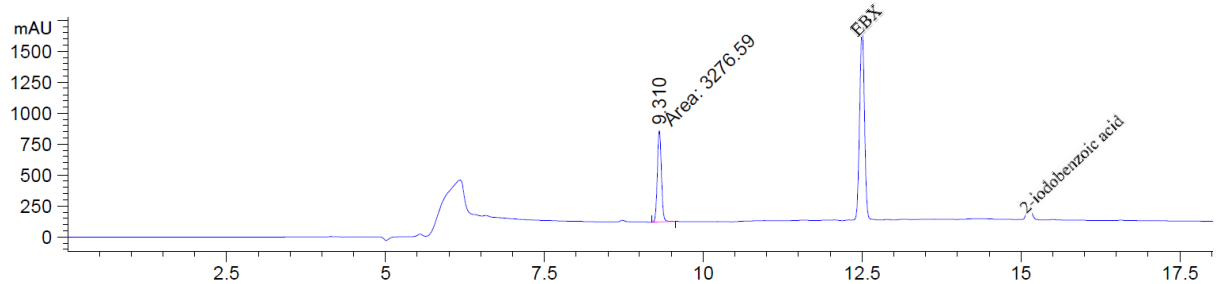
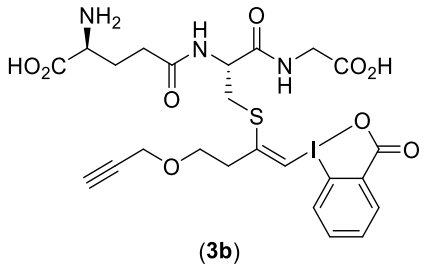


Figure S45: HPLC-UV chromatogram at 214 nm of **3a**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{21}H_{26}IN_6O_8S^+$ 649.0572; Found 649.0575.

S-Glutathione-(4-(prop-2-yn-1-yloxy- but-1-yn-1-yl))-1,2-vinylbenziodoxolone (**3b**)



(**3b**)

Following general procedure A, 4-(prop-2-yn-1-yloxy- but-1-yn-1-yl)-1,2-benziodoxol-3(1H)-one (**1b**) afforded the title compound **3b** in 86% yield (retention time: 9.342 minutes).

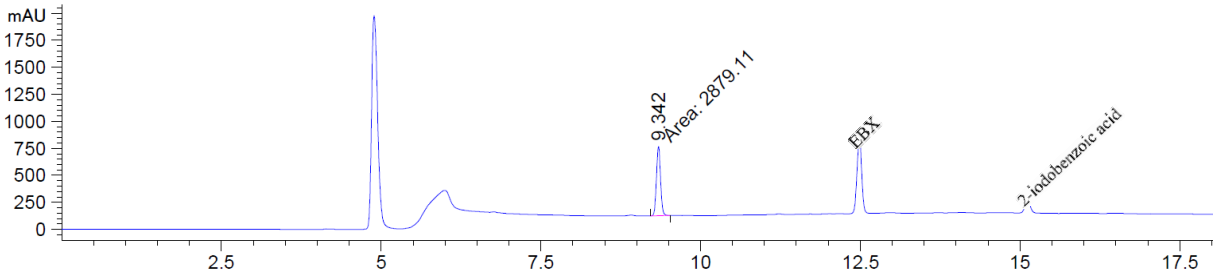


Figure S46: HPLC-UV chromatogram at 214 nm of **3b**.

Following general procedure B, 4-(prop-2-yn-1-yloxy- but-1-yn-1-yl)-1,2-benziodoxol-3(1H)-one (**1b**) afforded the title compound **3b** in 80% yield (retention time: 9.381 minutes).

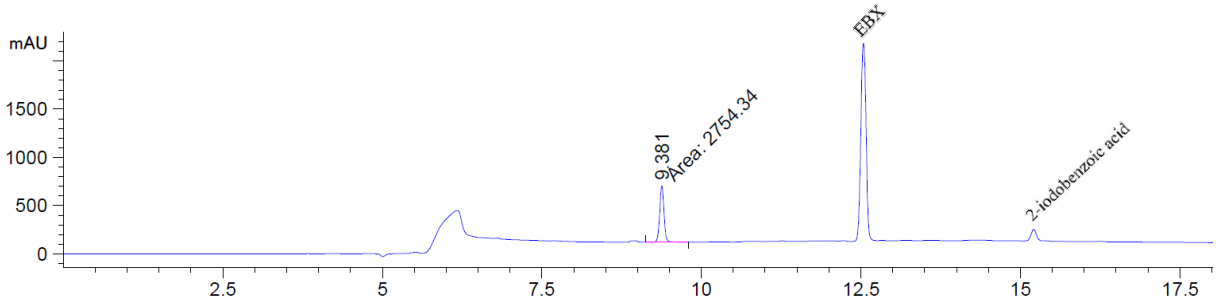
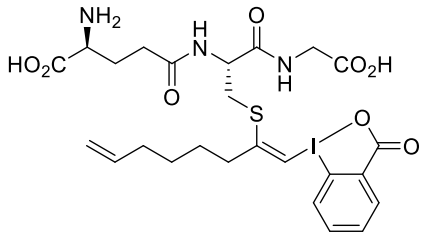


Figure S47: HPLC-UV chromatogram at 214 nm of **3b**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{24}H_{29}IN_3O_9S^+$ 662.0664; Found 662.1028.

S-Glutathione-(oct-6-en-1-ynyl)-1,2-vinylbenziodoxolone (**3c**)



(**3c**)

Following general procedure A, (oct-6-en-1-ynyl)-1,2-benziodoxol-3(1*H*)-one (**1c**) afforded the title compound **3c** in 84% yield (retention time: 11.405 minutes).

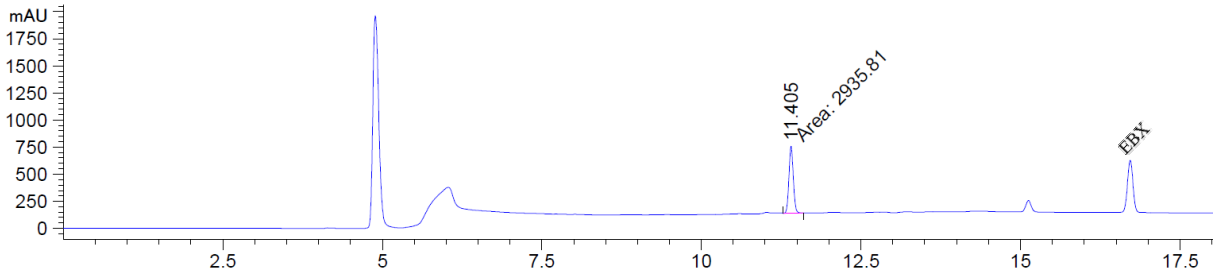


Figure S48: HPLC-UV chromatogram at 214 nm of **3c**.

Following general procedure B, (oct-6-en-1-ynyl)-1,2-benziodoxol-3(1*H*)-one (**1c**) afforded the title compound **3c** in 76% yield (retention time: 11.466 minutes).

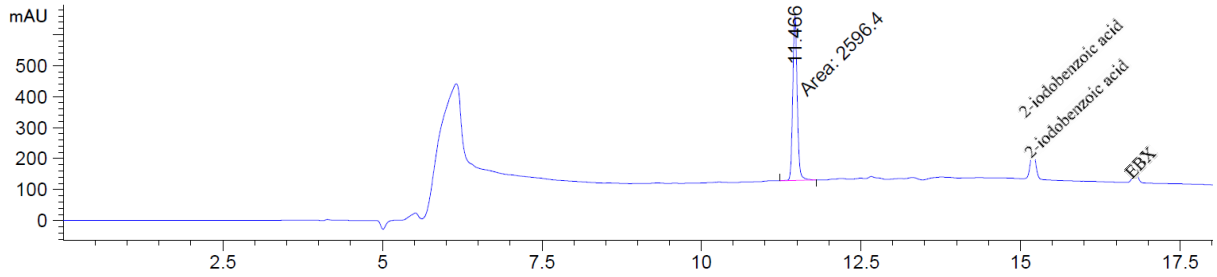
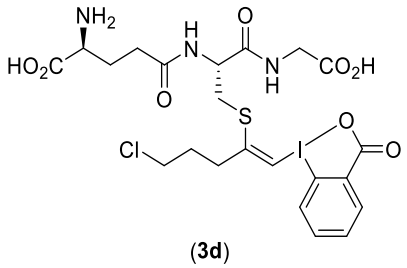


Figure S49: HPLC-UV chromatogram at 214 nm. of **3c**

HRMS (ESI) m/z: $[M + H]^+$ Calcd for $C_{25}H_{33i}N_3O_8S^+$ 662.1028; Found 662.1021.

S-Glutathione-(5-chloropent-1-ynyl)-1,2-vinylbenziodoxolone (**3d**)



(**3d**)

Following general procedure A, (5-chloropent-1-ynyl)-1,2-benziodoxol-3(1*H*)-one (**1d**) afforded the title compound **3d** in 99% yield (retention time: 9.735 minutes).

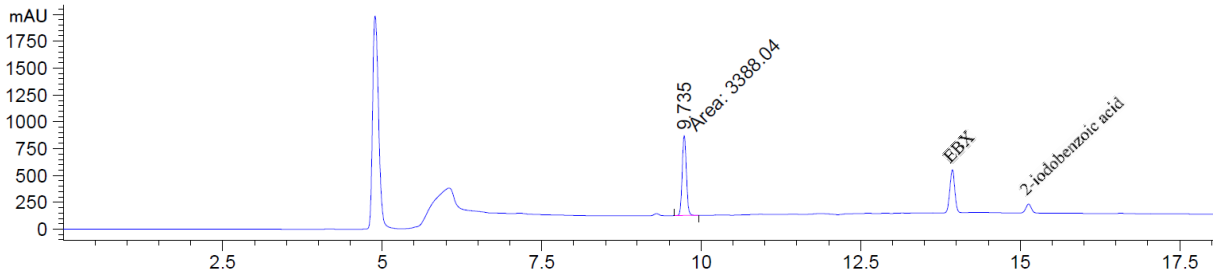


Figure S50: HPLC-UV chromatogram at 214 nm of **3d**.

Following general procedure B, (5-chloropent-1-ynyl)-1,2-benziodoxol-3(1*H*)-one (**1d**) afforded the title compound **3d** in 85% yield (retention time: 9.801 minutes).

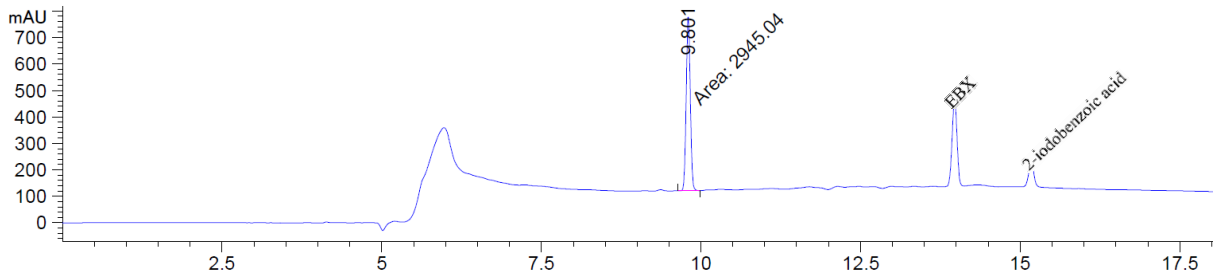
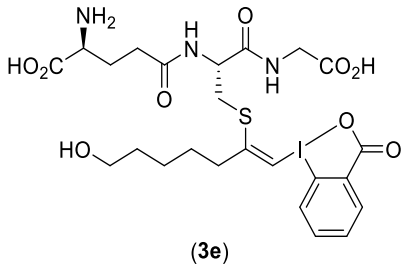


Figure S51: HPLC-UV chromatogram at 214 nm of **3d**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{22}H_{28cl}IIN_3O_8S^+$ 656.0325; Found 656.0333.

S-Glutathione-(5-pentanolethynyl)-1,2-vinylbenziodoxolone (**3e**)



(**3e**)

Following general procedure A, 5-pentanolethynyl-1,2-benziodoxol-3(1H)-one (**1e**) afforded the title compound **3e** in 93% yield (retention time: 8.240 minutes).

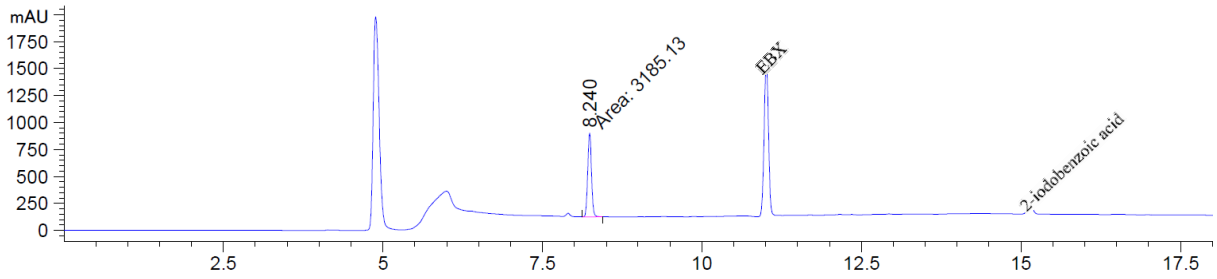


Figure S52: HPLC-UV chromatogram at 214 nm of **3d**.

Following general procedure B, 5-pentanolethynyl-1,2-benziodoxol-3(1H)-one (**1e**) afforded the title compound **3e** in 92% yield (retention time: 8.282 minutes).

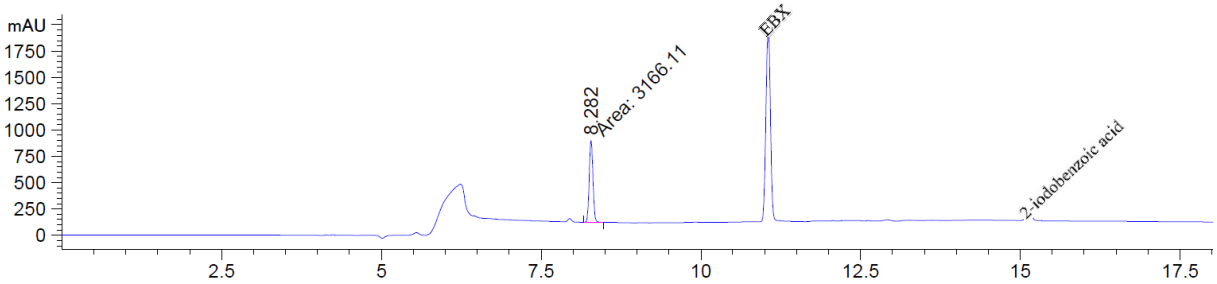
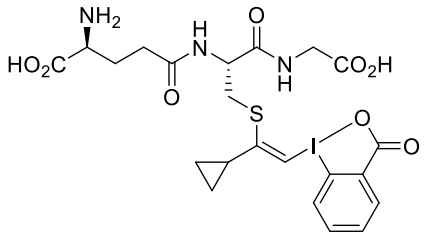


Figure S53: HPLC-UV chromatogram at 214 nm of **3d**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{24}H_{33i}N_3O_9S^+$ 666.0977; Found 666.0978.

S-Glutathione-(2-cyclopropyl)-1,2-vinylbenziodoxolone (**3f**)



(**3f**)

Following general procedure A, 2-cyclopropylethynyl-1,2-benziodoxol-3(1*H*)-one (**1f**) afforded the title compound **3f** in 96% yield (retention time: 8.684 minutes).

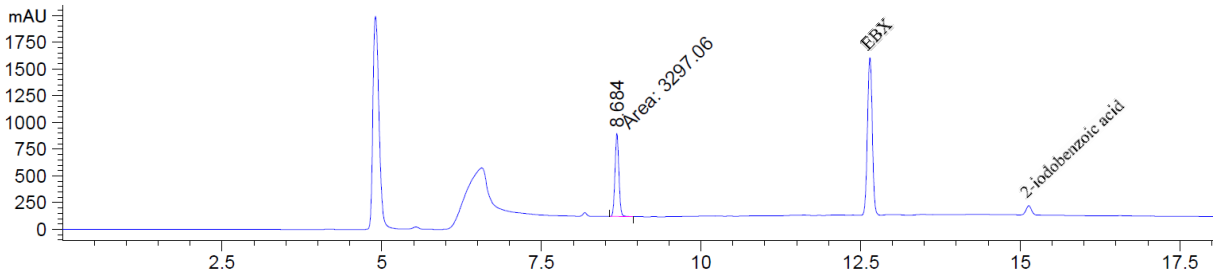


Figure S54: HPLC-UV chromatogram at 214 nm of **3f**.

Following general procedure B, 2-cyclopropylethynyl-1,2-benziodoxol-3(1*H*)-one (**1f**) afforded the title compound **3f** in 86% yield (retention time: 8.719 minutes).

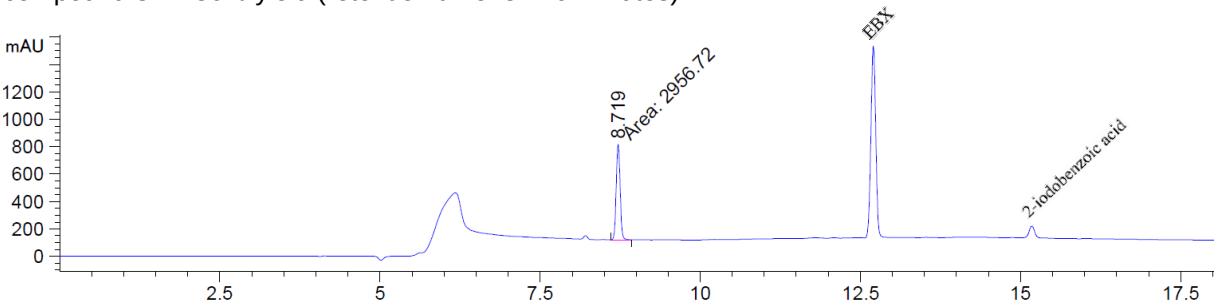
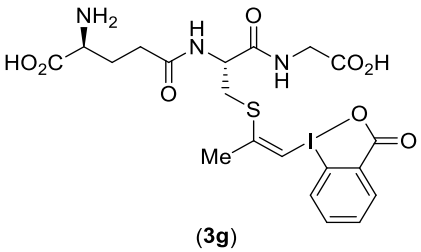


Figure S55: HPLC-UV chromatogram at 214 nm of **3f**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{22}H_{27}IN_3O_8S^+$ 620.0558; Found 620.0565.

S-Glutathione-(propynyl)-1,2-vinylbenziodoxolone (**3g**)



(**3g**)

Following general procedure A, propynyl-1,2-benziodoxol-3(1*H*)-one (**1g**) afforded the title compound **3g** in 89% yield (retention time: 7.675 minutes).

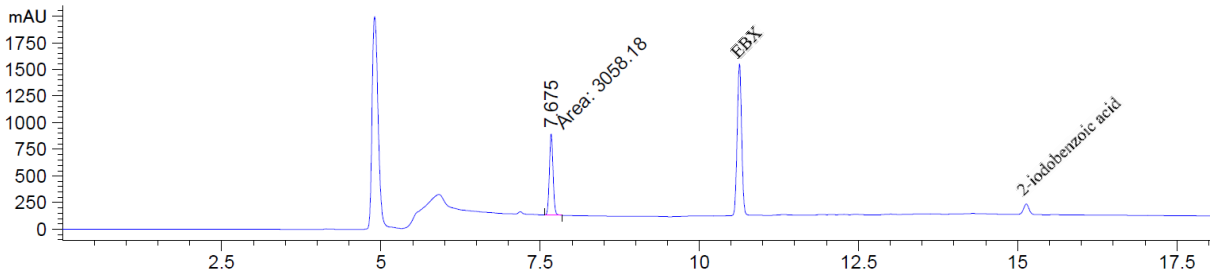


Figure S56: HPLC-UV chromatogram at 214 nm of **3g**.

Following general procedure B, propynyl-1,2-benziodoxol-3(1*H*)-one (**1g**) afforded the title compound **3g** in 82% yield (retention time: 7.730 minutes).

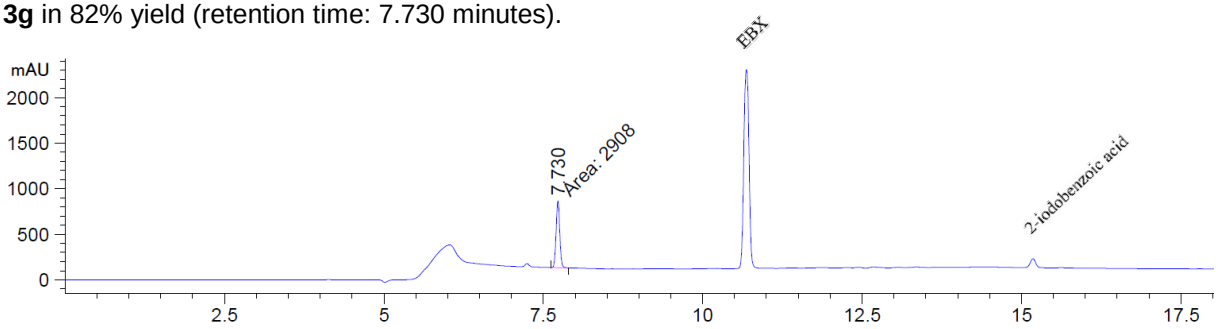
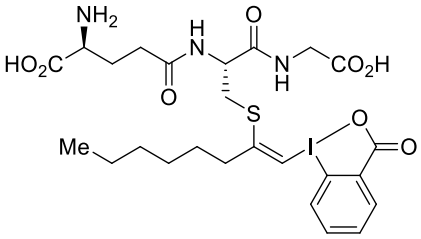


Figure S57: HPLC-UV chromatogram at 214 nm of **3g**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{20}H_{25}IN_3O_8S^+$ 594.0402; Found 594.0408.

S-Glutathione-(octenyl)-1,2-benziodoxolone (**3h**)



(**3h**)

Following general procedure A, octynyl-1,2-benziodoxol-3(1*H*)-one (**1h**) afforded the title compound **3h** in 89% yield (retention time: 12.110 minutes).

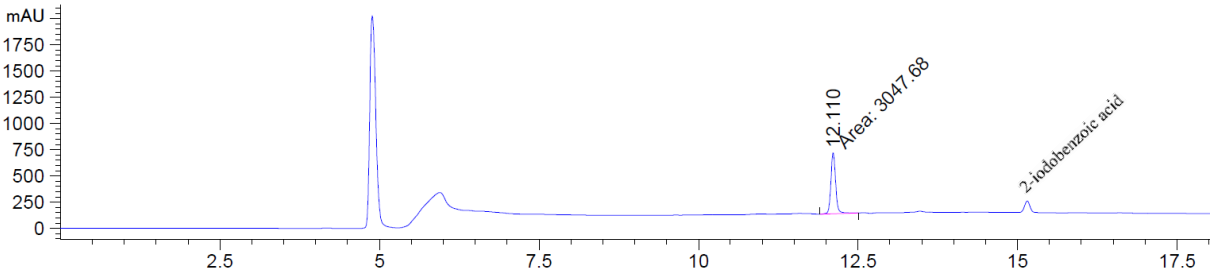


Figure S58: HPLC-UV chromatogram at 214 nm of **3g**.

Following general procedure B, octynyl-1,2-benziodoxol-3(1*H*)-one (**1h**) afforded the title compound **3h** in 49% yield (retention time: 12.141 minutes).

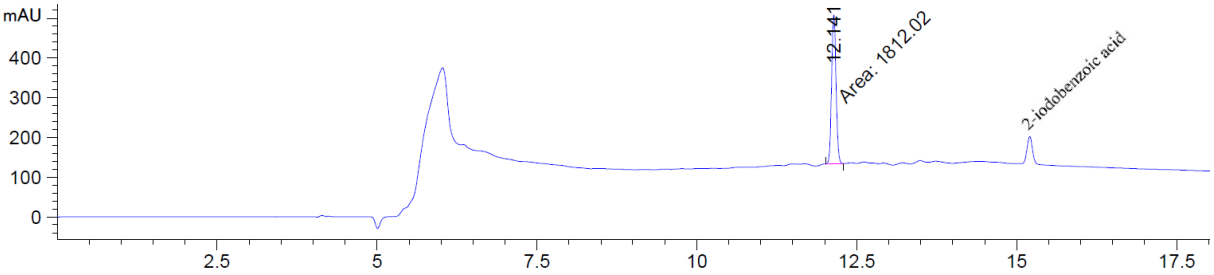
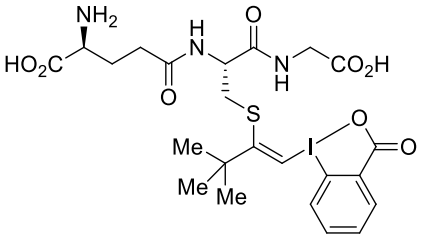


Figure S59: HPLC-UV chromatogram at 214 nm of **3g**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{25}H_{35}IN_3O_8S^+$ 664.1184; Found 664.1185.

S-Glutathione-(3,3-dimethylbutynyl)-1,2-vinylbenziodoxolone (**3i**)



(**3i**)

Following general procedure A, 3,3-dimethylbutynyl-1,2-benziodoxol-3(1*H*)-one (**1i**) afforded the title compound **3i** in 43% yield (retention time: 10.032 minutes).

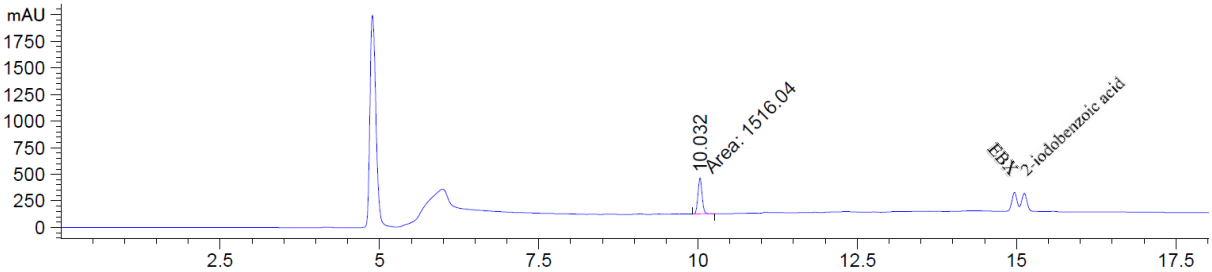


Figure S60: HPLC-UV chromatogram at 214 nm of **3i**.

Following general procedure B, 3,3-dimethylbutynyl-1,2-benziodoxol-3(1*H*)-one (**1i**) afforded the title compound **3i** in 37% yield (retention time: 10.089 minutes).

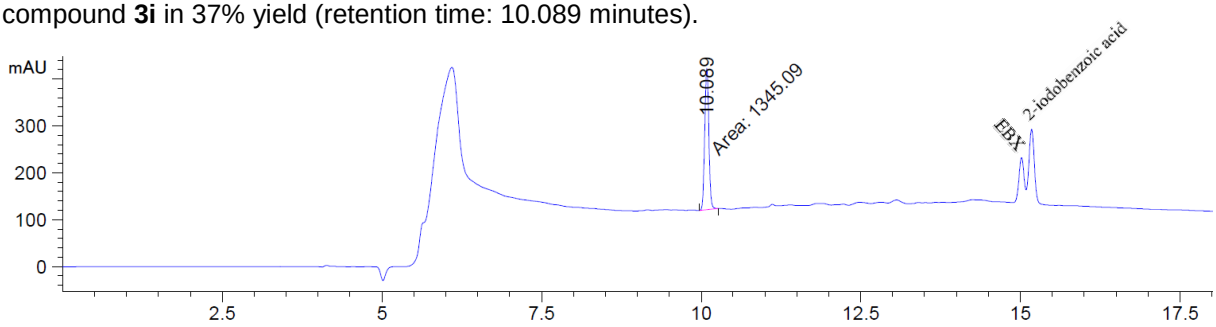
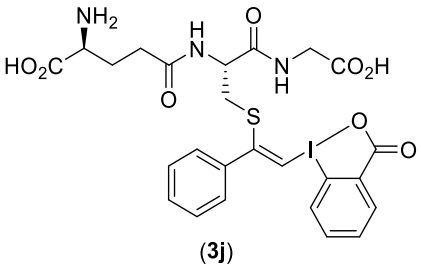


Figure S61: HPLC-UV chromatogram at 214 nm of **3i**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{23}H_{31}IN_3O_8S^+$ 636.0871; Found 636.0871.

S-Glutathione-(1-phenylethynyl)-1,2-vinylbenziodoxolone (**3j**)



(**3j**)

Following general procedure A, 1-phenylethynyl-1,2-benziodoxol-3(1*H*)-one (**1j**) afforded the title compound **3j** in 28% yield (retention time: 10.214 minutes).

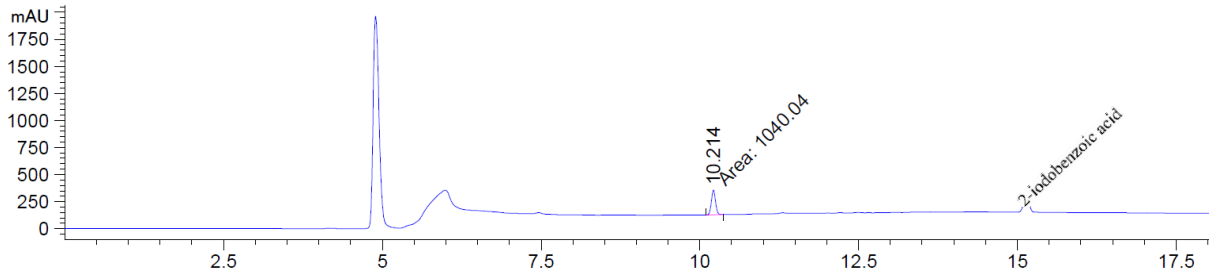
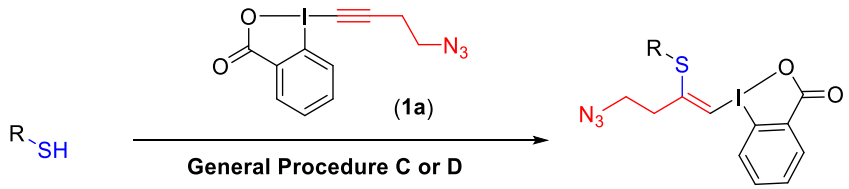


Figure S62: HPLC-UV chromatogram at 214 nm of **3j**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{25}H_{27}IN_3O_8S^+$ 656.0558; Found 656.0557.

9. Substrate scope of thiols

a. Small molecules



(**5-7, 11-13**) (**8-10, 78-80**)

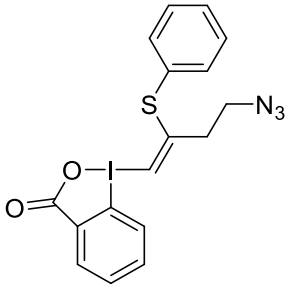
General procedure C:

In a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 100 mM solution of the thiol substrate (**5-7**) in 10 mM Tris buffer pH 8.2 (10.0 µL, 1.00 µmol) was diluted with Tris buffer (10 mM, pH 8.2, 480 µL). The resulting solution was vortexed few seconds and a 300 mM solution of N_3-EBX reagent (**1a**) in DMSO (10.0 µL, 3.00 µmol, 3.00 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS. The peak areas for all-relevant thiol-containing species on the chromatogram were integrated and the yield was determined using slightly modified equation introduced by Li *et al.*:[20] yield % = $I_{product}/(I_{starting} + I_{product} + I_{oxidation} + I_{side\ product})$, where $I_{starting}$, $I_{product}$, $I_{oxidized\ starting}$ and $I_{side\ product}$ respectively represent the average ion counts of the remaining starting material, product, oxidized starting material and side product, if any.

General procedure D:

The organosulfur compound (1.2 equiv.) and cesium carbonate (10 mol%) were dissolved in ethanol (0.08 M) at room temperature, under air. After 5 minutes stirring, EBX reagent (1.0 equiv.) was added in one portion. After 60 minutes stirring at room temperature, the reaction mixture was concentrated in vacuo. The resulting residue was dissolved in dichloromethane, then washed with a saturated aqueous solution of sodium bicarbonate and brine. The organic layer was dried over magnesium sulfate, filtered and concentrated in vacuo. The crude mixture was purified by boiling in acetonitrile.

(Z)-1-(4-azido-2-(phenylthio)but-1-en-1-yl)-1l3-benzo[d][1,2]iodaoxol-3(1H)-one (**8**):



(**8a**)

Following general procedure D, compound **8a** was synthesized in 56 % yield. The structure was confirmed by crystallography (CCDC 1912430).

m.p.: 149-151 °C.

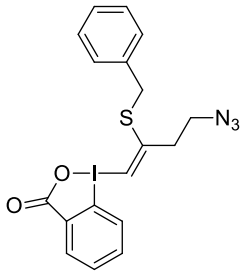
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (m, 1H, Ar H), 7.59-7.65 (m, 3H, Ar H), 7.30-7.38 (m, 5H, Ar H), 6.99 (s, 1H, H_{alkene}), 3.50 (t, *J* = 6.3 Hz, 2H, CH_2N_3), 2.71 (t, *J* = 6.3 Hz, 2H, CH_2-vinyl).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0 (*C*O), 157.6 (C_{alkene}-S), 134.0 (C_{Ar}-H), 133.7 (C_{Ar}-H), 133.7 (C_{Ar}-H), 133.1 (C_{Ar}-H), 130.1 (C_{Ar}-H), 130.0 (C_{Ar}-H), 129.9 (C_{Ar}-H), 129.7 (C_{Ar}-H), 126.2 (C_{Ar}-H), 114.8 (C_{Ph}-I), 104.6 (C_{alkene}-I), 49.5 (*C*H_2-N_3), 36.9 (*C*$H_2CH_2N_3$).

IR (vmax, cm-1): 3038 (w), 2931(w), 2125(w), 2087(m), 1602(S), 1552(m), 1441(w), 1334(m), 1297(m), 1002(w), 827(m), 749(S), 689(m), 638(m).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{17}H_{15}IN_3O_2S^+$ 451.9924; Found 451.9925.

(*Z*)-1-(4-azido-2-(benzylthio)but-1-en-1-yl)-1l3-benzo[d][1,2]iodaoxol-3(1H)-one (**9**)**:**



(**9a**)

Following general procedure D, compound **9a** was synthesized in 47 % yield. The structure was confirmed by crystallography (CCDC 1912427).

m.p.: 131-133 °C.

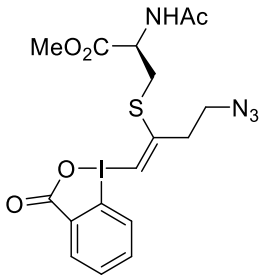
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (dd, *J* = 7.5, 1.8 Hz, 1H, C_{Ar}-*H*), 7.61 (t, *J* = 7.3 Hz, 1H, C_{Ar}-*H*), 7.53 (ddd, *J* = 8.9, 7.1, 1.8 Hz, 1H, C_{Ar}-*H*), 7.24-7.35 (m, 6H, C_{Ar}-*H*), 6.88 (s, 1H, H_{alkene}), 4.02 (s, 2H, S*C*H_2), 3.70 (t, *J* = 6.3 Hz, 2H, N_3C*H*$_2$), 2.92 (t, *J* = 6.3 Hz, 2H, *C*$H_2CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7 (CO), 160.0 (C_{alkene}-S), 135.9 (C_{Ar}-H), 133.6 (C_{Ar}-H), 133.6 (C_{Ar}-H), 133.1 (C_{Ar}-H), 130.8 (C_{Ar}-H), 129.2 (C_{Ar}-H), 128.9 (C_{Ar}-H), 128.3 (C_{Ar}-H), 125.8 (C_{Ar}-H), 114.4 (C_{Ph}-I), 108.9 (C_{alkene}-I), 49.5 (*C*H_2-N_3), 37.7 (S*C*H_2), 32.3 (*C*H_2).

IR (vmax, cm-1): 2975 (*m*), 2915 (*m*), 2124 (*m*), 2085 (*m*), 1585 (*s*), 1553 (*s*), 1450 (*m*), 1346 (*s*), 1283 (*m*), 1222 (*m*), 1074 (*m*), 912 (*m*), 828 (*m*), 743 (*s*), 706 (*s*), 680 (*s*), 626 (*w*).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{18}H_{17}IN_3O_2S^+$ 466.0081; Found 466.0082.

Methyl (Z)-N-acetyl-S-(4-azido-1-(3-oxo-1l3-benzo[d][1,2]iodaoxol-1(3H)-yl)but-1-en-2-yl)-L-cysteinate (**10**)**:**



(**10**)

Following general procedure D, compound **10** was isolated in 41 % yield as white solid. Around 13% of transesterification to the ethyl ester was observed. The *Z* configuration of the double bond was confirmed by a ROESY NMR experiment. An interaction was observed between the C_{alkene}-*H* and the $C_{allylic}$-H_2.

R.f. (10% MeOH/DCM): 0.2.

m.p.: 135-137 °C.

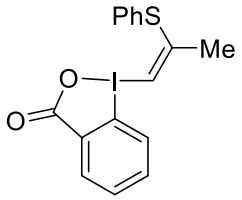
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (d, *J* = 7.2 Hz, 1H, N*H*), 8.33 (d, *J* = 7.4 Hz, 1H, C_{Ar}-*H*), 7.56 (dq, *J* = 9.2, 5.1, 4.6 Hz, 1H, C_{Ar}-*H*), 7.51 (d, *J* = 4.0 Hz, 2H, C_{Ar}-*H*), 6.94 (s, 1H, C_{alkene}-*H*), 4.63 (td, *J* = 6.9, 3.9 Hz, 1H, NC*H*), 3.69-3.72 (m, 5H, CO_2CH_3), 3.44 (dd, *J* = 14.7, 4.1 Hz, 1H, SCH_2), 3.33 (dd, *J* = 14.6, 7.0 Hz, 1H, SCH_2), 2.95 (q, *J* = 6.3 Hz, 2H, CH_2N_3), 2.02 (s, 3H, $COCH_3$), 1.18-1.36 (m, 2H, $CH_2CH_2N_3$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 171.5 (*C*O), 170.4 (*C*O), 168.1 (C_{Ar}-H), 157.1 (C_{Ar}-H), 134.0 (C_{Ar}-H), 133.9 (C_{Ar}-H), 132.9 (C_{Ar}-H), 130.6 (C_{Ar}-H), 127.0 (C_{Ar}-H), 114.7 (C_{Ph}-I), 106.7 (C_{alkene}-I), 53.8 (N*C*H/O*C*H_3), 53.0 (O*C*H_3/N*C*H), 49.6 (*C*H_2-N_3), 36.0 (S*C*H_2), 33.2 (*C*$H_2CH_2N_3$)) 22.9 (CO*C*H_3).

IR (vmax, cm-1): 2956 (*s*), 2925 (*s*), 2859 (*m*), 2353 (*w*), 2097 (*m*), 1740 (*m*), 1604 (*m*), 1549 (*m*), 1438 (*w*), 1346 (w), 1259 (*s*), 1221 (*s*), 1183 (*s*), 1102 (*s*), 1027 (*w*), 809 (*w*), 748 (*m*), 686 (*m*), 643 (*w*), 630 (*w*), 605 (*m*).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{17}H_{20}IN_4O_5S^+$ 519.0194; Found 519.0198.

(*Z*)-1-(2-(Phenylthio)prop-1-en-1-yl)-1λ^3-benzo[*d*][1,2]iodaoxol-3(1*H*)-one (**8b**):



(8b)

Following general procedure D, compound **8b** (0.25g, 0.64 mmol) was isolated in 64 % yield as white solid.

m.p.: 151 – 152 °C.

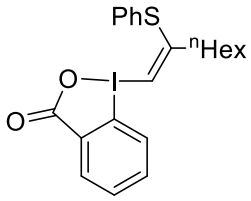
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 – 8.46 (m, 1H, Ar*H*), 7.68 – 7.60 (m, 2H, 2 x Ar*H*), 7.54 – 7.49 (m, 1H, Ar*H*), 7.46 – 7.36 (m, 5H, 5 x Ar*H*), 6.54 (d, *J* = 1.2 Hz, 1H, C*H*I), 2.22 (s, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 160.9, 135.1, 134.0, 133.5, 133.4, 131.0, 130.2, 129.8, 129.6, 125.3, 113.9, 96.0, 25.7.

IR (vmax, cm-1) 1601 (*s*), 1549 (*m*), 1478 (*w*), 1438 (*m*), 1354 (*s*), 1222 (*m*), 1090 (*m*), 1003 (*m*), 829 (*m*), 749 (*s*).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{16}H_{14}IO_2S^+$ 396.9754; Found 396.9758.

(Z)-1-(2-(Phenylthio)oct-1-en-1-yl)-1λ^3-benzo[*d*][1,2]iodaoxol-3(1*H*)-one (**8c**):



(8c)

Following general procedure D, compound **8c** (1.77 g, 3.79 mmol) was isolated in 68 % yield as white solid.

m.p.: 127 – 128 °C.

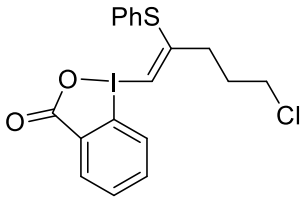
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.52 – 8.43 (m, 1H, Ar*H*), 7.69 – 7.57 (m, 2H, 2 x Ar*H*), 7.53 – 7.46 (m, 1H, Ar*H*), 7.44 – 7.32 (m, 5H, 5 x Ar*H*), 6.64 (s, 1H, C*H*I), 2.47 – 2.41 (m, 2H, CH_2), 1.55 (p, *J* = 7.6 Hz, 2H, CH_2), 1.28 – 1.14 (m, 6H, 3 x CH_2), 0.85 (t, *J* = 7.0 Hz, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.7, 164.3, 134.3, 134.0, 133.4, 133.4, 130.9, 130.0, 129.8, 125.4, 114.2, 98.7, 38.0, 31.4, 29.0, 28.6, 22.6, 14.1.

IR (vmax, cm-1) 2958 (*s*), 2926 (*s*), 2855 (*m*), 1633 (*m*), 1604 (*s*), 1551 (*m*), 1471 (*w*), 1440 (*m*), 1343 (*m*), 1229 (*m*), 1068 (*m*), 828 (*m*), 746 (*s*).

HRMS (APPI/LTQ-Orbitrap) m/z: $[M]^+$ Calcd for $C_{21}H_{23}IO_2S^+$ 466.0458; Found 466.0476.

(Z)-1-(2-(Phenylthio)-5-chloropent-1-en-1-yl)-1λ3-benzo[d][1,2]iodaoxol-3(1H)-one (**8d**):



(8d)

Following general procedure D, compound **8d** (3.99 g, 8.70 mmol) was isolated in 79 % yield as white solid.

m.p.: 181 – 182 °C.

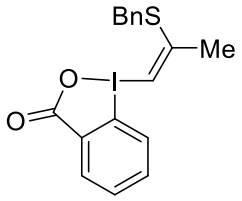
^{1}H NMR (400 MHz, MeOH-d_4) δ 8.34 – 8.29 (m, 1H, Ar*H*), 7.81 – 7.70 (m, 3H, 3 x Ar*H*), 7.52 – 7.39 (m, 5H, 5 x Ar*H*), 7.06 (s, 1H, C*H*I), 3.54 (t, *J* = 6.3 Hz, 2H, CH_2), 2.73 (ddd, *J* = 8.7, 6.0, 1.0 Hz, 2H, CH_2), 2.09 – 2.00 (m, 2H, CH_2).

^{13}C NMR (101 MHz, MeOH-d_4) δ 170.1, 164.0, 135.5, 135.3, 134.7, 133.7, 132.0, 131.1, 131.0, 128.6, 114.6, 100.5, 44.4, 35.5, 32.6.

IR (vmax, cm-1) 1602 (*s*), 1556 (*m*), 1441 (*w*), 1350 (*m*), 1020 (*w*), 828 (*w*), 752 (*m*).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{18}H_{17}ClIO_2S^+$ 458.9677; Found 458.9684

(Z)-1-(2-(Benzylthio)prop-1-en-1-yl)-1λ^3-benzo[*d*][1,2]iodaoxol-3(1*H*)-one (**9b**):



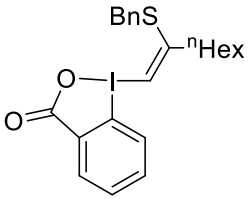
(9b)

Following general procedure D, compound **9b** (1.14g, 4.00 mmol) was isolated in 46 % yield as white solid. The structure was confirmed by crystallography (CCDC 1862137).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (dd, *J* = 7.4, 1.8 Hz, 1H, Ar*H*), 7.59 (td, *J* = 7.3, 1.0 Hz, 1H, Ar*H*), 7.50 (ddd, *J* = 8.8, 7.2, 1.8 Hz, 1H, Ar*H*), 7.33 – 7.21 (m, 6H, 6 x Ar*H*), 6.50 (q, *J* = 1.3 Hz, 1H, C*H*I), 4.10 (s, 2H, CH_2S), 2.54 (d, *J* = 1.3 Hz, 3H, CH_3).

Spectra data was consistent with the values reported in literature.

(Z)-1-(2-(Benzylthio)oct-1-en-1-yl)-1λ^3-benzo[*d*][1,2]iodaoxol-3(1*H*)-one (**9c**):



(9c)

Following general procedure D, compound **9c** (2.00g, 5.60 mmol) was isolated in 56 % yield as white solid.

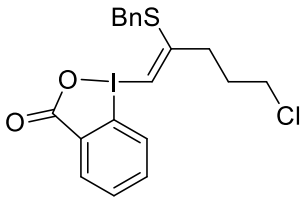
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (dd, *J* = 7.5, 1.7 Hz, 1H, Ar*H*), 7.61 (td, *J* = 7.4, 0.8 Hz, 1H, Ar*H*), 7.53 – 7.48 (m, 1H, Ar*H*), 7.31 – 7.21 (m, 6H, 6 x Ar*H*), 6.62 (s, 1H, C*H*I), 4.04 (s, 2H, SCH_2), 2.76 – 2.67 (m, 2H, CH_2), 1.71 (p, J = 7.4 Hz, 2H, CH_2), 1.45 – 1.34 (m, 6H, 3 x CH_2), 0.98 – 0.92 (m, 3H, CH_3).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.6, 162.3, 136.0, 133.9, 133.3, 133.1, 130.7, 129.0, 128.8, 128.1, 125.4, 114.0, 102.0, 38.0, 37.2, 31.6, 28.9, 28.8, 22.7, 14.2.

IR (vmax, cm-1) 3059 (*w*), 3027 (*w*), 2957 (*w*), 2925 (*m*), 2853 (*m*), 1638 (*m*), 1600 (*s*), 1555 (*m*), 1543 (*m*), 1455 (*m*), 1437 (*m*), 1343 (*m*), 1005 (*m*), 829 (*m*), 746 (*s*).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{22}H_{26}IO_2S^+$ 481.0693; Found 481.0696.

(Z)-1-(2-(Benzylthio)-5-chloropent-1-en-1-yl)-1λ^3-benzo[*d*][1,2]iodaoxol-3(1*H*)-one (**9d**):



(9d)

Following general procedure D, compound **9d** (3.81 g, 8.07 mmol) was isolated in 73 % yield as white solid.

m.p.: 168 – 169 °C.

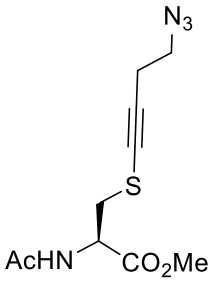
^{1}H NMR (400 MHz, DMSO-d_6) δ 8.11 (dd, *J* = 7.3, 1.8 Hz, 1H, Ar*H*), 7.65 – 7.55 (m, 2H, 2 x Ar*H*), 7.36 (dd, *J* = 7.9, 1.0 Hz, 1H, Ar*H*), 7.28 – 7.16 (m, 5H, 5 x Ar*H*), 7.00 (s, 1H, C*H*I), 4.16 (s, 2H, SCH_2), 3.76 (t, *J* = 6.5 Hz, 2H, ClCH_2), 2.94 – 2.87 (m, 2H, CH_2), 2.10 (p, *J* = 6.5 Hz, 2H, CH_2).

^{13}C NMR (101 MHz, DMSO-d_6) δ 166.0, 159.6, 137.4, 135.0, 133.6, 132.0, 130.6, 129.2, 129.0, 127.8, 127.5, 114.3, 103.6, 44.8, 35.9, 34.2, 31.8.

IR (vmax, cm-1) 2987 (*m*), 2362 (*s*), 2349 (*s*), 2337 (*m*), 1594 (*s*), 1430 (*m*), 1354 (*s*), 1242 (*m*), 1114 (*m*), 1035 (*m*), 825 (*m*), 747 (*s*), 718 (*s*).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{19}H_{19}ClIO_2S^+$ 472.9834; Found 472.9834.

Methyl N-acetyl-S-(4-azidobut-1-yn-1-yl)-L-cysteinate (**74**):



(10)

To a solution of protected cysteine (**7**) (0.113 mmol, 20.0 mg. 1.0 equiv.) in DMSO (0.2 M), *N,N*-tetramethyl guanidine (0.124 mmol, 15.5 µL, 1.1 equiv.) was added. After 5 minutes stirring at room temperature, Azido-EBX (**1a**) (0.135 mmol, 46.2 mg, 1.3 equiv.) was added. After 15 minutes, the reaction was diluted with sodium bicarbonate and extracted with DCM. The organic layer was washed with sodium bicarbonate and a saturated sodium chloride solution, and concentrated to dryness under reduced pressure. The crude product was purified on a preparative TLC (eluent 3% MeOH/DCM) to afford compound **74** as white solid (13.0 mg, 43%).

R.f. (10% MeOH/DCM): 0.2

m.p.: 155-157 °C

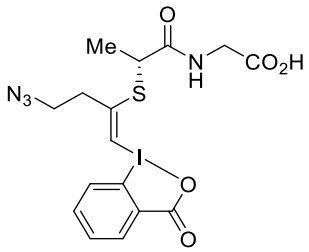
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.39 (d, *J* = 7.4 Hz, 1H, N*H*), 4.94 (dt, *J* = 7.6, 4.7 Hz, 1H, NC*H*CO_2Me), 3.80 (s, 3H, CO_2CH_3), 3.39 (td, *J* = 6.7, 1.7 Hz, 2H, CH_2N_3), 3.24 (dd, *J* = 13.9, 4.7 Hz, 1H, CH_2S), 3.17 (dd, *J* = 13.9, 4.8 Hz, 1H, CH_2S), 2.57 (t, *J* = 6.7 Hz, 2H, $CH_2CH_2N_3$), 2.09 (s, 3H, CH_3CO).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.6 (*C*O), 169.9 (*C*O), 90.5 (S*Csp*), 70.5 (SCsp-*C*sp), 52.9 (CO_2CH_3), 52.2 (NCHCO$_2$Me), 49.9 ($CH_2CH_2N_3$), 37.0 (CH_2S), 23.2 (CH_3CO), 21.4 ($CH_2CH_2N_3$).

IR (vmax, cm-1) 3842 (m), 3625 (w), 2858 (w), 2346 (w), 2105 (w), 1758 (w), 1696 (w), 1542 (m), 1333 (w), 1169 (m), 1060 (s), 795 (m), 723 (w), 673 (m), 603 (s).

HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_{10}H_{14}N_4NaO_3S^+$ 293.0679; Found 293.0683.

S-Tiopronin-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**78**)



(**78**)

Following general procedure C, tiopronin (**11**) afforded the title compound **78** in 95% yield (retention time: 10.719 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{16}H_{18}IN_4O_5S^+$ 505.0037; Found 505.0044.

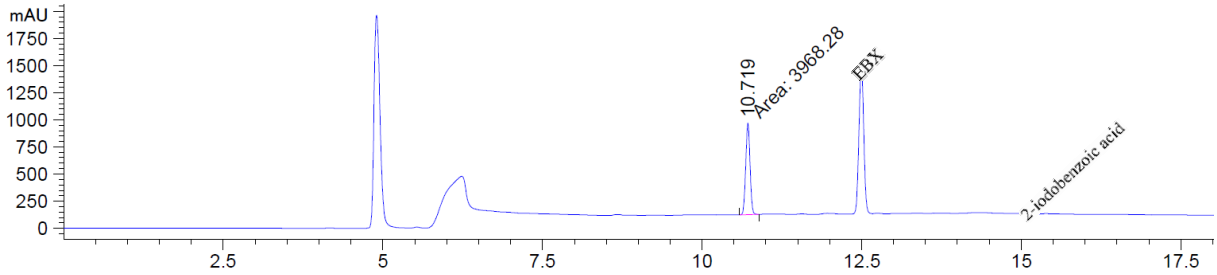


Figure S63: HPLC-UV chromatogram at 214 nm of **78**.

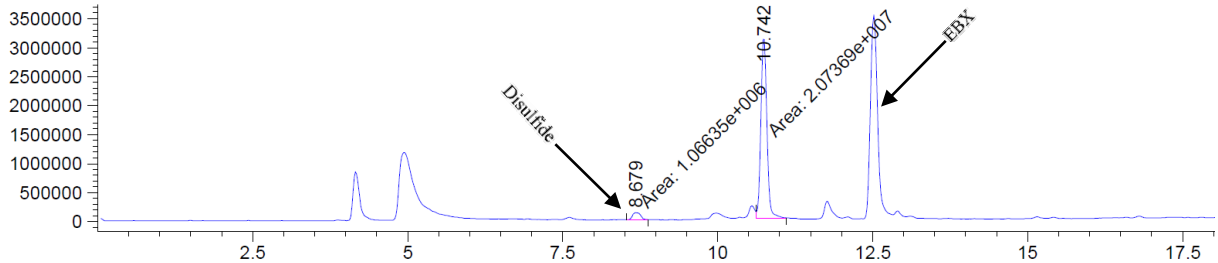
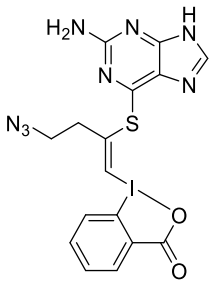


Figure S64: HPLC-MS chromatogram of **78**.

S-6-Thioguanine-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**79**)



(**79**)

Following general procedure C, 6-thioguanine (**12**) afforded the title compound **79** in 100% yield (retention time: 10.399 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{16}H_{14}IN_8O_2S^+$ 509.0000; Found 508.9997.

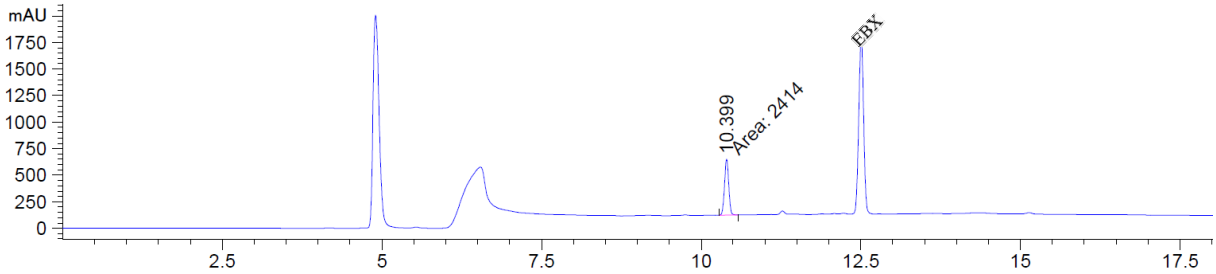


Figure S65: HPLC-UV chromatogram at 214 nm of **79**.

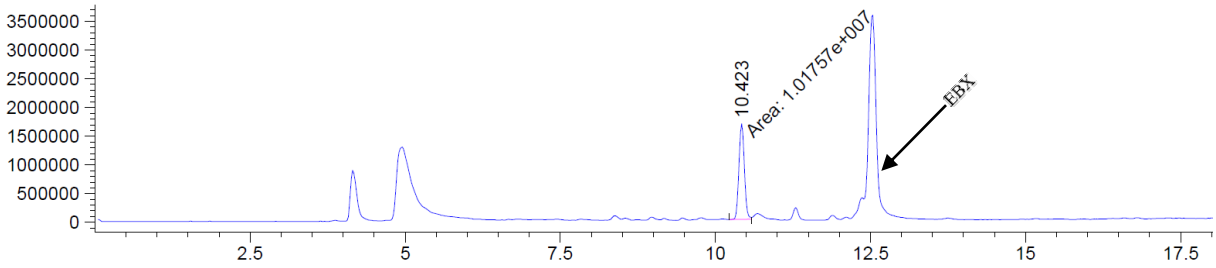
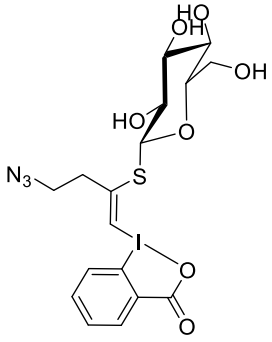


Figure S66: HPLC-MS chromatogram of **79**.

S-Thioglucose-(4-azidobut-1-ynyl)-1,2-vinylbenziodoxolone (**80**)



(**80**)

Following general procedure C, thioglucose (**13**) afforded the title compound **80** in 100% yield (retention time: 9.170 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{17}H_{21}IN_3O_7S^+$ 538.0139; Found 538.0152.

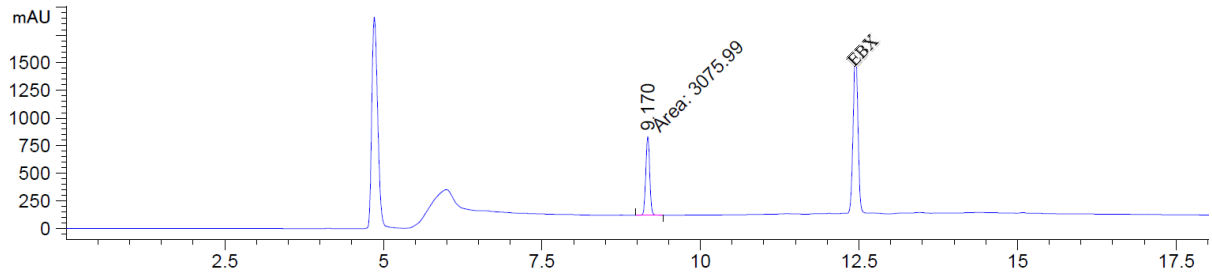


Figure S67: HPLC-UV chromatogram at 214 nm of **80**.

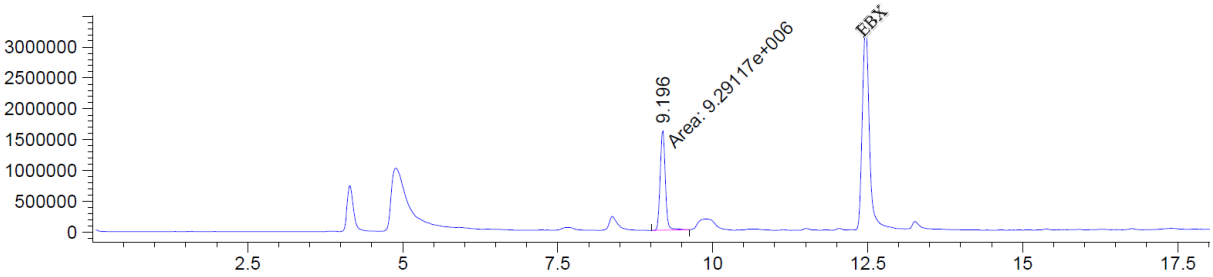
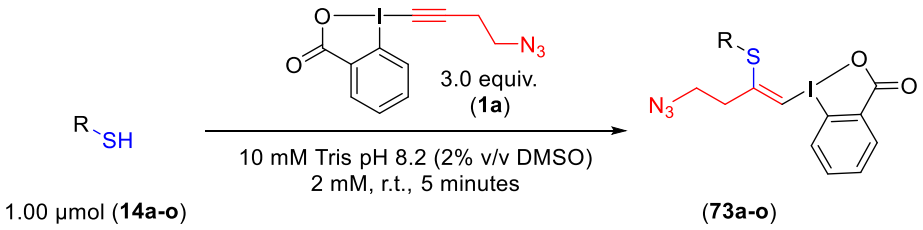


Figure S68: HPLC-MS chromatogram of **80**.

b. Substrate scope of cysteine-containing tetramers (**14**)



General procedure E:

In a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 20.0 mM solution of cysteine-containing tetramer in 10 mM Tris buffer pH 8.2 (50.0 µL, 1.00 µmol) was diluted with Tris buffer (10 mM, pH 8.2, 440 µL). The resulting solution was vortexed few seconds and a 300 mM solution of N_3-EBX reagent (**1a**) in DMSO (10.0 µL, 3.00 µmol, 3.00 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

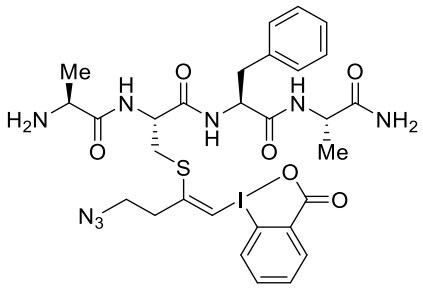
General procedure F:

A 0.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with N_3-EBX reagent (**1a**) (3.00 µmol, 3.00 equiv.) and Tris buffer (10 mM, pH 8.2, 450 µL). The resulting solution was vigorously shaken over 5 minutes and a 20.0 mM solution of cysteine-containing tetramer in 10 mM Tris buffer pH 8.2 (50.0 µL, 1.00 µmol) was added in one portion. The resulting mixture was vortexed few seconds and slowly shaken at room temperature for 5 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS.

Yield calculation:

The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using a slightly modified equation used by Li *et al.*:[20] yield % = $I_{product}/(I_{starting} + I_{product} + I_{oxidation} + I_{side\ product})$, where $I_{starting}$, $I_{product}$, $I_{oxidation}$ and $I_{side\ product}$ respectively represent the average ion counts of the remaining starting material, product, oxidized starting material and side product, if any. The tetramers being easily oxidized upon storage, they were diluted to the usual 2 mM reaction molarity and analyzed by HPLC-MS prior to the reaction. The area of oxidized tetramer already present prior to the reaction was removed from the yield calculation such as: $I_{oxidation} = I_{oxidation\ reaction} - I_{oxidation\ t0}$ where $I_{oxidation\ reaction}$ and $I_{oxidation\ t0}$ respectively represent the average ion counts of oxidized tetramer present at the end of the reaction and oxidized tetramer present prior to the reaction.

Azido-VBX conjugate of Ala-Cys-Phe-Ala (**73[a]**)



(**73a**)

Following general procedure E, Ala-Cys-Phe-Ala (**14a**) afforded the title compound **73a** in 97% yield (retention time: 10.545 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{29}H_{36}IN_8O_6S^+$ 751.1518; Found 751.1514.

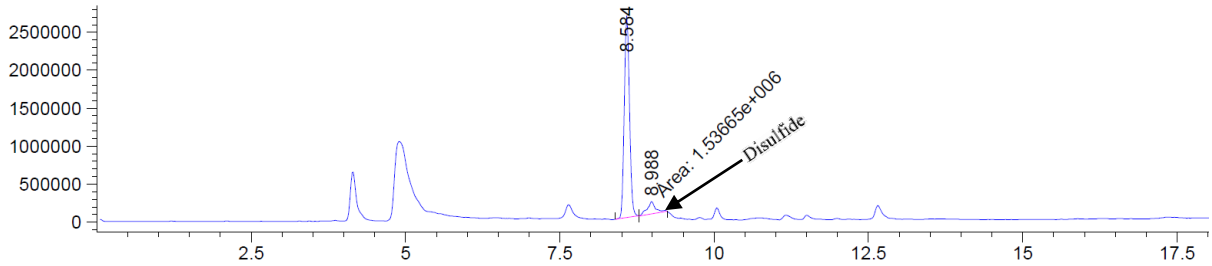


Figure S69: Ala-Cys-Phe-Ala HPLC-MS chromatogram.

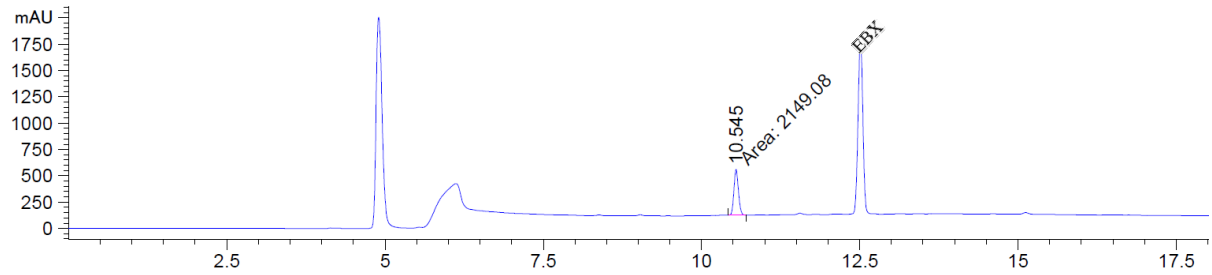


Figure S70: HPLC-UV chromatogram at 214 nm of the reaction of **73a**.

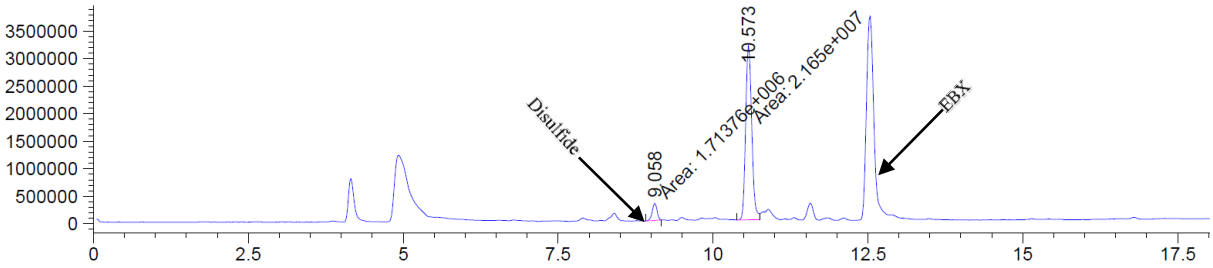
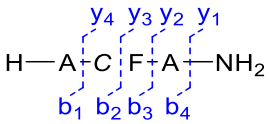


Figure S71: HPLC-MS chromatogram of the reaction of **73a**.

MS/MS of $[M + H]^+$ 751.14 @cid22.00[205.00-1200.00] (LTQ-Orbitrap).



	b	y
1	-	-
2	not found	-
3	663.0881	236.1394
4	734.1252	-

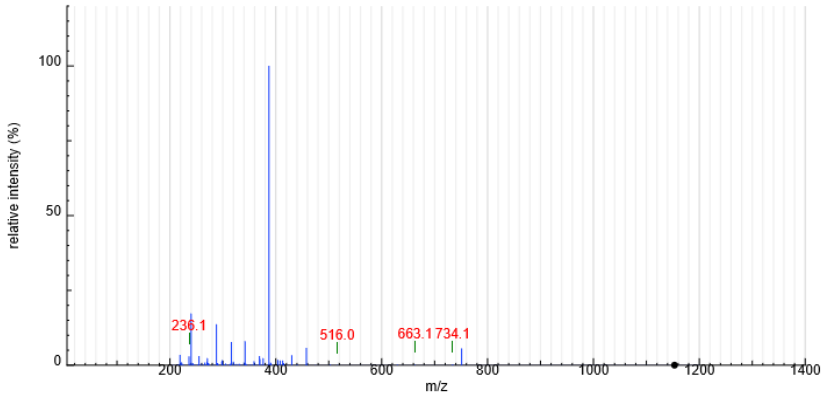
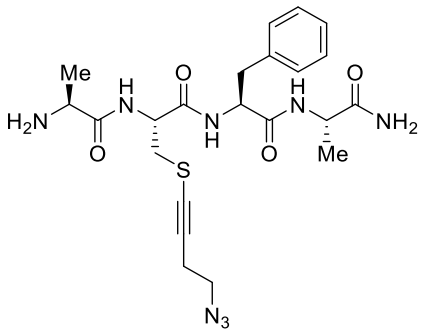


Figure S72: MS/MS found fragments, table and graphic, upon fragmentation of the molecular ion of product **73a**.

Thioalkynynalted Ala-Cys-Phe-Ala (**76**)



This product was synthesized to confirm the HPLC chromatographic analysis that matches the data in the buffer/DMSO ratio influence on the reactivity with a tetramer.

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M]^+$ Calcd for $C_{22}H_{31}N_8O_4S^+$ 503.2183; Found 503.2178.

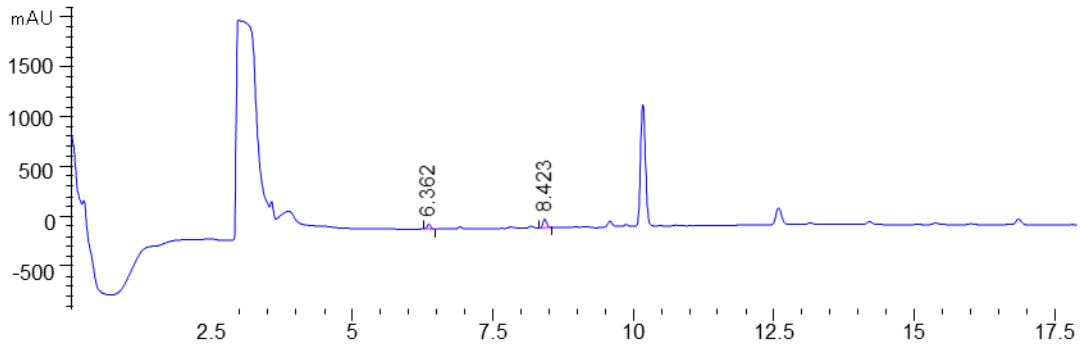


Figure S73: HPLC-UV chromatogram at 214 nm of the reaction of **76**.

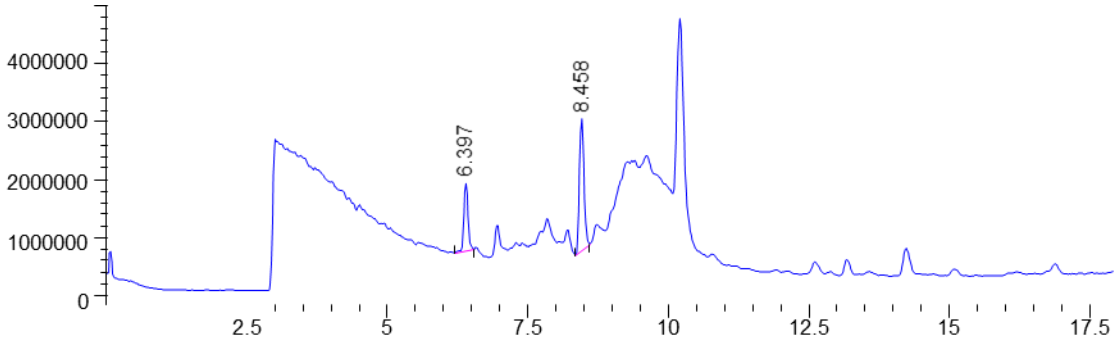
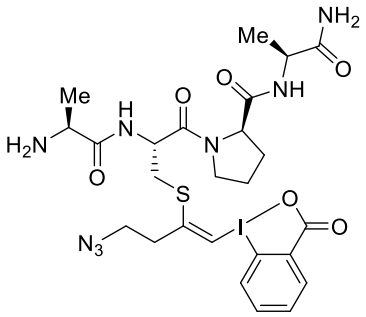


Figure S74: HPLC-MS chromatogram of the reaction of **76**.

Azido-VBX conjugate of Ala-Cys-Pro-Ala (**73b**)



(**73b**)

Following general procedure E, Ala-Cys-Pro-Ala (**14b**) afforded the title compound **73b** in 97% yield (retention time: 8.972 minutes).

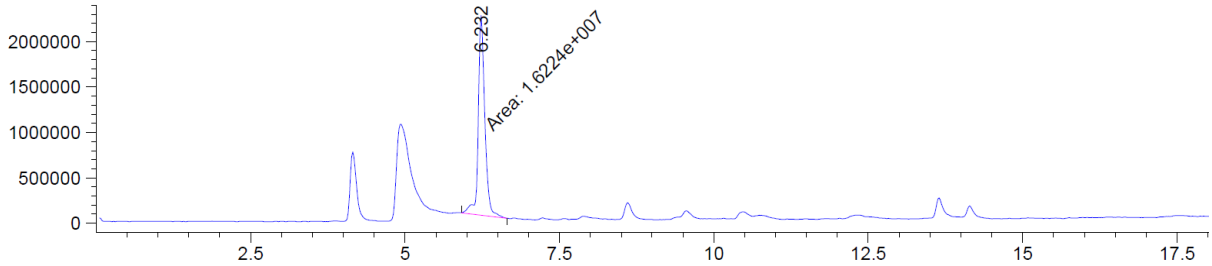


Figure S75: Ala-Cys-Pro-Ala HPLC-MS chromatogram.

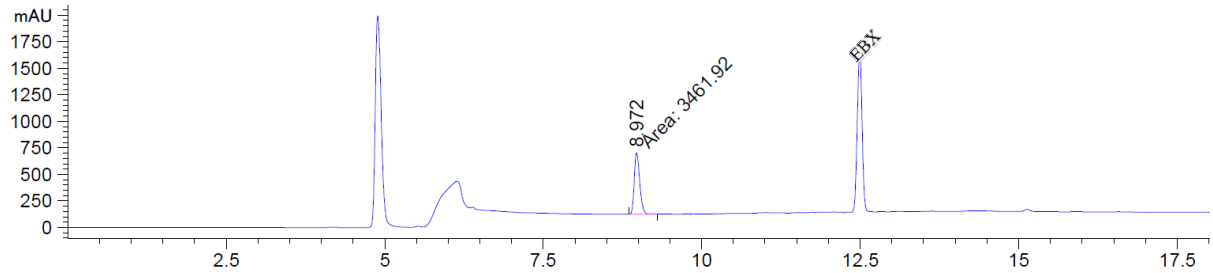


Figure S76: HPLC-UV chromatogram at 214 nm of the reaction of **73b**.

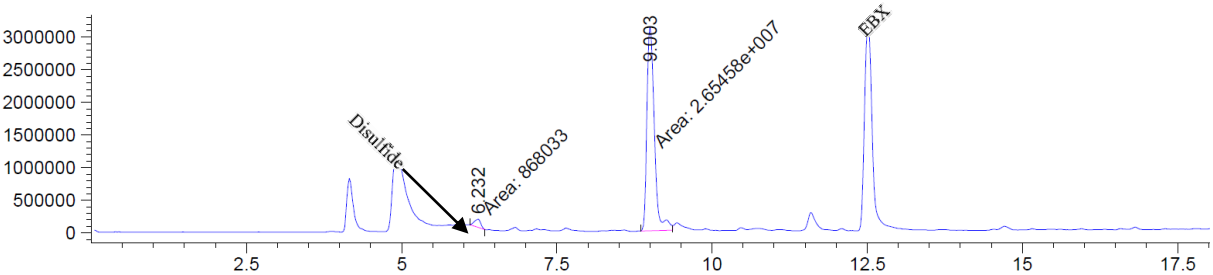


Figure S77: HPLC-MS chromatogram of the reaction of **73b**.

Following general procedure F, Ala-Cys-Pro-Ala (**14b**) afforded the title compound **73b** in 98% yield (retention time: 8.906 minutes).

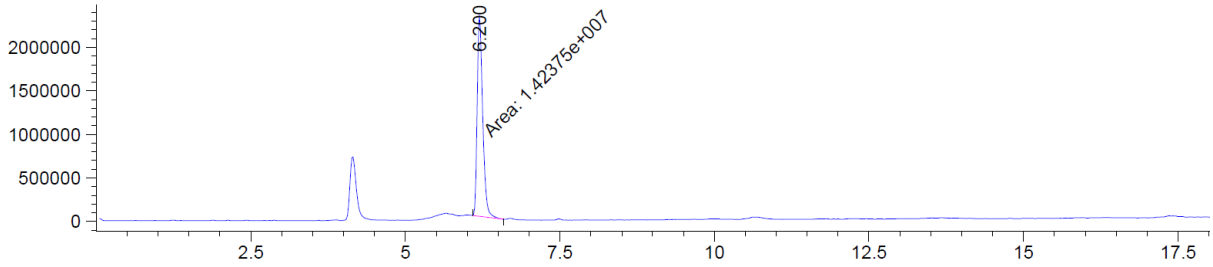


Figure S78: Ala-Cys-Pro-Ala HPLC-MS chromatogram.

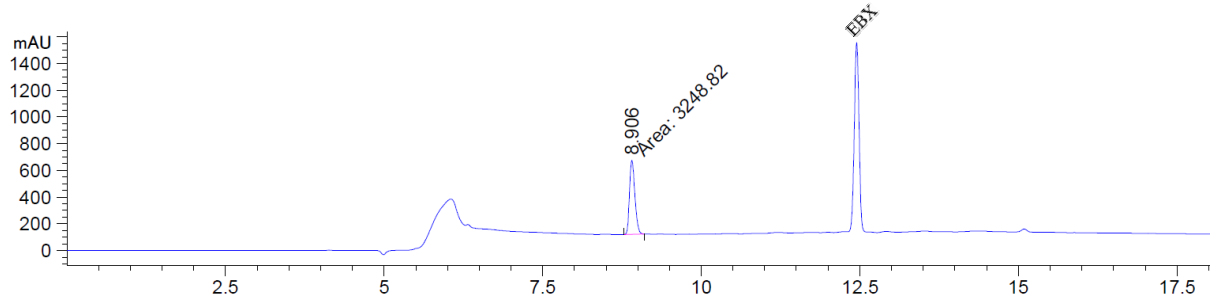


Figure S79: HPLC-UV chromatogram at 214 nm of the reaction of **73b**.

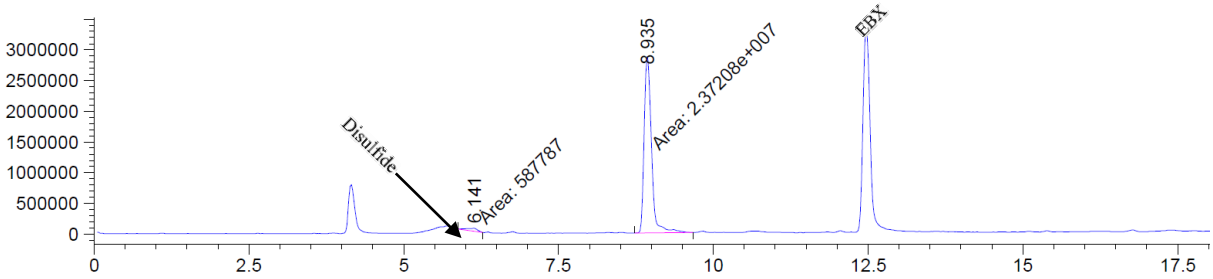
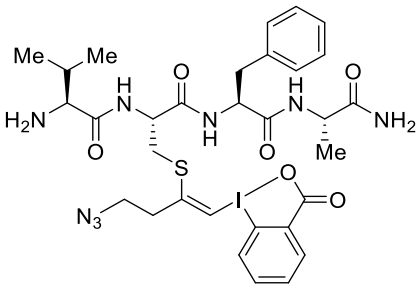


Figure S80: HPLC-MS chromatogram of the reaction of **73b**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{25}H_{34}IN_8O_6S^+$ 701.1361; Found 701.1361.

Azido-VBX conjugate of Val-Cys-Phe-Ala (**73c**)



(**73c**)

Following general procedure E, Val-Cys-Phe-Ala (**14c**) afforded the title compound **73c** in 95% yield (retention time: 10.805 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{31}H_{40}IN_8O_6S^+$ 779.1831; Found 779.1820.

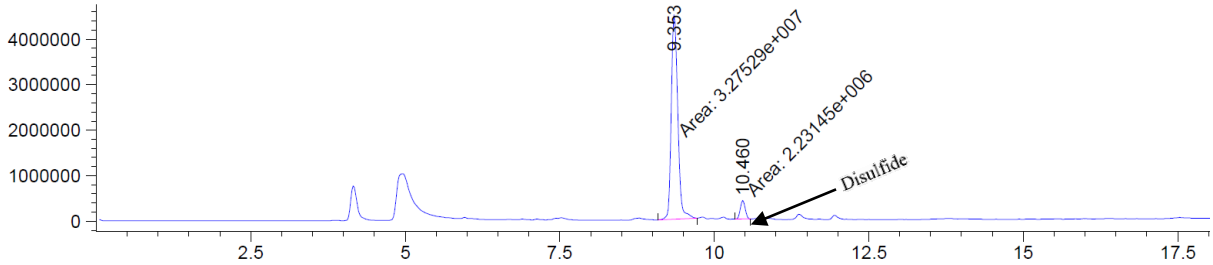


Figure S81: Val-Cys-Phe-Ala HPLC-MS chromatogram.

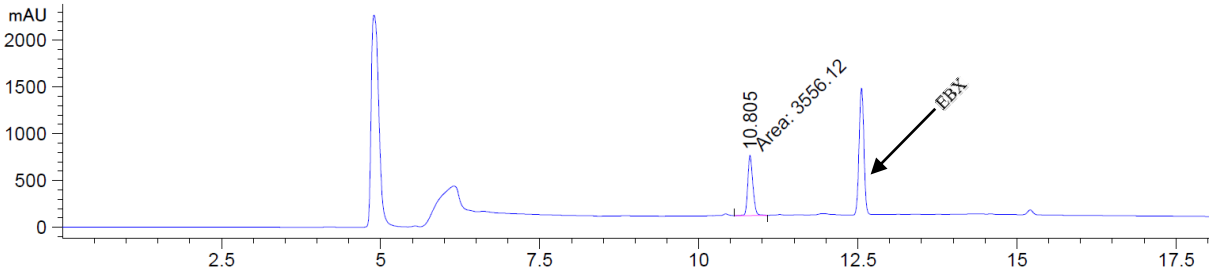


Figure S82: HPLC-UV chromatogram at 214 nm of the reaction of **73c**.

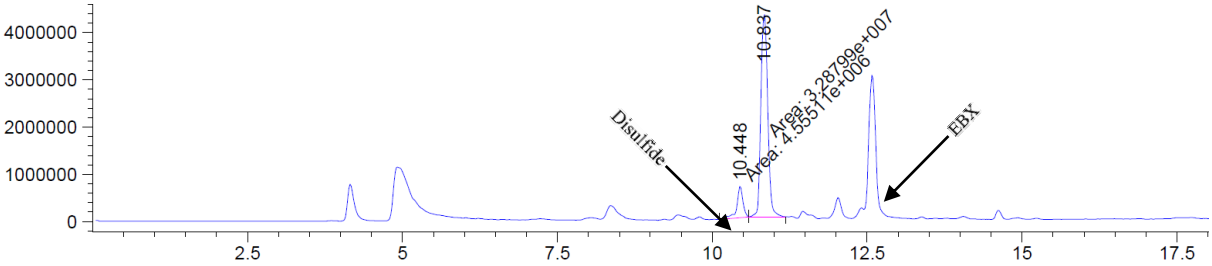
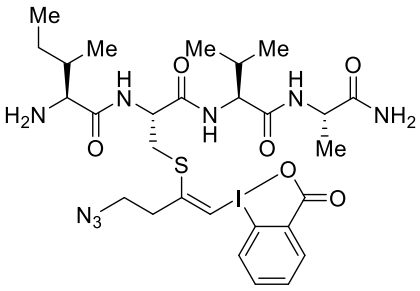


Figure S83: HPLC-MS chromatogram of **73c**.

Azido-VBX conjugate of Ile-Cys-Val-Ala (**73d**)



(**73d**)

Following general procedure E, Ile-Cys-Val-Ala (**14d**) afforded the title compound **73d** in 99% yield (retention time: 9.898 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{42}IN_8O_6S^+$ 745.1987; Found 745.1978.

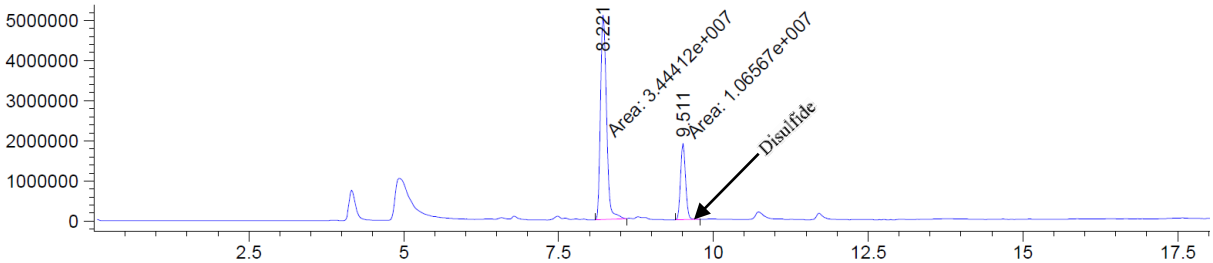


Figure S84: Ile-Cys-Val-Ala HPLC-MS chromatogram.

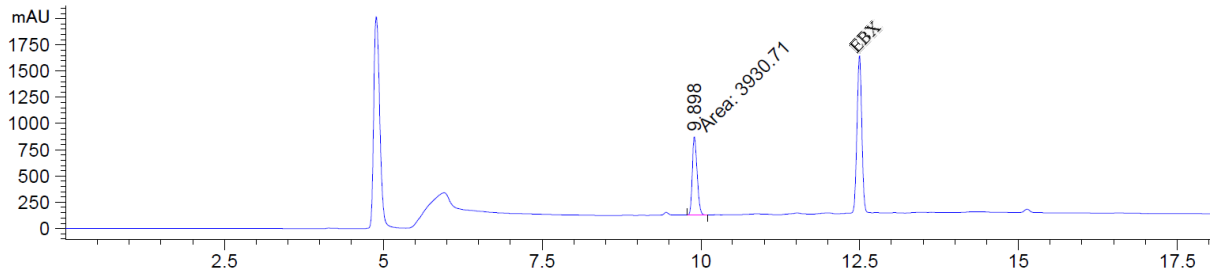


Figure S85: HPLC-UV chromatogram at 214 nm of the reaction of **73d**.

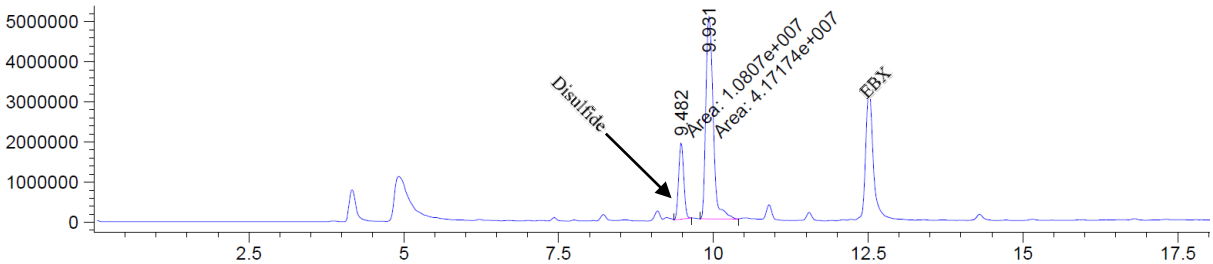
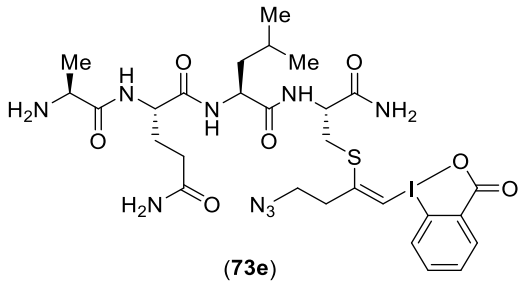


Figure S86: HPLC-MS chromatogram of the reaction of **73d**.

Azido-VBX conjugate of Ala-Gln-Leu-Cys (**73e**)



(**73e**)

Following general procedure E, Ala-Gln-Leu-Cys (**14e**) afforded the title compound **73e** in 79% yield (retention time: 10.062 minutes).

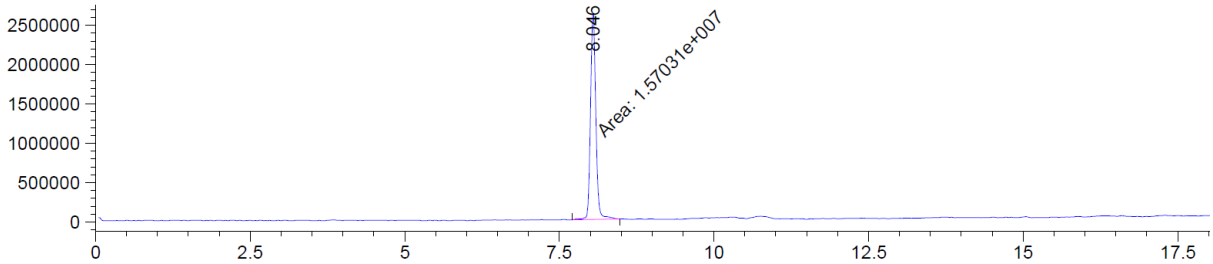


Figure S87: Ala-Gln-Leu-Cys HPLC-MS chromatogram.

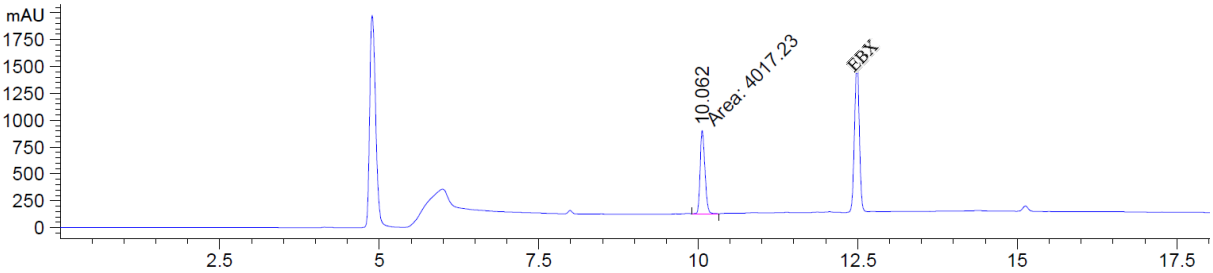


Figure S88: HPLC-UV chromatogram at 214 nm of the reaction of **73e**.

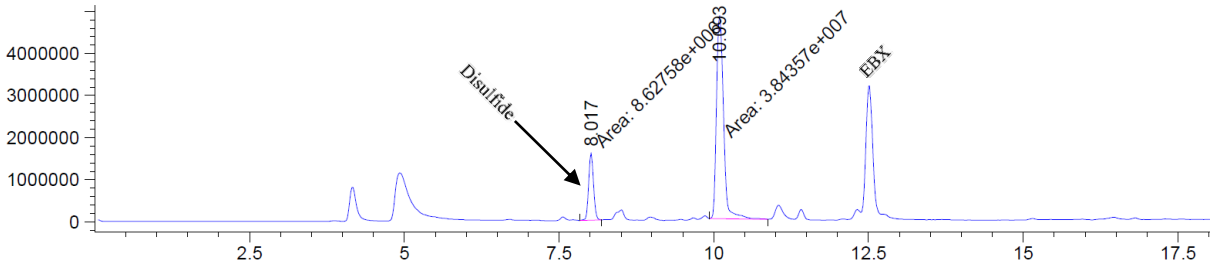


Figure S89: HPLC-MS chromatogram of the reaction of **73e**.

Following general procedure F, Ala-Gln-Leu-Cys (**14e**) afforded the title compound **73e** in 69% yield (retention time: 10.019 minutes).

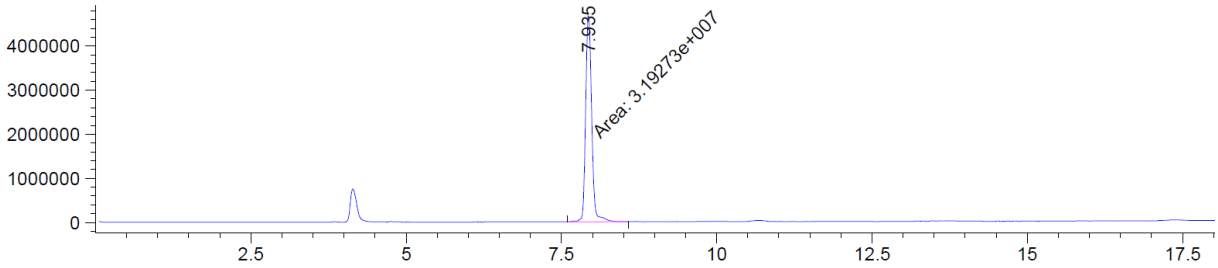


Figure S90: Ala-Gln-Leu-Cys HPLC-MS chromatogram.

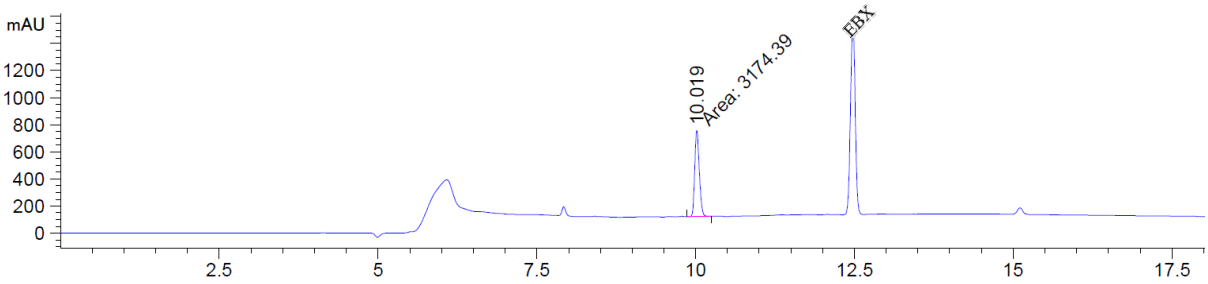


Figure S91: HPLC-UV chromatogram at 214 nm of the reaction of **73e**.

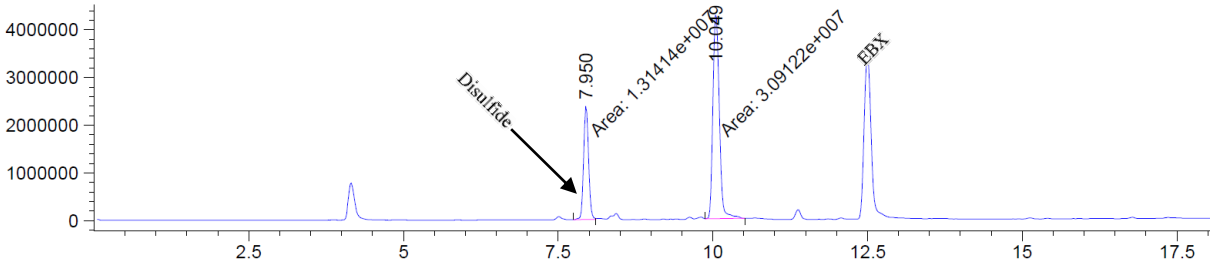
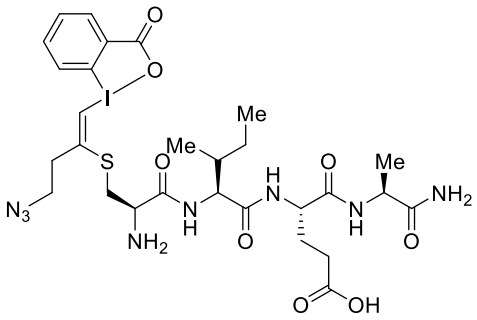


Figure S92: HPLC-MS chromatogram of the reaction of **73e**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{41}IN_9O_7S^+$ 774.1889; Found 774.1874.

Azido-VBX conjugate of Cys-Ile-Glu-Ala (**73f**)



(**73f**)

Following general procedure E, Cys-Ile-Glu-Ala (**14f**) afforded the title compound **73f** in 92% yield (retention time: 9.103 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{28}H_{40}IN_8O_8S^+$ 775.1729; Found 775.1717.

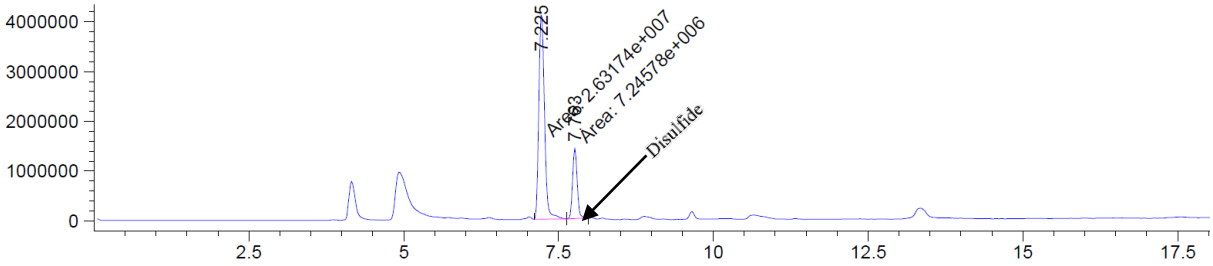


Figure S93: Cys-Ile-Glu-Ala HPLC-MS chromatogram.

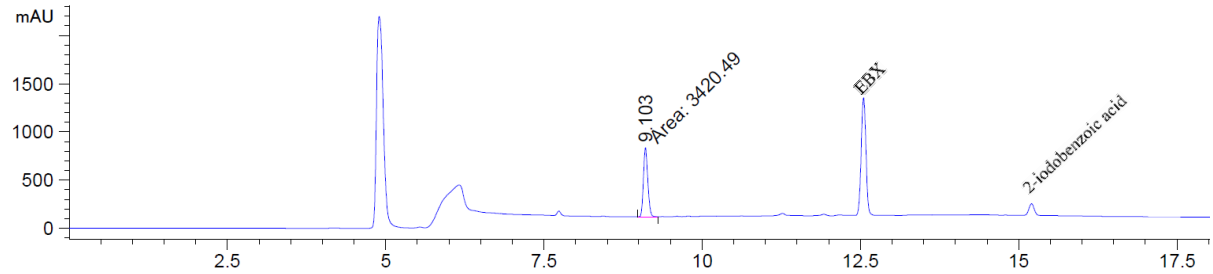


Figure S94: HPLC-UV chromatogram at 214 nm of the reaction of **73f**.

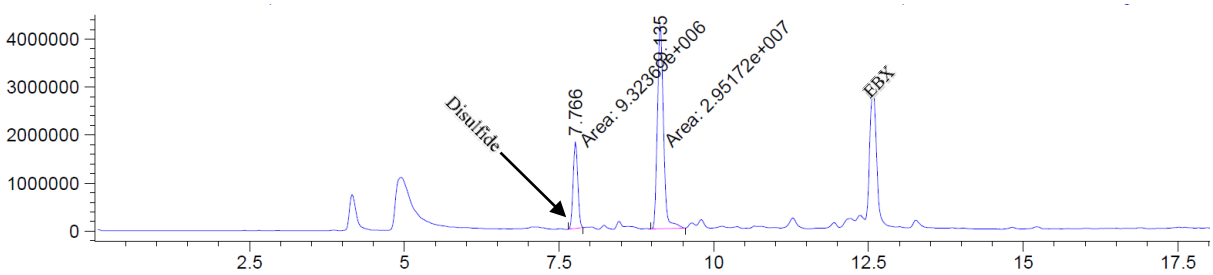
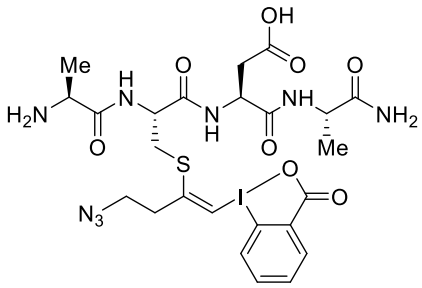


Figure S95: HPLC-MS chromatogram of the reaction of **73f**.

Azido-VBX conjugate of Ala-Cys-Asp-Ala (**73g**)



(**73g**)

Following general procedure E, Ala-Cys-Asp-Ala (**14g**) afforded the title compound **73g** in 96% yield (retention time: 8.987 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{24}H_{32}IN_8O_8S^+$ 719.1103; Found 719.1095.

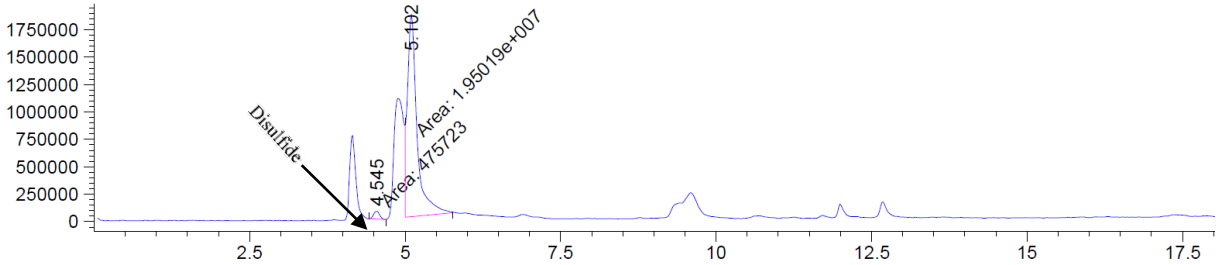


Figure S96: Ala-Cys-Asp-Ala HPLC-MS chromatogram.

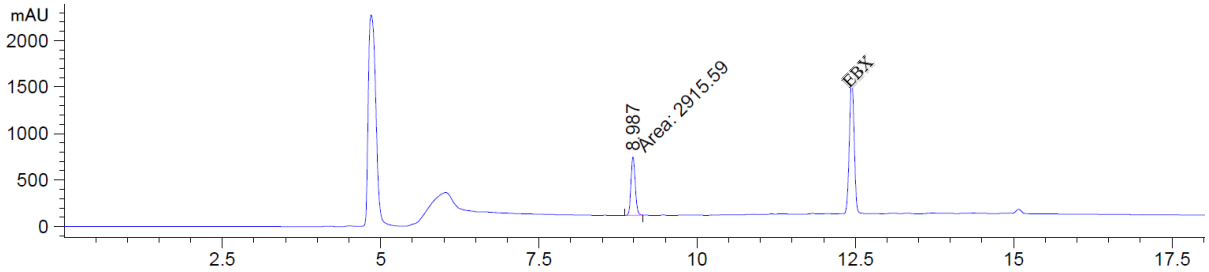


Figure S97: HPLC-UV chromatogram at 214 nm of the reaction **73g**.

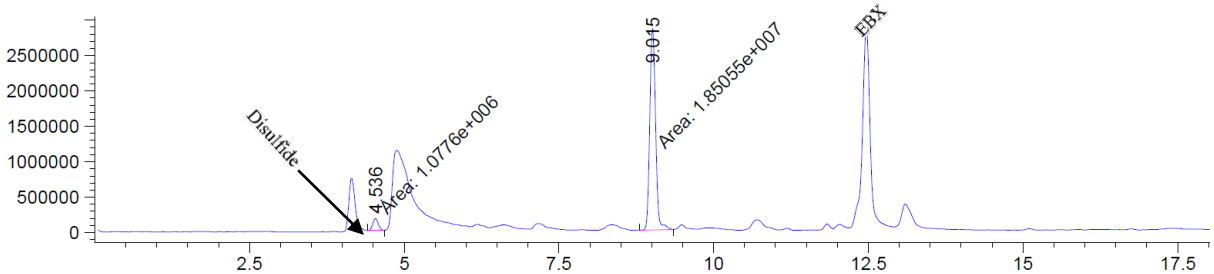
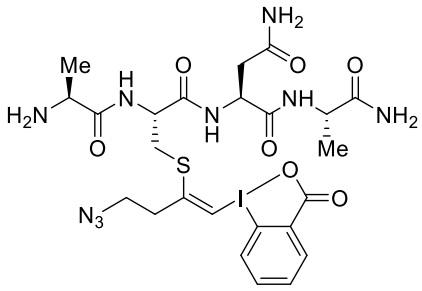


Figure S98: HPLC-MS chromatogram of the reaction of **73g**.

Azido-VBX conjugate of Ala-Cys-Asn-Ala (**73h**)



(**73h**)

By HPLC-MS, oxidized Ala-Cys-Asn-Ala elutes with Tris buffer and none of our attempts to separate them were successful. In order to get a viable area of oxidized Ala-Cys-Asn-Ala, blank samples of the reaction (only containing 10 mM Tris buffer, with or without 2% v/v DMSO) were prepared and analyzed by HPLC-MS. Then, the following equation was applied: $I_{\text{oxidation t0}} = I_{\text{oxidation+buffer t0}} - I_{\text{buffer}}$ and $I_{\text{oxidation reaction}} = I_{\text{oxidation+buffer reaction}} - I_{\text{buffer}}$ where $I_{\text{oxidation+buffer t0}}$, $I_{\text{oxidation+buffer reaction}}$ and I_{buffer} represent the average ion counts of oxidized tetramer with buffer prior to the reaction, oxidized tetramer with buffer after the reaction and buffer in the reaction.

Following general procedure E, Ala-Cys-Asn-Ala (**14h**) afforded the title compound **73h** in 98% yield (retention time: 8.917 minutes).

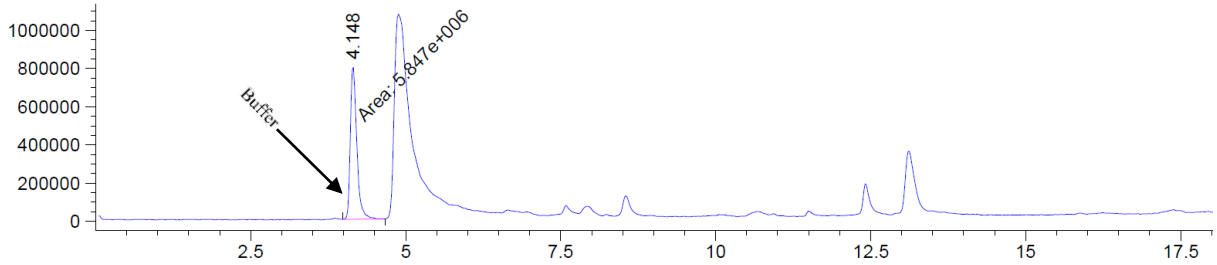


Figure S99: Tris buffer with DMSO HPLC-MS chromatogram.

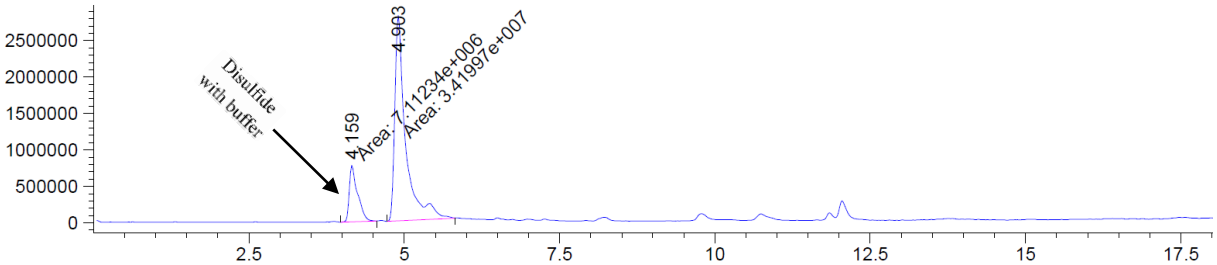


Figure S 100: Ala-Cys-Asn-Ala HPLC-MS chromatogram.

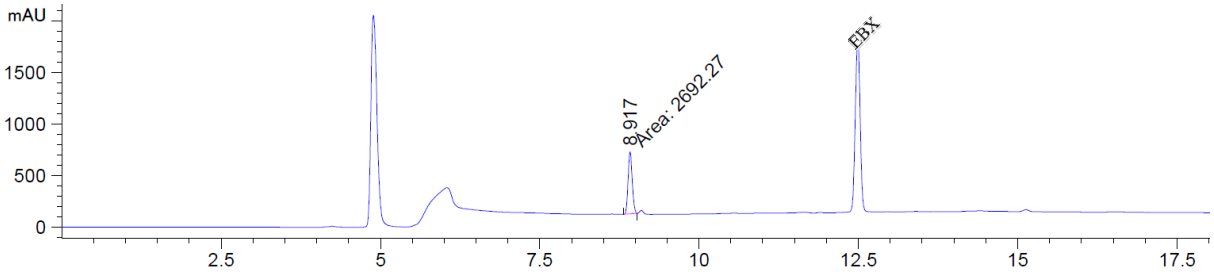


Figure S101: HPLC-UV chromatogram at 214 nm of the reaction of **73h**.

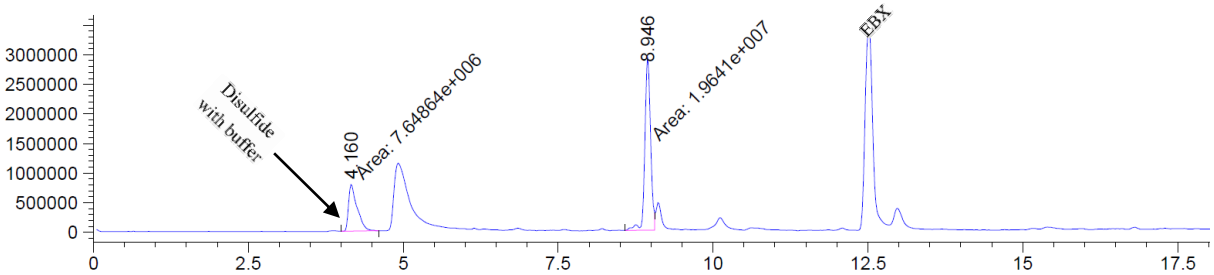


Figure S102: HPLC-MS chromatogram of the reaction of **73h**.

Following general procedure F, Ala-Cys-Asn-Ala (**14h**) afforded the title compound **73h** in 96% yield (retention time: 8.980 minutes).

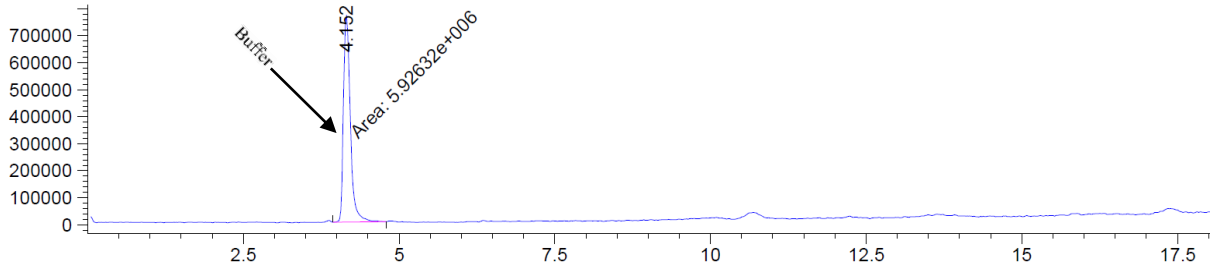


Figure S103: Tris buffer HPLC-MS chromatogram.

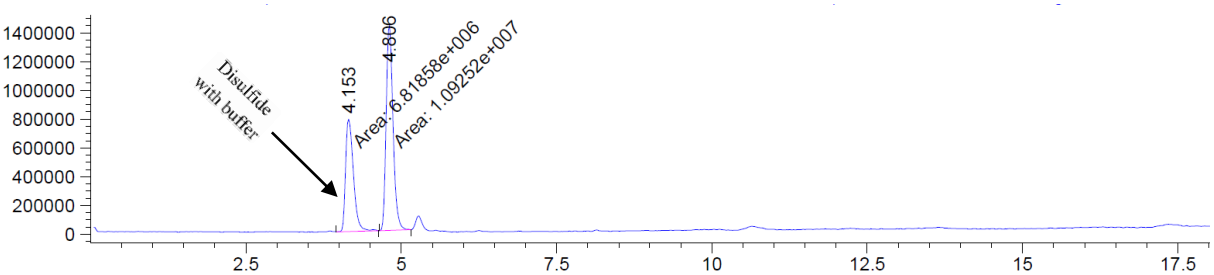


Figure S104: Ala-Cys-Asn-Ala HPLC-MS chromatogram.

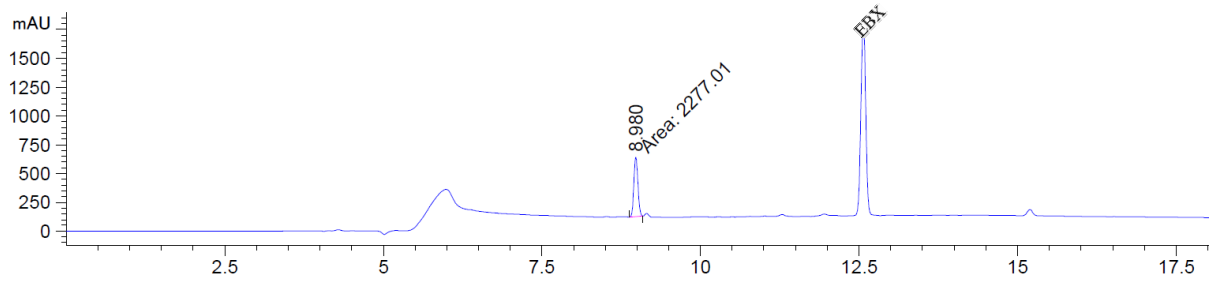


Figure S105: HPLC-UV chromatogram at 214 nm of the reaction of **73h**.

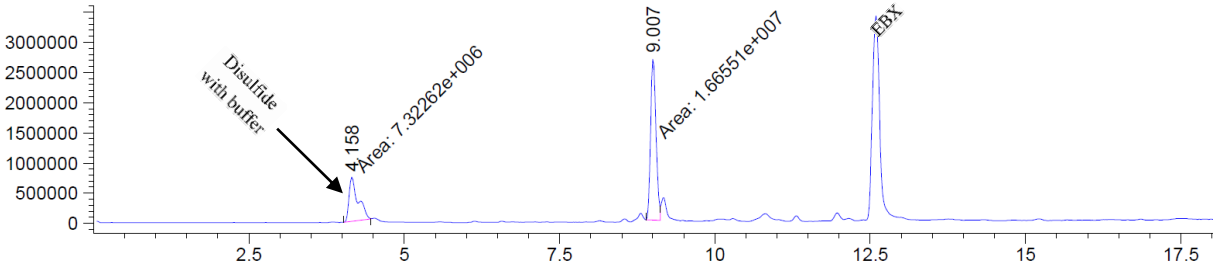
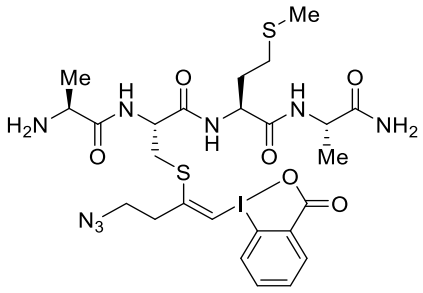


Figure S106: HPLC-MS chromatogram of the reaction of **73h**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{24}H_{33i}N_9O_7S^+$ 718.1263; Found 718.1259.

Azido-VBX conjugate of Ala-Cys-Met-Ala (**73i**)



(**73i**)

Following general procedure E, Ala-Cys-Met-Ala (**14i**) afforded the title compound **73i** in 97% yield (retention time: 9.709 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{25}H_{36}IN_8O_6S_2^+$ 735.1238; Found 735.1228.

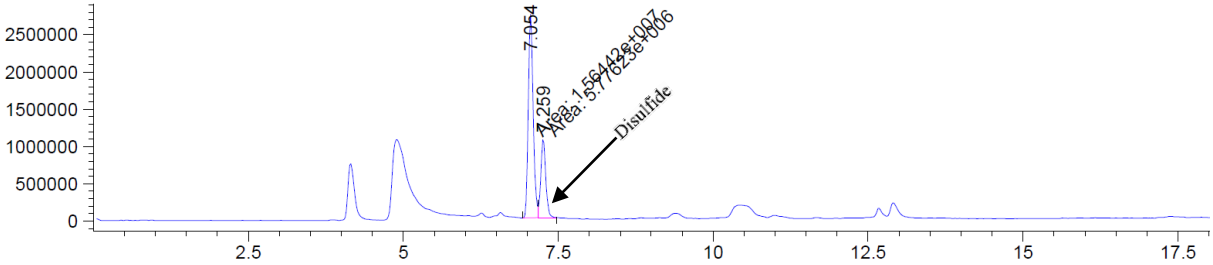


Figure S107: Ala-Cys-Met-Ala HPLC-MS chromatogram.

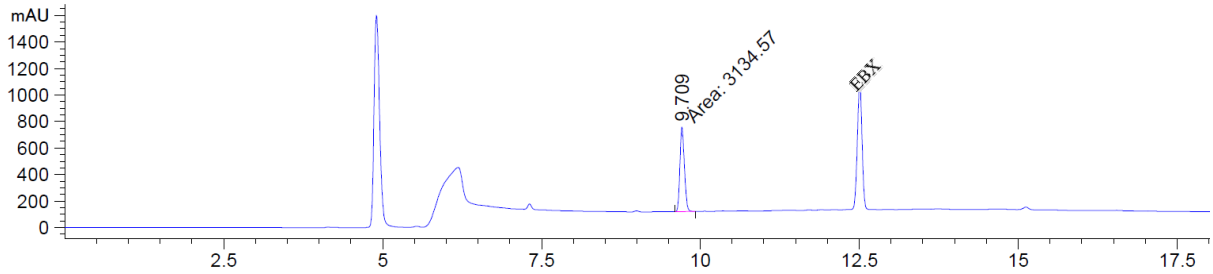


Figure S108: HPLC-UV chromatogram at 214 nm of the reaction of **73i**.

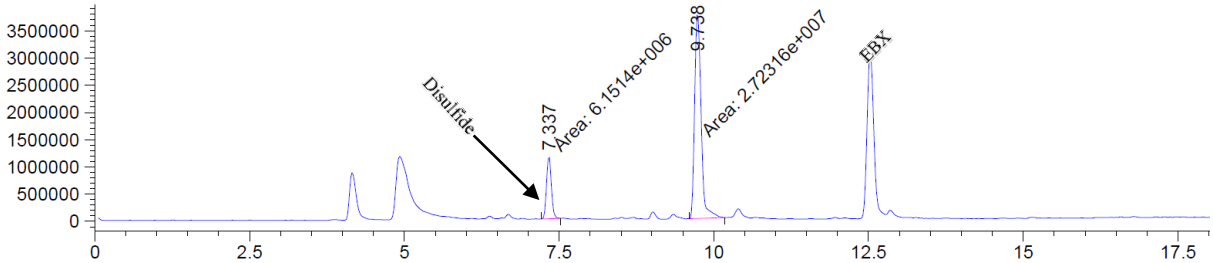
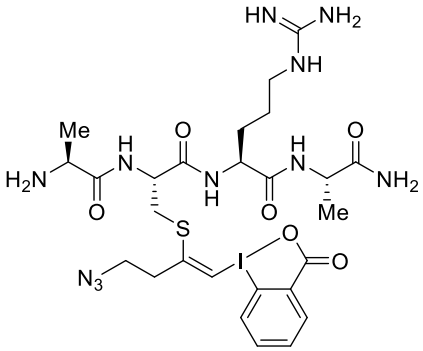


Figure S109: HPLC-MS chromatogram of the reaction of **73i**.

Azido-VBX conjugate of Ala-Cys-Arg-Ala (73j)



(**73j**)

Following general procedure E, Ala-Cys-Arg-Ala (**14j**) afforded the title compound **73j** in 84% yield (retention time: 8.411 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{26}H_{39}IN_{11o6}S^+$ 760.1845; Found 760.1830.

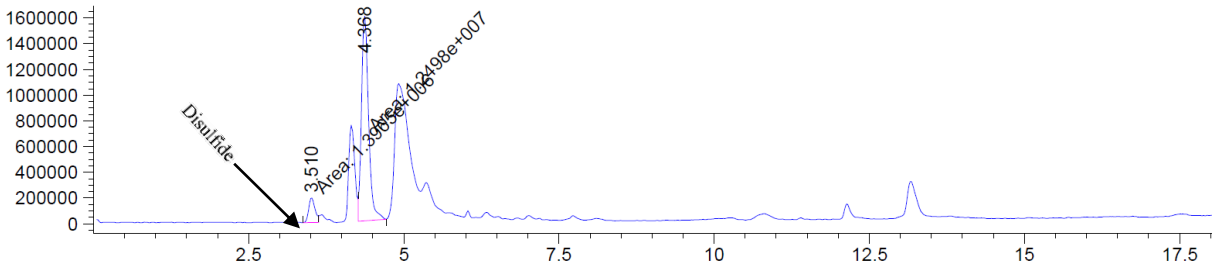


Figure S110: Ala-Cys-Arg-Ala HPLC-MS chromatogram.

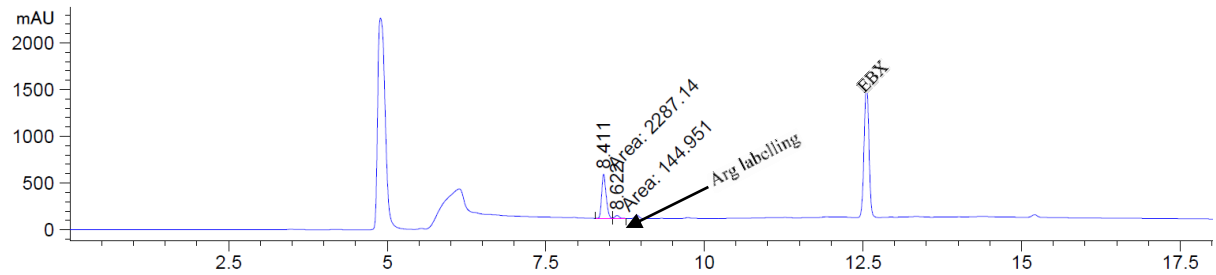


Figure S111: HPLC-UV chromatogram at 214 nm of the reaction of **73j**.

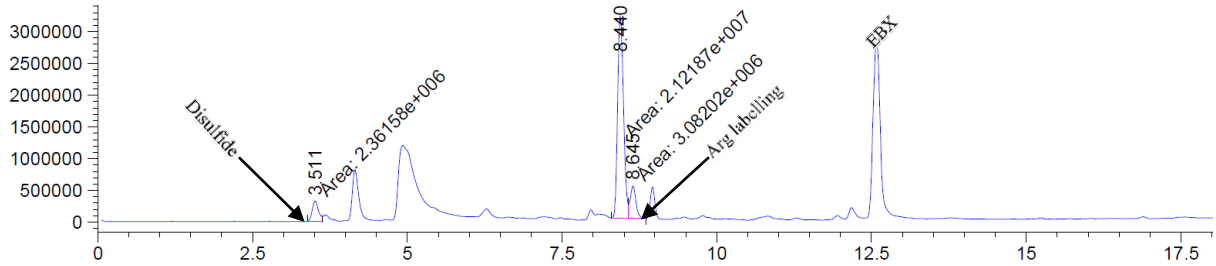
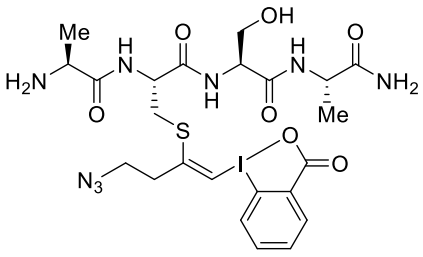


Figure S112: HPLC-MS chromatogram of the reaction of **73j**.

Azido-VBX conjugate of Ala-Cys-Ser-Ala (**73k**)



(**73k**)

Following general procedure E, Ala-Cys-Ser-Ala (**14k**) afforded the title compound **73k** in 96% yield (retention time: 9.003 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{23}H_{32}IN_8O_7S^+$ 691.1154; Found 691.1157.

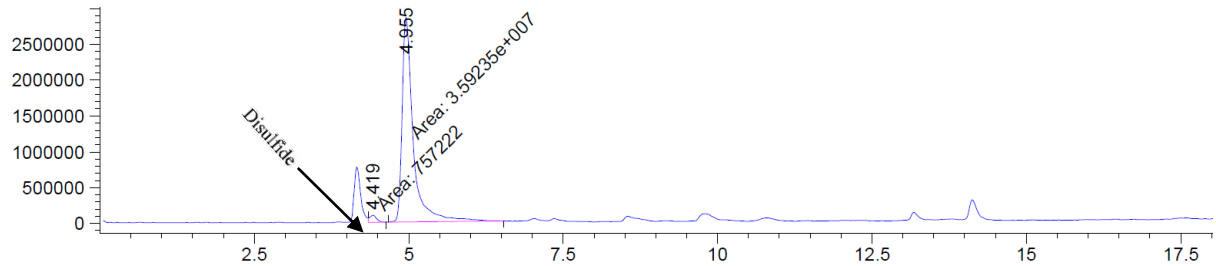


Figure S113: Ala-Cys-Ser-Ala HPLC-MS chromatogram.

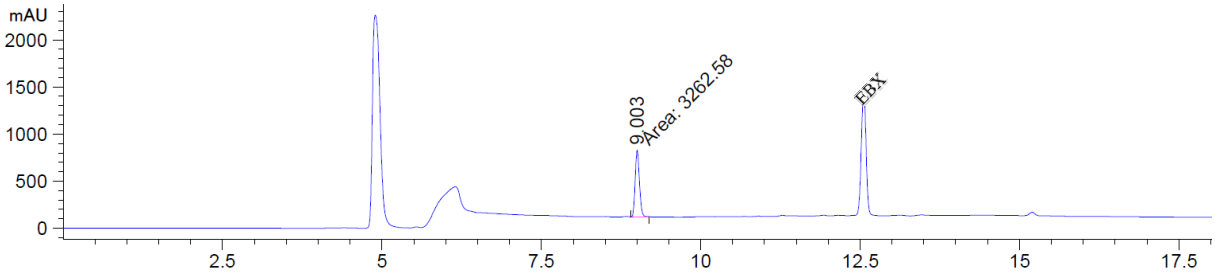


Figure S114: HPLC-UV chromatogram at 214 nm of the reaction of **73k**.

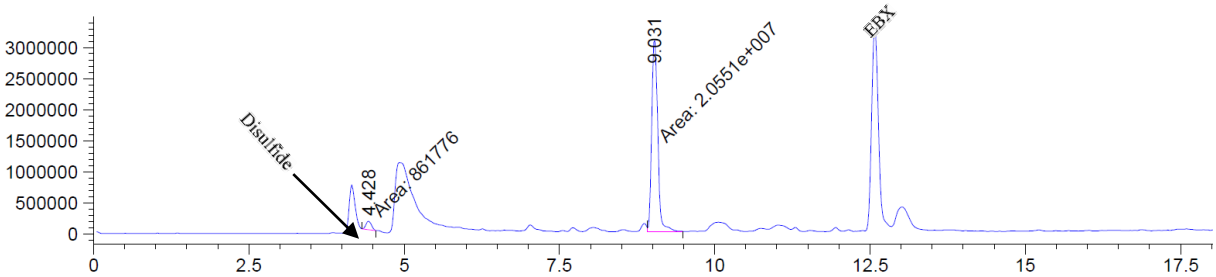
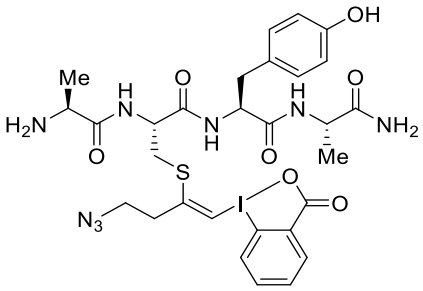


Figure S115: HPLC-MS chromatogram of the reaction of **73k**.

Azido-VBX conjugate of Ala-Cys-Tyr-Ala (**73l**)



(**73l**)

Following general procedure E, Ala-Cys-Tyr-Ala (**14l**) afforded the title compound **73l** in 95% yield (retention time: 9.581 minutes).

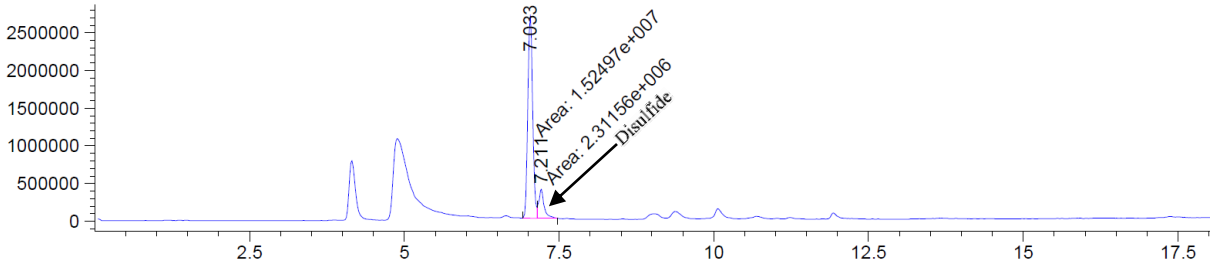


Figure S116: Ala-Cys-Tyr-Ala HPLC-MS chromatogram.

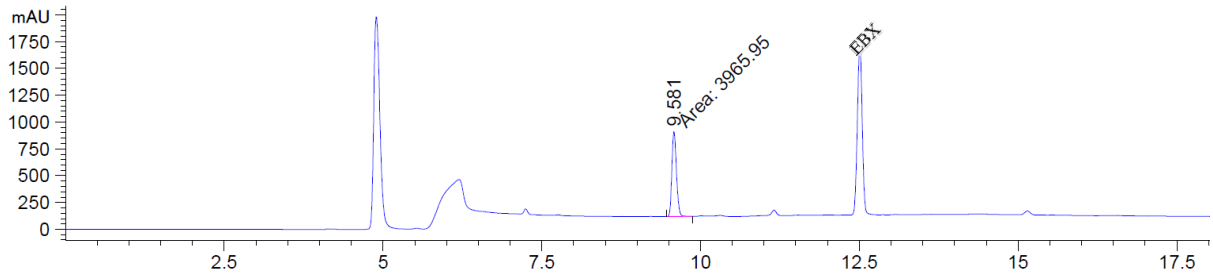


Figure S117: HPLC-UV chromatogram at 214 nm of the reaction of **73l**.

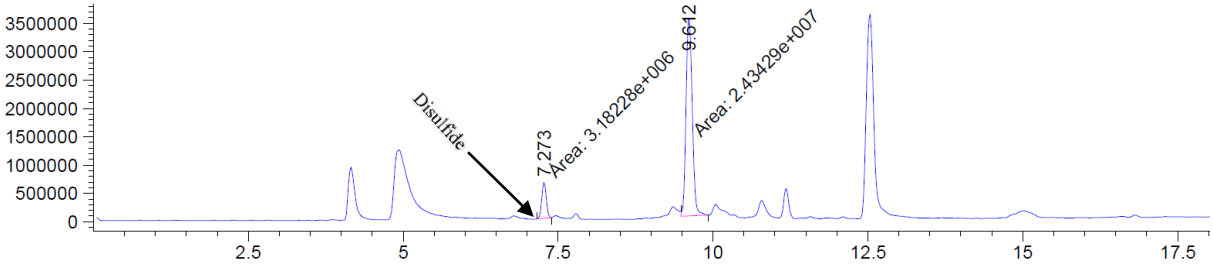


Figure S118: HPLC-MS chromatogram of the reaction of **73l**.

ACYA TIC/µmol 8.777e09 TIC/µmol

Following general procedure F, Ala-Cys-Tyr-Ala (**14l**) afforded the title compound **73l** in 95% yield (retention time: 9.547 minutes).

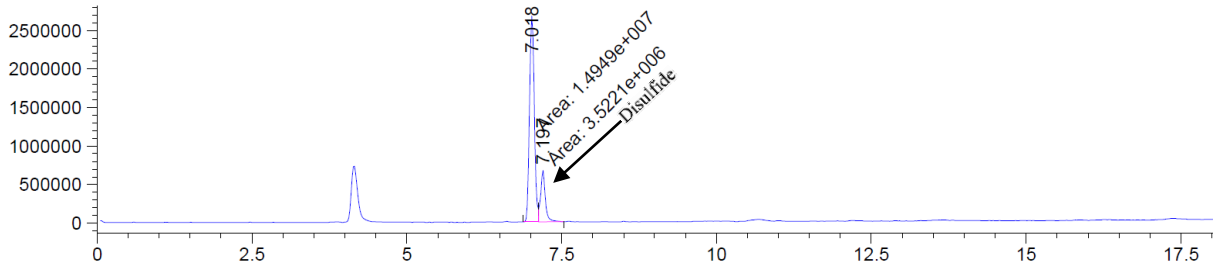


Figure S119: Ala-Cys-Tyr-Ala HPLC-MS chromatogram.

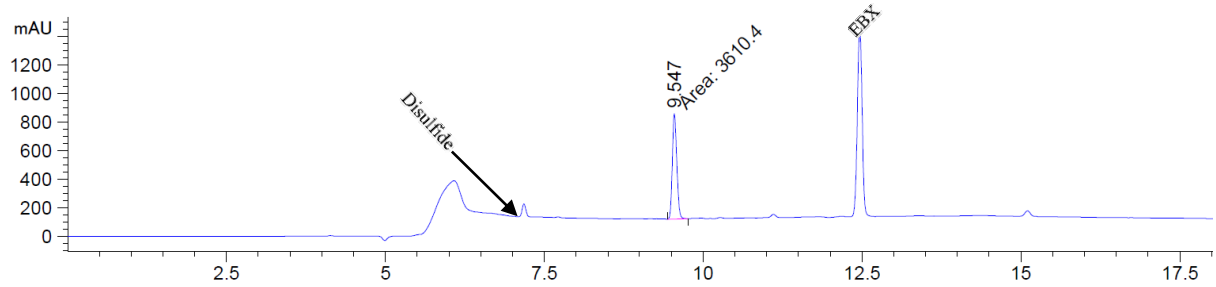


Figure S120: HPLC-UV chromatogram at 214 nm of the reaction of **73l**.

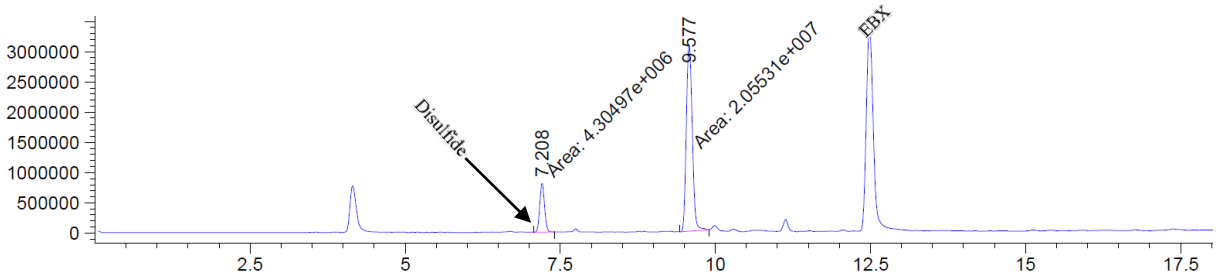
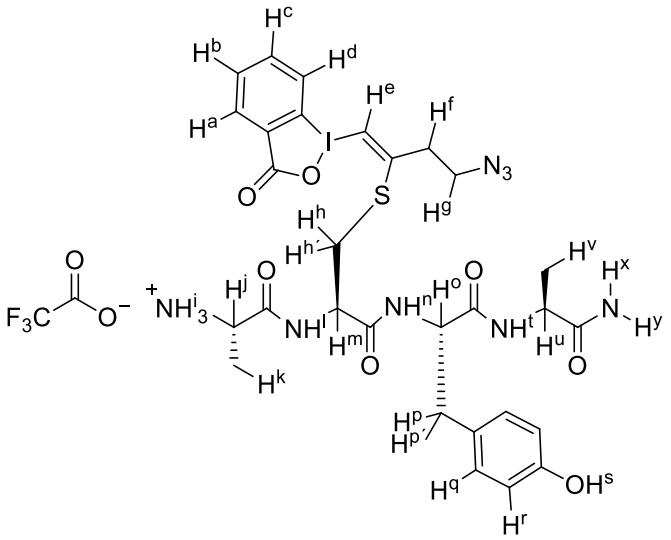
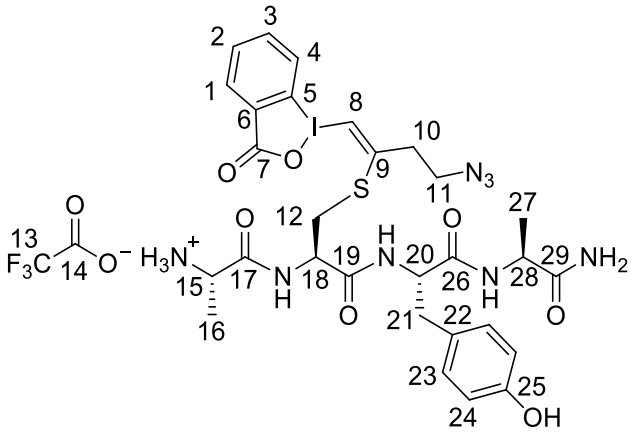


Figure S121: HPLC-MS chromatogram of the reaction of **73l**.

ACYA-VBX TIC/µmol 11.377e09 TIC/µmol



^{1}H NMR (600 MHz, DMSO-d_6) δ 9.18 (bs, 1H, H^s or H^x or H^y), 8.70 (d, J = 8.1 Hz, 1H, H^l), 8.38 (d, J = 8.1 Hz, 1H, H^n), 8.15-8.17 (m, 2H, H^a and H^t), 8.07 (bs, 3H, H^i), 7.63-7.69 (m, 2H, H^b and H^c), 7.59 (d, J = 7.28 Hz, 1H, H^d), 7.30 (s, 1H, H^s or H^x or H^y), 7.13 (s, 1H, H^e), 7.04 (s, 1H, H^x or H^y), 6.99 (d, J = 8.0 Hz, 2H, H^q), 6.60 (d, J = 8.0 Hz, 2H, H^r), 4.46-4.48 (m, 2H, H^m and H^o), 4.22 (p, J = 6.64 Hz, 1H, H^u), 3.72-3.80 (m, 3H, H^g and H^j), 3.11-3.16 (m, 1H, H^h or $H^{h'}$), 2.89-3.03 (m, 4H, H^h or $H^{h'}$, H^p or $H^{p'}$ and H^f), 2.63-2.68 (m, 1H, H^p or $H^{p'}$), 1.26 (d, J = 6.7 Hz, 3H, H^k), 1.21 (d, J = 6.7 Hz, 3H, H^v).

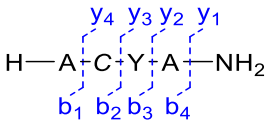


13C NMR (600 MHz, DMSO-*d6*) δ 174.0 (C^{29}), 170.3 (C^{26}), 169.6 (C^{17}), 168.4 (C^{19}), 165.9 (C^7), 158.0 (q, J = 32.1 Hz, C^{14}), 156.4 (C^9), 155.8 (C^{25}), 134.0 (C^6), 133.7 (C^3), 131.9 (C^1), 130.5 (C^2), 130.1 (C^{23}), 127.4 (C^4 or C^{22}), 127.3 (C^{22} or C^4), 116.8 (q, J = 297.7 Hz, C^{13}), 114.8 (C^{24}), 114.0 (C^5), 105.2 (C^8), 54.1 (C^{18} or C^{20}), 52.8 (C^{20} or C^{18}), 49.2 (C^{11}), 48.1 (C^{15} or C^{28}), 48.0 (C^{28} or C^{15}), 36.8 (C^{21}), 35.3 (C^{10}), 33.0 (C^{12}), 18.5 (C^{27}), 17.2 (C^{16}).

The assignment was done based on the interpretation of the 1H, 13C, COSY, HSQC, DEPT-135 and HMBC spectra (see Section: 21).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{29}H_{36}IN_8O_7S^+$ 767.1467; Found 767.1450.

MS/MS of $[M + H]^+$ 767.14 @cid22.00 [210.00-800.00] (LTQ-Orbitrap):



	b	y
1	-	-
2	516.0197	-
3	679.0830	252.1343
4	750.1201	-

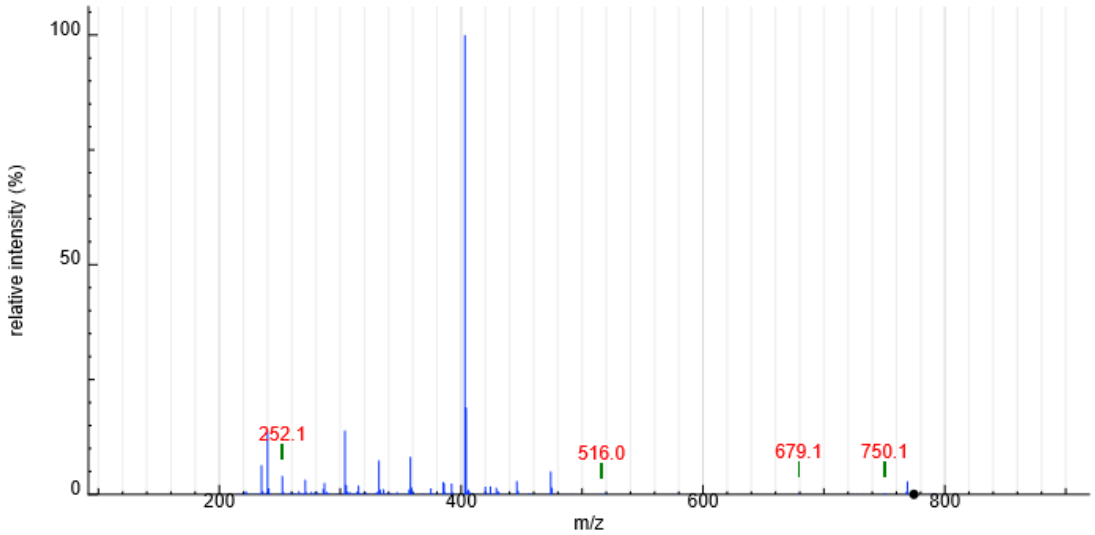
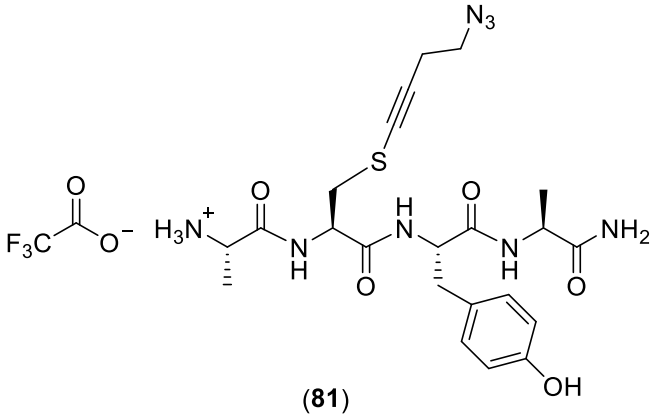


Figure S122: MS/MS found fragments, table and graphic, upon fragmentation of the molecular ion of product **73l**.

Thioalkynynlated Ala-Cys-Tyr-Ala(**81**)



(**81**)

A small cylindrical vial was charged with a solution of Ala-Cys-Tyr-Ala (**14l**) (1.63 mL, 2.0 mM 1.0 equiv.) in DMSO, a solution of TMG in DMSO (35.9 µL, 0.1 M, 1.1 equiv.) was then added and the mixture was then vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes before adding a final solution of N_3-EBX reagent (**1a**) in DMSO (39.2 µL, 0.1M, 1.2 equiv.). After 1 hour the solution was sampled and analyzed by LC-MS and the reaction was purified by preparative HPLC using method D.

Reaction chromatogram:

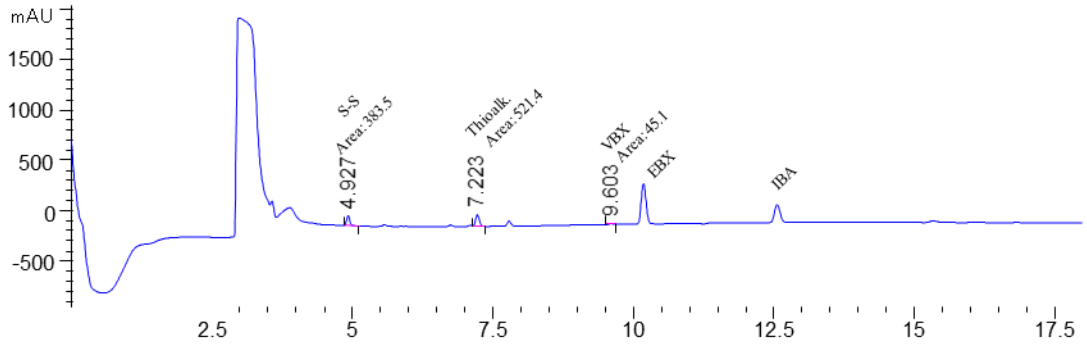


Figure S123: HPLC-UV chromatogram at 214 nm of the reaction of **81**.

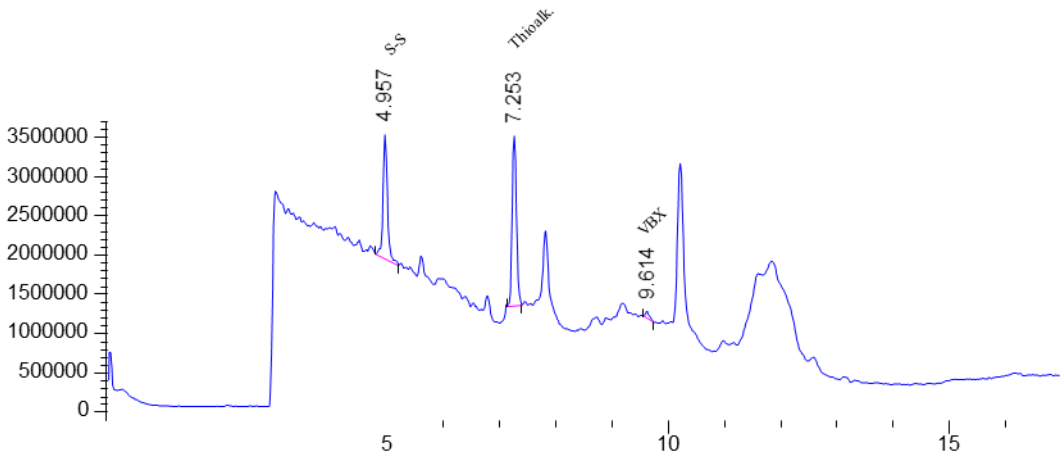
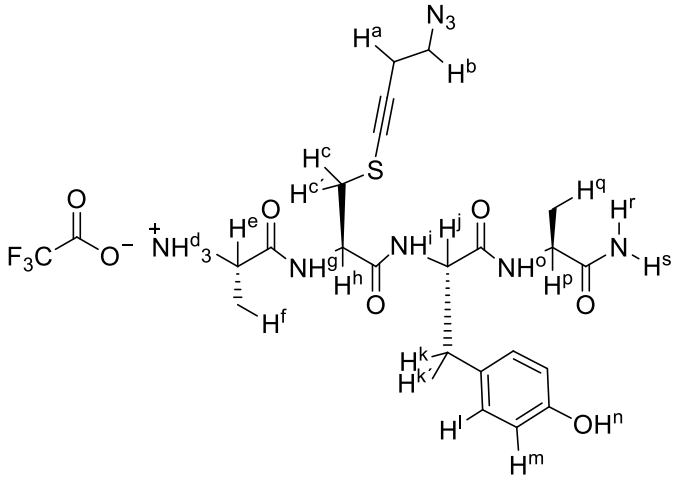
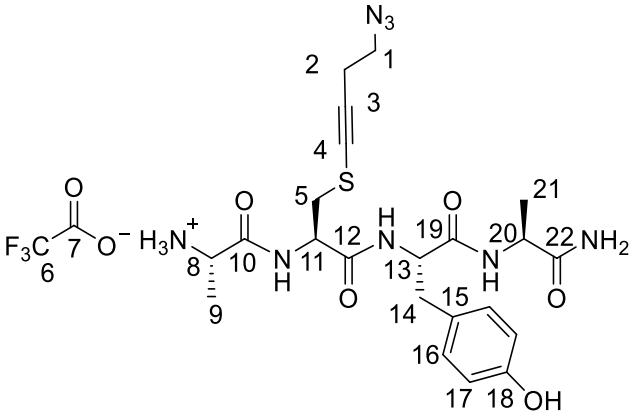


Figure S124: HPLC-MS chromatogram of the reaction of **81**.

ACYA-thioalkynylation TIC/µmol 5.832e09 TIC/µmol



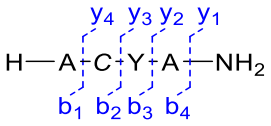
^{1}H NMR (600 MHz, DMSO-*d*$_6$) δ 9.20 (d, *J* = 8.9 Hz, 1H, H^n), 8.70 (d, *J* = 8.2 Hz, 1H, H^g), 8.15 (d, *J* = 7.9 Hz, 1H, H^i), 8.07 (bs, 3H, H^d), 7.66 (bs, 1H, H^r or H^s or H^o), 7.26 (bs, 1H, H^r or H^s or H^o), 7.04 (bs, 1H, H^r or H^s), 7.00 (d, *J* = 6.9 Hz, 2H, H^l), 6.61 (d, *J* = 6.2 Hz, 2H, H^m), 4.53-4.56 (m, 1H, H^j or H^h), 4.41-4.44 (m, 1H, H^h or H^j), 4.18 (p, *J* = 8.3, 7.7 Hz, 1H, H^e or H^p), 3.83 (m, 1H, H^p or H^e), 3.43 (td, *J* = 6.6, 2.0 Hz, 2H, H^b), 3.13 (d, *J* = 16.7 Hz, 1H, H$^{c'}$ or H^c), 2.93 (d, *J* = 16.9 Hz, 1H, H^c or H$^{c'}$), 2.82 (t, *J* = 11.1 Hz, 1H, H^k or H$^{k'}$), 2.68 (dd, *J* = 14.1, 9.1 Hz, 1H, H^k or H$^{k'}$), 2.62 (tjd, *J* = 6.5, 2.2 Hz, 2H, H^a), 1.30 (d, *J* = 6.7 Hz, 3H, H^f), 1.21 (d, *J* = 6.8 Hz, 3H, H^q).



13C NMR (600 MHz, DMSO-*d6*) δ 174.4 (C^{22}), 170.0 (C^{12} or C^{19}), 169.8 (C^{10}) 169.0 (C^{19} or C^{12}), 155.9 (C^{18}), 130.6 (C^{16}), 128.0 (C^{15}), 115.2 (C^{17}), 91.9 (C^4), 70.0 (C^3), 54.7 (C^{11} or C^{13}), 52.6 (C^{13} or C^{11}), 49.5 (C^1), 48.5 (C^8 or C^{20}), 48.4 (C^{20} or C^8), 37.4 (C^5), 36.9 (C^{14}), 20.8 (C^2), 18.9 (C^{21}), 17.6 (C^9) (Due to the weak signals the carbons of the trifluoroacetate are not observed and some of the 13C signals were inferred from the HMBC and HSQC spectra).

The assignment was done based on the interpretation of the 1H, 13C, HSQC and HMBC spectra (see Section: 21).**HRMS** (APPI/LTQ-Orbitrap) m/z: [M + H]$^+$ Calcd for $C_{22}H_{31}N_8O_4S^+$ 503.2183; Found 503.2184

MS/MS of $[M + H]^+$ 503.21 @cid18.00 [135.00-1200.00] (LTQ-Orbitrap):



	b	y
1	-	-
2	268.0863	-
3	415.1547	236.1394
4	486.1918	432.1812

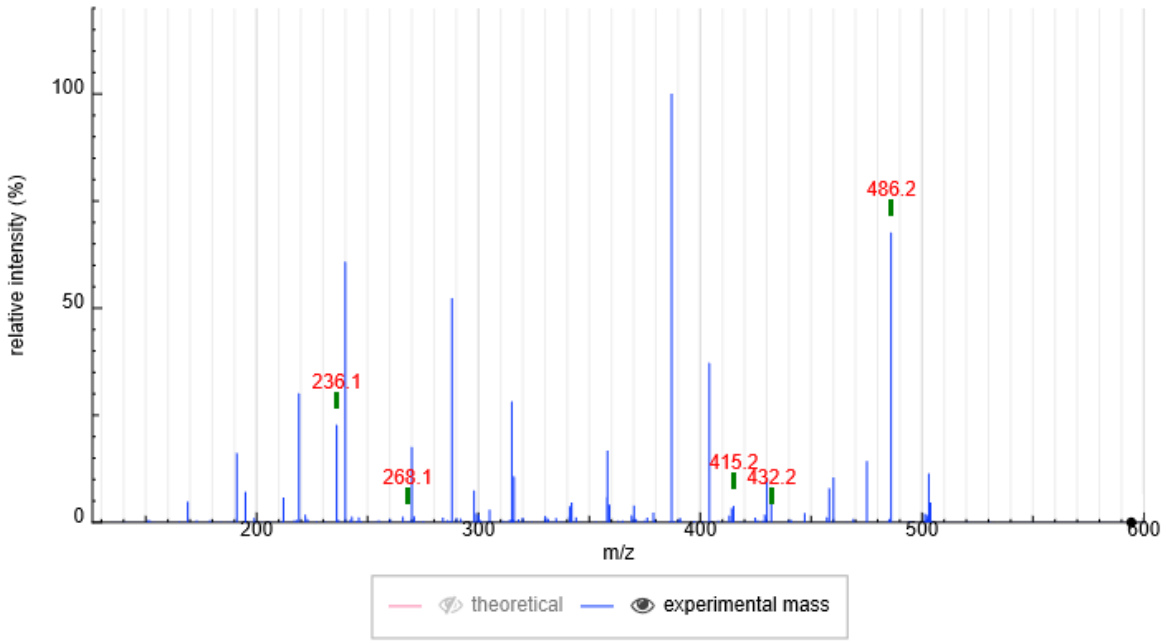
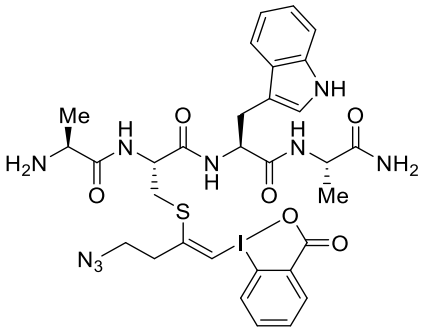


Figure S125: MS/MS found fragments, table and graphic, upon fragmentation of the molecular ion of product **76**.

Ala-Cys-Trp-Ala (**73m**)



(**73m**)

Following general procedure E, Ala-Cys-Trp-Ala (**14m**) afforded the title compound **73m** in 99% yield (retention time: 10.893 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{31}H_{37}IN_9O_6S^+$ 790.1627; Found 790.1620.

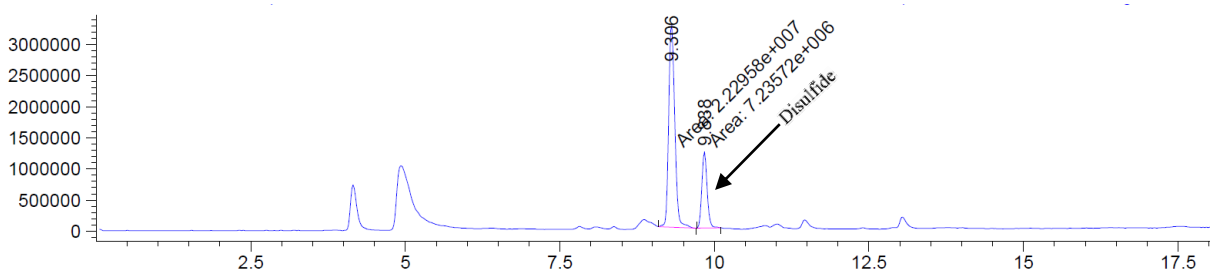


Figure S126: Ala-Cys-Trp-Ala HPLC-MS chromatogram.

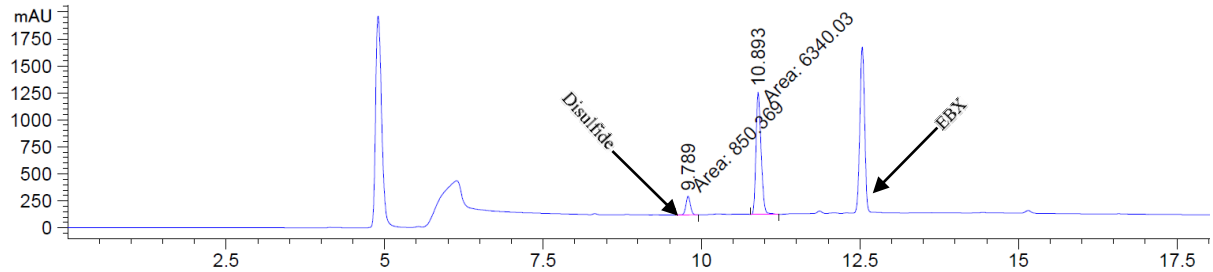


Figure S127: HPLC-UV chromatogram at 214 nm of the reaction of **73m**.

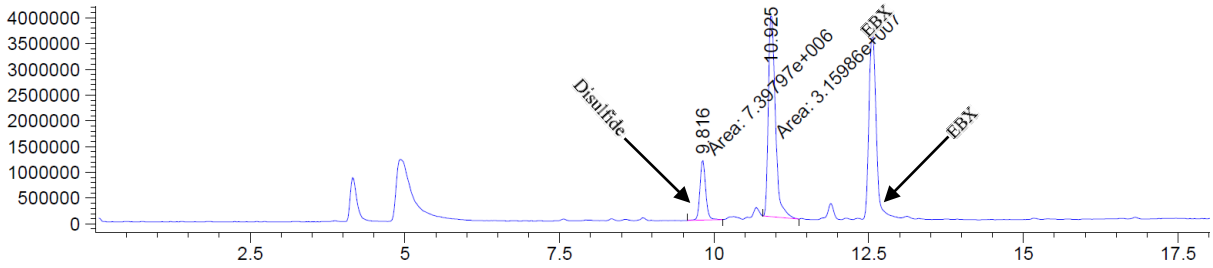
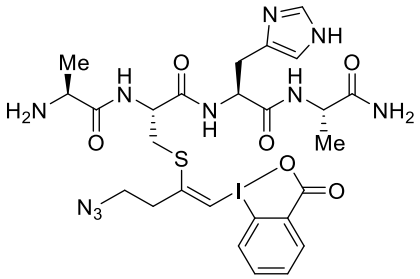


Figure S128: HPLC-MS chromatogram of the reaction of **73m**.

Ala-Cys-His-Ala (**73n**)



(**73n**)

Following general procedure E, Ala-Cys-His-Ala (**14n**) afforded the title compound **73n** in 92% yield (retention time: 8.352 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{26}H_{34}IN_{10}O_6S^+$ 741.1423; Found 741.1423.

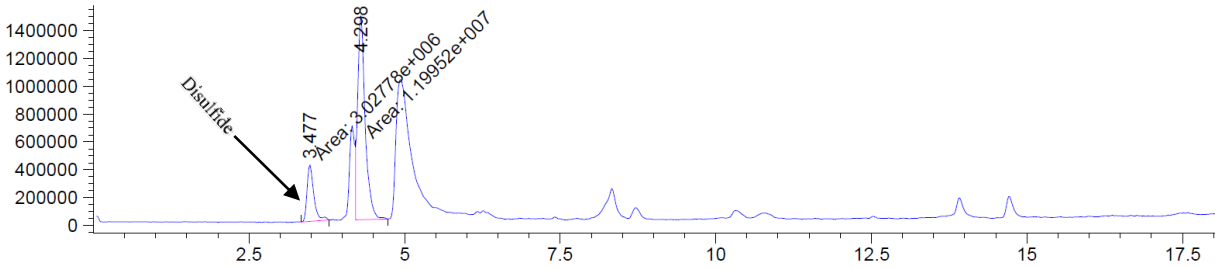


Figure S129: Ala-Cys-His-Ala HPLC-MS chromatogram.

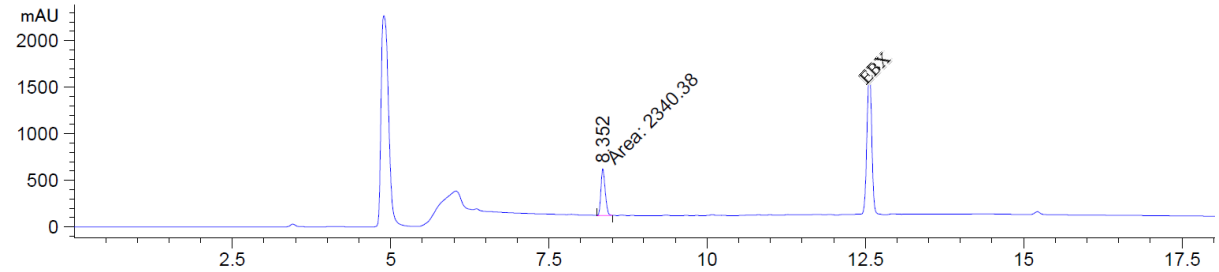


Figure S130: HPLC-UV chromatogram at 214 nm of the reaction of **73n**.

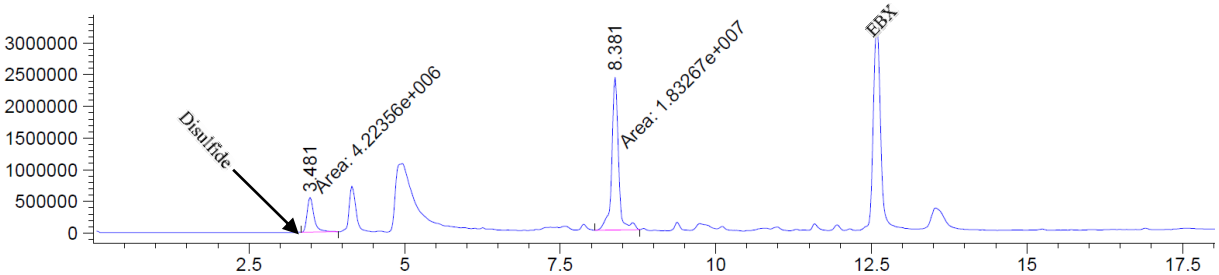
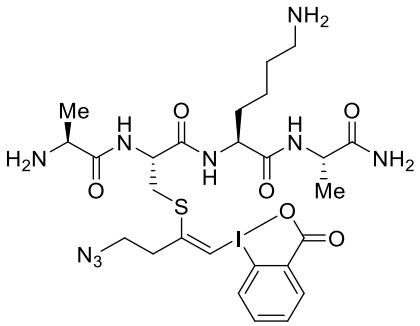


Figure S131: HPLC-MS chromatogram of the reaction of **73n**.

Ala-Cys-Lys-Ala (**73o**)



(**73o**)

Following general procedure E, Ala-Cys-Lys-Ala (**14o**) afforded the title compound **73o** in 99% yield (retention time: 8.285 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{26}H_{39}IN_9O_6S^+$ 732.1783; Found 732.1776.

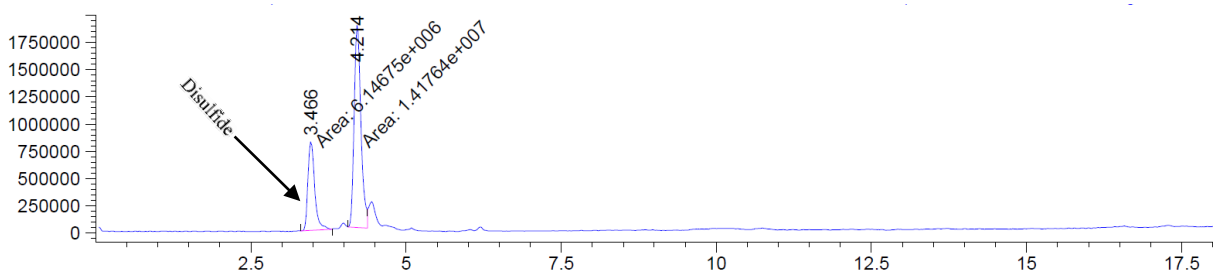


Figure S132: Ala-Cys-Lys-Ala HPLC-MS chromatogram.

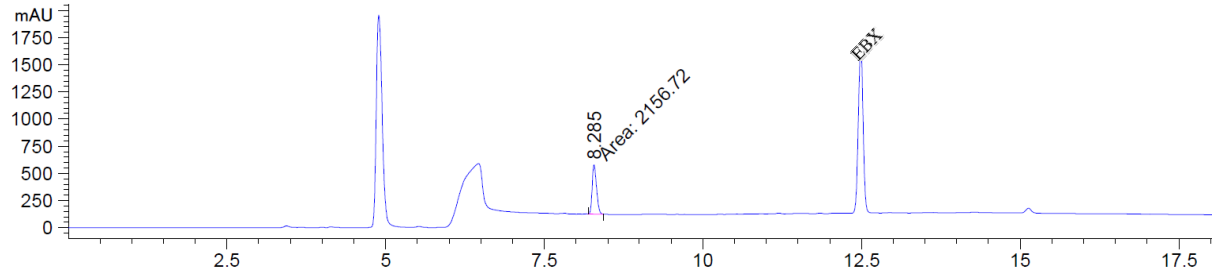


Figure S133: HPLC-UV chromatogram at 214 nm of the reaction of **73o**.

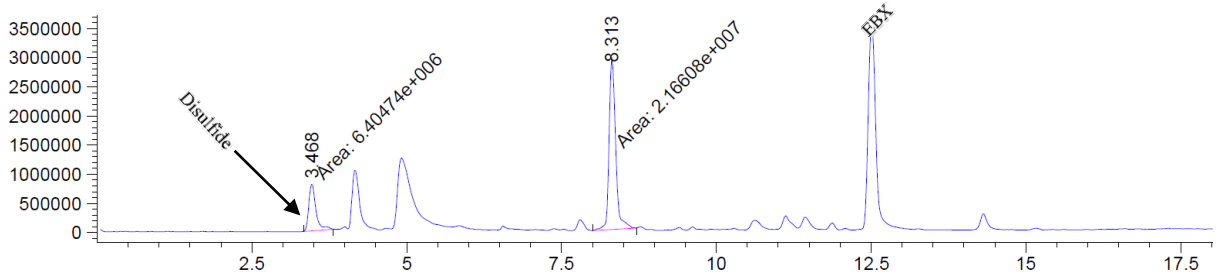
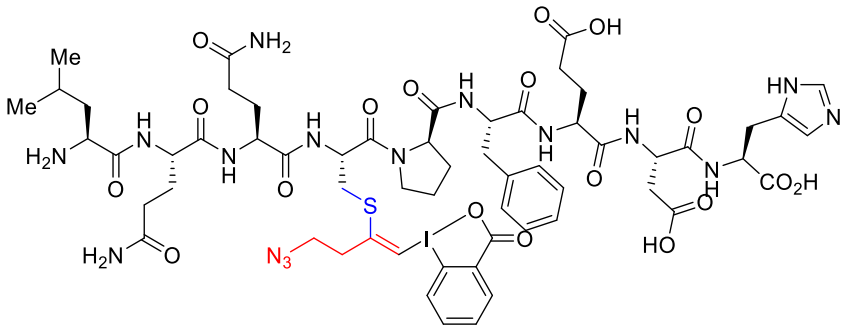


Figure S134: HPLC-MS chromatogram of the reaction of **73o**.

c. Substrate scope on cysteine-containing nonamer (**15)**

Leu-Gln-Gln-Cys-Pro-Phe-Glu-Asp-His



(**16**)

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 20.0 mM solution of nonamer **15** in water (66.0 μL, 1.32 μmol) was diluted with Tris buffer (10 mM, pH 8.2, 328 μL). The resulting solution was vortexed few seconds and a 200 mM solution of N_3-EBX reagent (**1a**) in DMSO (6.00 μL, 1.20 μmol, 0.91 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **16** in 88% yield (retention time: 16.667 minutes). No effort was made to exclude oxygen. The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product} + A_{oxidation})$, where $A_{starting}$, $A_{product}$ and $A_{oxidation}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material, product and oxidized starting material.

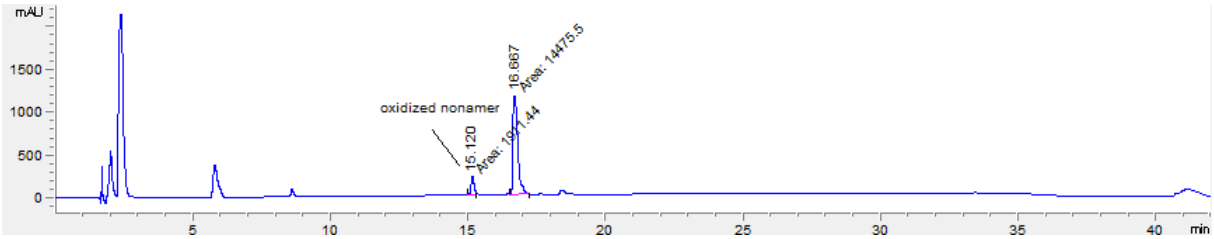


Figure S135: HPLC-UV chromatogram at 214 nm of **16**.

ESI-MS Calcd mass 1457.4 Da; Found 1457.1 Da.

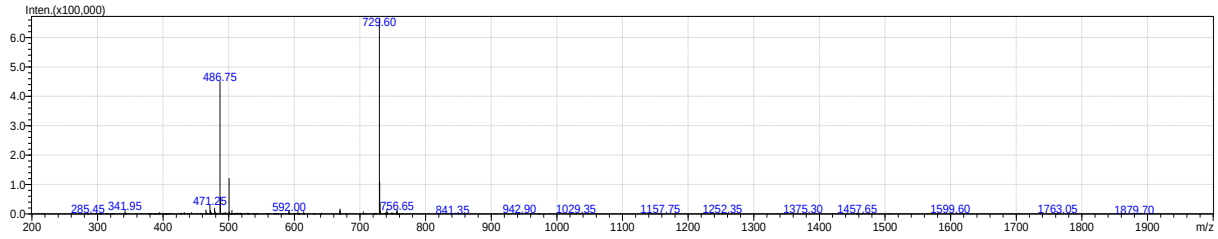
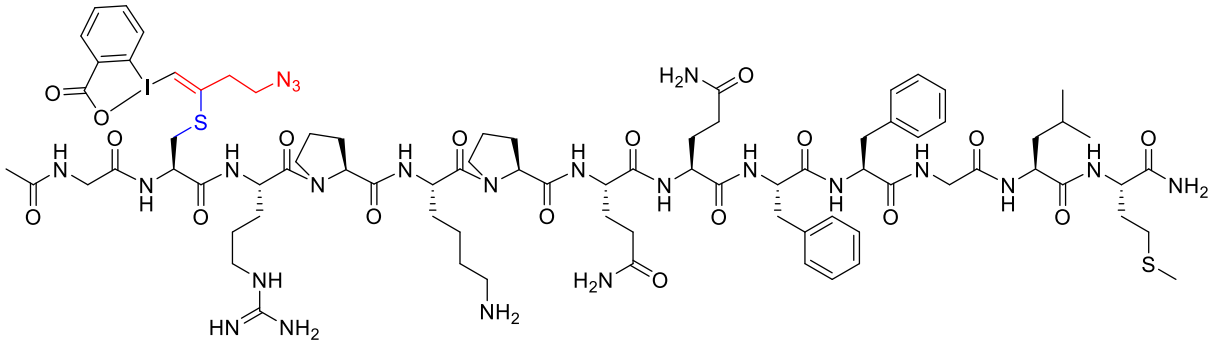


Figure S136: ESI-MS spectrum of **16**.

d. Substrate scope on Substance P analogue (**35**)



(**82**)

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, Substance P (**35**) (1.00 mg, 645 nmol, 1.00 equiv.) was dissolved in 1.3 mL phosphate buffer (50.0 mM, pH 8.2) containing 10% (v/v) DMSO. The resulting solution was vortexed few seconds and a 200 mM solution of N_3-EBX reagent (**1a**) in DMSO (4.20 µL, 838.5 nmol, 1.30 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **82** in 81 % yield (retention time: 20.714 minutes). No effort was made to exclude oxygen. The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product} + A_{oxidation} + A_{side\ products})$, where $A_{starting}$, $A_{product}$, $A_{oxidation}$ and $A_{side\ products}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material, product, oxidized starting material and side products.

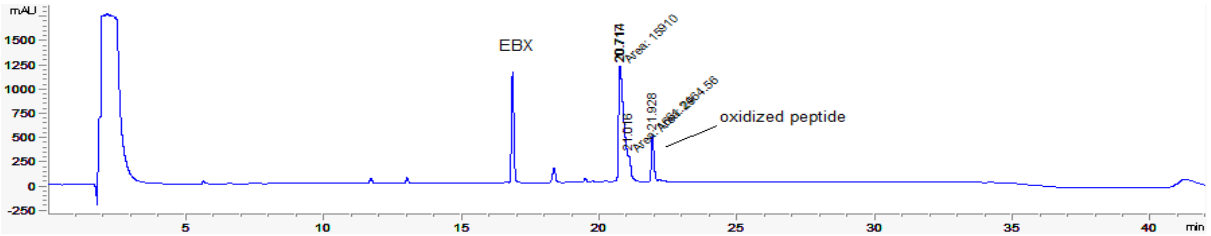


Figure S 137: HPLC-UV chromatogram of **82**.

ESI-MS Calcd mass 1891.0 Da; Found 1890.4 Da.

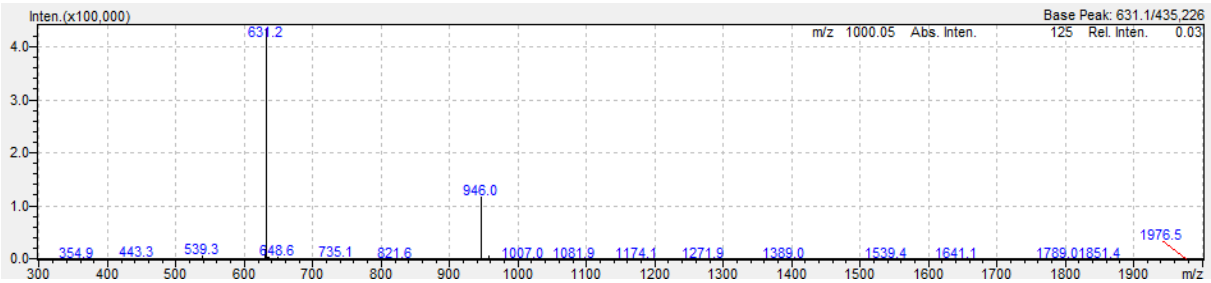
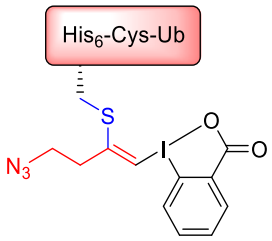


Figure S138: ESI-MS spectrum of **82**.

e. Substrate scope on cys-ubiquitin (**17**)

Native cys-ubiquitin procedure



(**18**)

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 1.00 mM solution of cys-ubiquitin (**17**) in water (50.0 μL, 50.0 nmol) was diluted with Tris buffer (10 mM, pH 8.2, 40.0 μL). The resulting solution was vortexed few seconds and a 10.0 mM solution of N_3-EBX reagent (**1a**) in Tris buffer (10.0 mM, pH 8.2) containing 5% (v/v) DMSO (10.0 μL, 100 nmol, 2.00 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **18** in 99% yield (retention time: 21.395 minutes). No effort was made to exclude oxygen. The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product} + A_{oxidation})$, where $A_{starting}$, $A_{product}$ and $A_{oxidation}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material, product and oxidized starting material. The product was further subjected to top-down analysis to confirm the cysteine-specific labeling

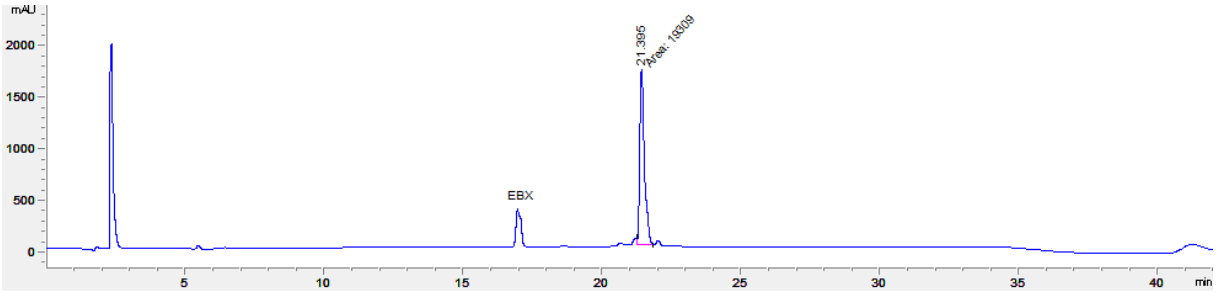


Figure S139: HPLC-UV chromatogram at 214 nm of **18**.

ESI-MS Calcd mass 11041.2 Da; Found 11041.0 Da.

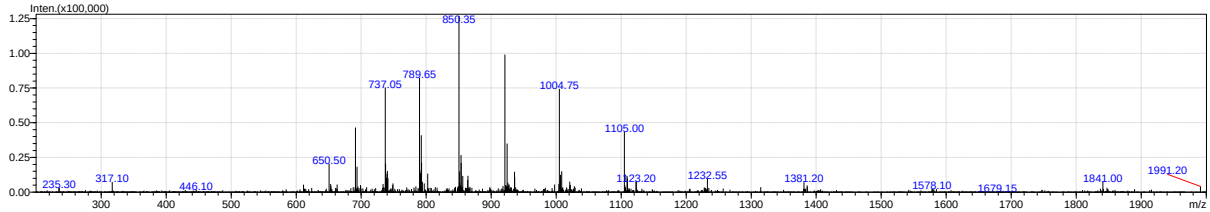


Figure S140: ESI-MS spectrum of **18**.

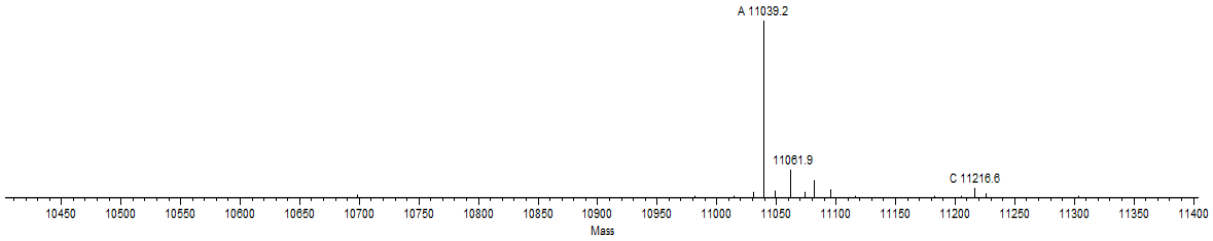


Figure S141: Deconvoluted spectrum of **18**.

Top-down MS analysis:

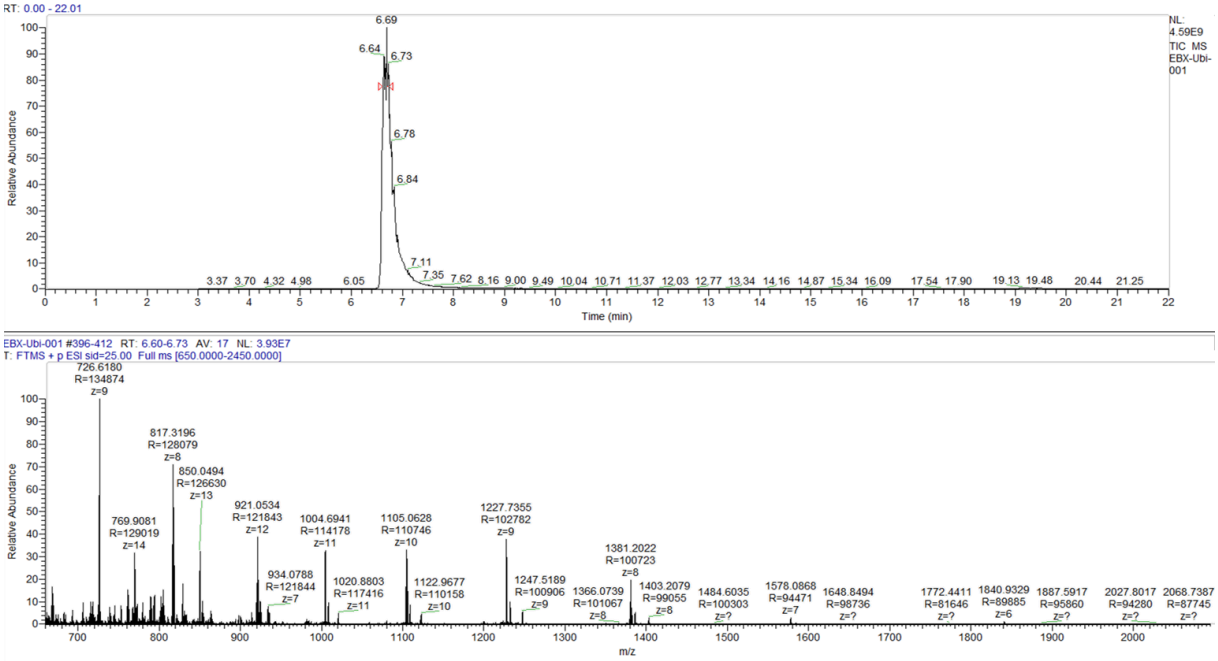


Figure S142: LC-MS of **18.**

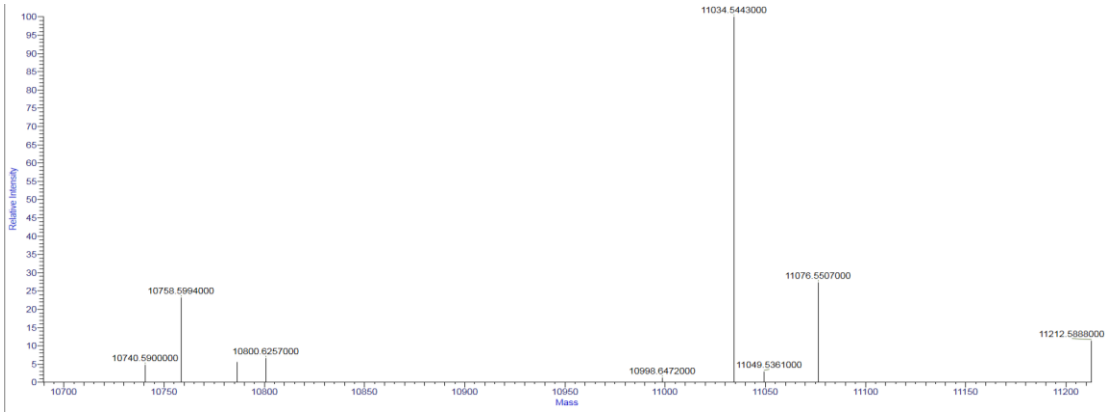
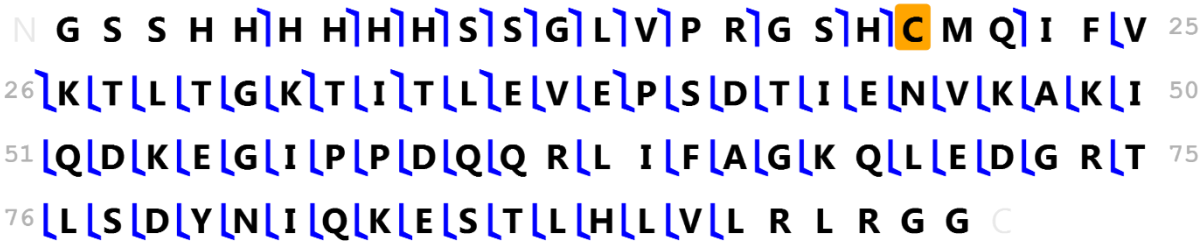


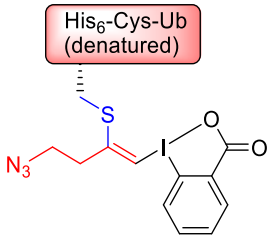
Figure S143: Deconvoluted spectrum of **18**.

MS2:



Obtained sequence coverage was 80% with p-score of 7e-24. Assigned *b*- and *y*-fragmentation ions (in blue) confirm the Cys20 modification by EBX molecule. Presence of key fragments b_{19}, b_{22}, y_{72} and a pair b_{25}/y_{71} was validated manually.

Denatured cys-ubiquitin procedure



(**18´**)

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, a 1.00 mM solution of cys-ubiquitin (**17**) in water (50.0 μL, 50.0 nmol) was diluted with denaturing phosphate buffer (6.00 M GdmHCl), 200 mM phosphate, pH 8.2, 44.5 μL). The resulting solution was vortexed few seconds and a 10.0 mM solution of N_3-EBX reagent (**1a**) in denaturing phosphate buffer (6.00 M GdmHCl, 200 mM phosphate, pH 8.2) containing 5% (v/v) DMSO (5.50 μL, 55.0 nmol, 1.10 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **18´** in 77% yield (retention time: 21.629 minutes). No effort was made to exclude oxygen. The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product} + A_{oxidation})$, where $A_{starting}$, $A_{product}$ and $A_{oxidation}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material, product and oxidized starting material.

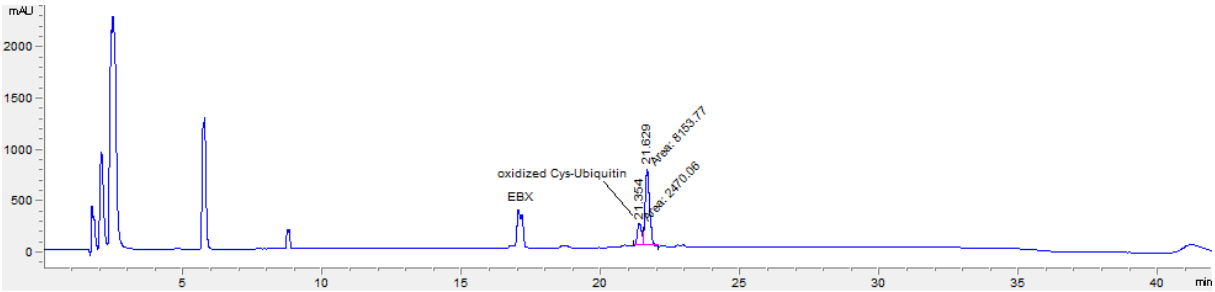


Figure S144: HPLC-UV chromatogram at 214 nm of **18'**.

ESI-MS Calcd mass 11041.2 Da; Found 11041.1 Da.

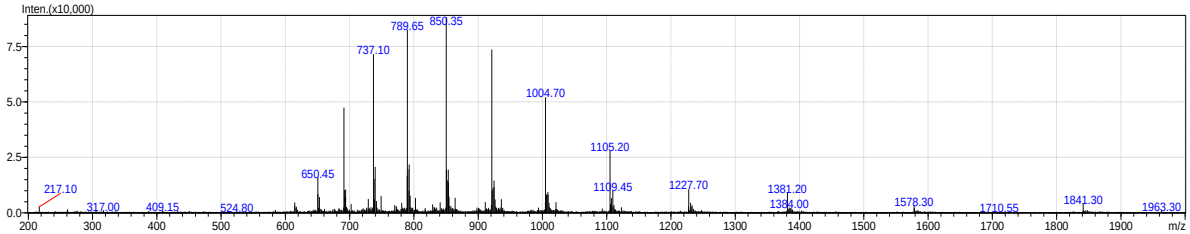
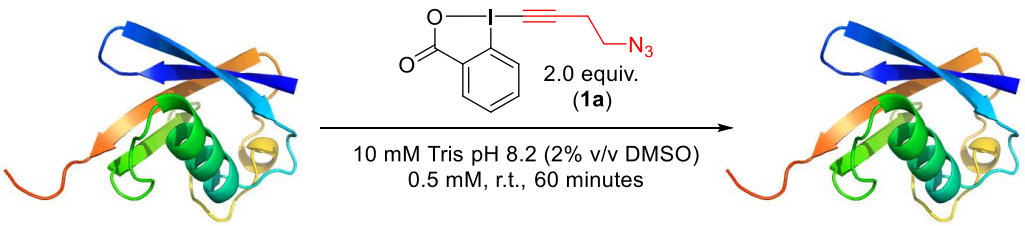


Figure S145: ESI-MS spectra of **18'**.

f. Control experiment with non-mutated ubiquitin (**83**)



(**83**) (**83**), no reaction

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, a 1.00 mM solution of non-mutated ubiquitin (**83**) in water (50.0 μL, 50.0 nmol) was diluted with Tris buffer (10.0 mM, pH 8.2, 40.0 μL). The resulting solution was vortexed few seconds and a 10.0 mM solution of N_3-EBX reagent (**1a**) in Tris buffer (10.00 mM, pH 8.2) containing 5% (v/v) DMSO (10.0 μL, 100 nmol, 2.00 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 120 minutes. No reactivity was observed. The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis.

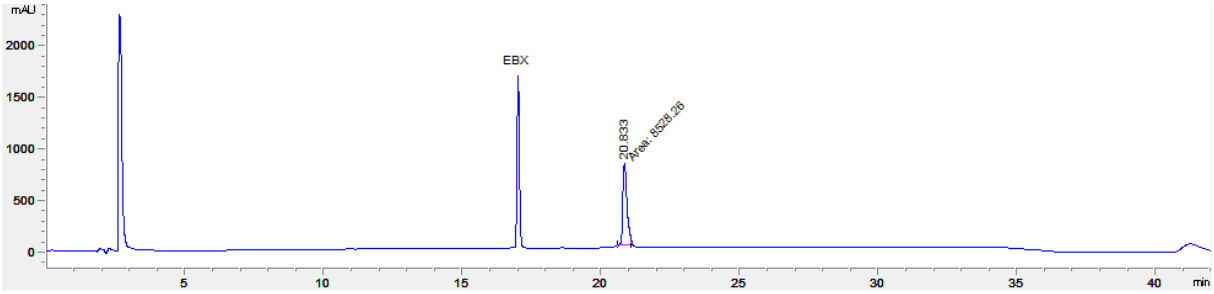


Figure S146: HPLC-UV chromatogram at 214 nm.

ESI-MS Calcd mass 8564.8 Da; Found 8565.3.

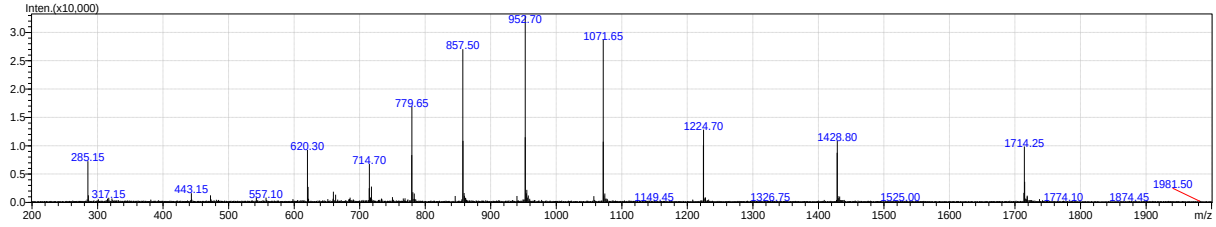
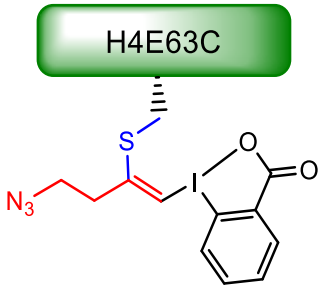


Figure S147: ESI-MS spectrum.

g. Substrate scope on histone H4E63C (**42**)



(**84**)

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, 1.00 mg solution of histone H4E63C (**42**) (89.2 nmol, 1.00 equiv.) was dissolved in denaturing phosphate buffer (6.00 M GdmHCl, 200 mM phosphate, pH 8.2, 178 μL). The resulting solution was vortexed few seconds and a 100 mM solution of DTT in water (0.89 μL, 89.2 nmol, 1.00 equiv.) was added and the solution incubated at room temperature 10 min. Subsequently, a 100 mM solution of N_3-EBX reagent (**1a**) in DMSO (2.67 μL, 267.7 nmol, 3.00 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford the EBX-labeled H4 histone protein (**84**) in 95 % yield (retention time: 26.444 minutes). No effort was made to exclude oxygen. The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product} + A_{oxidation})$, where $A_{starting}$, $A_{product}$ and $A_{oxidation}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material, product and oxidized starting material.

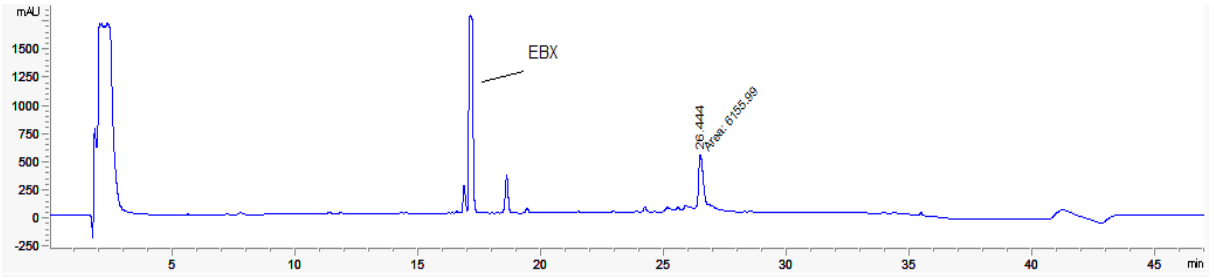


Figure S148: LC-MS spectrum of **84**.

ESI-MS Calcd mass 11550 Da; Found 11550 Da

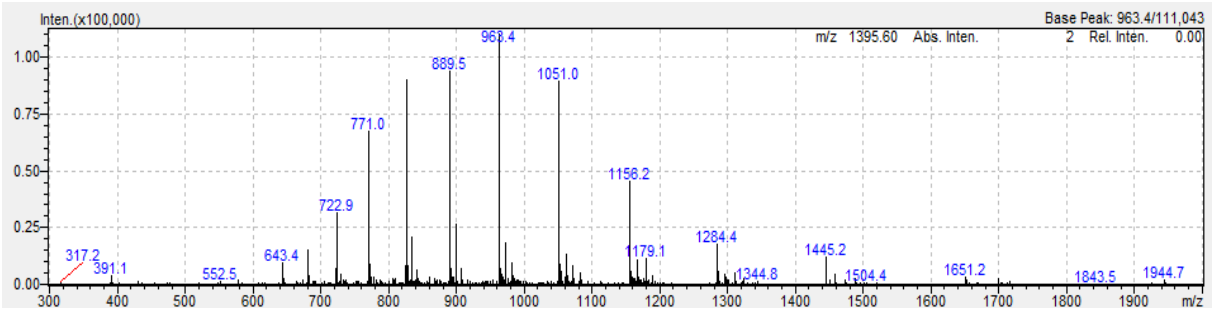


Figure S149: ESI-MS spectrum of **84**.

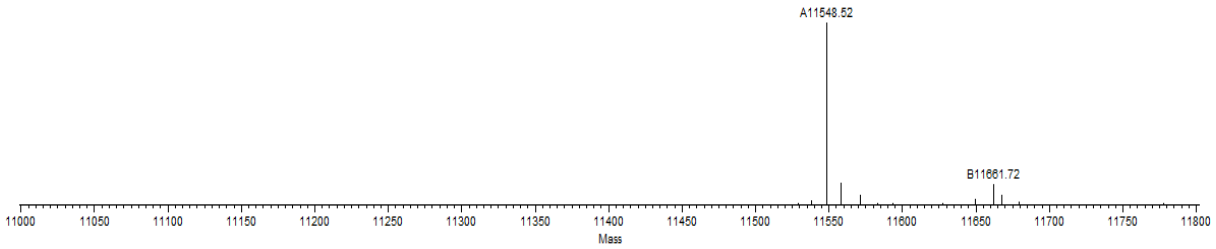


Figure S150: Deconvoluted spectrum of **84**.

Top-down MS analysis:

LC-MS1:

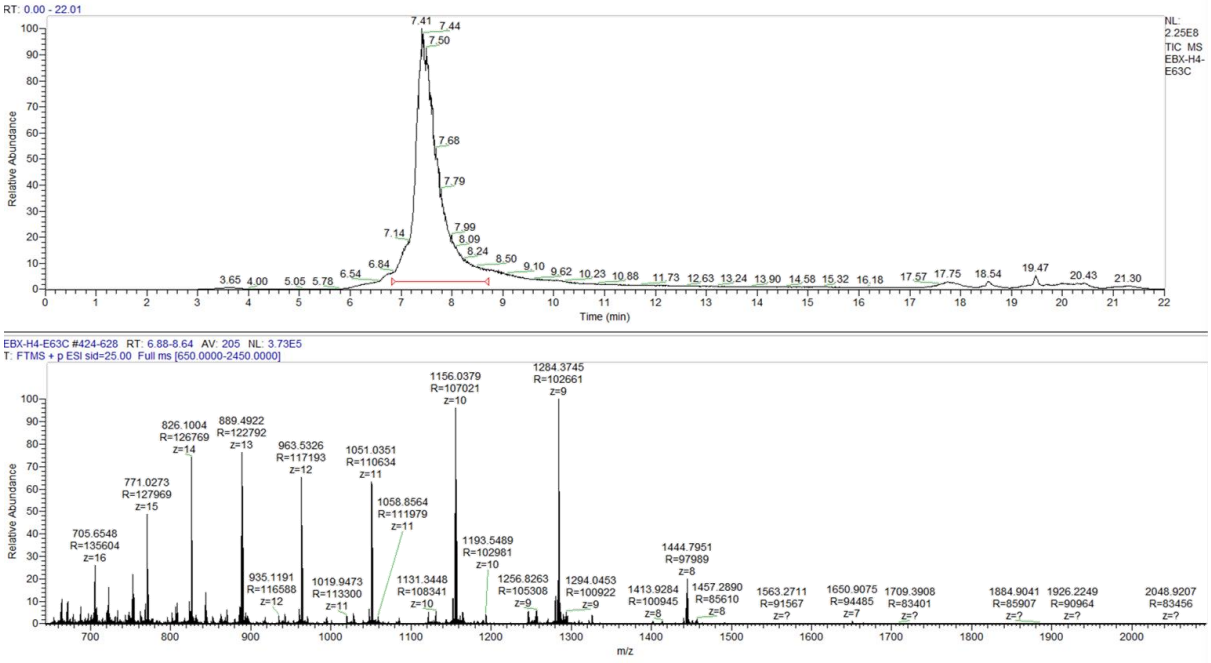


Figure S151: LC-MS of **84**.

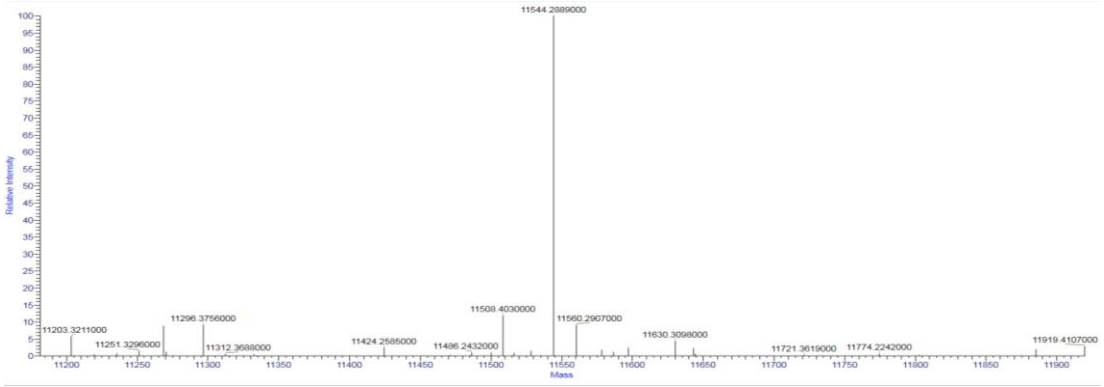


Figure S152: Deconvoluted spectrum of **84**.

MS2:

N S[G R[G K]G[G]K]G]L]G]K]G]G A]K]R]H]R]K V]L]R]D]N 25

26]I]Q]G]I T K P A I]R]R]L A]R]R]G G V K R]I S G]L I 50

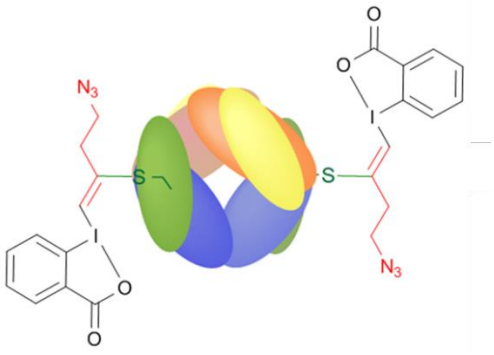
51 Y]E]E]T R]G V]L]K]V]F]L]C]N]V]I]R]D]A]V]T]Y T]E]H 75

76]A]K]R]K T]V]T]A]M]D]V]V]Y]A]L]K]R]Q]G]R]T]L]Y]G F 100

101 G G C

Obtained sequence coverage was 75% with p-score of 6e-16. Assigned *b*- and *y*-fragmentation ions (in blue) confirm the Cys63 modification by EBX molecule. Presence of key fragments b_{62}, y_{39}, b_{98}, y_{101} and y_{99} was validated manually.

h. Substrate scope on H4E63C octamer (**27**)



EBX-labeled H4E63C octamer (**28**)

In a 1.50 mL Eppendorf microcentrifuge tube, 40.0 μM H4E63C octamers (**27**) solution stock (60.0 μL, 2.40 nmol) were incubated with a 1.00 mM DTT solution in water (2.40 μL, 2.40 nmol, 1.00 equiv.) for 20 min at room temperature. Subsequently, a 10.0 mM N_3-EBX stock solution in DMSO (**1a**) (0.96 μL,

9.60 nmol, 4.00 equiv.) were added to the reaction mixture and incubated for 1 h at room temperature. The reaction was monitored by analytical RP-HPLC and product identity confirmed by ESI-MS analysis (retention time: 22.668 minutes, yield 99%). When the reaction was complete, excess N_3-EBX reagent was quenched with the addition of a 10.0 mM GSH aqueous solution (2.40 μL, 24.0 nmol, 10.0 equiv.). EBX-labeled octamers (**28**) were transferred to a Slide-A-Lyzer™ MINI dialysis device and dialyzed overnight against refolding buffer (2.00 M NaCl, 10.0 mM Tris-HCl, 1.00 mM EDTA, 1.00 mM DTT, pH 8.3 at 4°C). H4 EBX-labeled octamers (**28**) were stored at 4°C.

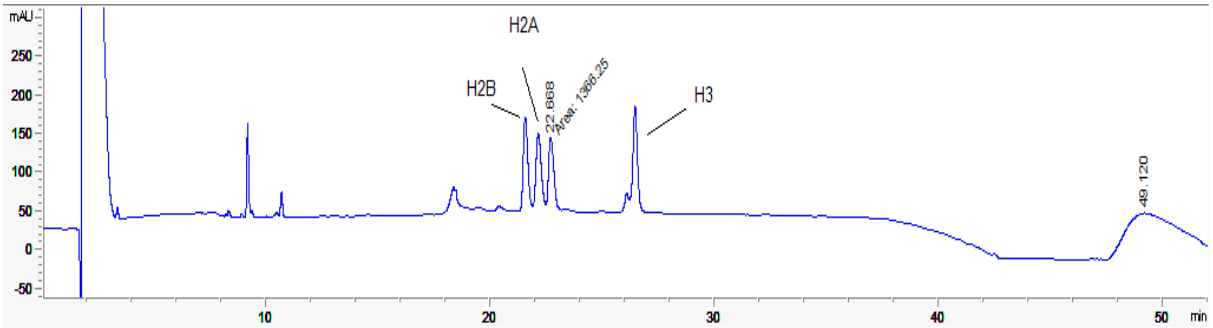


Figure S153: HPLC-UV chromatogram at 214 nm of **28**.

ESI-MS Calcd mass 11550 Da; Found 11549 Da

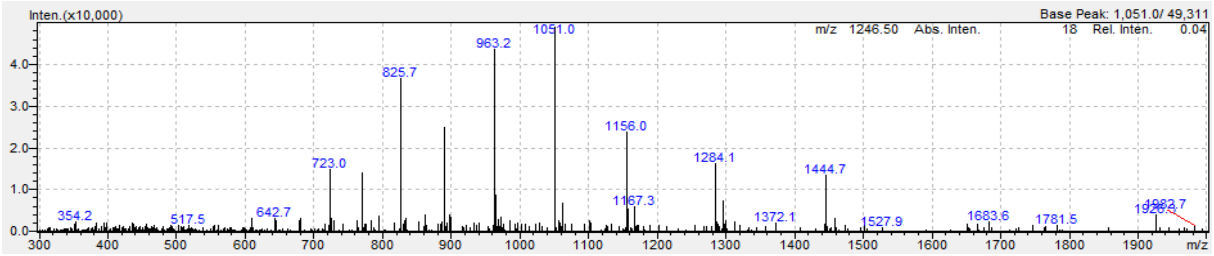
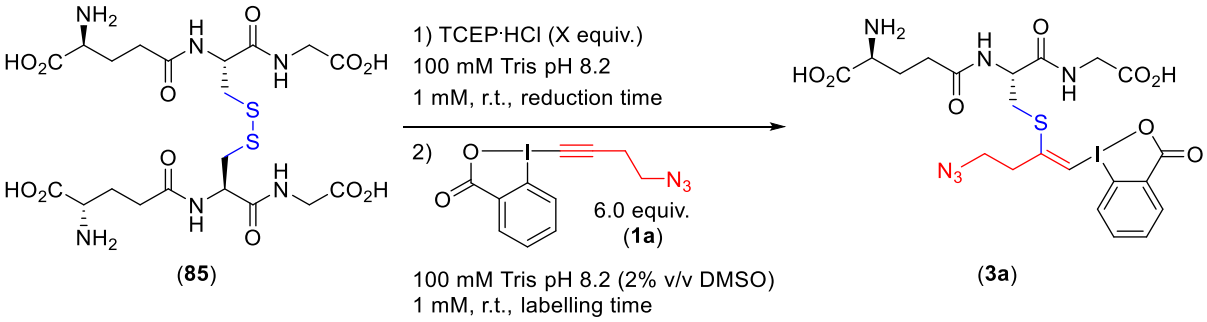


Figure S154: ESI-MS spectrum of **28.**

10. Reaction of disulfide bond-containing molecules

a. Application on oxidized glutathione (**85**)

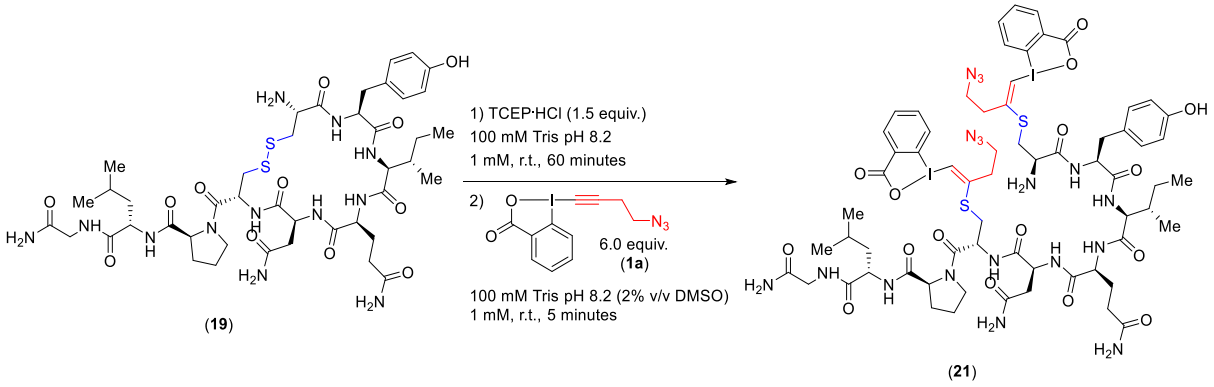


In a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 50.0 mM solution of oxidized glutathione (**85**) in 100 mM Tris buffer pH 8.2 (10.0 µL, 0.50 µmol) was diluted with Tris buffer (100 mM, pH 8.2, 470 µL). The resulting solution was vortexed few seconds and a solution of TCEP reagent in 100 mM Tris buffer pH 8.2 (10.0 µL, 0.75/2.50/5.00 µmol, respectively 1.50/5.00/10.0 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 10 or 60 minutes. Then, a 300 mM solution of N_3-EBX reagent (**1a**) in DMSO (10.0 µL, 3.00 µmol, 6.00 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes to afford **3a**. 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.

Table S13: Screened conditions for the labeling of oxidized glutathione (**85**) with **1a**.

Entry	Buffer	TCEP equiv.	Reduction time	Labeling Time	Yield
1	10 mM Tris	1.50 equiv.	24 hours	24 hours	< 5%
2	100 mM Tris	1.50 equiv.	60 minutes	5 minutes	92%
				24 hours	90%
3	100 mM Tris	5.00 equiv.	10 minutes	5 minutes	82%
				24 hours	80%
4	100 mM Tris	10.0 equiv.	10 minutes	5 minutes	60%
				24 hours	57%

b. Application on oxytocin (**19**)



In a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 10.0 mM solution of oxytocin (**19**) in 100 mM Tris buffer pH 8.2 (50 µL, 0.50 µmol) was diluted with Tris buffer (100 mM, pH 8.2, 365 µL). The resulting solution was vortexed few seconds and a 10.0 mM solution of TCEP reagent in 100 mM Tris buffer pH 8.2 (75 µL, 0.75 µmol, 1.50 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 60 minutes. Then, a 300 mM solution of N_3-EBX reagent (**1a**) in DMSO (10.0 µL, 3.00 µmol, 6.00 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 5 minutes to afford **21** in 90% yield (retention time: 11.672 minutes). No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product} + A_{side\ products})$, where $A_{starting}$, $A_{product}$ and $A_{side\ products}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material, product and side products.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{65}H_{85}I_2N_{18}O_{16}S_2^+$ 1691.3916; Found 1691.3932.

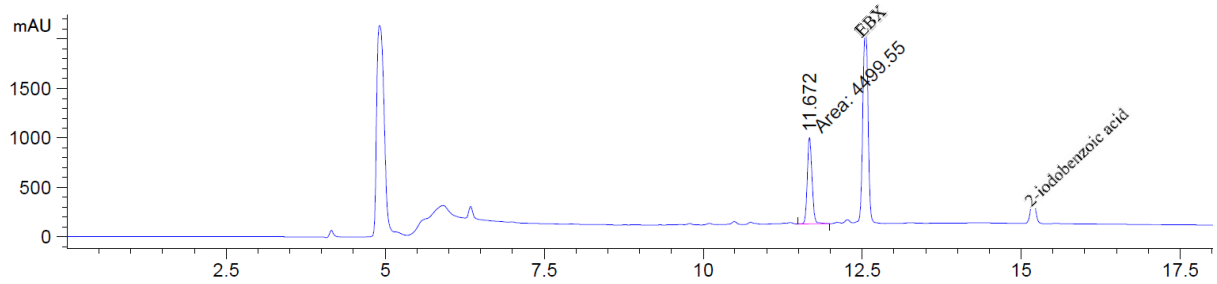
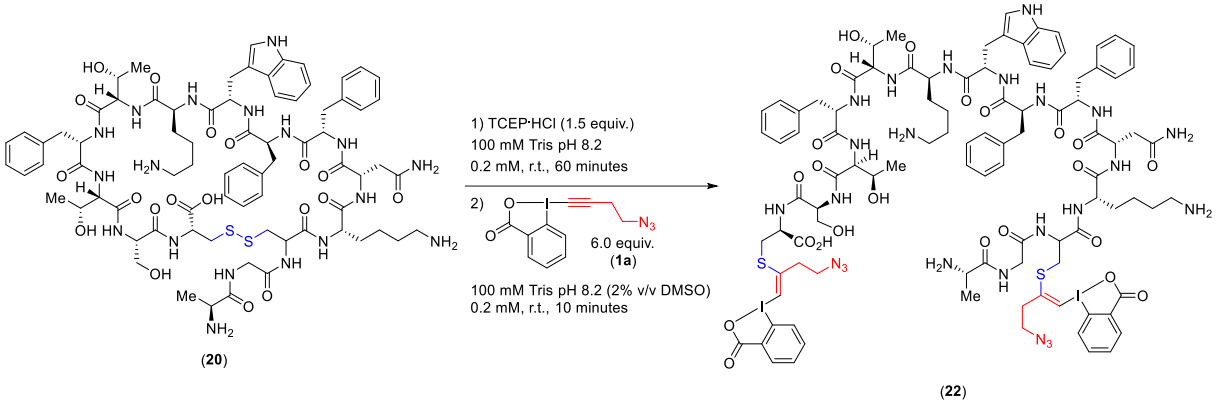


Figure S155: HPLC-UV chromatogram at 214 nm of **21**.

c. Application on somatostatin (**20**)



In a 0.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 10.0 mM solution of somatostatin (**20**) in 100 mM Tris buffer pH 8.2 (10.0 µL, 0.10 µmol) was diluted with Tris buffer (100 mM, pH 8.2, 465 µL). The resulting solution was vortexed few seconds and a 10.0 mM solution of TCEP reagent in 100 mM Tris buffer pH 8.2 (15.0 µL, 0.15 µmol, 1.50 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 60 minutes. Then, a 60.0 mM solution of N_3-EBX reagent (**1a**) in DMSO (10.0 µL, 0.60 µmol, 6.00 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 10 minutes to afford **22** in 70% yield (retention time: 19.346 minutes). No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product} + A_{side\ products})$, where $A_{starting}$, $A_{product}$ and $A_{side\ products}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material, product and side products.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{98}H_{123i2}N_{24}O_{23}S_2^+$ 2321.6718; Found 2321.6767.

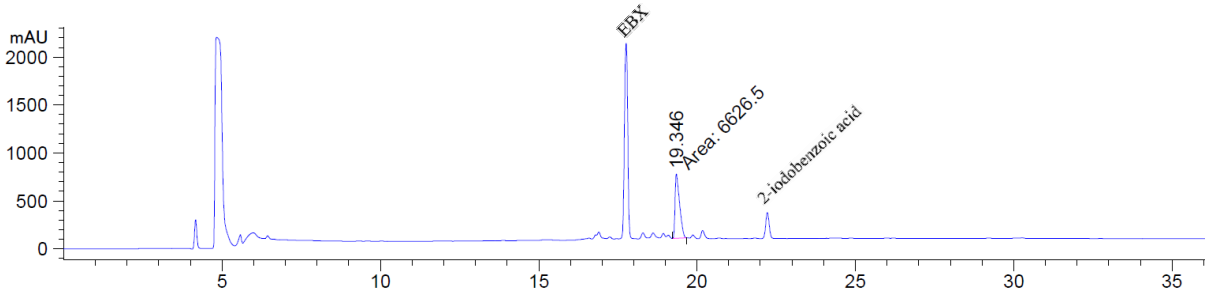
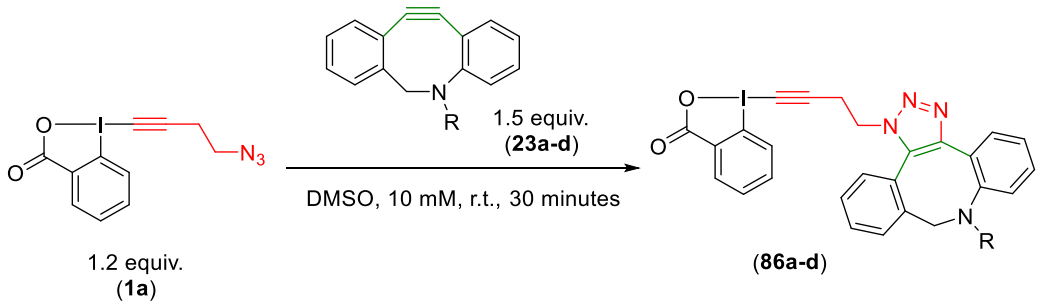


Figure S156: HPLC-UV chromatogram at 214 nm of **22**.

11. Click chemistry

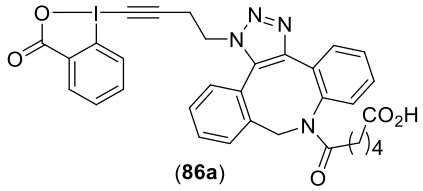
a. Strain-promoted azide-alkyne cycloaddition (SPAAC) on EBX reagents



General procedure G:

In a 0.2 mL Eppendorf PCR tube, a 24.0 mM solution of N_3-EBX reagent (**1a**) in DMSO (1.00 µL, 24.0 nmol, 1.20 equiv.) and a 30.0 mM solution of dibenzocyclooctyne reagent (**23a-d**) in DMSO (1.00 µL, 30.0 nmol, 1.50 equiv.) were combined. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 30 minutes. No effort was made to exclude oxygen. The reaction was diluted with Tris buffer (10 mM, pH 8.2, 98.0 µL) and analyzed by HPLC-MS. The desired products **86a-d** were delivered as regioisomers mixture (both peaks were confirmed to be regioisomers by LRMS).

Using DBCO-Acid (**23a**):



Following general procedure G, dibenzocyclooctyne-acid (**23a**) afforded the title compound **86a** (retention time: 13.611 & 13.994 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{32}H_{28}IN_4O_5^+$ 675.1099; Found 675.1100.

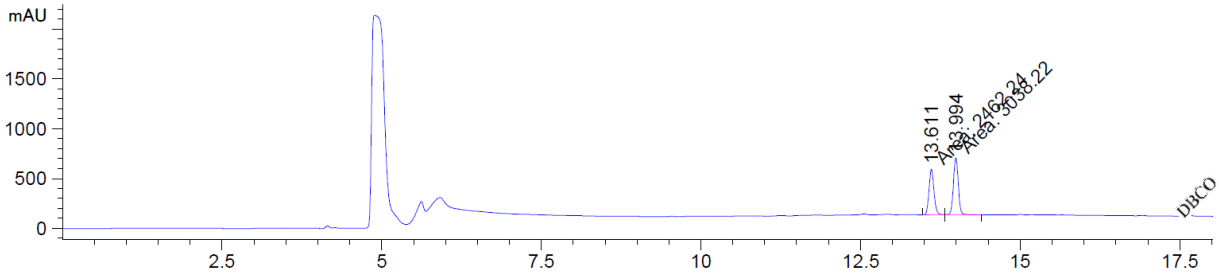
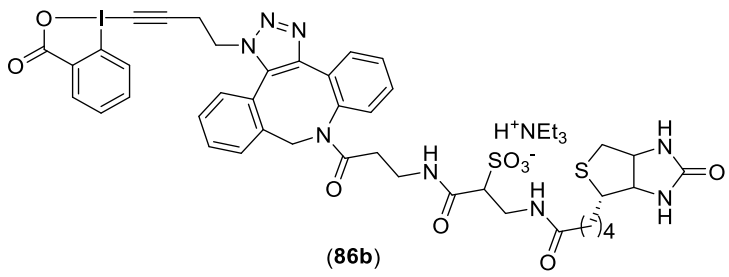


Figure S157: HPLC-UV chromatogram at 214 nm **86a**.

Using Sulfo-DBCO-Biotin (**23b**):



(**86b**)

Following general procedure G, sulfo-dibenzocyclooctyne-biotin conjugate (**23b**) afforded the title compound **86b** (retention time: 11.171 & 11.329 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{42}H_{44}IN_8O_9S_2^+$ 995.1712; Found 995.1711.

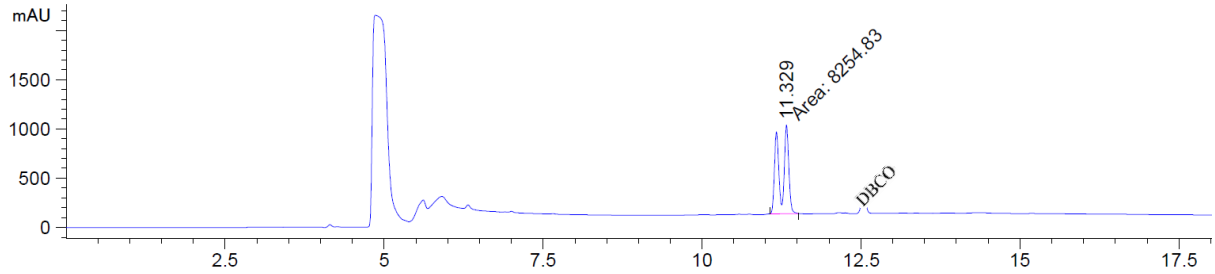
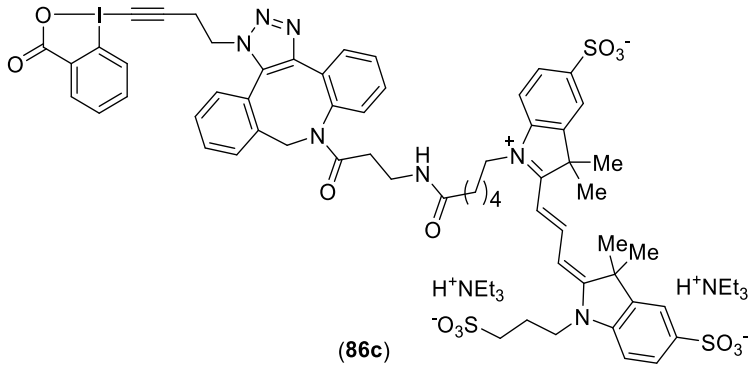


Figure S158: HPLC-UV chromatogram at 214 nm of **86b**.

Using DBCO-Cy3 (**23c**):



(**86c**)

Following general procedure G, dibenzocyclooctyne-Cy3 (**23c**) afforded the title compound **86c** (retention time: 10.252 & 10.423 minutes).

HRMS (ESI/QTOF) m/z: $[M]^+$ Calcd for $C_{61}H_{63i}N_7O_{13}S_3^+$ 1324.2685; Found 1324.2678.

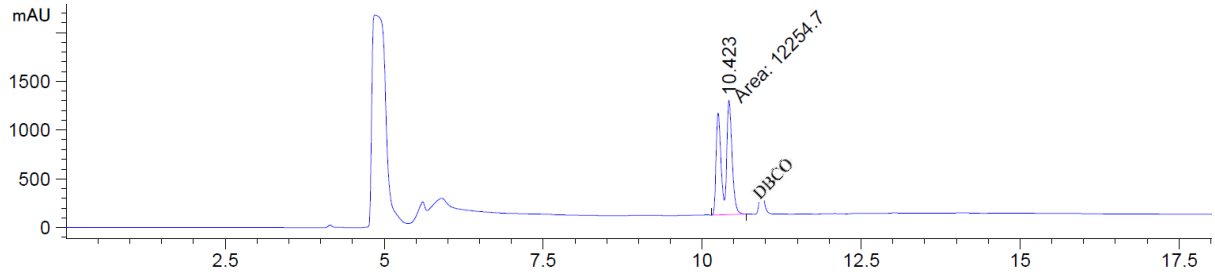
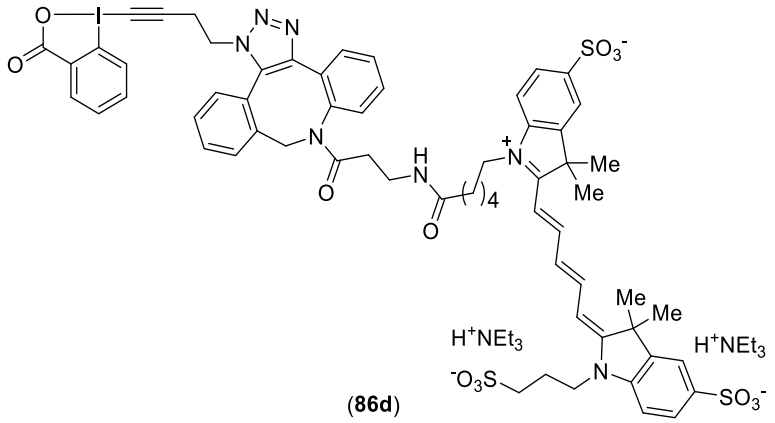


Figure S159: HPLC-UV chromatogram at 214 nm of **86c**.

Using DBCO-Cy5 (**23d**):



(**86d**)

Following general procedure G, dibenzocyclooctyne-Cy5 (**23d**) afforded the title compound **86d** (retention time: 10.385 & 10.527 minutes).

HRMS (ESI/QTOF) m/z: $[M]^+$ Calcd for $C_{63}H_{65}IN_7O_{13}S_3^+$ 1350.2842; Found 1350.2844.

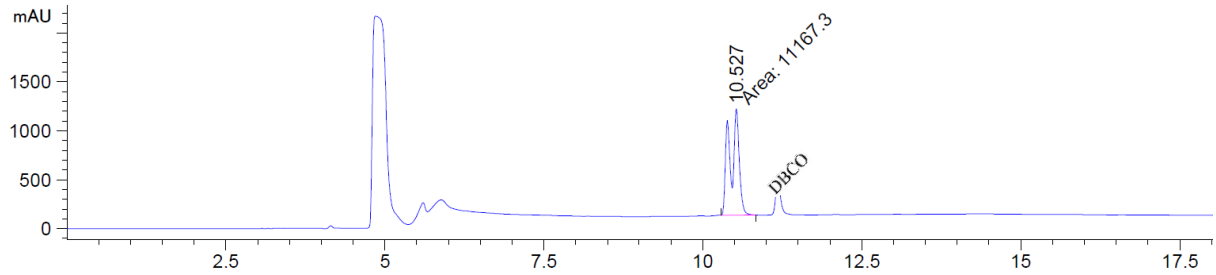
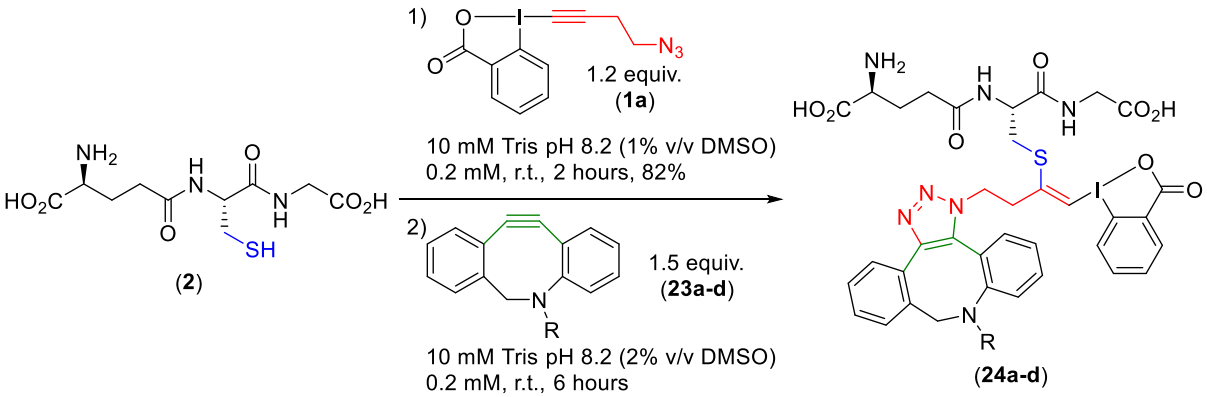


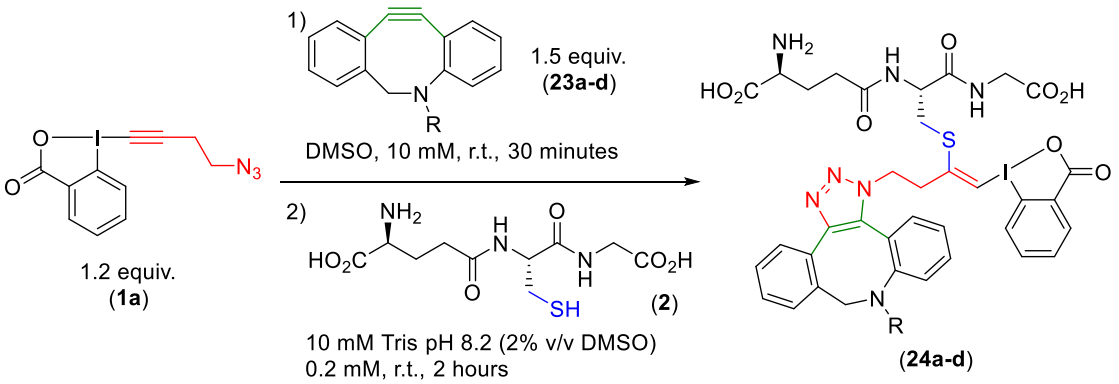
Figure S160: HPLC-UV chromatogram at 214 nm **86d**.

b. Labeling/SPAAC one-pot on GSH



General procedure H for post-labeling strain-promoted azide-alkyne cycloaddition:

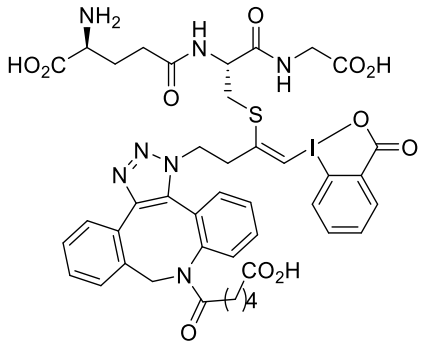
In a 0.2 mL Eppendorf PCR tube, a 10.0 mM solution of glutathione (**2**) in 10 mM Tris buffer pH 8.2 (2.00 µL, 20.0 nmol) was diluted with Tris buffer (10 mM, pH 8.2, 96.0 µL). The resulting solution was vortexed few seconds and a 24.0 mM solution of N_3-EBX reagent (**1a**) in DMSO (1.00 µL, 24.0 nmol, 1.20 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 2 hours. Then, a 30.0 mM solution of dibenzocyclooctyne reagent (**23a-d**) in DMSO (1.00 µL, 30.0 nmol, 1.50 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 6 hours. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS. The desired products **24a-d** were delivered as regioisomers mixture (both peaks were confirmed to be regioisomers by LRMS).



General procedure I for pre-labeling strain-promoted azide-alkyne cycloaddition:

In a 0.2 mL Eppendorf PCR tube, a 24.0 mM solution of N_3-EBX reagent (**1a**) in DMSO (1.00 µL, 24.0 nmol, 1.20 equiv.) and a 30.0 mM solution of dibenzocyclooctyne reagent (**23a-d**) in DMSO (1.00 µL, 30.0 nmol, 1.50 equiv.) were combined. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 30 minutes. Then, the reaction mixture was diluted with Tris buffer (10 mM, pH 8.2, 96.0 µL) and a 10.0 mM solution of glutathione (**2**) in 10 mM Tris buffer pH 8.2 (2.00 µL, 20.0 nmol) was added. The resulting solution was vortexed few seconds to ensure proper reagent mixing and left on the bench at room temperature for 2 hours. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS. The desired products **24a-d** were delivered as regioisomers mixture (both peaks were confirmed to be regioisomers by LRMS).

Using DBCO-Acid (**23a**):



(**24a**)

Following the post-labeling general procedure H, dibenzocyclooctyne-acid (**23a**) afforded the title compound **24a** (retention time: 11.673 & 11.752 minutes).

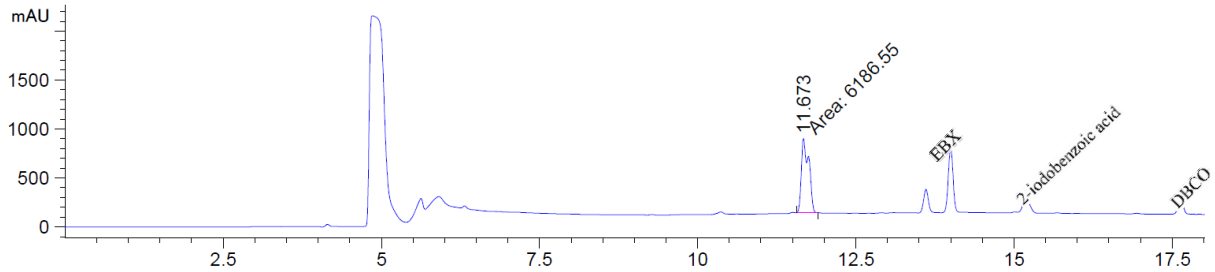


Figure S161: HPLC-UV chromatogram at 214 nm of **24a**.

Following the pre-labeling general procedure I, dibenzocyclooctyne-acid (**23a**) afforded the title compound **24a** (retention time: 11.680 & 11.723 minutes).

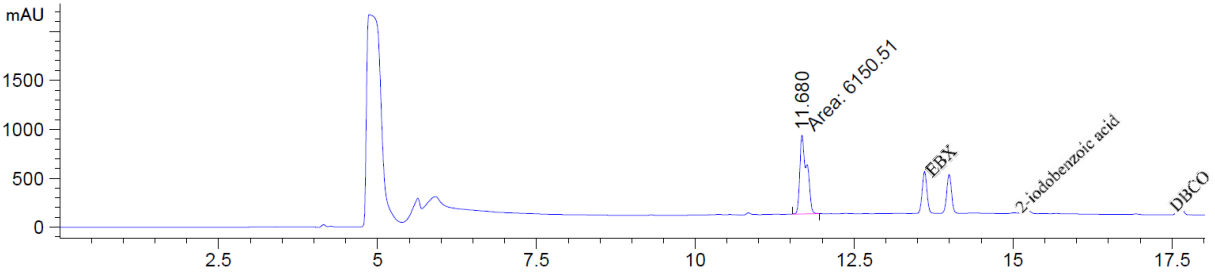
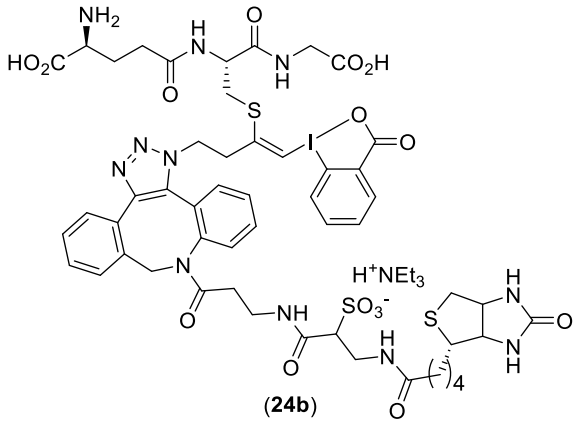


Figure S162: HPLC-UV chromatogram at 214 nm of **24a**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{42}H_{45}IN_7O_{11}S^+$ 982.1937; Found 982.1928.

Using Sulfo-DBCO-Biotin (**23b**):



(**24b**)

Following the post-labeling general procedure H, sulfo-dibenzocyclooctyne-biotin conjugate (**23b**) afforded the title compound **24b** (retention time: 10.275 & 10.574 minutes).

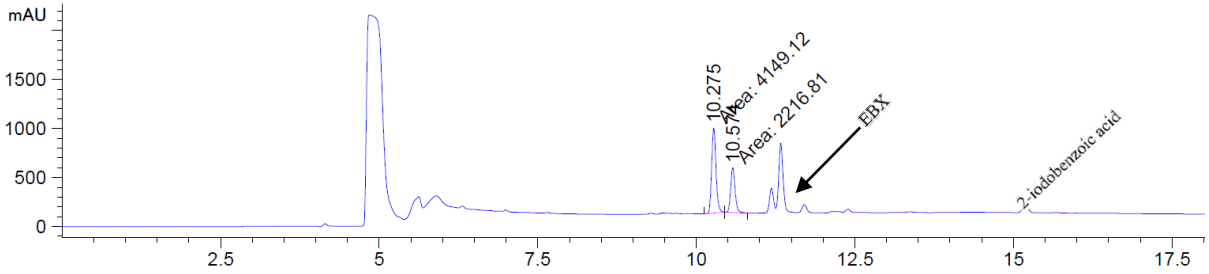


Figure S163: HPLC-UV chromatogram at 214 nm of **24b**.

Following the pre-labeling general procedure I, sulfo-dibenzocyclooctyne-biotin conjugate (**23b**) afforded the title compound **24b** (retention time: 10.281 & 10.567 minutes).

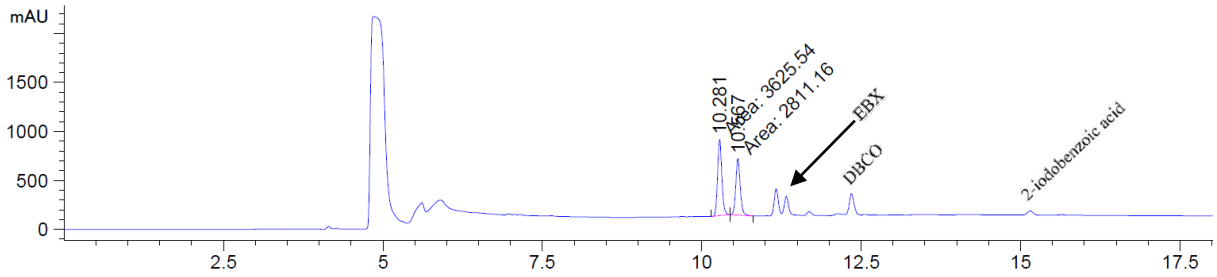
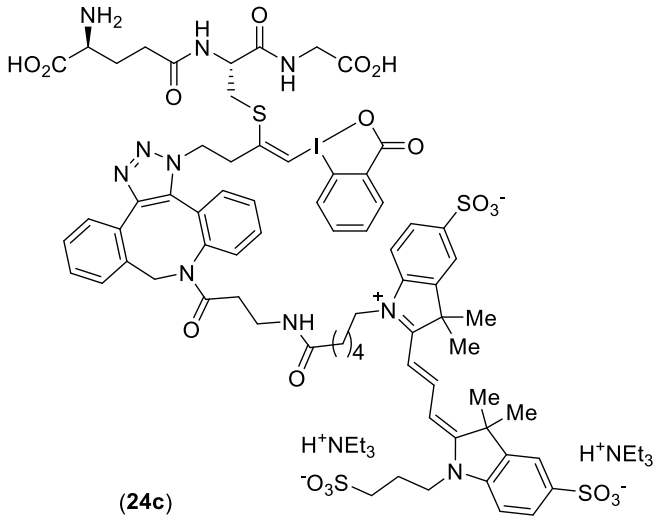


Figure S164: HPLC-UV chromatogram at 214 nm of **24b**.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{52}H_{61}IN_{11}O_{15}S_3^+$ 1302.2550; Found 1302.2541.

Using DBCO-Cy3 (**23c**):



(**24c**)

Following the post-labeling general procedure H, dibenzocyclooctyne-Cy3 (**23c**) afforded the title compound **24c** (retention time: 9.730 & 9.893 minutes).

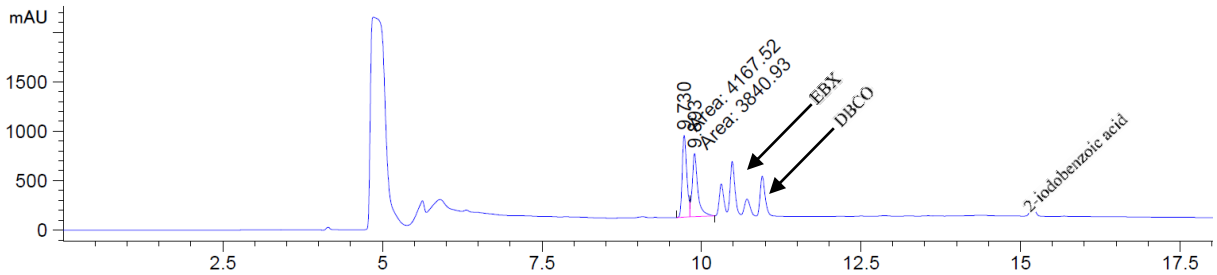


Figure S165: HPLC-UV chromatogram at 214 nm of **24c**.

Following the pre-labeling general procedure I, dibenzocyclooctyne-Cy3 (**23c**) afforded the title compound **24c** (retention time: 9.705 & 9.852 minutes).

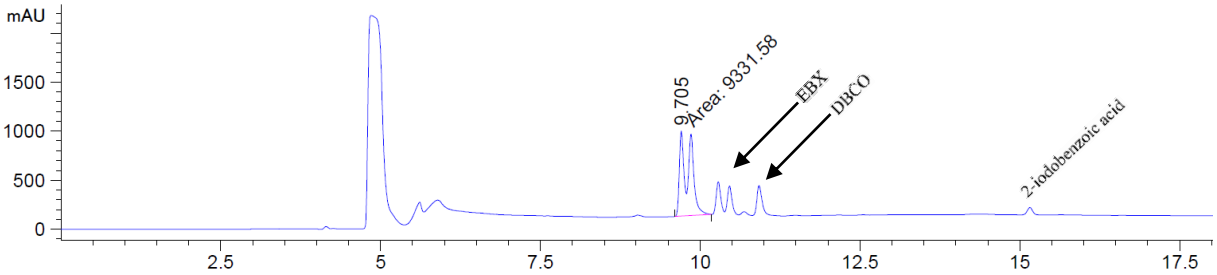
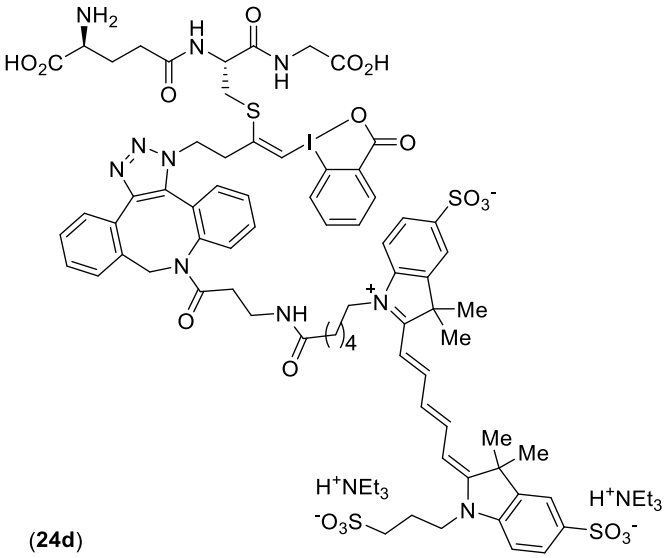


Figure S166: HPLC-UV chromatogram at 214 nm of **24c**.

HRMS (ESI/QTOF) m/z: $[M + H]^{+2}$ Calcd for $C_{71}H_{81}IN_{10}O_{19}S_4^{+2}$ 816.1798; Found 816.1814.

Using DBCO-Cy5 (**23d**):



(**24d**)

Following the post-labeling general procedure H, dibenzocyclooctyne-Cy5 (**23d**) afforded the title compound **24d** (retention time: 9.871 & 9.998 minutes).

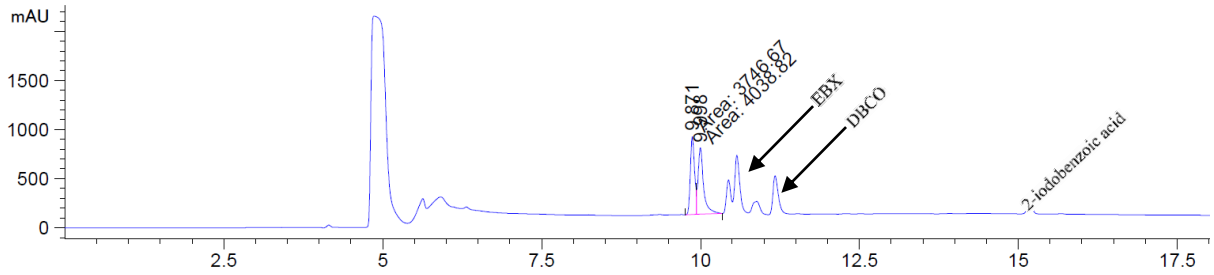


Figure S167: HPLC-UV chromatogram at 214 nm of **24d**.

Following the pre-labeling general procedure I, dibenzocyclooctyne-Cy5 (**23d**) afforded the title compound **24d** (retention time: 9.858 & 9.985 minutes).

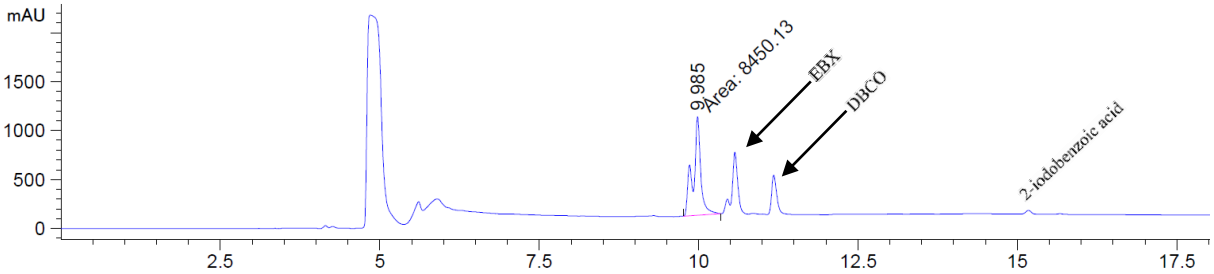
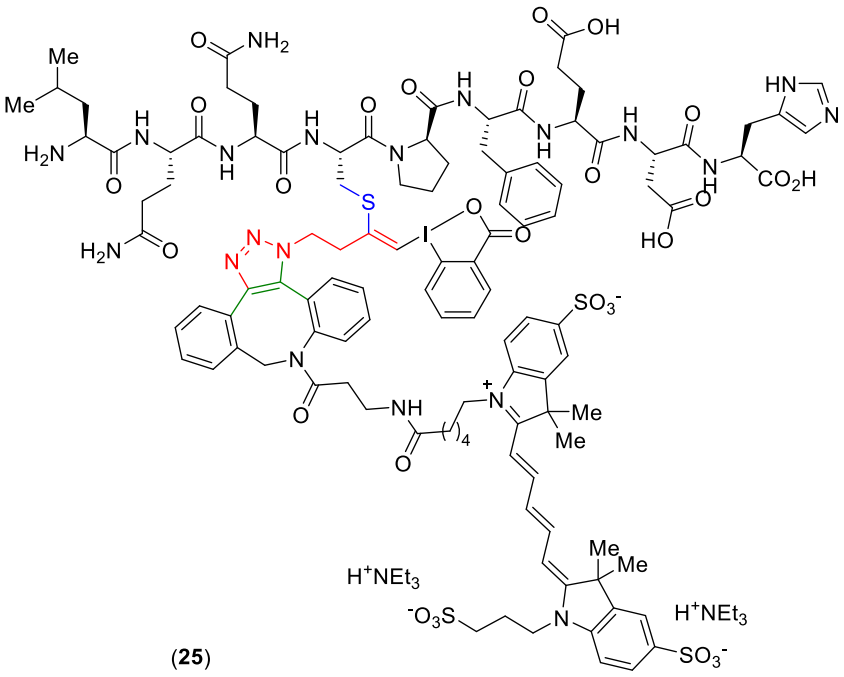


Figure S168: HPLC-UV chromatogram at 214 nm of **24d**.

HRMS (ESI/QTOF) m/z: $[M + H]^{+2}$ Calcd for $C_{73}H_{83}IN_{10}O_{19}S_4^{+2}$ 829.1876; Found 829.1899.

c. Post-labeling SPAAC on nonamer HSA Leu$_{55}$-His$_{63}$ (**15**)



(**25**)

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, a 20.0 mM solution of nonamer **15** in water (66.0 µL, 1.32 µmol) was diluted with Tris buffer (10.0 mM, pH 8.2, 328 µL). The resulting solution was vortexed few seconds and a 200 mM solution of N_3-EBX reagent (**1a**) in DMSO (6.00 µL, 1.20 µmol, 0.91 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **16**. Subsequently, a 20.0 mM solution of dibenzocyclooctyne-Cy5 (**23d**) in DMSO (60.0 µL, 1.20 µmol, 1.00 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **25** (retention time: 18.285 & 18.447 minutes, 99% yield). The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. No effort was made to exclude oxygen.

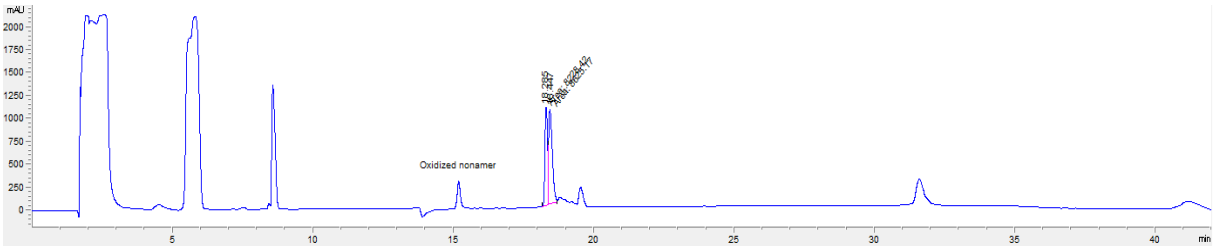


Figure S169: HPLC-UV chromatogram at 214 nm of **25**.

ESI-MS Calcd mass 2464.5 Da; Found 2466.7 Da.

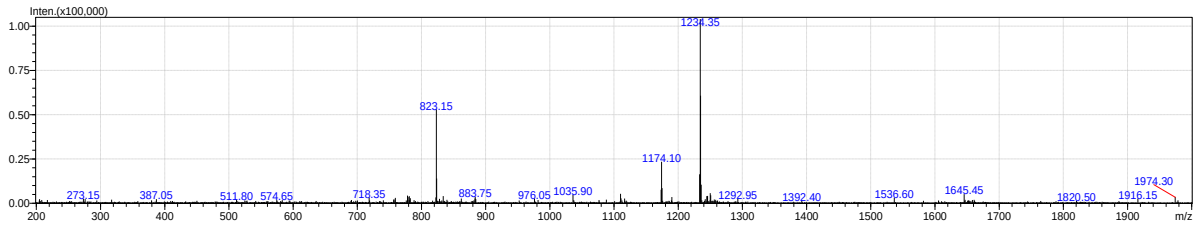
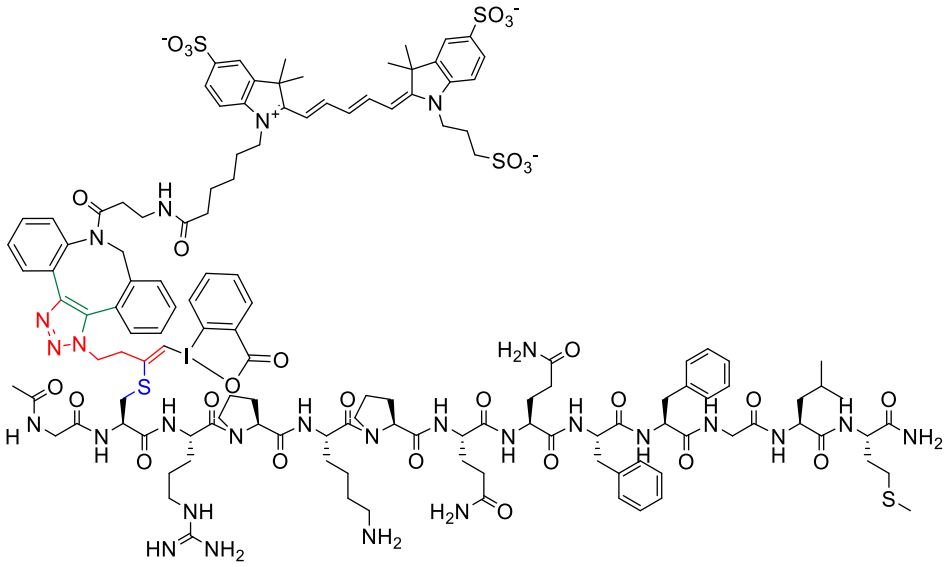


Figure S170: ESI-MS spectrum of **25**.

d. Post-labeling SPAAC on Substance P analogue (**35**)



(**36**)

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, Substance P (**35**) (1.00 mg, 645 nmol, 1.00 equiv.) was dissolved in 1286 µL phosphate buffer (50.0 mM, pH 8.2) containing 10% (v/v) DMSO. The resulting solution was vortexed few seconds and a 200 mM solution of N_3-EBX reagent (**1a**) in DMSO (4.20 µL, 838.5 nmol, 1.30 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes. Subsequently, a 20.0 mM solution of DBCO-Cy5 (**23d**) in DMSO (48.3 µL, 967.5 nmol, 1.50 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 15 min to afford **36** (retention time: 21.368 minutes, yield 82%). The reaction mixture was analysed by analytical RP-HPLC and products identity characterised by ESI-MS. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product} + A_{oxidation})$, where $A_{starting}$, $A_{product}$ and $A_{oxidation}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material, product and oxidized starting material. The reaction mixture was diluted to a total volume of 3.50 mL with 6M GdmHCl, pH 3 and purified by semipreparative RP-HPLC employing a linear gradient from 30 to 60% solvent B over 30 min. Fractions were analysed, pooled accordingly and lyophilised (yield: 43 %).

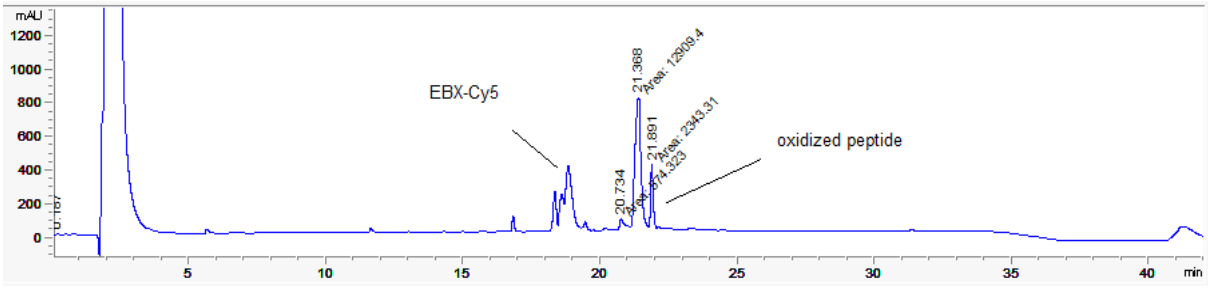


Figure S171: HPLC-UV chromatogram at 214 nm of **36**.

ESI-MS Calcd mass 2898.2 Da; Found 2899.7 Da.

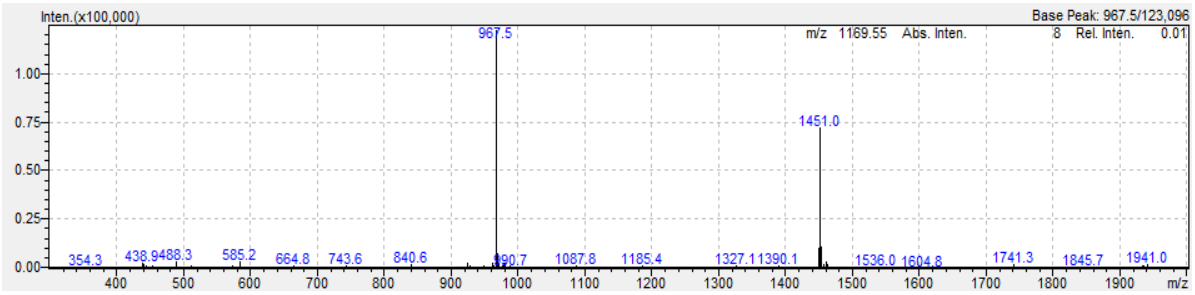
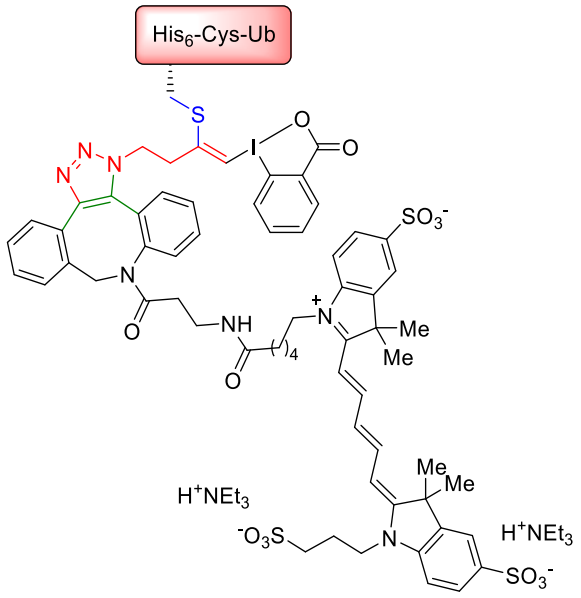


Figure S172: ESI-MS spectrum of **36**.

e. Pre-labeling SPAAC on native cys-ubiquitin (**17**)



(**26**)

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, 200 mM solution of N_3-EBX reagent (**1a**) in DMSO (0.50 µL, 100 nmol, 2.00 equiv.) was diluted with Tris buffer (10.0 mM, pH 8.2, 43.3 µL). The resulting solution was vortexed few seconds and a 20.0 mM solution of dibenzocyclooctyne-Cy5 (**23d**) in DMSO (6.25 µL, 125 nmol, 2.50 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes. Subsequently, a 1.00 mM solution of cys-ubiquitin (**17**) in water (50.0 µL, 50.0 nmol) was added to the reaction mixture. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **26** (retention time: 21.984 & 22.130 minutes, yield 95%). The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product})$, where $A_{starting}$, $A_{product}$ and respectively represent the area of absorption peak at 214 nm of the remaining starting material and product. No effort was made to exclude oxygen.

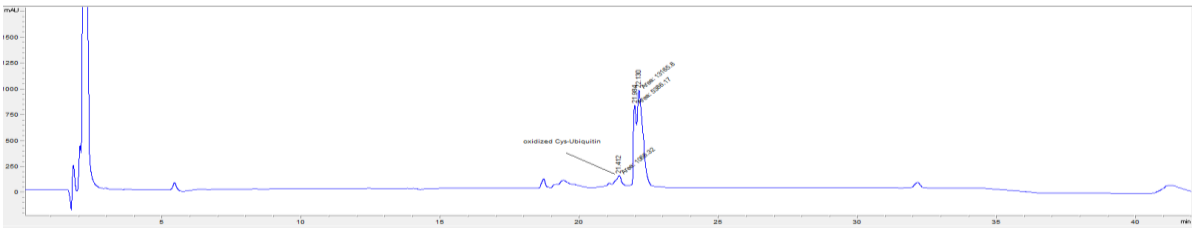


Figure S173: HPLC-UV chromatogram at 214 nm of **26**.

ESI-MS Calcd mass 12048.5 Da; Found 12049.4 Da.

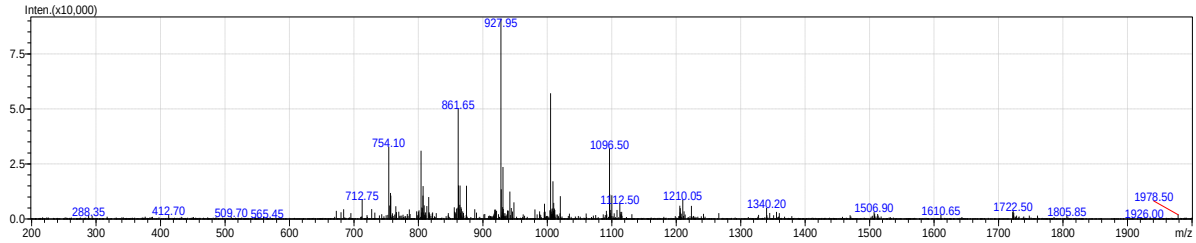
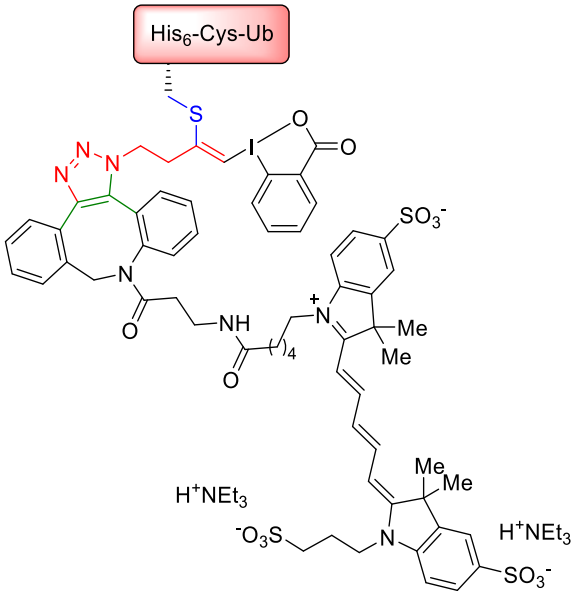


Figure S174: ESI-MS spectrum of **26**.

f. Post-labeling SPAAC on native cys-ubiquitin (**17**)



(**26**)

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, a 1.00 mM solution of cys-ubiquitin (**17**) in water (50.0 μL, 50.0 nmol) was diluted with Tris buffer (10.0 mM, pH 8.2, 40.0 μL). The resulting solution was vortexed few seconds and a 10.0 mM solution of N_3-EBX reagent (**1a**) in Tris buffer (10.0 mM, pH 8.2) containing 5% (v/v) DMSO (10.0 μL, 100 nmol, 2.00 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **18**. Subsequently, a 20.0 mM solution of dibenzocyclooctyne-Cy5 (**23d**) in DMSO (2.50 μL, 50.0 nmol, 1.00 equiv.) was added in one portion. The resulting mixture was vortexed few

seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **26** (retention time: 21.959 & 22.109 minutes, yield 99%). No effort was made to exclude oxygen. The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. Labeling was further confirmed by SDS-PAGE (17% polyacrylamide gel) and fluorescence microscopy analysis.

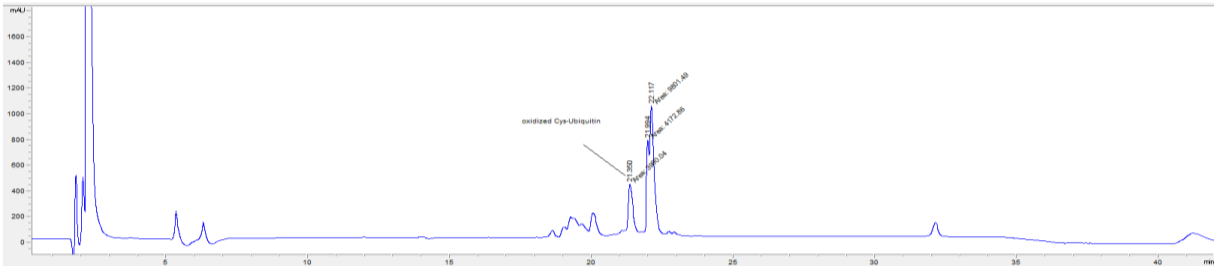


Figure S175: HPLC-UV chromatogram at 214 nm of **26**.

ESI-MS Calcd mass 12048.5 Da; Found 12051.0 Da.

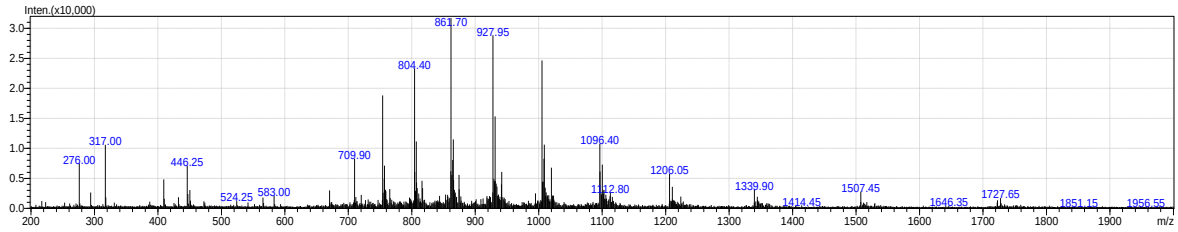
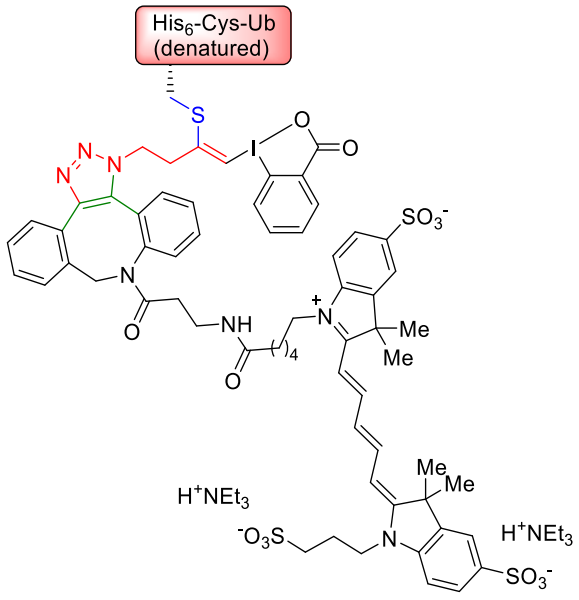


Figure S176: ESI-MS spectrum of **26**.

g. Post-labeling SPAAC on denatured cys-ubiquitin (**17´**)



(**26´**)

In a 1.5 mL Eppendorf Safe-Lock microcentrifuge tube, a 1.00 mM solution of cys-ubiquitin (**17**) in water (50.0 μL, 50.0 nmol) was diluted with denaturing phosphate buffer (6.00 M GdmHCl, 200 mM phosphate, pH 8.2, 44.5 μL). The resulting solution was vortexed few seconds and a 10.0 mM solution of N_3-EBX

reagent (**1a**) in denaturing phosphate buffer (6.00 M GdmHCl, 200 mM phosphate, pH 8.2) containing 5% (v/v) DMSO (5.50 μL, 55.0 nmol, 1.10 equiv.) was added. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **18´**. Subsequently, a 20.0 mM solution of dibenzocyclooctyne-Cy5 (**23d**) in DMSO (2.50 μL, 50.0 nmol, 1.00 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **26´** (retention time: 21.994 & 22.117 minutes, yield 77%). The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product} + A_{oxidation})$, where $A_{starting}$, $A_{product}$ and $A_{oxidation}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material, product and oxidized starting material. No effort was made to exclude oxygen.

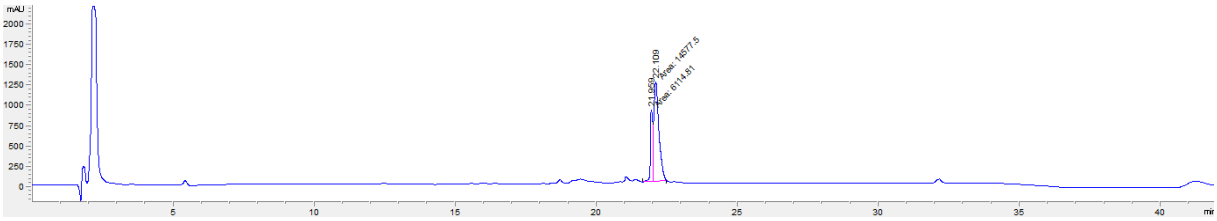


Figure S177: HPLC-UV chromatogram at 214 nm of **26'**.

ESI-MS Calcd mass 12048.5 Da; Found 12049.4 Da.

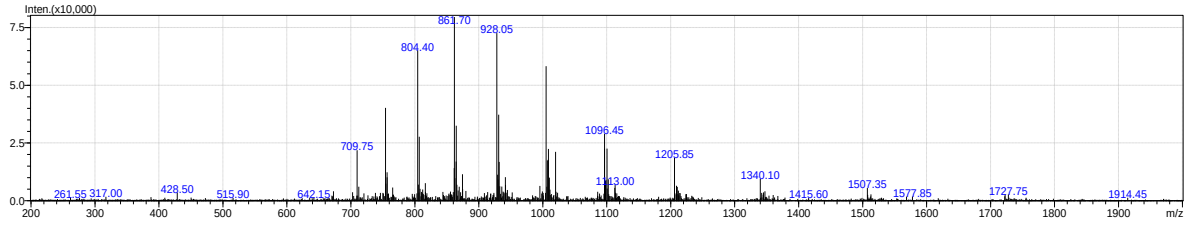


Figure S178: ESI-MS spectra of **26'**.

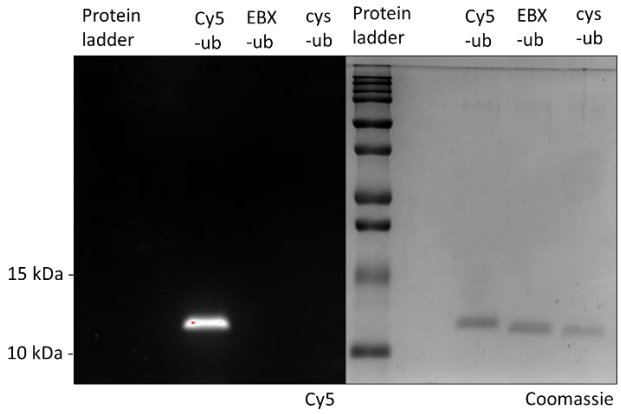
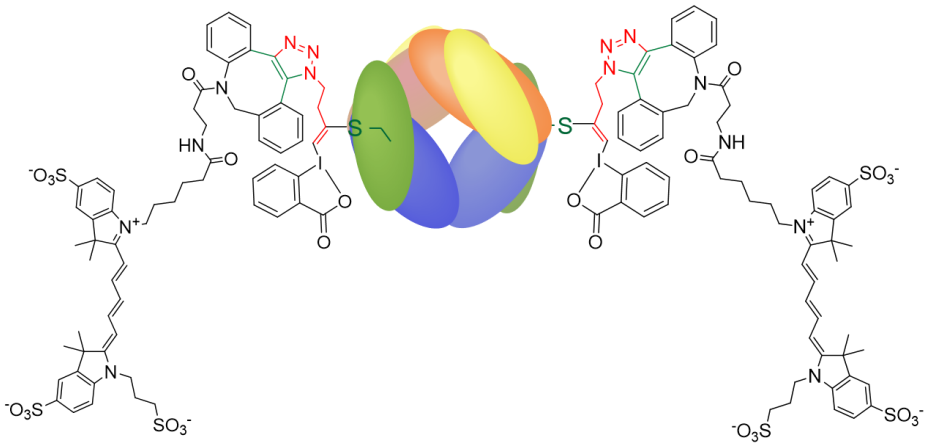


Figure S179: SDS-PAGE of **17**, **18** and **26**.

h. Post-labeling SPAAC on H4E63C octamers (**27**) and nucleosomes reconstitution



(**29**)

In a 1.50 mL Eppendorf microcentrifuge tube, 40 μM H4E63C octamers (**27**) solution stock (20.0 μL, 0.80 nmol) in refolding buffer (2.00. M NaCl, 10.0 mM Tris-HCl, 1.00 mM EDTA, pH 8.3 at 4°C) were incubated with a 1.00 mM DTT solution in water (0.80 μL, 0.80 nmol, 1.00 equiv.) for 20 min at room temperature. Subsequently, a 5.00 mM N_3-EBX stock solution in DMSO (**1a**) (0.48 μL, 3.20 nmol, 4.00 equiv.) was added to the reaction mixture and incubated for 1 h at room temperature. The reaction was monitored by analytical RP-HPLC and product identity confirmed by ESI-MS analysis. When the reaction was complete, excess N_3-EBX reagent was quenched with the addition of a 10.0 mM GSH aqueous solution (0.80 μL, 8.00 nmol, 10.0 equiv.). Subsequently, to 10.0 μL of the crude H4-EBX octamers, a 10.0 mM DBCO-Cy5 (**23d**) stock solution in DMSO (0.64 μL, 6.40 nmol, 8.00 equiv.) was added and the reaction mixture and incubated for 15 min at room temperature. The reaction was monitored by analytical RP-HPLC and product identity (**29**) confirmed by ESI-MS analysis (retention time: 22.708 minutes, yield 95%). Specific cysteine labeling was confirmed by SDS-PAGE (17% polyacrylamide gel) and fluorescence microscopy analysis.

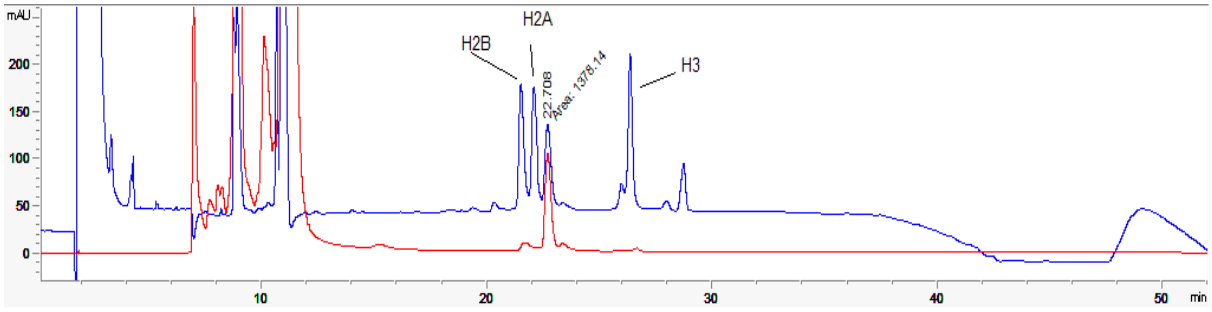


Figure S180: HPLC-UV chromatogram at 214 nm (blue) and 647nm (red) of **29**.

ESI-MS Calcd mass 12557 Da; Found 12559 Da.

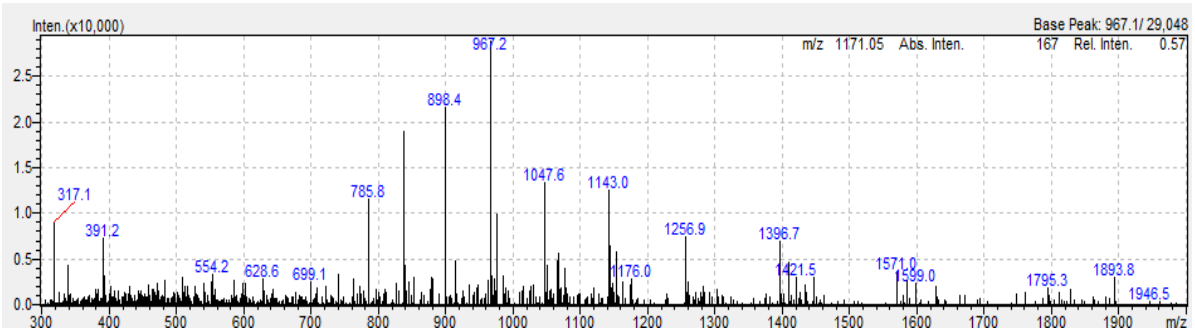


Figure S181: ESI-MS spectrum of **29**.

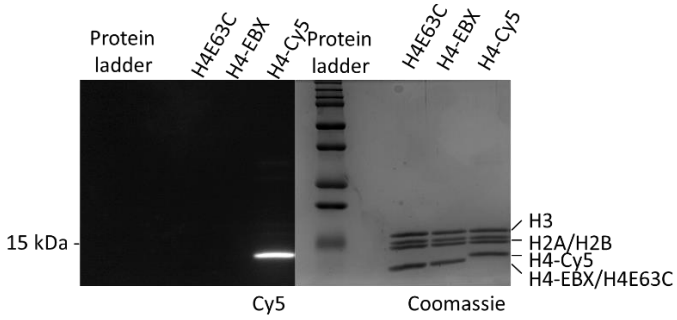


Figure S182: SDS-PAGE of **27**, **28** and **29**.

Nucleosomes were reconstituted as described before.[1] Typically, a 170 base pair 601 nucleosome positioning DNA sequence[21] (10.0 pmol, 1.00 eq.) was mixed with the refolded octamers (unmodified octamers (**43**) 1.50 eq.; EBX-H4 octamers (**28**) 2.20 eq.; Cy5-H4 octamers (**29**) 2.20 eq.) at high salt concentration and reconstitution was achieved by gradient dialysis into low salt conditions (10.0 mM KCl, 10 mM Tris-HCl, 0.10 mM EDTA, pH 7.5). Dialysis was performed in Slide-A-Lyzer™ MINI dialysis devices using a peristaltic pump at a flow rate of 1ml/min over 18h at 4°C. The reconstitution of **28'**, **29'** and unmodified nucleosomes (**43´**) was confirmed by native gel electrophoresis and fluorescent microscopy analysis.

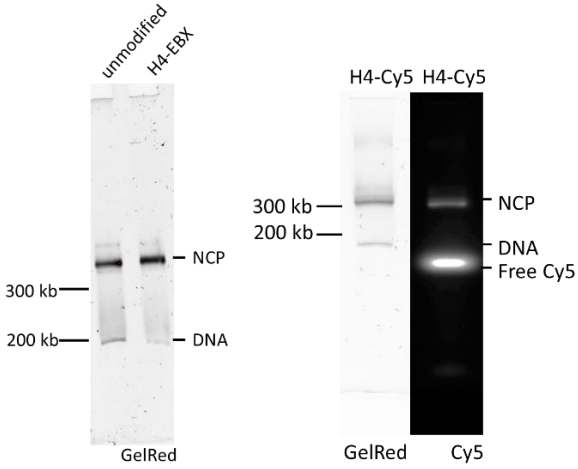
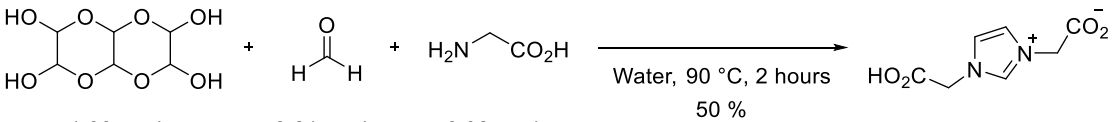


Figure S183: Native PAGE gel of **43, 28**, and Cy5-labeled **29**.

12. Synthesis of ligands and preparation of palladium complexes

a. Synthesis of ligands

2-(1-(Carboxymethyl)-1H-imidazol-3-ium-3-yl)acetate (**90**)



1.00 equiv. (**87**) + 3.21 equiv. (**88**) + 6.02 equiv. (**89**) → (**90**)

Following a reported procedure,[22] glyoxal trimer dehydrate (**87**) (0.350 g, 1.66 mmol, 1.00 equiv.), paraformaldehyde (**88**) (0.160 g, 5.33 mmol, 3.21 equiv.) and glycine (**89**) (0.750 g, 9.99 mmol, 6.02 equiv.) were added to a round-bottom flask containing water (7.0 mL, 0.238 M). The resulting mixture was heated at 90 °C for 2 hours. The solvent was then removed under reduced pressure, and the crude was washed with a small amount of water. Recrystallization of the solid residue from methanol (15 mL) yielded product **90** as a brown solid (0.153 g, 0.831 mmol, 50%).

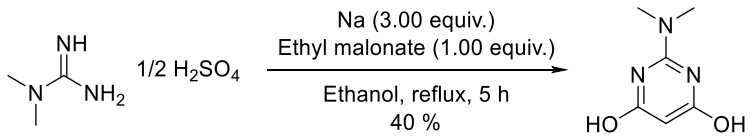
^{1}H NMR (400 MHz, D_2O) δ 8.87 (s, 1H, (-N)$_2$C*H*), 7.54 (d, *J* = 1.7 Hz, 2H, alkene-*H*), 5.02 (s, 4H, -CH_2).

^{13}C NMR (101 MHz, D_2O) δ 171.1, 137.6, 123.3, 51.1.

HRMS (ESI/QTOF) m/z: $[M - H]^-$ Calcd for $C_7H_7N_2O_4^-$ 183.0411; Found 183.0406.

Spectroscopic data was consistent with that previously reported in the literature.[22]

2-(Dimethylamino)pyrimidine-4,6-diol (**92**)



(**91**) → (**92**)

Following a reported procedure,[23] to a solution of sodium (Na, 93.0 mg, 4.04 mmol, 1.10 equiv.) in ethanol (1.9 mL, 2.12 M), dimethyl guanidine sulfate (**91**) (1.00 g, 3.67 mmol, 1.00 equiv.) was added. The resulting solution was added to another solution of sodium (Na, 0.160 g, 6.98 mmol, 1.90 equiv.) and diethyl malonate (0.560 mL, 3.67 mmol, 1.00 equiv.) in ethanol (1.96 mL, 1.87 M). The combined solution was refluxed for 5 hours. The reaction was then evaporated to dryness, dissolved in water (5 mL) and taken to pH 6 with acetic acid. Collection of the white solid formed by filtration under vacuum afforded product **92** (0.225 g, 1.45 mmol, 40%).

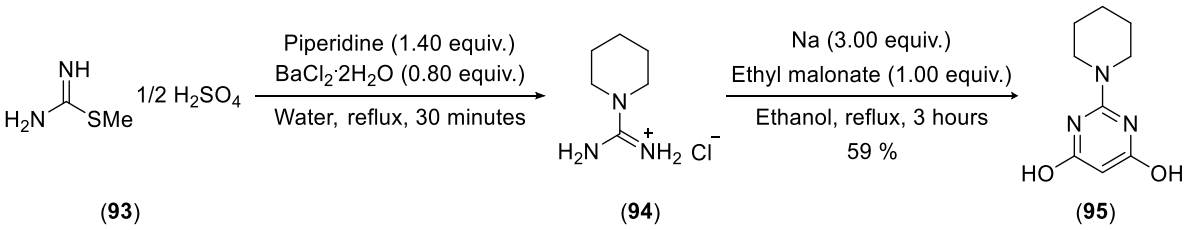
^{1}H NMR (400 MHz, $CDCl_3$) δ 10.50 (s, 2H, O*H*), 4.65 (s, 1H, Ar*H*), 3.00 (s, 6H,-N(CH_3)$_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 168.0, 155.0, 78.3, 37.1.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_6H_{10}N_3O_2^+$ 156.0768; Found 156.0766.

Spectroscopic data was consistent with that previously reported in the literature.[23]

2-(Piperidin-1-yl)pyrimidine-4,6-diol (**95**)



(**93**) (**94**) (**95**)

Following a reported procedure,[20] S-methyl*iso*thiourea sulfate (**93**) (2.00 g, 10.6 mmol, 1.00 equiv.), water (5.26 mL, 2.02 M) and piperidine (1.47 mL, 14.9 mmol, 1.40 equiv.) were added to a flask fitted with a reflux condenser. The mixture was slowly heated to reflux and the reflux was maintained for 5 minutes. A 2.36 M hot solution of barium chloride in water ($BaCl_2$, 3.60 mL, 8.50 mmol, 0.80 equiv.) was added, and the mixture was then heated for additional 30 minutes. After filtration, the solvent was removed by applying a stream of dry nitrogen over 72 hours. The residue was heated in hot ethanol and acetone and allowed to cool down, the precipitate was collected after overnight standing to give white solid **94** (1.03 g, 6.29 mmol, 59%) which was used in the next step without further purification.

Sodium (Na, 0.211 g, 9.06 mmol, 3.00 equiv.) was cut into small pieces and added to ethanol (6.04 mL, 0.5 M). After all the sodium has reacted, amino(piperidin-1-yl)methaniminium chloride (**94**) (500 mg, 3.02 mmol, 1.00 equiv.) and diethylmalonate (0.466 mL, 3.02 mmol, 1.00 equiv.) were added and the reaction was refluxed for 3 hours. The solvents were then evaporated and 1.0 N hydrochloric acid (10 mL) was added. The solid dissolved initially but gradually reappeared over 1 hour. The resulting white solid was filtered, washed with water (3 x 5 mL) and dried to give 2-morpholinopyrimidine-4,6-diol (**95**) (71.0 mg, 0.364 mmol, 12%) as a white solid.

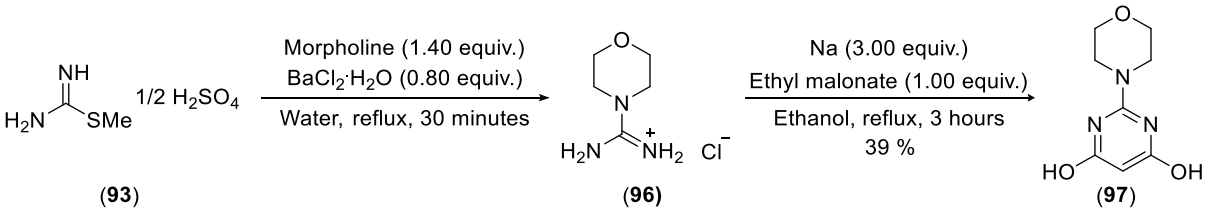
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.54 (br, 2H, O*H*), 4.69 (s, 1H, Ar*H*), 3.56 (t, *J* = 5.4 Hz, 4H, N(CH_2-)$_2$), 1.47-1.59 (m, 6H, -CH_2CH_2CH_2-).

^{13}C NMR (101 MHz, DMSO) δ 168.2, 154.3, 78.5, 45.0, 25.2, 23.9.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_9H_{14}N_3O_2^+$ 196.1081; Found 196.1085.

Spectroscopic data was consistent with that previously reported in the literature.[20]

2-Morpholinopyrimidine-4,6-diol (**97**)



(**93**) (**96**) (**97**)

Following a reported procedure,[20] S-methyl*iso*thiourea sulfate (**93**) (2.00 g, 10.6 mmol, 1.00 equiv.), water (5.26 mL, 2.02 M) and morpholine (1.28 mL, 14.9 mmol, 1.40 equiv.) were added to a flask fitted with a reflux condenser. The mixture was slowly heated to reflux and the reflux was maintained for 5 minutes. A 2.36 M hot solution of barium chloride in water ($BaCl_2$, 3.60 mL, 8.50 mmol, 0.80 equiv.) was added, and the mixture was then heated for additional 30 minutes. After filtration, the solvent was removed by applying a stream of dry nitrogen over 72 hours. The residue was heated in hot ethanol (1.5 mL) and acetone (11 mL) and allowed to cool down, the precipitate was collected after overnight standing to give white solid **96** (0.773 g, 5.94 mmol, 44%) which was used in the next step without further purification.

^{1}H NMR (400 MHz, D_2O) δ 3.82 (m, 4H, O(C*H*$_2$-)$_2$, 3.50 (m, 4H, N(C*H*$_2$-)$_2$).

^{13}C NMR (101 MHz, DMSO) δ 165.6, 65.3, 45.1.

HRMS (ESI/QTOF) m/z: $[M]^+$ Calcd for $C_5H_{12}N_3O^+$ 130.0975; Found 130.0977.

Sodium (Na, 0.208 g, 9.06 mmol, 3.00 equiv.) was cut into small pieces and added to ethanol (6.04 mL, 0.5 M). After all the sodium has reacted, amino(morpholino)methaniminium chloride (**96**) (500 mg, 3.02 mmol, 1.00 equiv.) and diethylmalonate (0.461 mL, 3.02 mmol, 1.00 equiv.) were added and the reaction was refluxed for 3 hours. The solvents were then evaporated and 1.0 N hydrochloric acid (10 mL) was added. The solid dissolved initially but gradually reappeared over 1 hour. The resulting white solid was filtered, washed with water (3 x 5 mL) and dried to give 2-morpholinopyrimidine-4,6-diol (**97**) (0.230 g, 1.17 mmol, 39%) as a white solid.

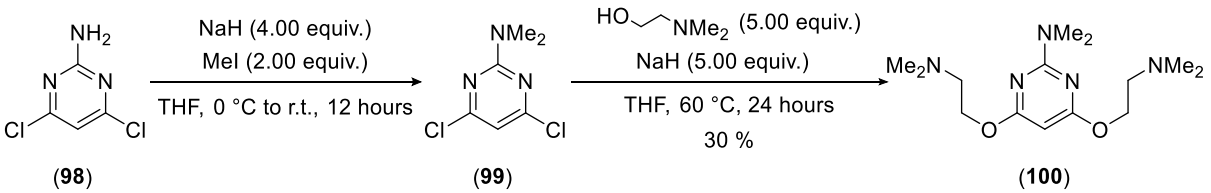
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.67 (br, 2H, O*H*), 4.79 (s, 1H, Ar*H*), 3.54-3.62 (m, 8H, morpholine-*H*).

^{13}C NMR (101 MHz, DMSO-d_6) δ 168.4, 155.5, 79.2, 65.7, 44.4.

HRMS (ESI/QTOF) m/z: $[M + Na]^+$ Calcd for $C_8H_{11n3}NaO_3^+$ 220.0693; Found 220.0693.

Spectroscopic data was consistent with that previously reported in the literature.[20]

2,2'-((2-(Dimethylamino)pyrimidine-4,6-diyl)bis(oxy))bis(N,N-dimethylethan-1-amine) (**100**)



(**98**) (**99**) (**100**)

Following a reported procedure,[20] a solution of 2-amino-4,6-dichloropyrimidine (**98**) (1.64 g, 10.0 mmol, 1.00 equiv.) in tetrahydrofuran (30 mL, 0.33 M) was treated with 60% sodium hydride (dispersed in oil) (NaH, 1.60 g, 40.0 mmol, 4.00 equiv.) at 0 °C, then the mixture was warmed to ambient temperature, and stirred for 1 hour. The mixture was cooled to 0 °C then iodomethane (MeI, 1.25 mL, 20.0 mmol, 2.00 equiv.) was added. The mixture was warmed to ambient temperature again and then stirred overnight before evaporating the solvent. The mixture was then dissolved in diethyl ether (30 mL) and washed with water (2 x 15 mL). Evaporation of the organic solvents yielded crude product **99**, which was used directly without further purification.

Sodium hydride (NaH, 0.610 g, 15.3 mmol, 5.00 equiv.) was added to an ice cooled solution of N,N-dimethylethanolamine (1.54 mL, 15.3 mmol, 5.00 equiv.) in anhydrous tetrahydrofuran (5.0 mL, 0.61 M), and the mixture was stirred for 30 minutes at 0 °C. 4,6-Dichloro-N,N-dimethylpyrimidin-2-amine (**99**) (0.500 g, 3.05 mmol, 1.00 equiv.) and a catalytic amount of N,N-di*iso*propylethylamine (DIPEA, 0.16 mL, 0.97 mmol, 0.32 equiv.) were added, and the mixture was stirred at 60 °C for 24 hours under nitrogen. The reaction mixture was quenched with saturated aqueous ammonium chloride solution (5 mL), and extracted with ethyl acetate (3 x 5 mL). The combined ethyl acetate layers were washed with brine (5 mL), dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was purified by flash chromatography (Dichloromethane:Methanol:Triethylamine 94:5:1) to give the desired product 2,2'-((2-(dimethylamino)pyrimidine-4,6-diyl)bis(oxy))bis(N,N-dimethylethan-1-amine) (**100**) (272 mg, 0.915 mmol, 30% over the two steps) as a white solid.

R_f 0.20 (Dichloromethane:Methanol 95:5).

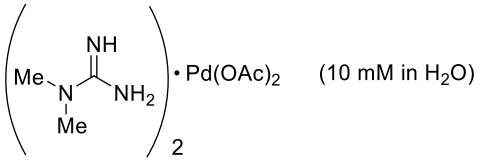
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38 (s, 1H, Ar*H*), 4.36 (td, *J* = 6.0, 1.1 Hz, 4H, OC*H*$_2$-), 3.12 (s, 6H, C(N-)$_2$N(C*H*$_3$)$_2$), 2.67 (td, *J* = 6.0, 1.6 Hz, 4H, NC*H*$_2$-), 2.31 (d, *J* = 1.4 Hz, 12H, -CH$_2$N(C*H*$_3$)$_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ 171.3, 161.5, 78.3, 63.5, 58.2, 45.9, 36.9.

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{14}H_{28n5}O_2^+$ 298.2238; Found 298.2237.

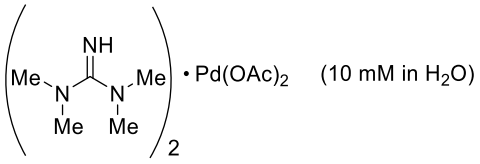
Spectroscopic data was consistent with that previously reported in the literature.[20]

b. Synthesis of palladium complexes



(**101**)

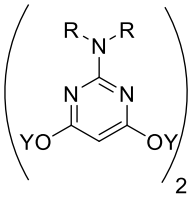
Following a reported procedure,[24] 1,1-dimethylguanidine sulfate (**91**) (2.72 mg, 20.0 µmol) was dissolved in an aqueous solution of sodium hydroxide (0.1 M, 0.4 mL) and deionized water (0.2 mL). Palladium acetate (2.25 mg, 10.0 µmol) was added and the mixture was heated at 65 °C for 45 minutes, vortexing it intermittently, to afford a clear brown solution. The mixture was then diluted to 1.00 mL with deionized water to afford a 10.0 mM catalyst solution **101**.



(**102**)

Following a reported procedure,[24] 1,1,3,3-tetramethylguanidine (2.30 mg, 20.0 µmol) was dissolved in an aqueous solution of sodium hydroxide (0.1 M, 0.2 mL) and deionized water (0.4 mL). Palladium acetate (2.25 mg, 10.0 µmol) was added and the mixture was heated at 65 °C for 45 minutes, vortexing it intermittently, to afford a clear brown solution. The mixture was then diluted to 1.00 mL with deionized water to afford a 10.0 mM catalyst solution **102**.

General Procedure for the synthesis of pyrimidine-4,6-diol-based Palladium complexes:

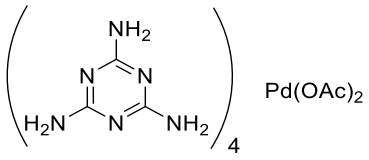


[Pd] (10 mM in H_2O)

31a: R=Me, Y=Li
31b: R=Me, Y=Na
31c: R=Me, Y=K
103: R=H , Y=Na
104: R= R=-$(CH_2)_5$- , Y=Na
105: R= R=-$(CH_2)_2O(CH_2)_2$- , Y=Na

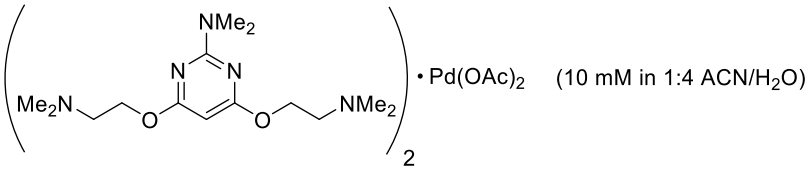
Palladium catalysts **31a-c, 103**, **104** and **105** were synthesized following reported procedures.[20,24] 2-amino-4,6-dihydroxypyrimidine or pyrimidine-4,6-diol based ligands (**95** and **97**) (20.0 µmol) were dissolved in an aqueous solution of YOH (0.4 mL, 0.1m) in an ultrasonic bath for 2 minutes. The palladium source (10.0 µmol) was added and the mixture was magnetically stirred at 65 °C for 30

minutes, deionized water (0.6 mL) was then added to afford a 10.0 mM catalyst solutions of **31a-c** and, **103-105**. The 40.0 mM catalyst solution was achieved with a 0.2 M solution of YOH.



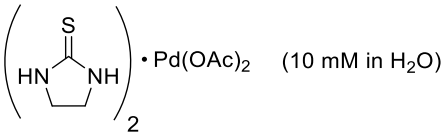
(**106**)

Following a reported procedure,[25] palladium acetate (2.25 mg, 10.0 µmol), melamine (5.04 mg, 40.0 µmol) were suspended in 10 mL of deionized water and the mixture was stirred at 80 °C for 2 hours to afford a clear 1 mM solution of **106**.



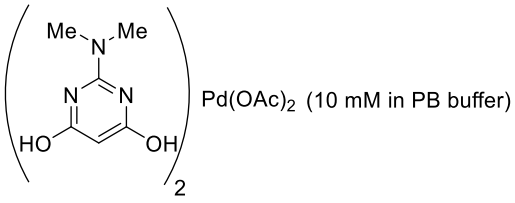
(**107**)

Following a reported procedure,[20] 2,2'-((2-(dimethylamino)pyrimidine-4,6-diyl)bis(oxy))bis(N,N-dimethylethanamine) (**100**) (21.4 mg, 72.0 µmol) was dissolved in acetonitrile (0.72 mL) and the mixture has heated at 65 °C for 5 minutes. Palladium acetate (8.08 mg, 36.0 µmol) was added and the heating continued for 30 minutes. The mixture was then diluted to 2.88 mL with deionized water to afford a 10.0 mM catalyst solution of **107**.



(**108**)

Imidazolidine-2-thione (2.04 mg, 0.020 mmol) and palladium acetate (2.25 mg, 10.0 µmol) were dissolved in 1.00 mL of degassed deionized water to afford a 10.0 mM black catalyst solution of **108**.

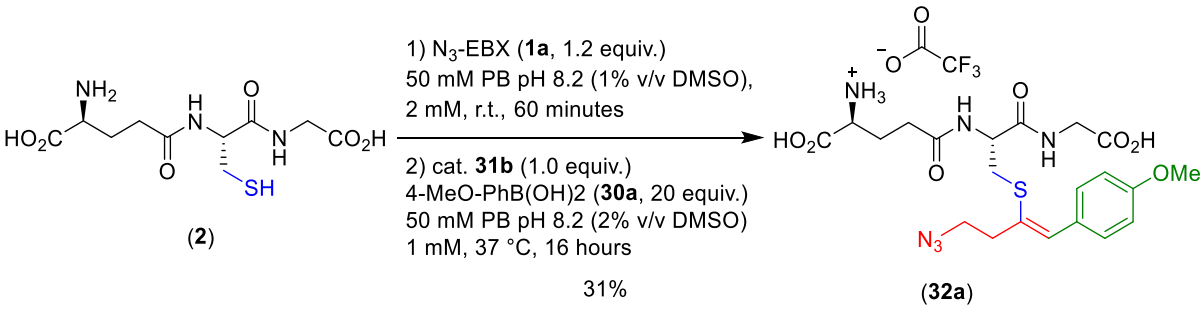


(**31d**)

Following a reported procedure,[24] 2-(dimethylamino)pyrimidine-4,6-diol (**92**) (3.10 mg, 20.0 µmol) was dissolved in 50 mM phosphate buffer solution (1.00 mL) in an ultrasonic bath for 2 minutes. Palladium acetate (2.25 mg, 10.0 µmol) was added and the mixture was magnetically stirred at 65 °C for 30 minutes to afford a 10.0 mM catalyst solution of **31d**.

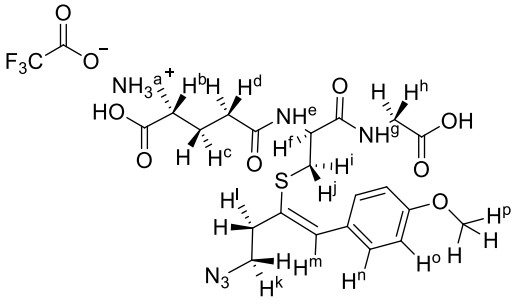
13. Isolation of Suzuki-Miyaura coupling product 32a

a. Large scale reaction

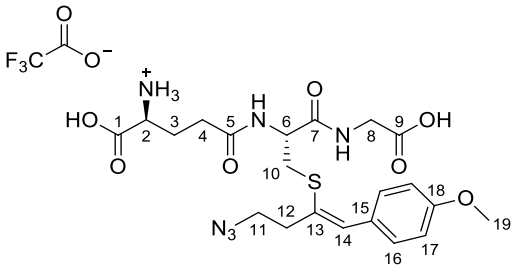


In a 250 mL one neck round bottom flask, glutathione (**2**) (30.7 mg, 100 µmol) was dissolved in a phosphate buffer (50 mM, pH 8.2, 49.0 mL). Then, a 120 mM solution of N_3-EBX reagent (**1a**) in DMSO (1.00 mL, 120 µmol, 1.20 equiv.) was added and the solution was stirred over 60 minutes at room temperature. Separately, a 100 mL one neck round bottom flask was charged with 4-methoxyphenyl boronic acid (**30a**) (152 mg, 1.00 mmol, 10.0 equiv.), phosphate buffer (63 mM, pH 8.2, 39.0 mL), DMSO (1.0 mL) and stirred at 50 °C, upon complete dissolution. After cooling down to 37 °C, the 25.0 mM solution of 4-methoxyphenyl boronic acid (**30a**) was added to intermediate **3a** at 37 °C and stirred over 10 minutes. Then, a 10.0 mM solution of **31b** in water (10.0 mL, 100 µmol, 1.00 equiv.) was added and the reaction mixture was stirred at 37 °C for 2 hours. Additional 4-methoxyphenyl boronic acid (**30a**) (152 mg, 1.00 mmol, 10.0 equiv.) was added, followed by another 16 hours stirring. The resulting reaction mixture was quenched with a 570 mM solution of 3-mercaptopropionic acid in water (1.00 mL, 570 µmol, 5.70 equiv.) and stirred for an additional 10 minutes at 37 °C. The crude mixture was directly lyophilized to afford an orange solid. The crude solid was washed with ethanol (2 x 50 mL) and purified by preparative RP-HPLC using method C (retention time: 19 – 19.5 minutes). Fractions containing the desired product were lyophilized to afford **32a** trifluoroacetic salt (16.9 mg, 30.0 µmol, 31% yield) as a pale yellow solid.

b. Characterization and structure analysis



^{1}H NMR (400 MHz, DMSO-*d_6*) δ 8.58 (t, *J* = 5.8 Hz, 1H, H^{g}), 8.41 (d, *J* = 8.3 Hz, 1H, H^{e}), 7.50 (d, *J* = 8.8 Hz, 2H, H^{n}), 6.94 – 6.82 (m, 2H, H^{o}), 6.68 (s, 1H, H^{m}), 4.36 (td, *J* = 9.3, 4.4 Hz, 1H, H^{f}), 3.78 – 3.61 (m, 5H, H^{h}+H^{p}), 3.56 (m, 3H, H^{b}+H^{k}), 3.11 (dd, *J* = 13.2, 4.4 Hz, 1H, H^{i}), 2.84 (dd, *J* = 13.2, 9.7 Hz, 1H, H^{j}), 2.70 (m, 2H, H^{l}), 2.54 (s, 3H, H^{a}), 2.38 – 2.23 (m, 2H, H^{d}), 1.91 (hept, *J* = 6.6 Hz, 2H, H^{c}).



^{13}C NMR (101 MHz, DMSO-*d_6*) δ 171.4 (C^{1}), 170.8 (C^{5}), 170.5 (C^{9}), 170.3 (C^{7}), 158.2 (C^{18}), 131.8 (C^{14}), 130.6 (C^{16}), 129.2 (C^{13}), 128.7 (C^{15}), 113.4 (C^{17}), 55.1 (C^{19}), 52.8 (C^{2}), 52.4 (C^{6}), 49.2 (C^{11}), 40.9 (C^{8}), 36.8 (C^{12}), 32.8 (C^{10}), 31.1 (C^{4}), 26.4 (C^{3}).

m.p.: 167-168 °C.

IR (vmax, cm-1) 3427 (w), 2987 (m), 2911 (w), 2094 (m), 1636 (s), 1603 (s), 1512 (s), 1416 (w), 1250 (s), 1182 (m), 1030 (m).

HRMS (ESI/QTOF) m/z: $[M + H]^{+}$ Calcd for $C_{21}H_{29}N_6O_7S^{+}$ 509.1813; Found 509.1826.

^{1}H NMR COSY, HSQC, and ROESY (DMSO-*d6*, 400 MHz) were consistent with this attribution.

(Z)-configuration attribution: H^{m} could be assigned due to its unique vinylic shift. Meanwhile, H^{l} could be assigned based on COSY and HMBC correlation with H^{k} and C^{11}. Finally, ROESY correlation between H^{m} and H^{l} confirmed the (Z) configuration of the double bond. No ROESY correlation could be observed with H^{j} nor H^{i}.

c. Calibration

Calibration of **32a** 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 = 0.00074229 x X - 0.05717175 and R = 0.99944004, where Y is the concentration in µmol/mL of **32a** and X the absorbance area of the peak at 214 nm.

14. Optimization of the Suzuki-Miyaura reaction

General procedure for Suzuki-Miyaura coupling using ligandless palladium catalysts:

A 1.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with (*para*-methoxyphenyl)boronic acid (**30a**) (1.52 mg, 10.0 µmol, 10.0 equiv.), phosphate buffer (63 mM, pH 8.2, 290 µL) and DMSO (10 µL). The resulting mixture was heat up to 50 °C in order to obtain a clear solution. After cooling down to 37 °C, a 10.0 mM solution of **3a** in 63 mM phosphate buffer pH 8.2 (100 µL, 1.00 µmol) was added to the 33.3 mM solution of phenyl boronic acid. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated at 37 °C over 10 minutes. Then, a 10.0 mM solution of PdX_2 or M_2PdX_4 in water (100 µL, 1.00 µmol, 1.00 equiv.) was added in one portion. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated at 37 °C. After 15, 30 and 60 minutes, aliquots of the reaction (100 µL) were quenched with a 570 mM solution of 3-mercaptopropionic acid in water (2.00 µL, 1.14 µmol, 1.14 equiv. per equiv. of palladium) and shaken at room temperature for 10 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.

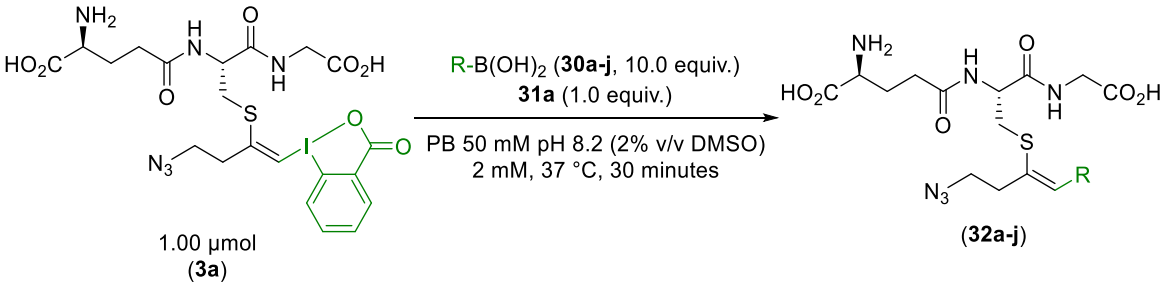
General procedure for Suzuki-Miyaura coupling using non-preformed palladium catalysts:

A 1.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with (*para*-methoxyphenyl)boronic acid (**30a**) (1.52 mg, 10.0 µmol, 10.0 equiv.), phosphate buffer (63 mM, pH 8.2, 290 µL) and DMSO (10 µL). The resulting mixture was heat up to 50 °C in order to obtain a clear solution. After cooling down to 37 °C, a 10.0 mM solution of **3a** in 63 mM phosphate buffer pH 8.2 (100 µL, 1.00 µmol) was added to the 33.3 mM solution of phenyl boronic acid. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated at 37 °C over 10 minutes. Separately, another 1.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with PdX_2 or M_2PdX_4 (1.00 µmol, 1.00 equiv.), **90** or **109**-**112** (1.00 or 2.00 µmol, 1.00 or 2.00 equiv.) and water (100 µL). The resulting mixture was vortexed few seconds and incubated at 37 °C over 10 minutes. Then, the solution of (*para*-methoxyphenyl)boronic acid and **3a** was added to the mixture containing the palladium catalyst. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated at 37 °C. After 15, 30 and 60 minutes, aliquots of the reaction (100 µL) were quenched with a 570 mM solution of 3-mercaptopropionic acid in water (2.00 µL, 1.14 µmol, 1.14 equiv. per equiv. of palladium) and shaken at room temperature for 10 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.

General procedure for Suzuki-Miyaura coupling using preformed palladium catalysts:

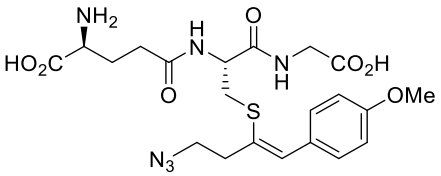
A 1.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with (*para*-methoxyphenyl)boronic acid (**30a**) (1.52 mg, 10.0 µmol, 10.0 equiv.), phosphate buffer (63 mM, pH 8.2, 290 µL) and DMSO (10 µL). The resulting mixture was heat up to 50 °C in order to obtain a clear solution. After cooling down to 37 °C, a 10.0 mM solution of **3a** in 63 mM phosphate buffer pH 8.2 (100 µL, 1.00 µmol) was added to the 33.3 mM solution of phenyl boronic acid. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated at 37 °C over 10 minutes. Then, a 10.0 mM solution of palladium complexes (**31a**-**d**, **101-108**) in water (100 µL, 1.00 µmol, 1.00 equiv.) was added in one portion. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated at 37 °C. After 15, 30 and 60 minutes, aliquots of the reaction (100 µL) were quenched with a 570 mM solution of 3-mercaptopropionic acid in water (2.00 µL, 1.14 µmol, 1.14 equiv. per equiv. of palladium) and shaken at room temperature for 10 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.

15. Scope of boronic acids



General procedure J:

A 1.5 mL Eppendorf Safe-Lock microcentrifuge tube was charged with phenyl boronic acid (10.0 µmol, 10.0 equiv.), phosphate buffer (63 mM, pH 8.2, 290 µL) and DMSO (10 µL). The resulting mixture was heat up to 50 °C in order to obtain a clear solution (except (*para*-hydroxyphenyl)boronic acid (**30c**) and furan-2-ylboronic acid (**30l**), dissolved at room temperature). After cooling down to 37 °C, a 10.0 mM solution of **3a** in 63 mM phosphate buffer pH 8.2 (100 µL, 1.00 µmol) was added to the 33.3 mM solution of phenyl boronic acid. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated at 37 °C over 10 minutes. Then, a 10.0 mM solution of **31a** in water (100 µL, 1.00 µmol, 1.00 equiv.) was added in one portion. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated 30 minutes at 37 °C. The reaction was quenched with a 570 mM solution of 3-mercaptopropionic acid in water (10.0 µL, 5.70 µmol, 5.70 equiv. per equiv. of palladium) and shaken at room temperature for 10 minutes. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS. For the (*para*-methoxyphenyl)boronic acid (**30a**), 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.



(**32a**)

Following general procedure J, (*para*-methoxyphenyl)boronic acid (**30a**) afforded the title compound **32a** in 70% yield (retention time: 12.706 minutes).

HRMS (ESI/QTOF) m/z: $[M + H]^+$ Calcd for $C_{21}H_{29}N_6O_7S^+$ 509.1813; Found 509.1826.

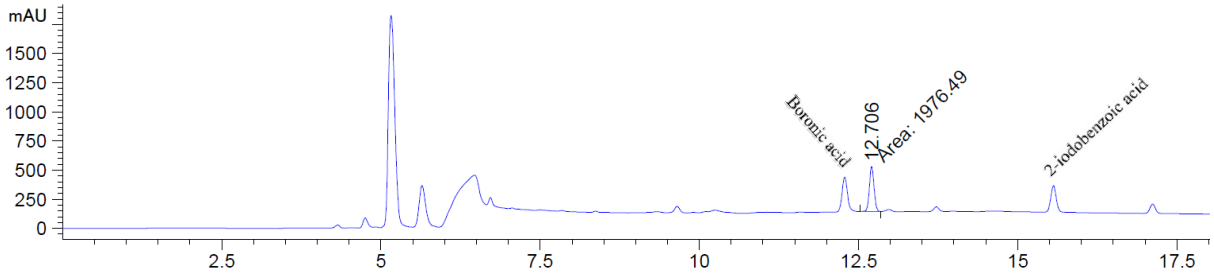


Figure S184: HPLC-UV chromatogram at 214 nm of **32a**.

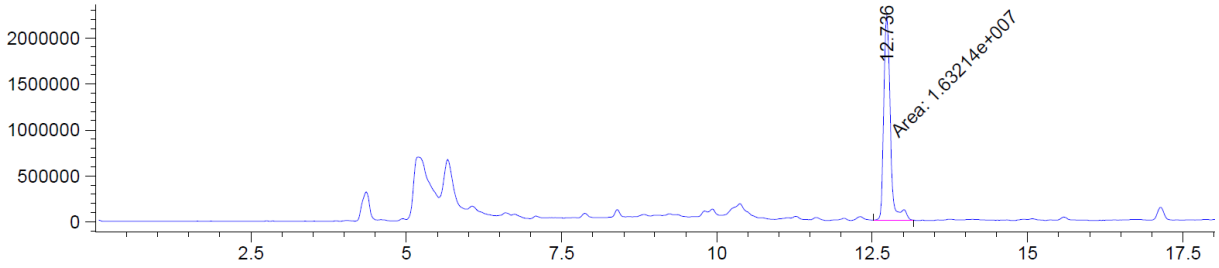
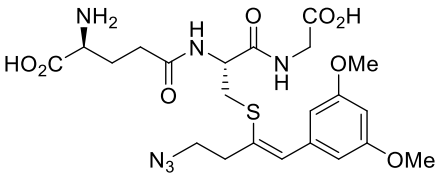


Figure S185: HPLC-MS chromatogram of **32a**.



(**32b**)

Following general procedure J, (3,5-dimethoxyphenyl)boronic acid (**30b**) afforded the title compound **32b** (retention time: 13.017 minutes).

HRMS (ESI/QTOF) m/z: $[M + H-1]^-$ Calcd for $C_{22}H_{29}N_6O_8S^-$ 537.1773; Found 537.1774.

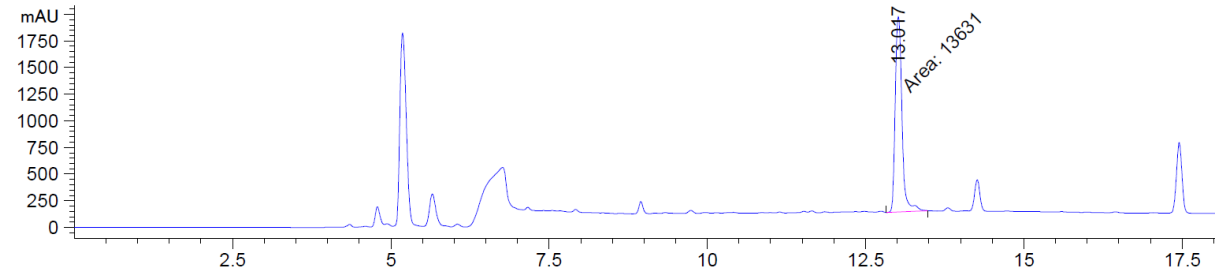


Figure S186: HPLC-UV chromatogram at 214 nm of **32b**.

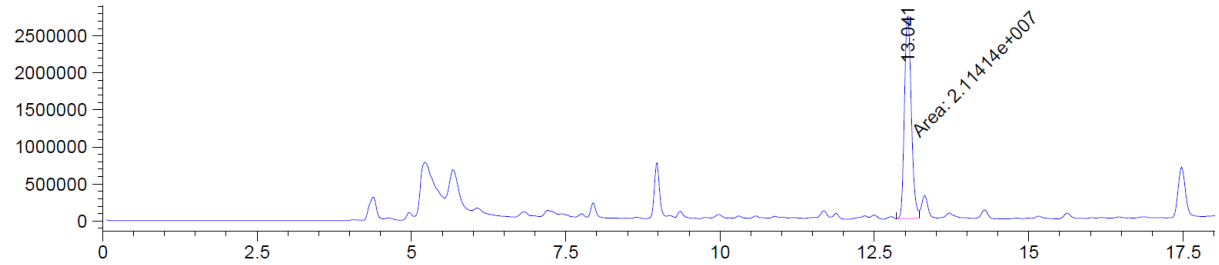
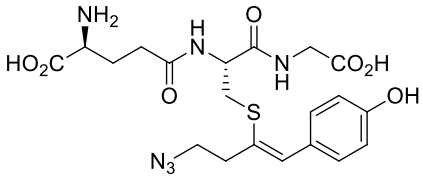


Figure S187: HPLC-MS chromatogram of **32b**.



(**23c**)

(**32c**)

Following general procedure J, (*para*-hydroxyphenyl)boronic acid (**30c**) afforded the title compound **32c** (retention time: 11.005 minutes).

HRMS (ESI/QTOF) m/z: $[M + H\text{-}1]^-$ Calcd for $C_{20}H_{25}N_6O_7S^-$ 493.1511; Found 493.1504.

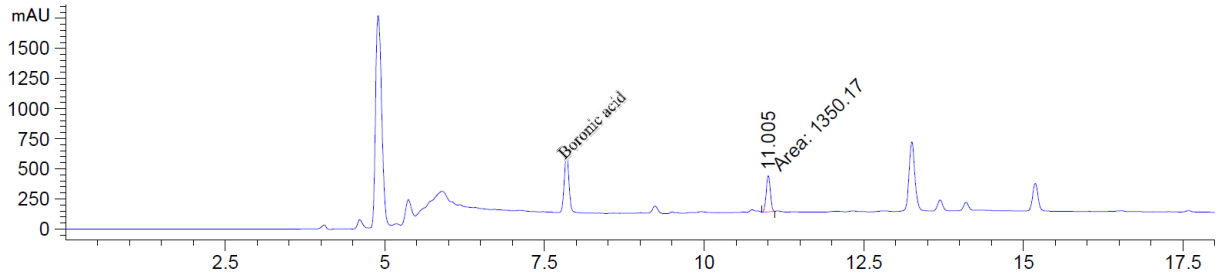


Figure S188: HPLC-UV chromatogram at 214 nm of **32c**.

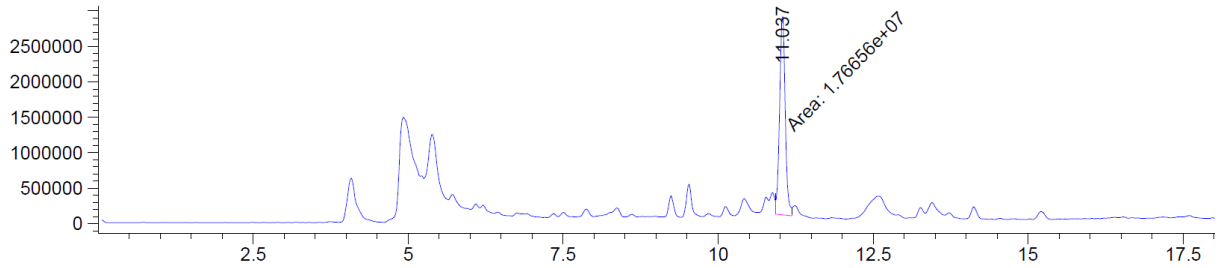
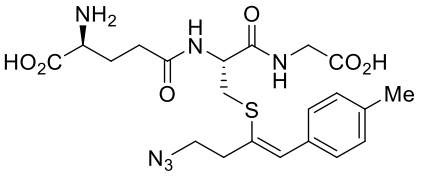


Figure S189: HPLC-MS chromatogram of **32c**.



(**32d**)

Following general procedure J, (*para*-methylphenyl)boronic acid (**30d**) afforded the title compound **32d** (retention time: 13.350 minutes).

HRMS (ESI/QTOF) m/z: $[M + H\text{-}1]^-$ Calcd for $C_{21}H_{27}N_6O_6S^-$ 491.1718; Found 491.1725.

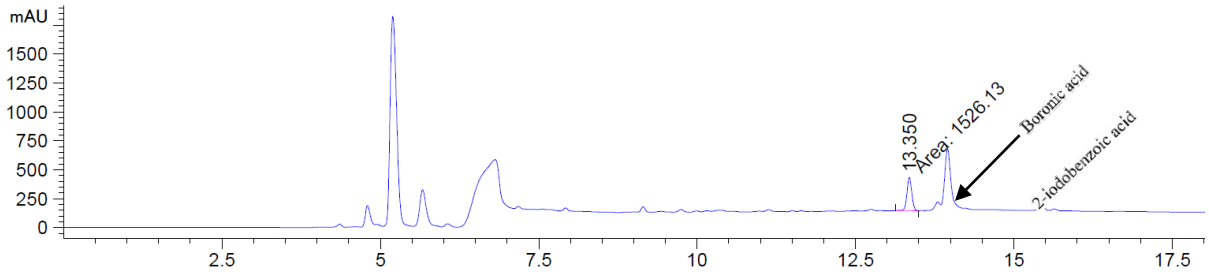


Figure S190: HPLC-UV chromatogram at 214 nm of **32d.**

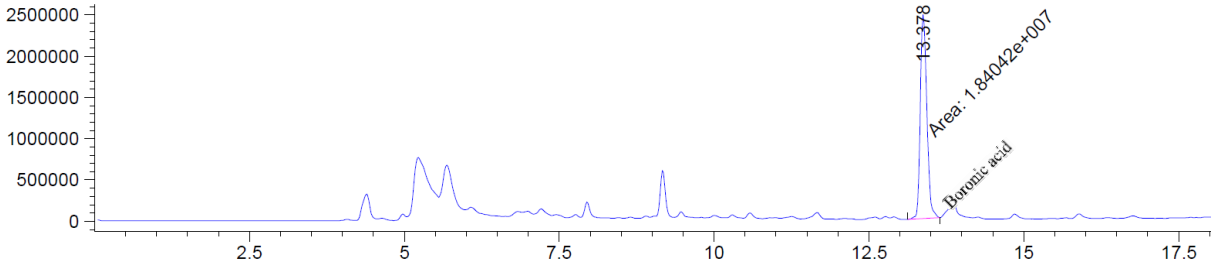
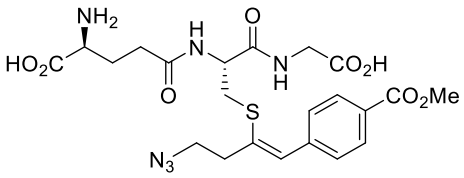


Figure S191: HPLC-MS chromatogram of **32d**.



(**32e**)

Following general procedure J, (*para*-methoxycarbonylphenyl)boronic acid (**30e**) afforded the title compound **32e** (retention time: 12.814 minutes).

HRMS (ESI/QTOF) m/z: $[M + H\text{-}1]^-$ Calcd for $C_{22}H_{27}N_6O_8S^-$ 535.1617; Found 535.1618.

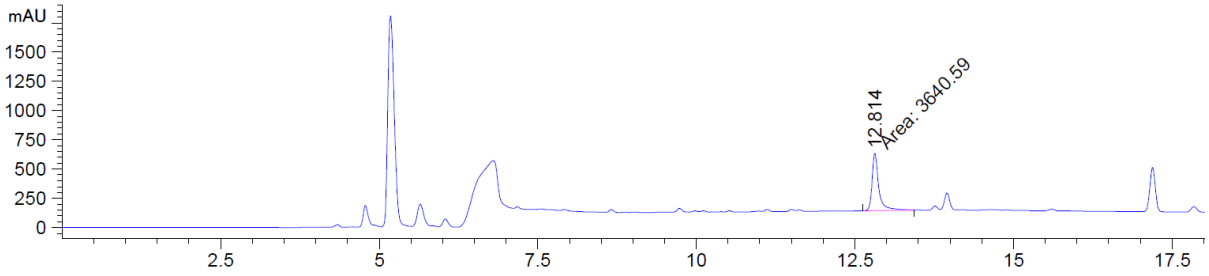


Figure S192: HPLC-UV chromatogram at 214 nm **32e**.

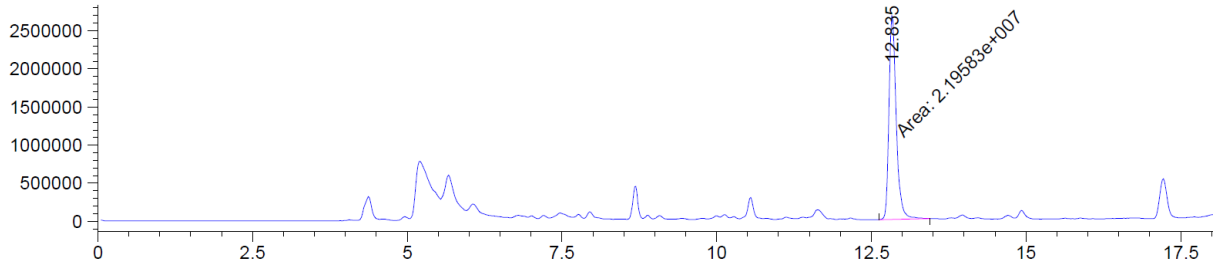
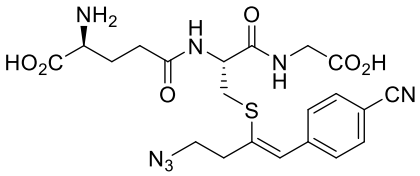


Figure S193: HPLC-MS chromatogram **32e.**



(**32f**)

Following general procedure J, (*para*-cyanophenyl)boronic acid (**30f**) afforded the title compound **32f** (retention time: 17.821 minutes).

HRMS (ESI/QTOF) m/z: $[M + H\text{-}1]^-$ Calcd for $C_{21}H_{24}N_7O_6S^-$ 502.1514; Found 502.1517.

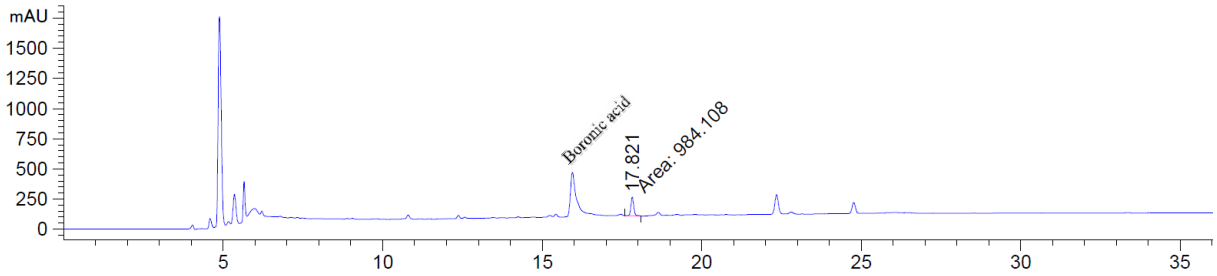


Figure S194: HPLC-UV chromatogram at 214 nm of **32f**.

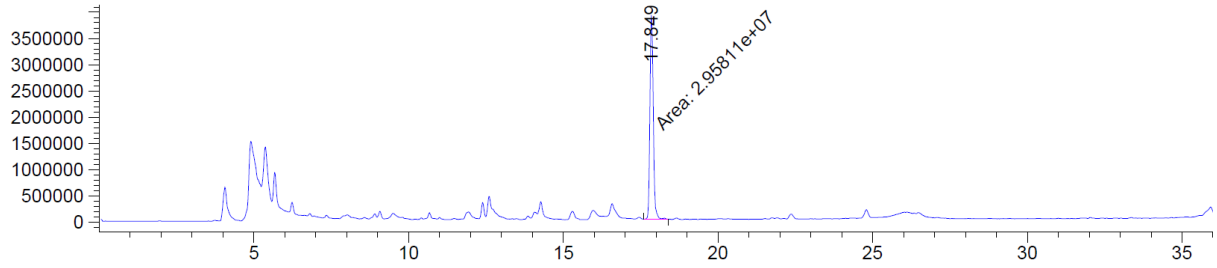
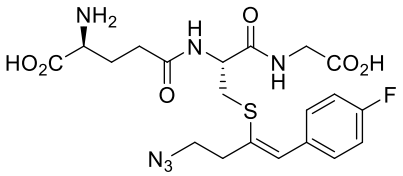


Figure S195: HPLC-MS chromatogram of **32f**.



(**32g**)

Following general procedure J, (*para*-fluorophenyl)boronic acid (**30g**) afforded the title compound **32g** (retention time: 12.948 minutes).

HRMS (ESI/QTOF) m/z: $[M + H\text{-}1]^-$ Calcd for $C_{20}H_{24}FN_6O_6S^-$ 495.1468; Found 495.1469.

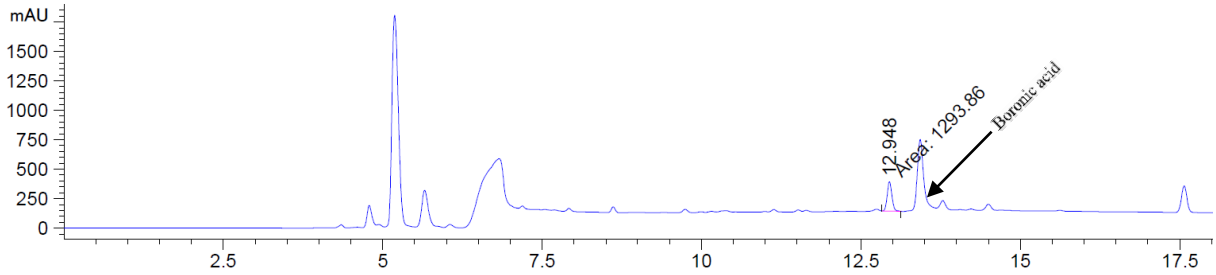


Figure S196: HPLC-UV chromatogram at 214 nm of **32g.**

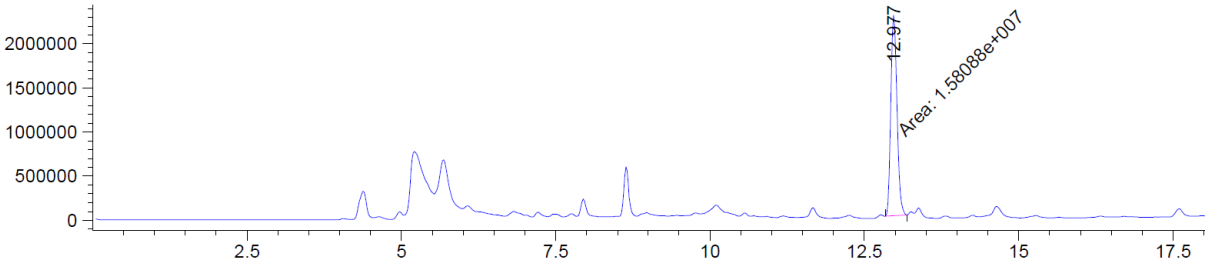
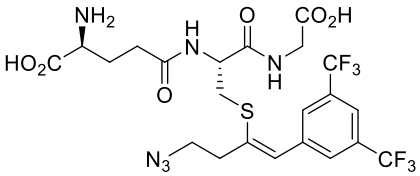


Figure S197: HPLC-MS chromatogram of **32g**.



(**32h**)

Following general procedure J, (3,5-bis(trifluoromethyl)phenyl)boronic acid (**30h**) afforded the title compound **32h** (retention time: 23.743 minutes).

HRMS (ESI/QTOF) m/z: $[M + H\text{-}1]^-$ Calcd for $C_{22}H_{23f6n6}O_6S^-$ 613.1309; Found 613.1311.

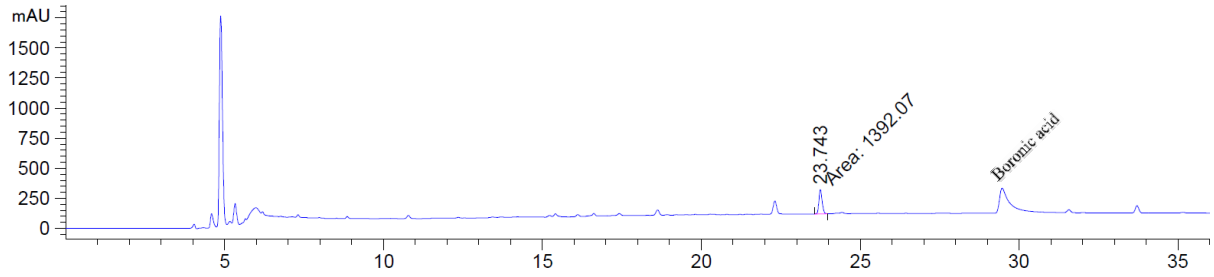


Figure S198: HPLC-UV chromatogram at 214 nm of **32h**.

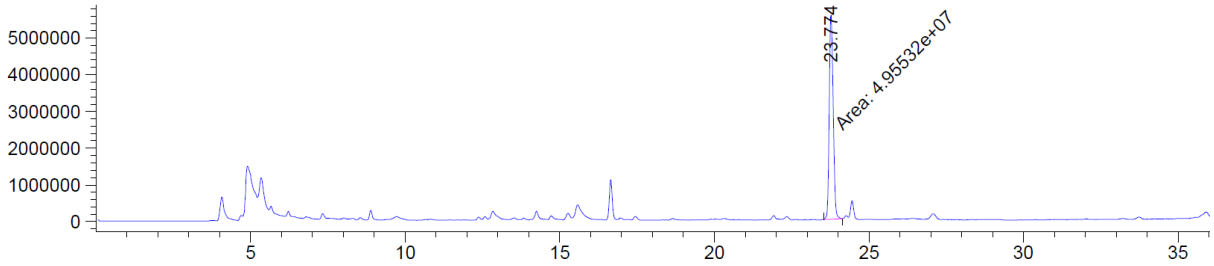
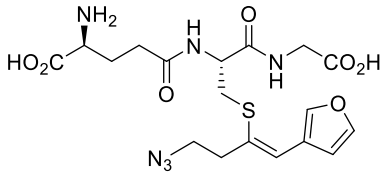


Figure S199: HPLC-MS chromatogram of **32h**.



(**32i**)

Following general procedure J, furan-2-ylboronic acid (**30i**) afforded the title compound **32i** (retention time: 16.158 minutes).

HRMS (ESI/QTOF) m/z: $[M + H\text{-}1]^-$ Calcd for $C_{18}H_{23}N_6O_7S^-$ 467.1354; Found 467.1354.

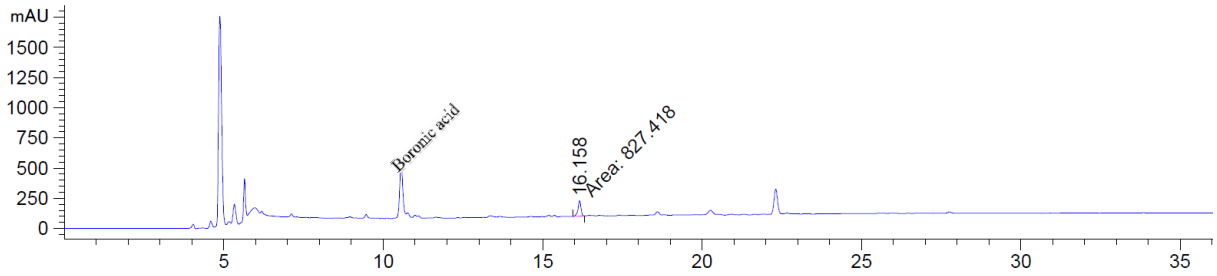


Figure S200: HPLC-UV chromatogram at 214 nm of **32i**.

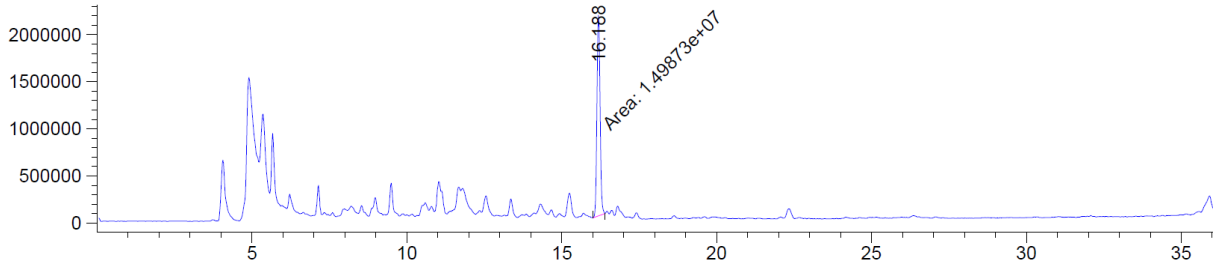
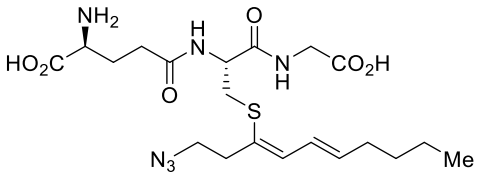


Figure S201: HPLC-MS chromatogram of **32i.**



(**32j**)

Following general procedure J, (E)-hex-1-en-1-ylboronic acid (**30j**) afforded the title compound **32j** (retention time: 14.069 minutes).

HRMS (ESI/QTOF) m/z: $[M + H-1]^-$ Calcd for $C_{20}H_{31n6}O_6S^-$ 483.2031; Found 483.2033.

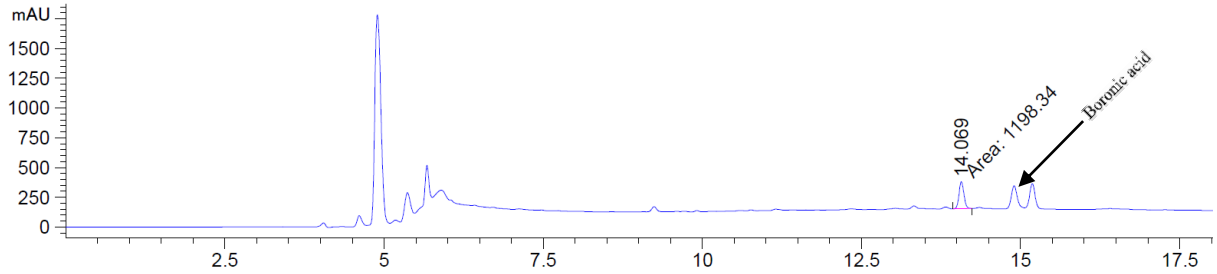


Figure S202: HPLC-UV chromatogram at 214 nm of **32j**.

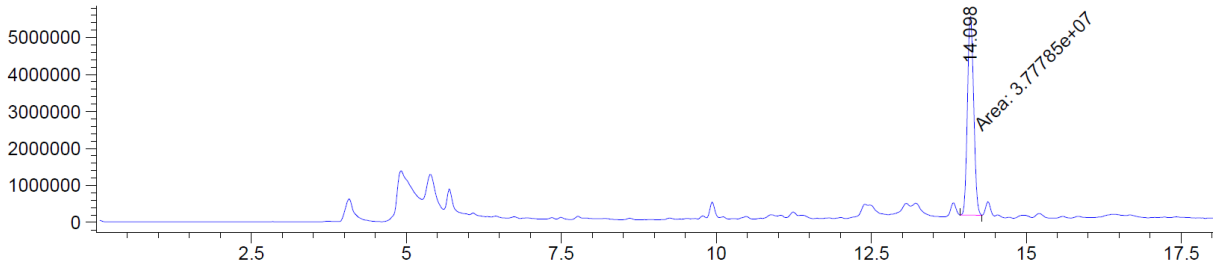
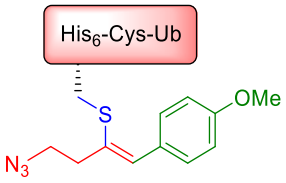


Figure S203: HPLC-MS chromatogram of **32j**.

16. Cross-coupling on cys-ubiquitin (17)

a. Labeling/Suzuki-Miyaura coupling one-pot on cys-ubiquitin:



(**29**)

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, a 1.6 mM solution of cys-ubiquitin (**17**) in water (4.38 μL, 7.00 nmol) was diluted with phosphate buffer (100 mM, pH 8.2, 4.38 μL). The resulting solution was vortexed few seconds and a 1.60 mM solution of N_3-EBX reagent (**1a**) in phosphate buffer (50.0 mM, pH 8.2) containing 5% (v/v) DMSO (8.75 μL, 14.0 nmol, 2.00 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes. Separately, a 25.0 mM stock solution of (*para*-methoxyphenyl)boronic acid (**30a**) in phosphate buffer (50.0 mM, pH 8.2) containing 2.5% (v/v) DMSO was prepared by heating up the mixture to 50 °C, until obtain a clear solution. After cooling down to 37 °C, an aliquot of the phenyl boronic acid solution (14.0 μL, 350 nmol, 50.0 equiv.) was added to the protein sample. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated at 37 °C over 10 minutes. Then, a 40.0 mM solution of **31a** in water (3.50 μL, 140 nmol, 20.0 equiv.) was added in one portion. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated 30 minutes at 37 °C to afford **33** (retention time: 23.165 minutes, yield 76%). The reaction was quenched with a 1.14 M solution of 3-mercaptopropionic acid in water (0.700 μL, 0.798 μmol, 5.70 equiv. per equiv. of palladium) and shaken at room temperature for 10 minutes. No effort was made to exclude oxygen. The reaction was monitored by analytical HPLC and products identity confirmed by ESI-MS analysis. The peak areas for all-relevant peptide-containing species on the chromatogram were integrated and the yield was determined using the following equation: yield % = $A_{product}/(A_{starting} + A_{product})$, where $A_{starting}$, and $A_{product}$ respectively represent the area of absorption peak at 214 nm of the remaining starting material and product.

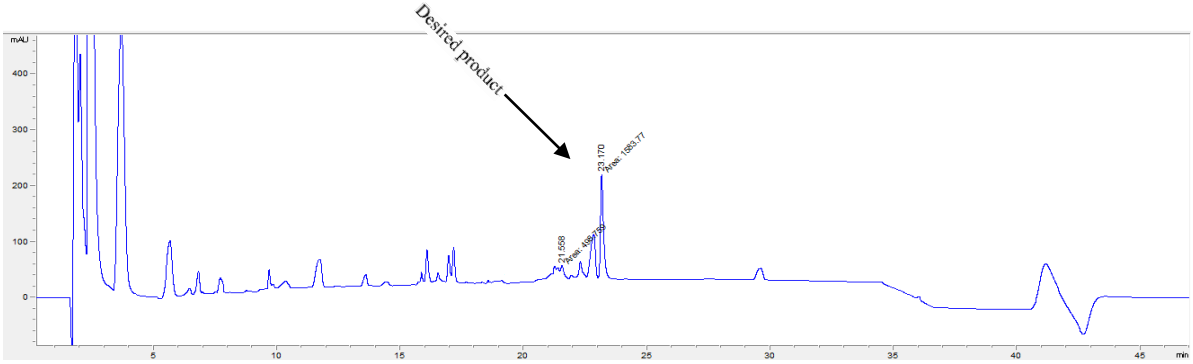


Figure S204: HPLC-UV chromatogram at 214 nm of **29**.

ESI-MS Calcd mass 10901.2 Da; Found 10901.5 Da.

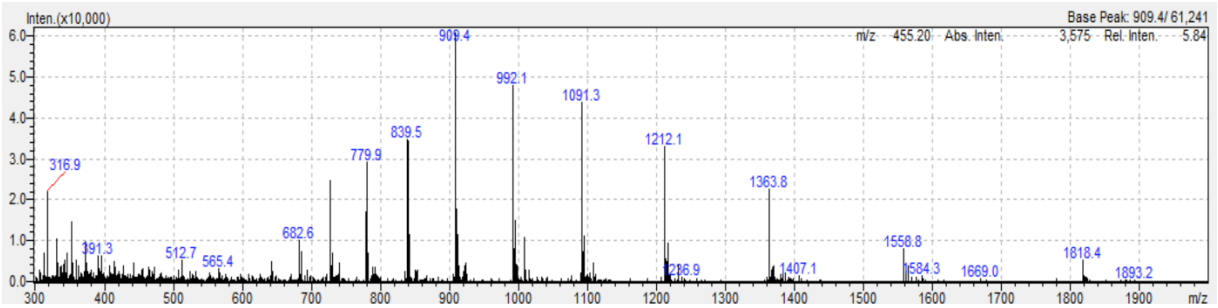
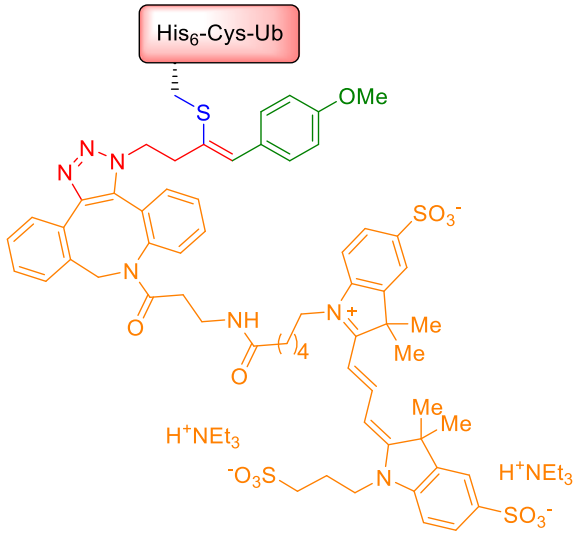


Figure S205: ESI-MS spectrum of **29**.

b. Labeling/SPAAC/Suzuki-Miyaura one-pot on cys-ubiquitin (**17**):



(**34**)

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, a 1.6 mM solution of cys-ubiquitin (**17**) in water (4.38 μL, 7.00 nmol) was diluted with phosphate buffer (100 mM, pH 8.2, 4.38 μL). The resulting solution was vortexed few seconds and a 1.60 mM solution of N_3-EBX reagent (**1a**) in phosphate buffer (50.0 mM, pH 8.2) containing 5% (v/v) DMSO (8.75 μL, 14.0 nmol, 2.00 equiv.) was added in one portion. The

resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes. Subsequently, a 20.0 mM solution of dibenzocyclooctyne-Cy3 (**23c**) in DMSO (1.75 µL, 35.0 nmol, 5.00 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 2 hours to afford **113** (retention time: 21.879 & 22.073 minutes). Separately, a 25.0 mM stock solution of (*para*-methoxyphenyl)boronic acid (**30a**) in phosphate buffer (50.0 mM, pH 8.2) containing 2.5% (v/v) DMSO was prepared by heating up the mixture to 50 °C, until obtain a clear solution. After cooling down to 37 °C, an aliquot of the phenyl boronic acid solution (14.0 µL, 350 nmol, 50.0 equiv.) was added to the protein sample. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated at 37 °C over 10 minutes. Then, a 40.0 mM solution of **31a** in water (3.50 µL, 140 nmol, 20.0 equiv.) was added in one portion. The resulting solution was vortexed few seconds to ensure proper reagent mixing and incubated 30 minutes at 37 °C to afford **34** (retention time: 22.993 minutes). The reaction was quenched with a 1.14 M solution of 3-mercaptopropionic acid in water (0.70 µL, 0.80 µmol, 5.70 equiv. per equiv. of palladium) and shaken at room temperature for 10 minutes. The reaction was monitored by analytical HPLC and product identity confirmed by ESI-MS analysis. No effort was made to exclude oxygen.

Chromatograms after labeling/SPAAC process (**113**):

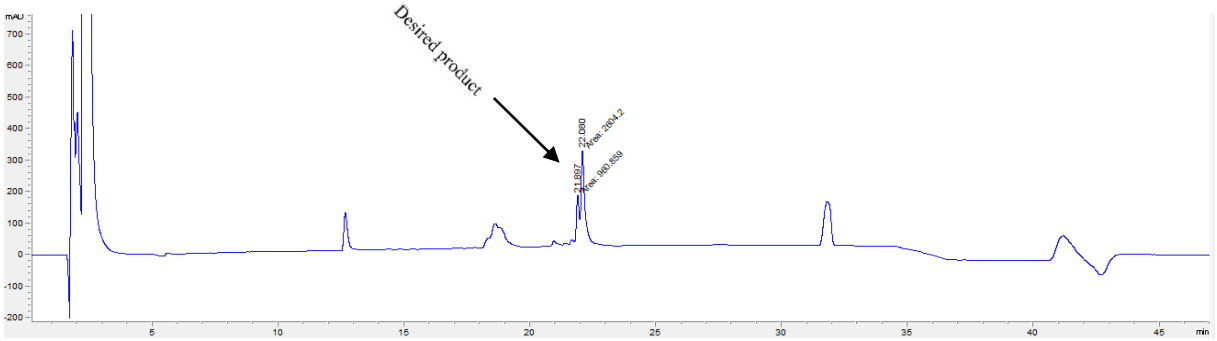


Figure S206: HPLC-UV chromatogram at 214 nm of **34**.

ESI-MS Calcd mass 12023.4 Da; Found 12023.4 Da.

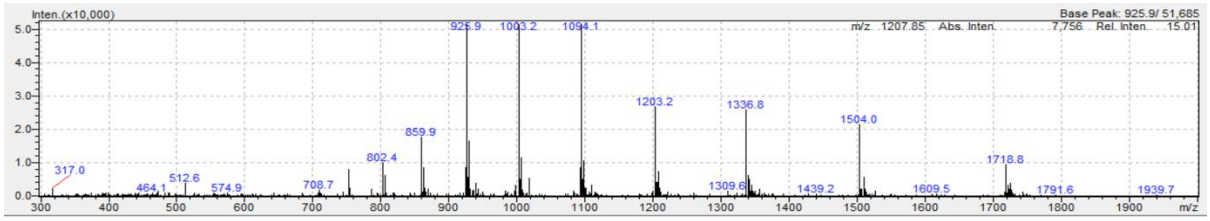


Figure S207: ESI-MS spectrum of **34.**

Chromatograms after labeling/SPAAC/Suzuki-Miyaura one-pot (**34**):

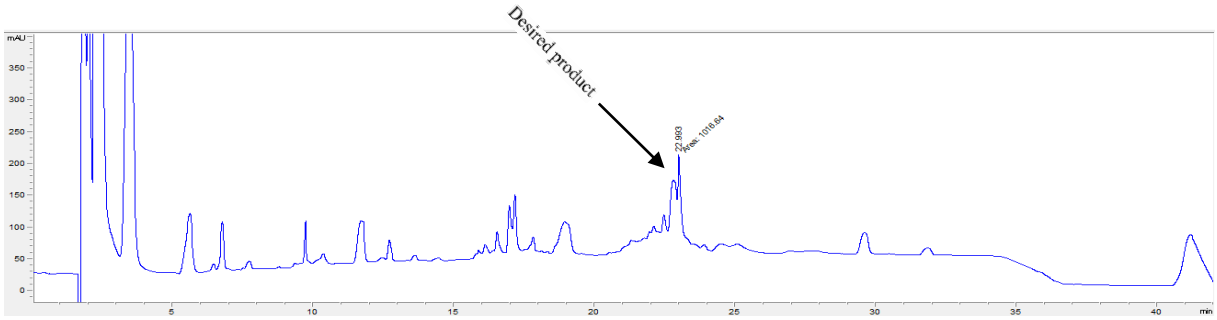


Figure S208: HPLC-UV chromatogram at 214 nm of **34**.

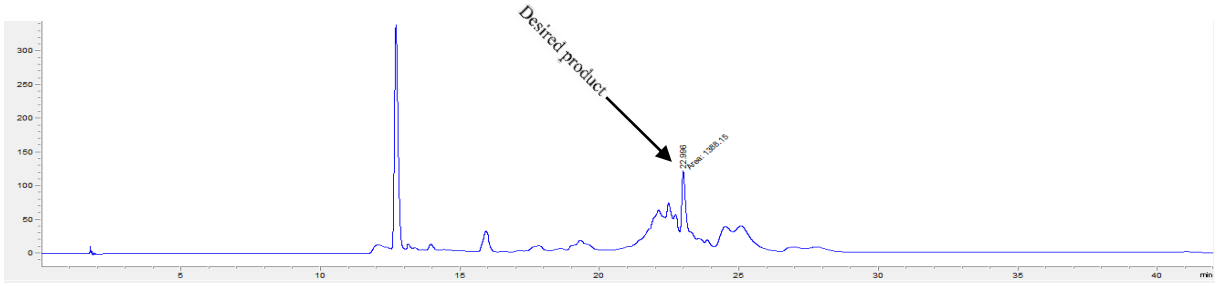


Figure S209: HPLC-UV chromatogram at 553 nm of **34**.

ESI-MS Calcd mass 11883.5 Da; Found 11882.9 Da.

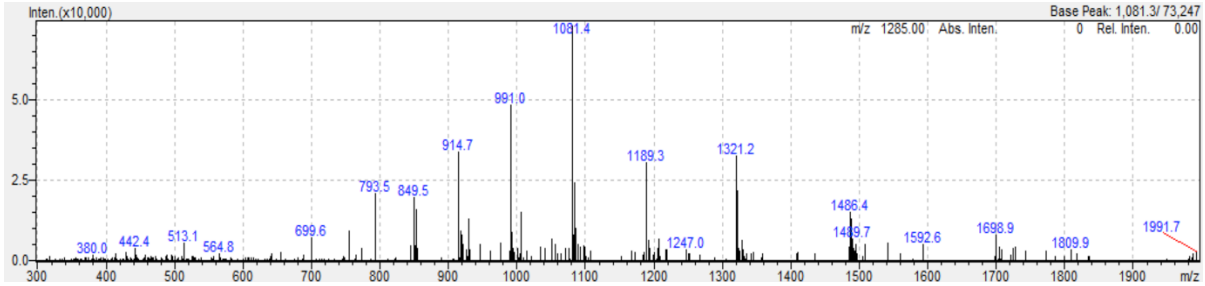
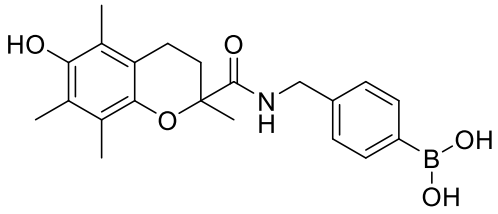


Figure S210: ESI-MS spectrum of **34.**

17. Trolox cross-coupling on Substance P analogue (35)

a. Synthesis of the Trolox-based boronic acid (**37**)



To a round bottom flask charged with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (20.0 mg, 0.08 mmol, 1.0 equiv) in DMF (0.47 mL, 0.17 M), TSTU (2-(2,5-dioxopyrrolidin-1-yl)-1,1,3,3-tetramethylisouronium tetrafluoroborate) (28.9 mg, 0.096 mmol, 1.20 equiv) and NEt_3 (32.3 µL, 0.320

mmol, 4.00 equiv) were added. 4-boronic acid phenylmethanamine (12.1 mg, 0.080 mmol, 1.00 equiv) at room temperature was then added and the reaction mixture was stirred for 16 hours. The reaction was then submitted to preparative HPLC to obtain (4-((6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxamido)methyl)phenyl)boronic acid as a white solid (15.3 mg, 0.040 mmol, 50%).

m.p.: 226-232 °C

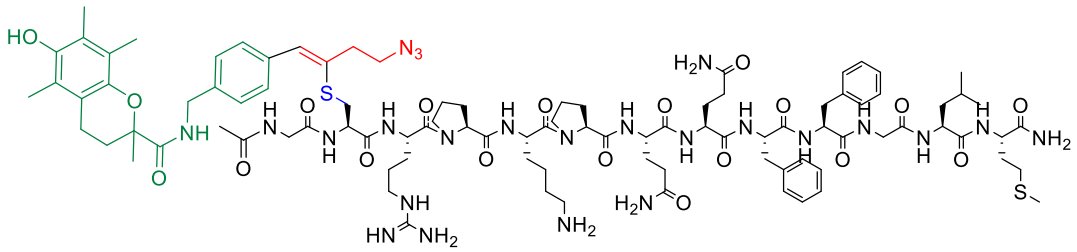
IR (vmax, cm-1) 3740 (w), 3559 (m), 3418 (m), 2932 (w), 1778 (w), 1657 (s), 1529 (m), 1414 (m), 1344 (s), 1206 (s), 1011 (s), 819 (m), 659 (s)

^{1}H NMR (400 MHz, DMSO) δ 7.83 (t, *J* = 6.3 Hz, 1H, N*H*), 7.62 (d, *J* = 8.0 Hz, 2H, $C_{Ar}H$), 6.96 (d, *J* = 7.8 Hz, 2H, $C_{Ar}H$), 4.35 (dd, *J* = 15.6, 6.6 Hz, 1H, NCH_2), 4.20 (dd, *J* = 15.6, 5.8 Hz, 1H, NCH_2), 2.57-2.53 (m, 1H, $C_{Ar}CH_2CH_2$), 2.47-2.39 (m, 1H, $C_{Ar}CH_2CH_2$), 2.24 (dt, *J* = 13.2, 5.8 Hz, 1H, $C_{Ar}CH_2CH_2$), 2.10 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 1.73 (ddd, *J* = 13.1, 9.0, 6.2 Hz, 1H, $C_{Ar}CH_2CH_2$), 1.43 (s, 3H, CH_3C-O).

^{13}C NMR (101 MHz, DMSO) δ 173.4 (*C*O), 145.9 (C_{Ar}), 144.0 (C_{Ar}), 141.5 (C_{Ar}), 134.0 (C_{Ar}), 132.1 (C_{Ar}), 125.4 (C_{Ar}), 122.7 (C_{Ar}), 121.3 (C_{Ar}), 120.3 (C_{Ar}), 117.1 (C_{Ar}), 77.4 (CH_3C-O), 41.9 (NCH_2), 29.6 ($C_{Ar}CH_2CH_2$) 24.5 (CCH_3), 20.3, 12.8 (C_{Ar}-CH_3), 12.1 (C_{Ar}-CH_3), 11.8 (C_{Ar}-CH_3).

HRMS (nanochip-ESI/LTQ-Orbitrap) m/z: $[M + Na]^+$ Calcd for $C_{21}H_{26}BNNaO_5^+$ 406.1796; Found 406.1784.

b. One-pot Labeling/Suzuki-Miyaura coupling with Trolox on Substance P (**35**)



(**38**)

Substance P (**35**) (4.12 mg, 2.66 µmol, 1.00 equiv.) was dissolved in a 5 mL vial with 2250 µL of miliQ water and 2246 µL of phosphate buffer pH 8.2 100 mM. A solution of Azido-EBX (**1a**) (89 µL, 30 mM, 1.00 equiv.) was added and the reaction was vortexed and shaken at room temperature for 3.0 hours. A solution of Palladium complex (**31a)** in water (665 µL, 20 mM, 5.0 equiv.) was added in one portion, followed by a solution of the Trolox boronic acid (**37**) in DMSO (3.54 mL, 7.5 mM, 10 equiv.) The reaction was then shaken for 4 hours at room temperature before quenching it with a solution of mercaptopropionic acid (6.60 µL, 7.95 mM, 28.5 equiv.) at 37 °C for 10 minutes. The reaction was then transferred to a 250 mL round-bottom flask, diluted with 100 mL of water and left to dry on the liophilizer. The crude product was resuspended in 2 mL of DMSO, passed through an HPLC filter and purified by preparative reverse-phase HPLC, using method D (see. Trolox-subtance P conjugate (**38**) (0.49 mg, 8%) was isolated as liophilized solid and transformed directly into a 20 mM DMSO solution. No effort was made to exclude oxygen at any step.

HRMS (ESI/QTOF) m/z: $[M + 2H]^{+2}$ Calcd for $C_{95}H_{138}N_{24}O_{19}S_2^{+2}$ 991.5000; Found 991.5007.

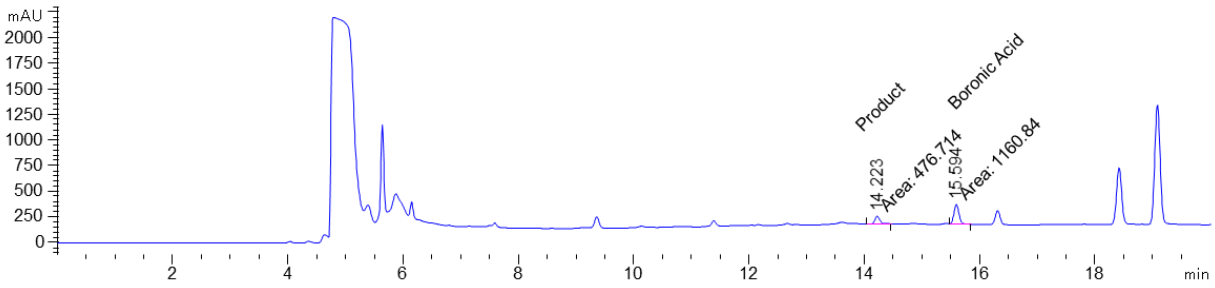


Figure S211: HPLC-UV chromatogram at 214 nm of **38**.

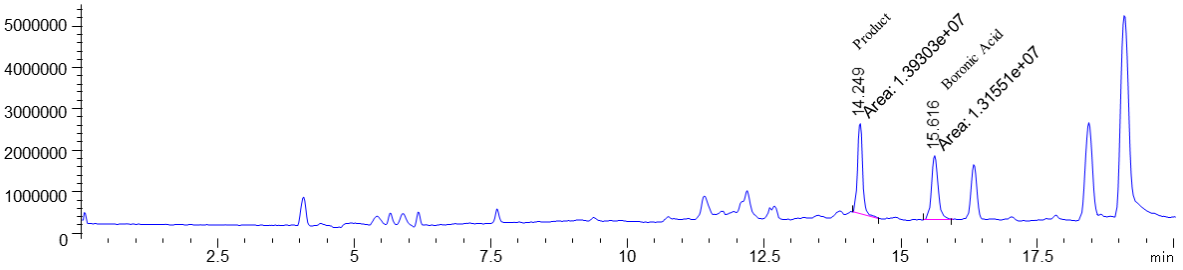
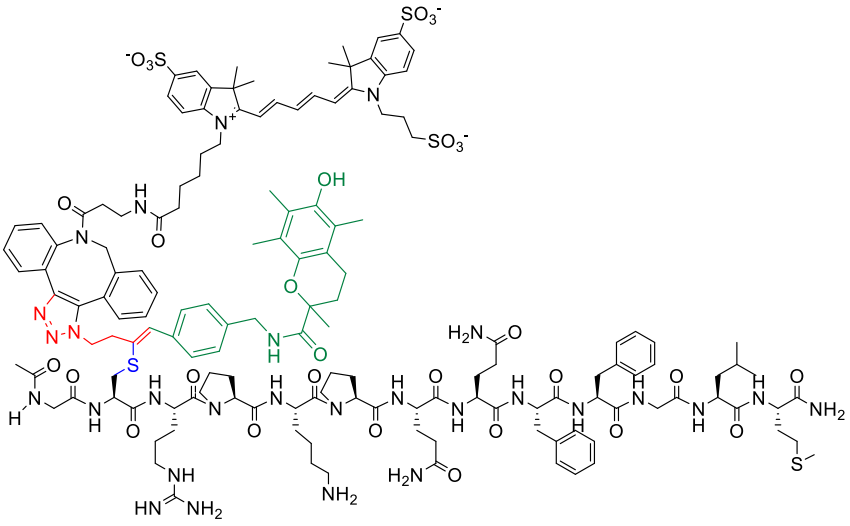


Figure S212: HPLC-MS chromatogram of **38**.

c. SPAAC on Trolox-Substance P conjugate (**38**)



(**114**)

To a solution of the Trolox-substance P (**38**) conjugate in dmso (11.1 µL, 20 mM), another solution of dibenzocyclooctyne-Cy5 (**23d**) in dmso (11.1 µL, 20 mM) was added. The reaction was vortexed and left on the bench at room temperature for 1.5 hours. No effort was made to exclude oxygen. The reaction was analyzed by HPLC-MS. The crude product was used for subsequent studies as a 10 mM solution without need for further purification.

HRMS (ESI/QTOF) m/z: $[M]^+$ Calcd for $C_{147}H_{190}N_{28}O_{30}S_5^{-2}$ 1493.6409; Found 1493.6242.

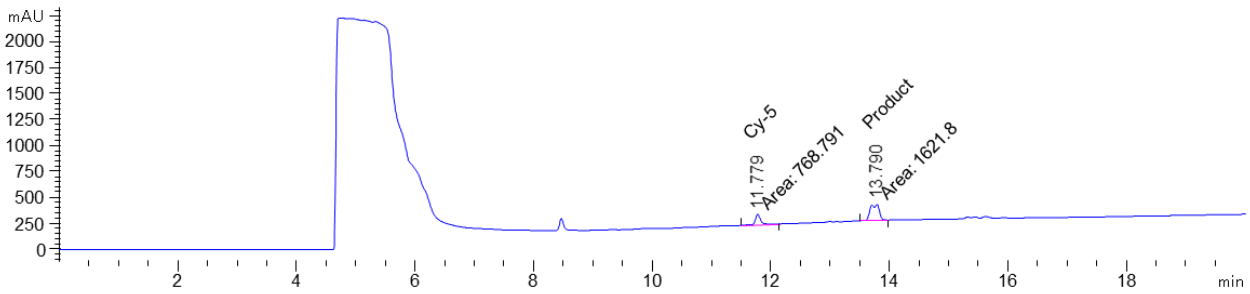


Figure S213: HPLC-UV chromatogram at 214 nm of **114**.

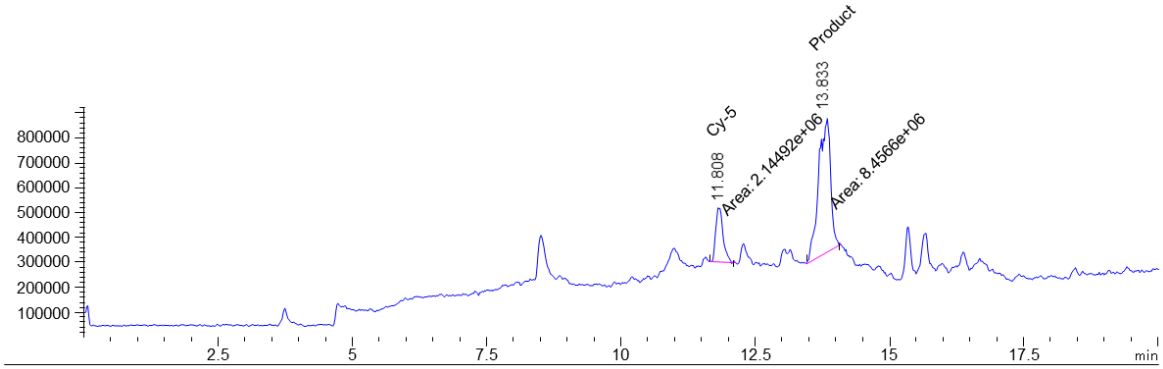
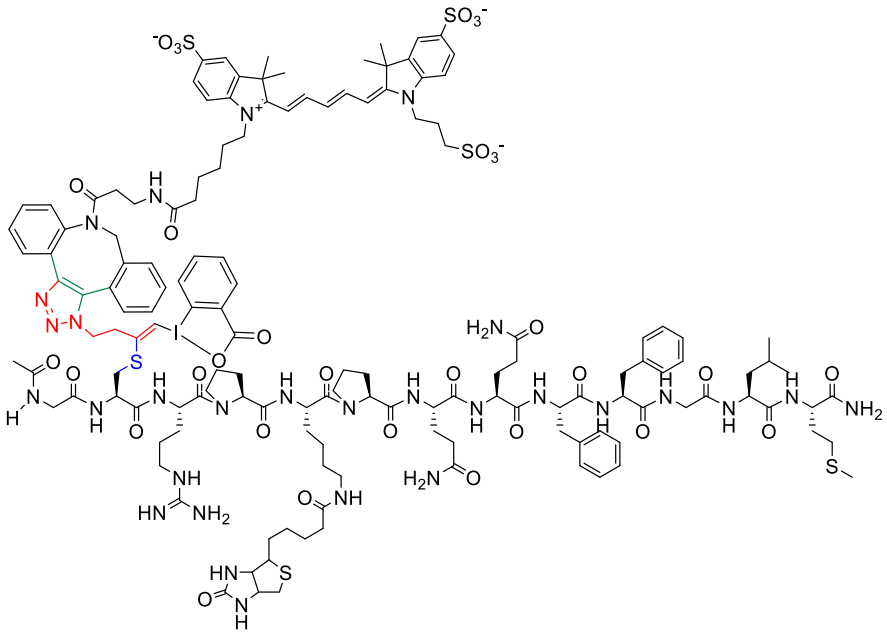


Figure S214: HPLC-MS chromatogram of **114**.

18. smTIRF experiments

a. Synthesis of biotinylated Cy5 Substance P conjugate (**40**)



In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, a 5.00 mM Cy5-Substance P (**36**) solution in DMSO (13.0 µL, 65.0 nmol, 1.00 equiv.) was diluted to 1.00 mM in phosphate buffer (51.6 µL, 50.0 mM, pH 8). The resulting solution was vortexed few seconds and a 10.0 mM solution of biotin-NHS (**39**) in DMSO (6.50 µL, 65.0 nmol, 1.00 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **40** (retention time: 22.322 minutes, yield 82%). 10.0 µL of the reaction mixture were diluted to 100 µL with 6.00 M GdmHCl, pH 3 and purified by analytical RP-HPLC employing a linear gradient from 0 to 70% solvent B over 30 min. Fractions were analysed, pooled accordingly and lyophilised. Purified Bt-Cy5-Substance P conjugate **40** was dissolved in DMSO to a final concentration of 1.00 mM, aliquoted and stored at -20°C.

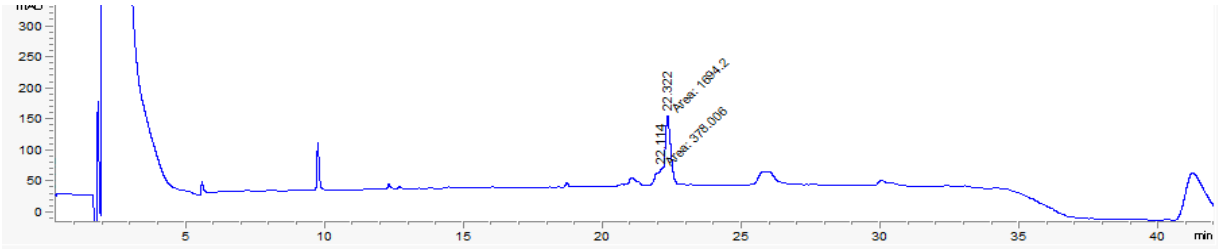


Figure S215: HPLC-UV chromatogram at 214 nm of **40.**

ESI-MS Calcd mass 3124.5 Da; Found 3125.2 Da.

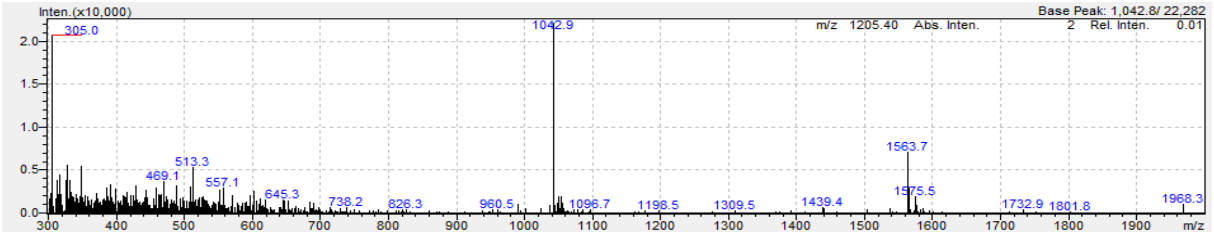
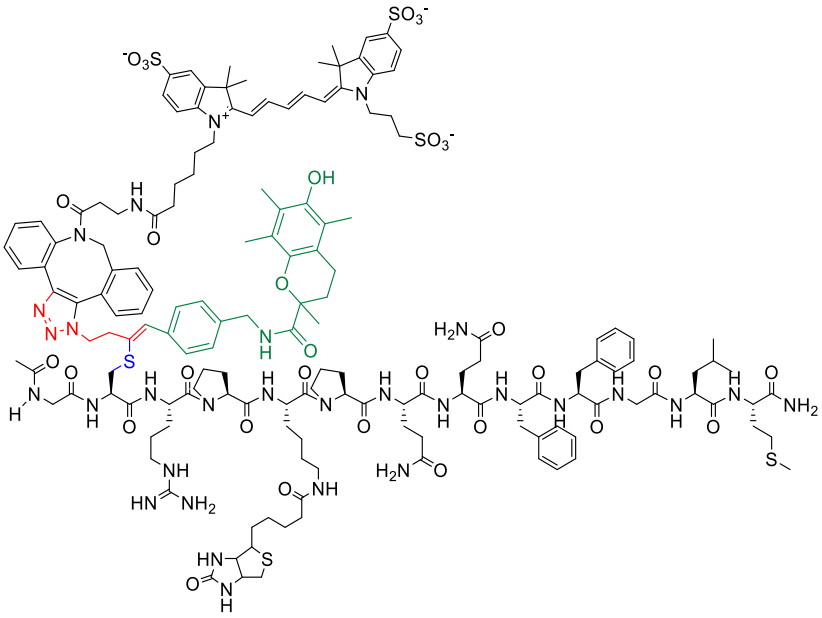


Figure S216: ESI-MS spectrum of **40.**

b. Synthesis of biotinylated Cy5-Trolox Substance P conjugate (**41**)



(**41**)

In a 1.50 mL Eppendorf Safe-Lock microcentrifuge tube, a 10.0 mM solution of crude Cy5-Trolox-Substance P in DMSO (**114**) (6.50 µL, 65.0 nmol, 1.00 equiv.) was diluted to 1.00 mM in phosphate buffer (58.2 µL, 50.0 mM, pH 8). The resulting solution was vortexed few seconds and a 10.0 mM solution of biotin-NHS (**39**) in DMSO (6.50 µL, 65.0 nmol, 1.00 equiv.) was added in one portion. The resulting mixture was vortexed few seconds to ensure proper reagent mixing and shaken at 300 rpm at room temperature for 60 minutes to afford **41** (retention time: 24.818 minutes, yield 97%). 10 µL of the reaction mixture were diluted to 100 µL with 6.00 M GdmHCl, pH 3 and purified by analytical RP-HPLC employing a linear gradient from 0 to 70% solvent B over 30 min. Fractions were analysed, pooled accordingly and lyophilised. Purified Bt-Cy5-Trolox-Substance P conjugate **41** was dissolved in DMSO to a final concentration of 1.00 mM, aliquoted and stored at -20°C.

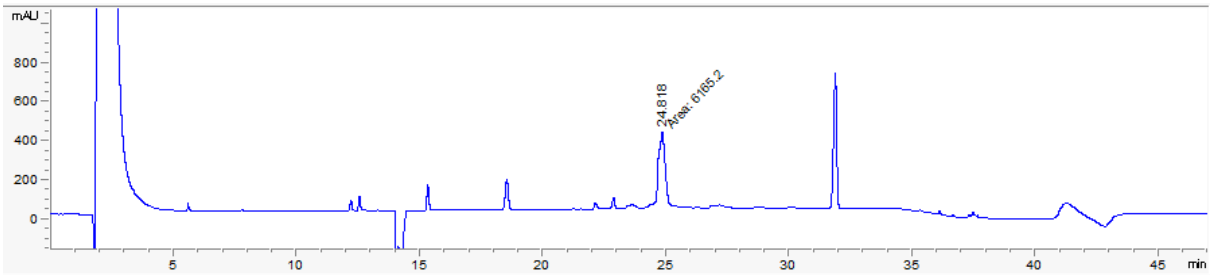


Figure S217: HPLC-UV chromatogram at 214 nm of **41**.

ESI-MS Calcd mass 3215.9 Da; Found 3217.2 Da.

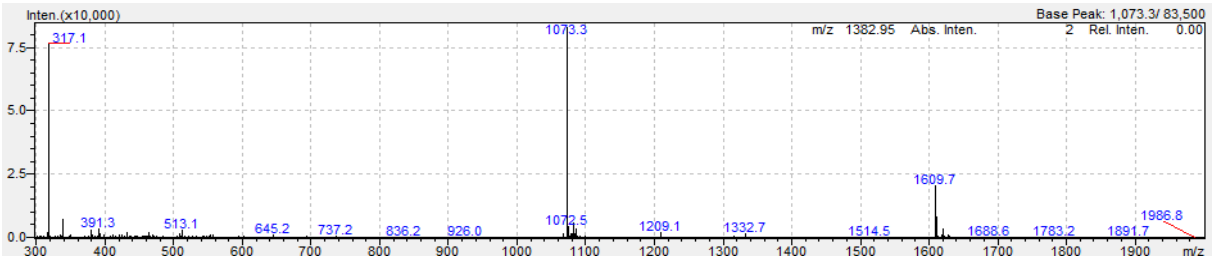


Figure S218: ESI-MS spectra of **41**.

c. smTIRF imaging and data processing

SP-Cy5-Trolox-bt (**41**) or SP-Cy5-bt (**40**) were immobilized within polyethylene-glycol (PEG) passivated flow channels using neutravidin–biotin attachment chemistry.[1] The imaging buffer composition was 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM 2-mercaptoethanol, 3.2% (w/v) glucose, 1 mg/mL glucose oxidase, 40 μg/mL catalase in ultrapure degassed H_2O, filtered. Surface-immobilized molecules were imaged using a fully automated Nikon Eclipse Ti-E inverted fluorescence microscope with a manually controlled TIRF illuminator arm and equipped with a CFI Apo TIRF 100x Oil immersion objective (NA 1.49). Data was acquired using an Andor iXon EMCCD camera. Movies were recorded with a 100-ms integration time per frame. Typical movies contained 1800 frames. SP-Cy5-Trolox-bt (**41**) or SP-Cy5-bt (**40**) photobleaching was observed using a 640-nm laser line (Coherent Obis). During measurements, a laser intensity 0.74 mW was employed.

For data analysis, a custom-made Matlab script was employed.[1] In short, a baseline correction and a drift correction was performed. A peak-finding algorithm was employed to detect individual chromatin array positions. Fluorescence intensity traces for each chromatin position were obtained by integrating over a circle of 2 pixel radius. Traces over 200-300 individual molecules were averaged and analyzed by a single-exponential decay to obtain bleaching rate- or time constants.

19. Confocal microscopy

HEK 293T NK1-GFP stable cell lines were plated on poly-lysine coated 12-well plates (seeding density 10^5 cell/well, surface area 3.5 cm^2) and cultured for 36 h at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and Penicillin-Streptomycin antibiotics (Penicillin G Sodium Salt: 100 lU/mL, Streptomycin Sulfate: 100 µg/mL). Cells were washed with Opti-MEM Reduced Serum Medium and incubated with 1 mL of 20 nM SP-Cy5 (**36**) solution in Opti-MEM. Time-lapse images were acquired on a confocal microscope LSM 510 Meta (Zeiss), using a C-Apochromat 63x objective. Imaging was performed using the following parameters: pixel time: 3.2 µs, excitation lasers: 488 nm (16% emission power) and 633 nm (26% emission power), detector gain: 696.

20. Spectra of new compounds

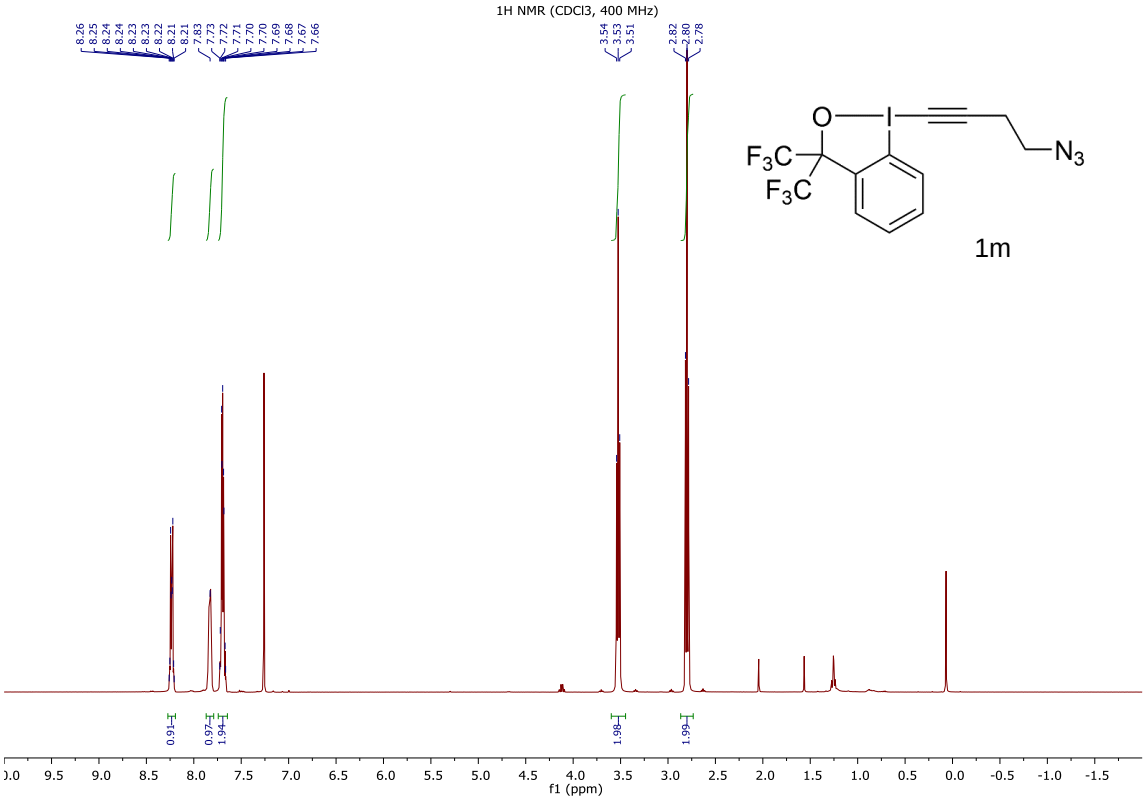


Figure S219: 1H NMR spectrum of **1a.**

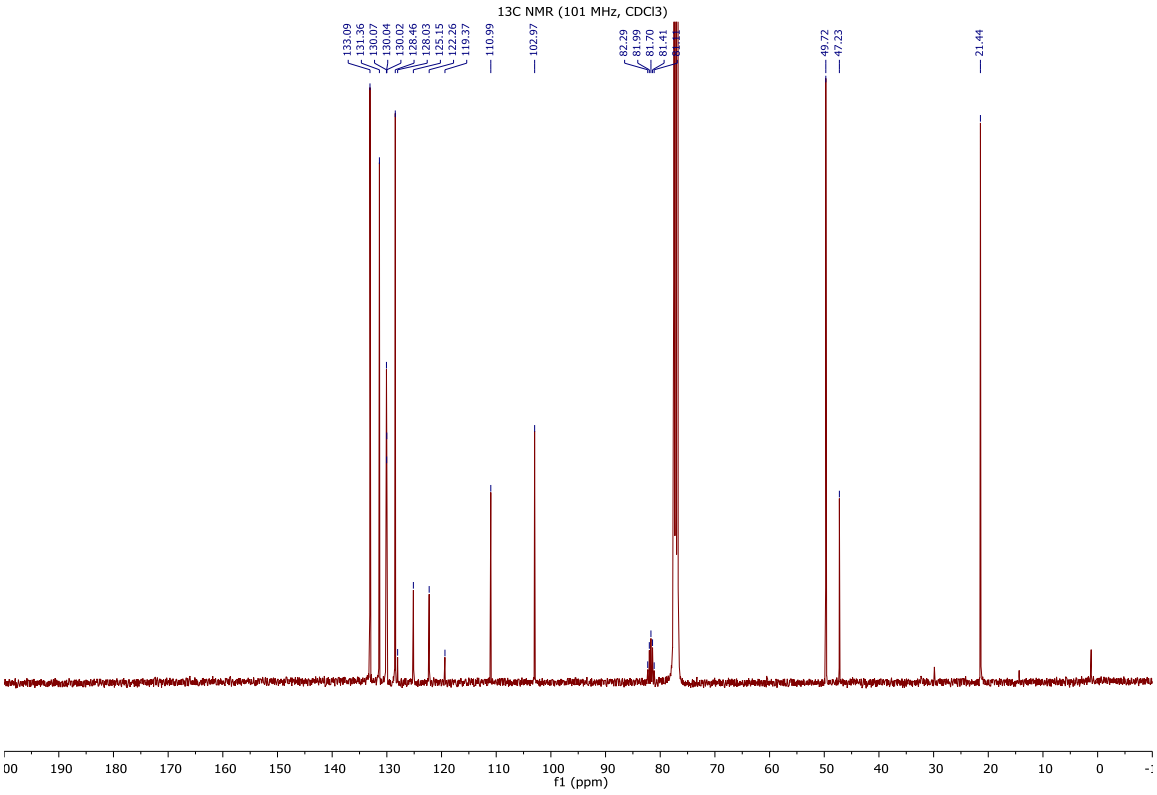


Figure S220: 13C NMR spectrum of **1a.**

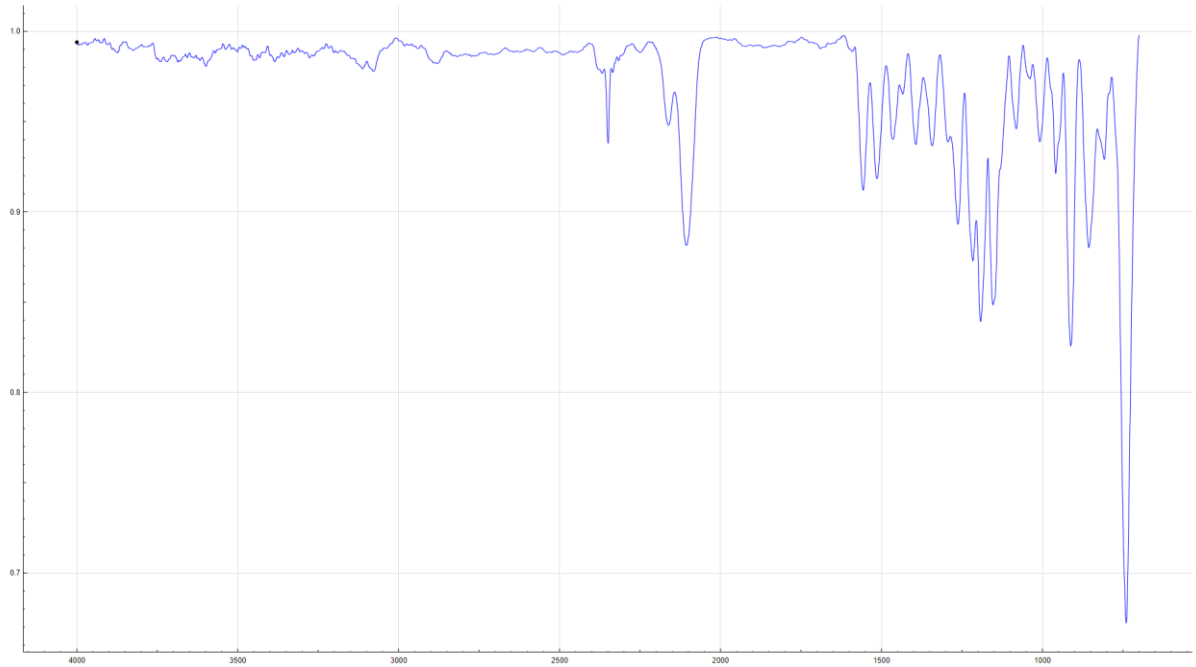


Figure S221: IR spectrum of **1a.**

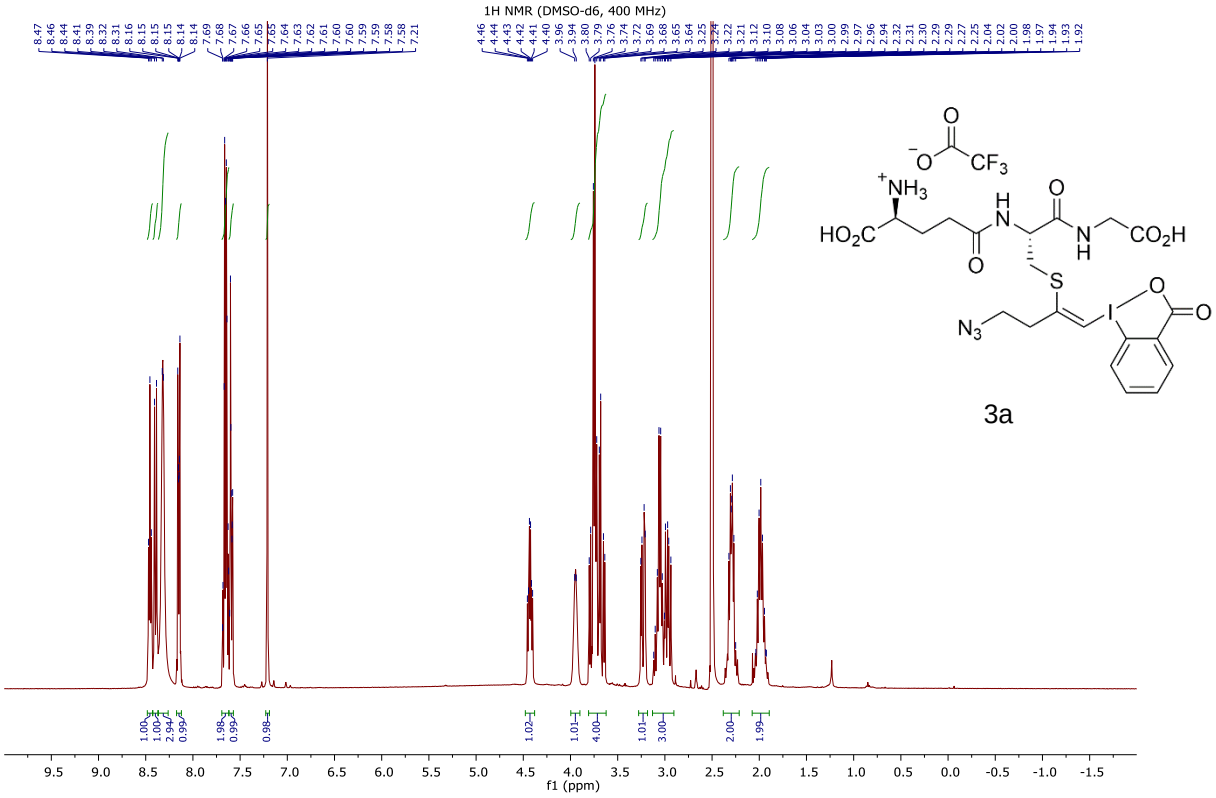


Figure S222: 1H NMR spectrum of **3a**.

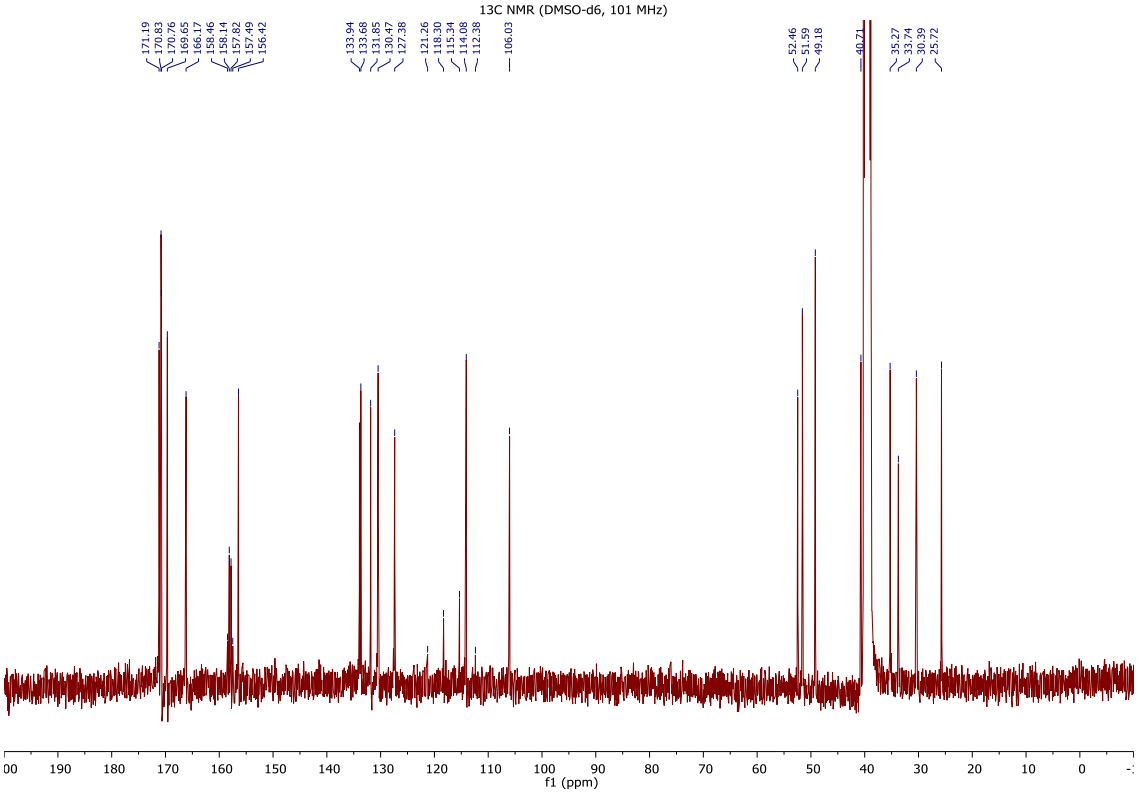


Figure S223: 13C NMR spectrum of **3a**,

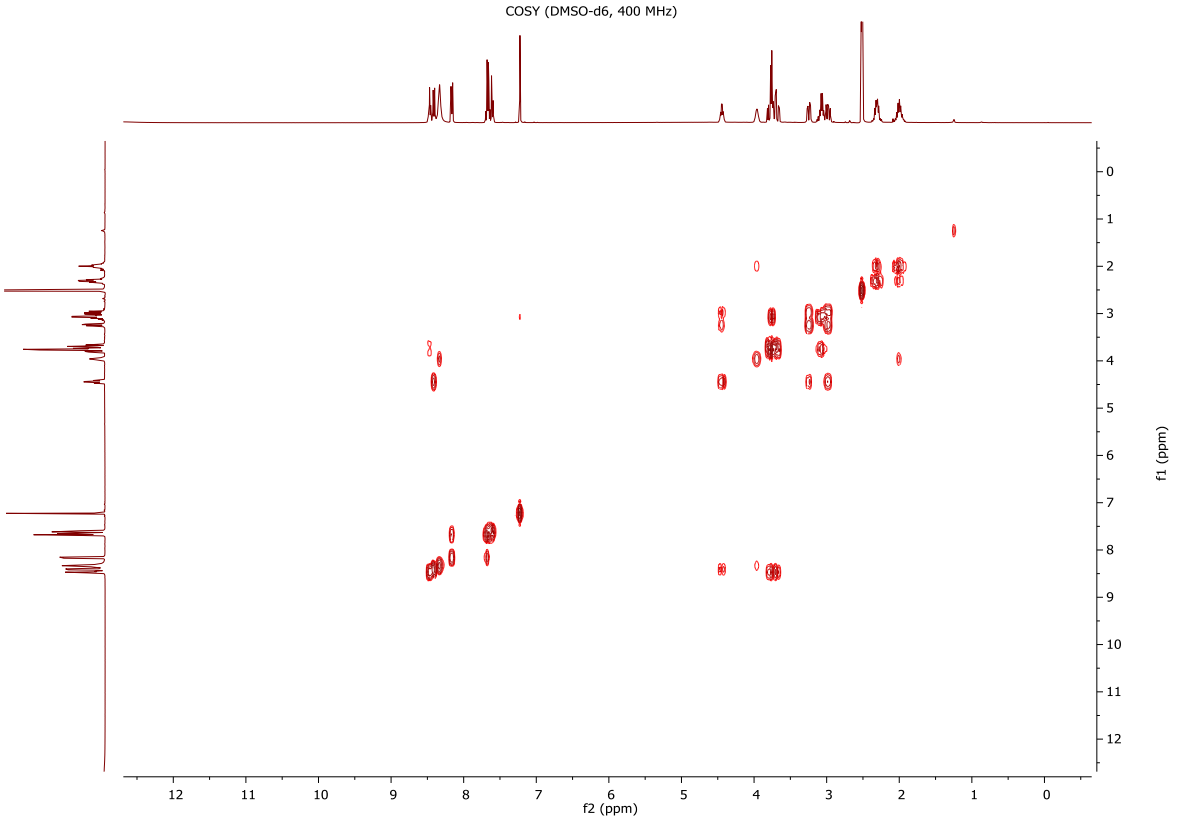


Figure S224: COSY NMR spectrum of **3a**.

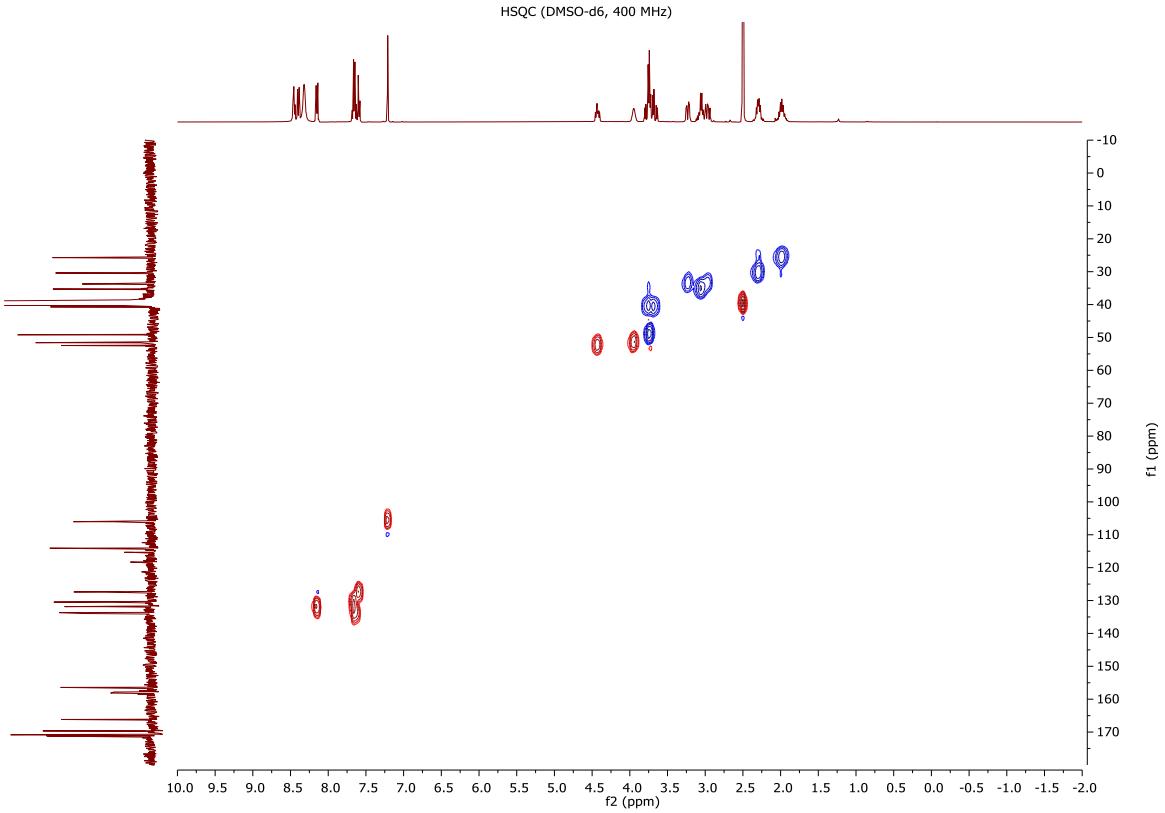


Figure S225: HSQC NMR spectrum of **3a.**

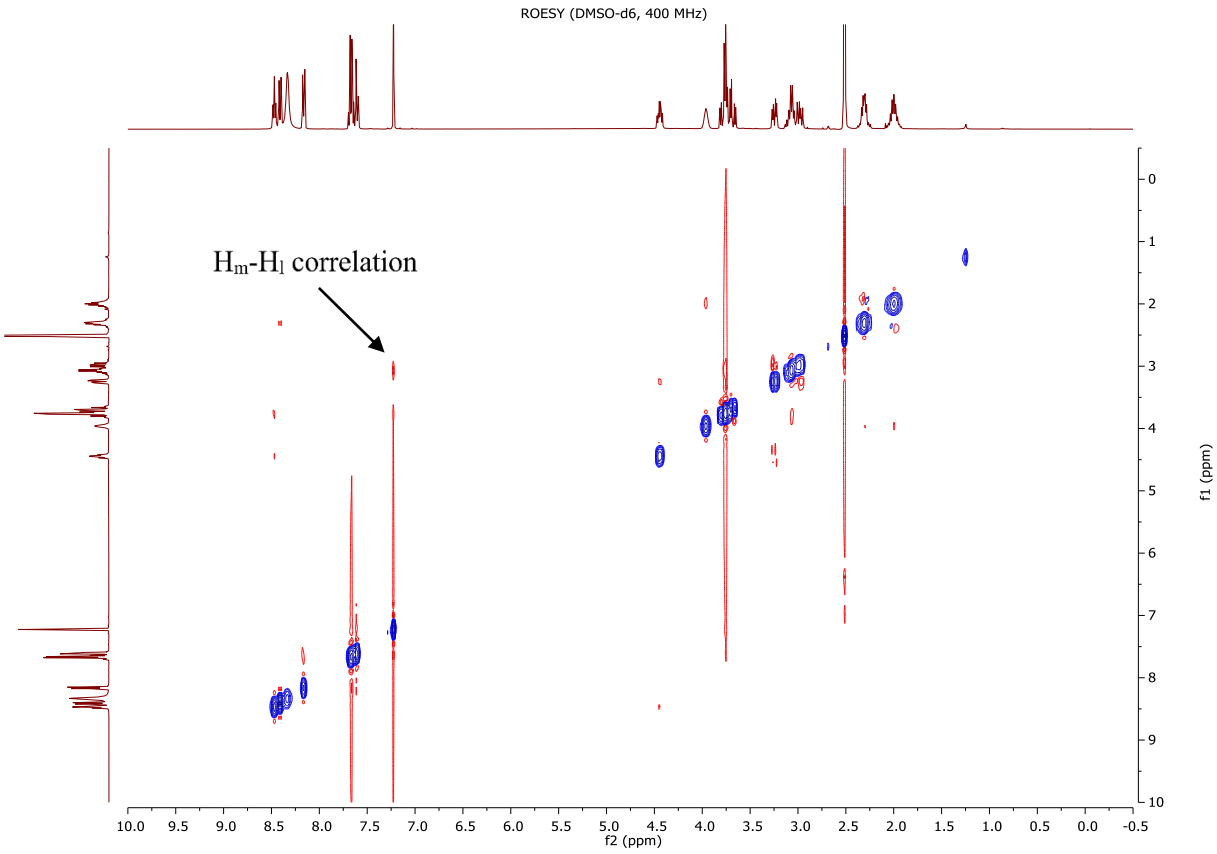


Figure S226: ROESY NMR spectrum of **3a**.

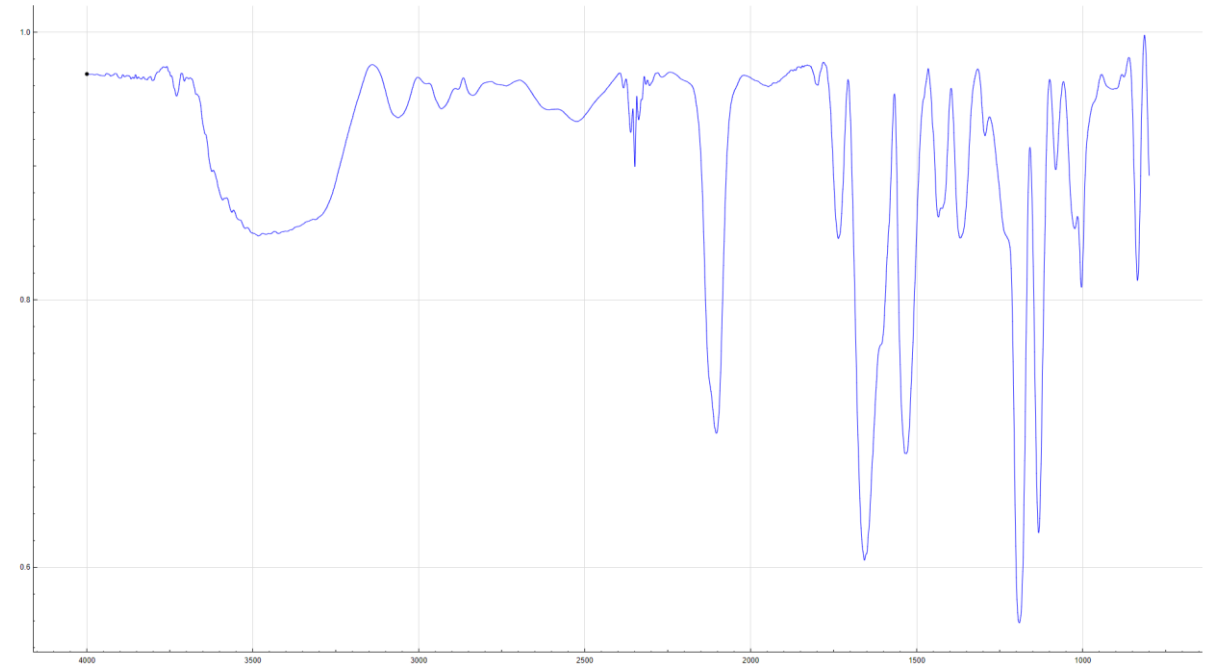


Figure S227: IR spectrum of **3a**.

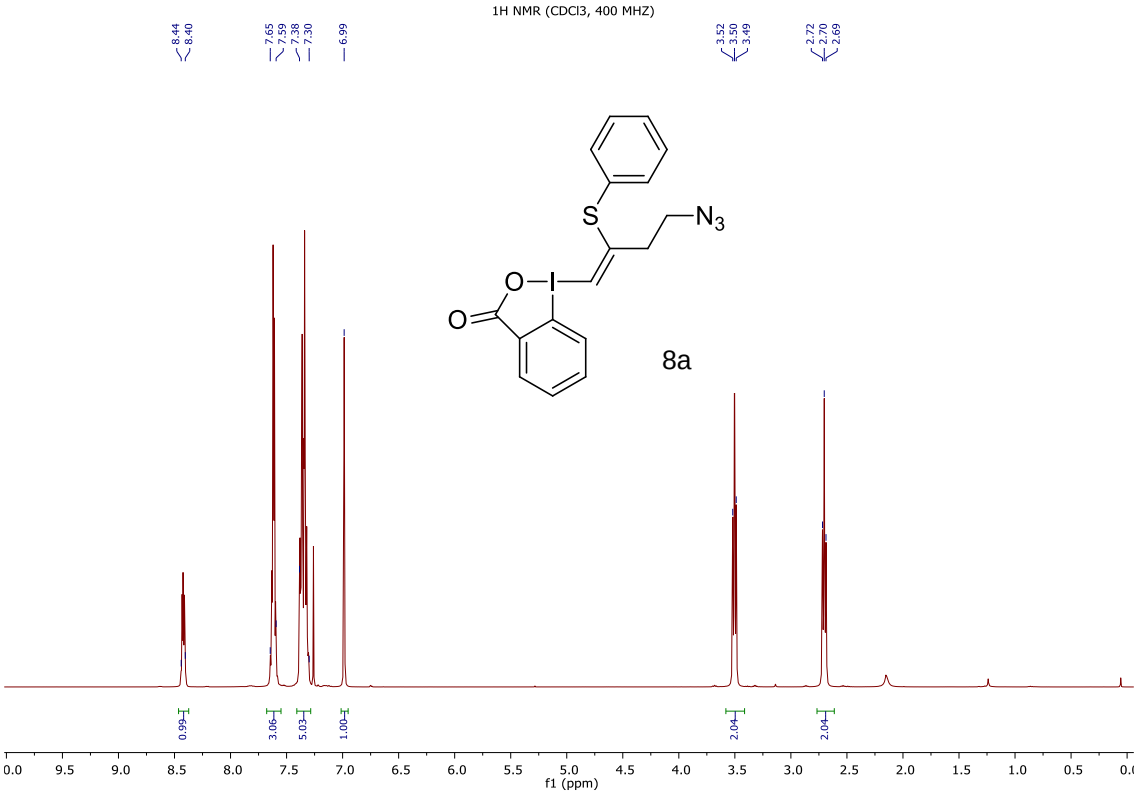


Figure S228: 1H NMR spectrum of **8a**.

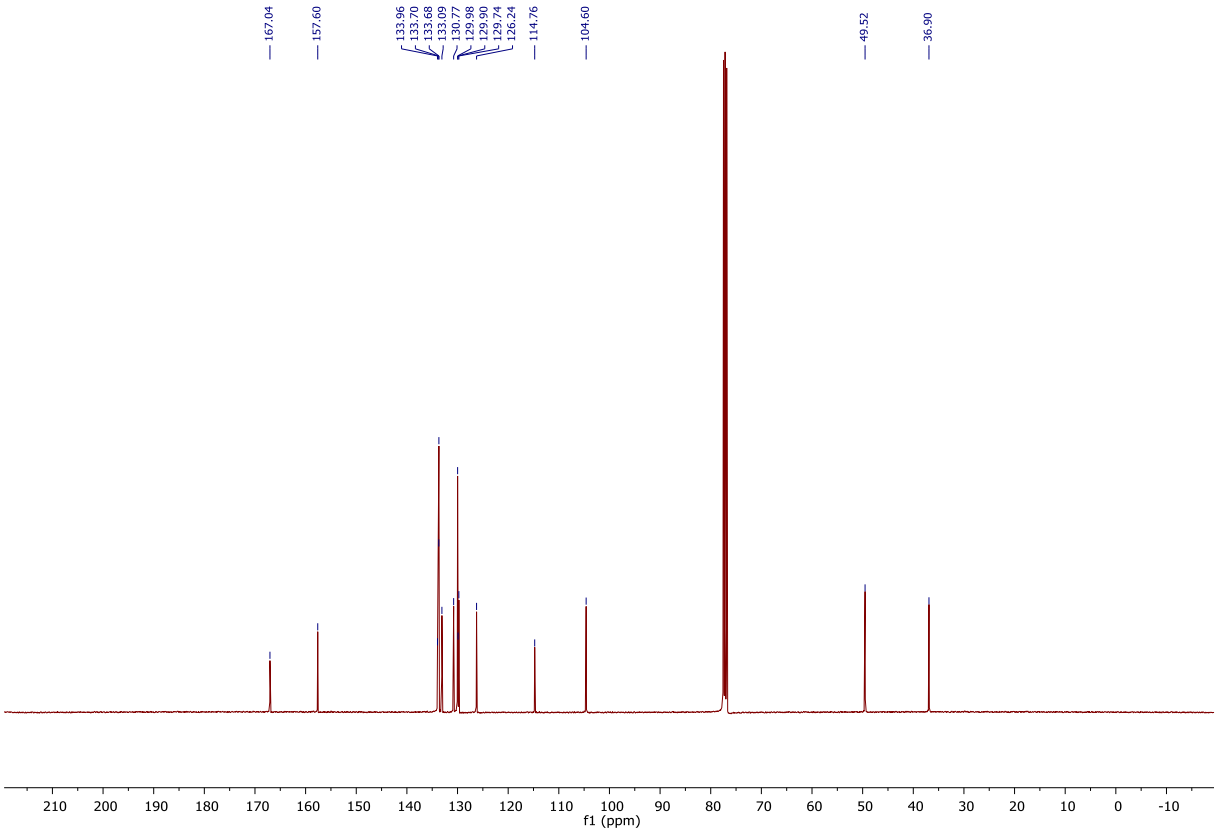


Figure S229: 13C NMR spectrum of **8a**.

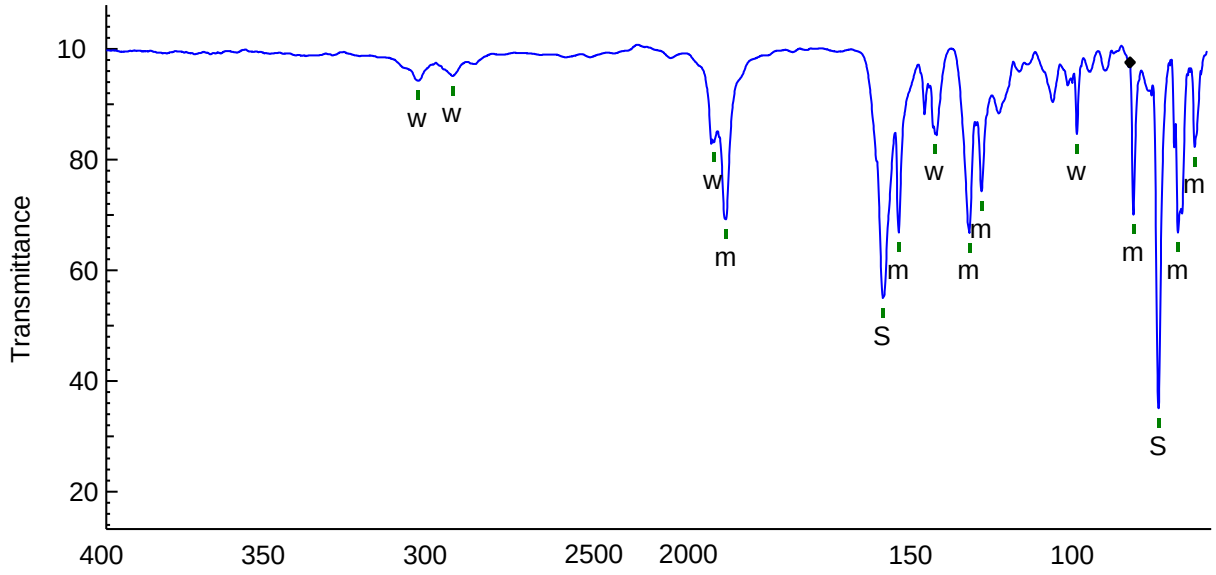


Figure S230: IR spectrum of **8a.**

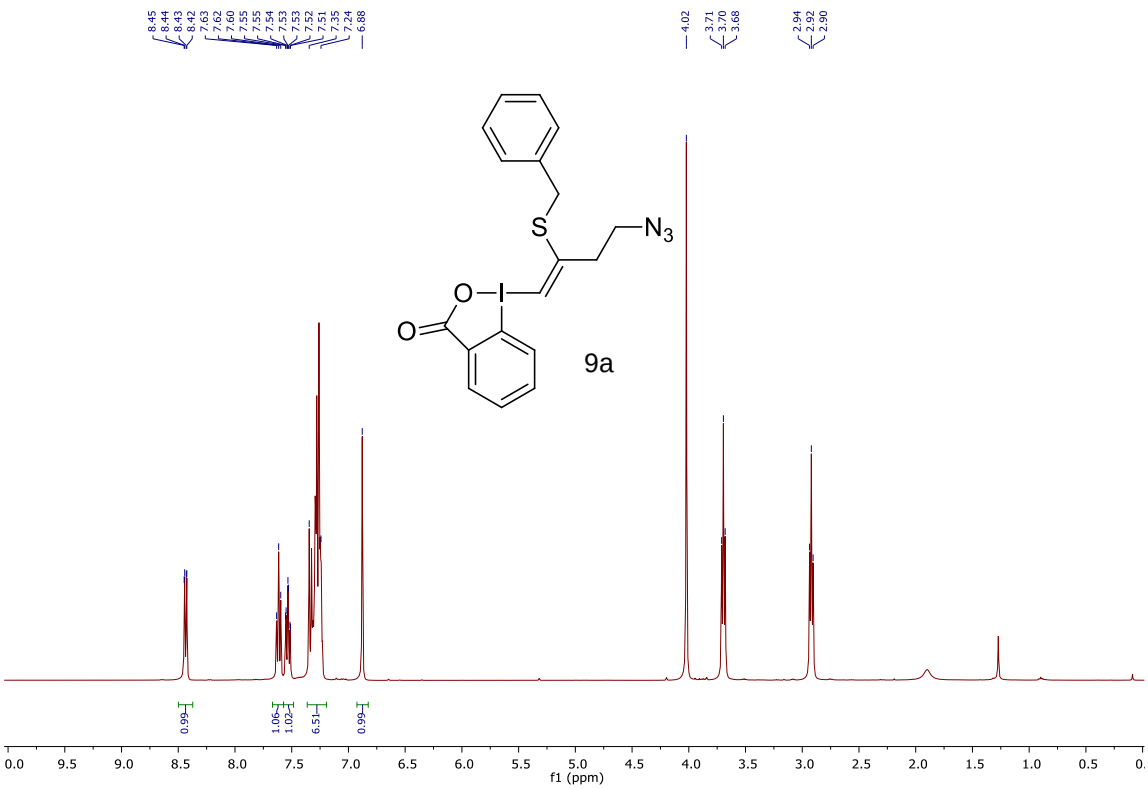


Figure S231: 1H NMR spectrum of **9a.**

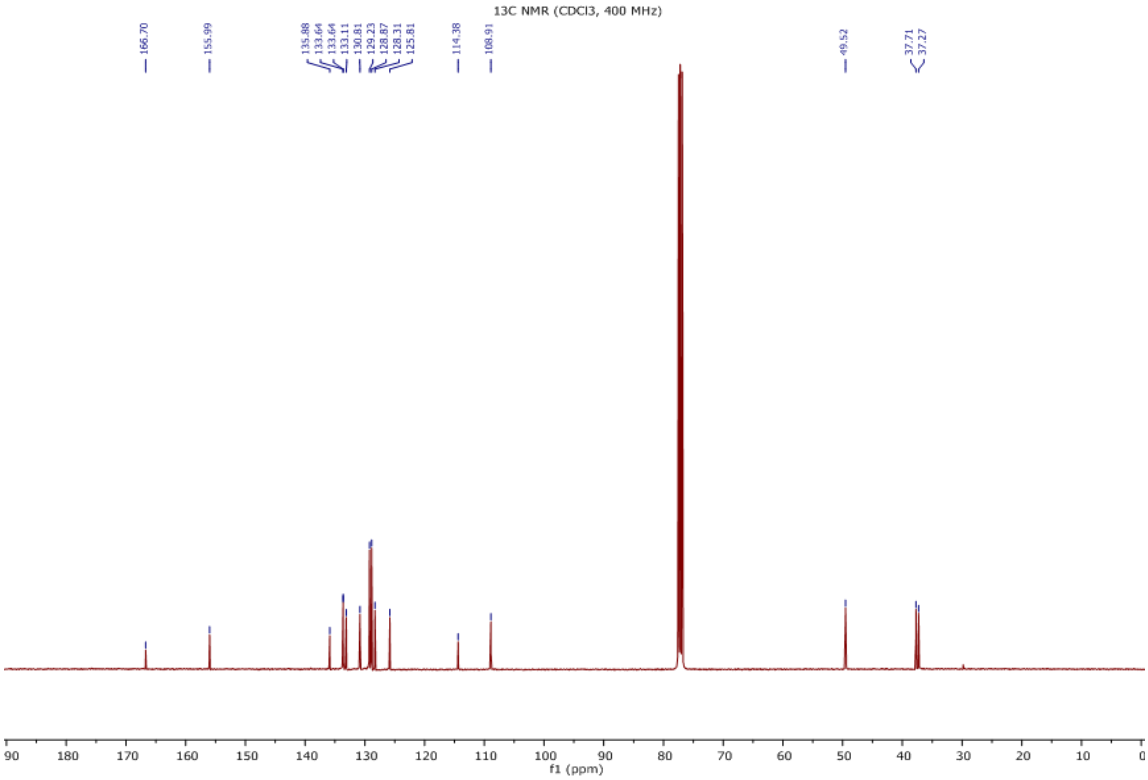


Figure S232: 13C NMR spectrum of **9a.**

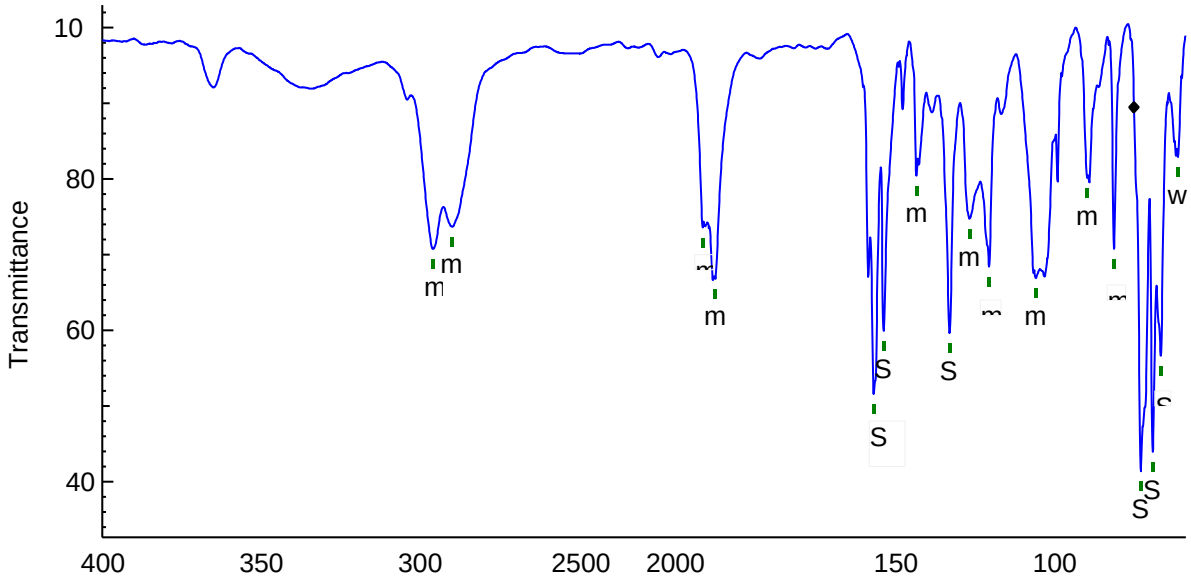


Figure S233: IR spectrum of **9a**.

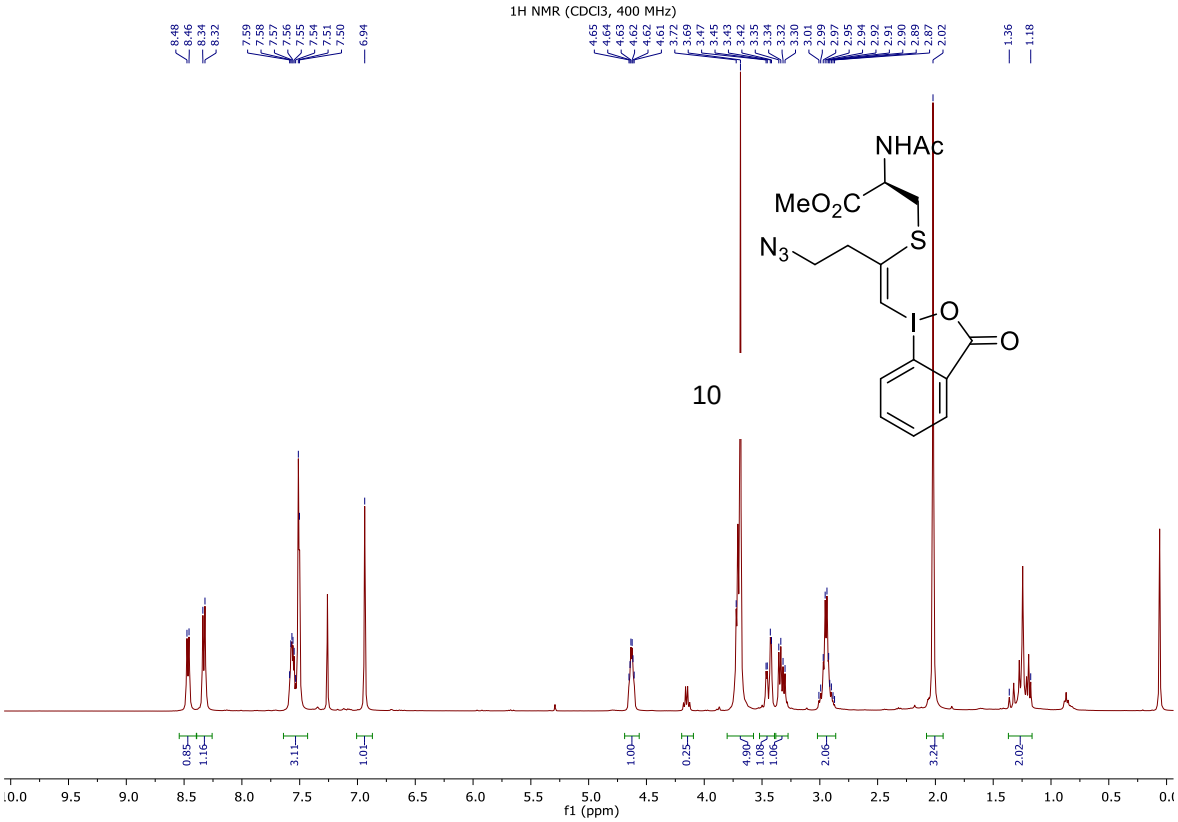


Figure S234: 1H NMR spectrum of **10.**

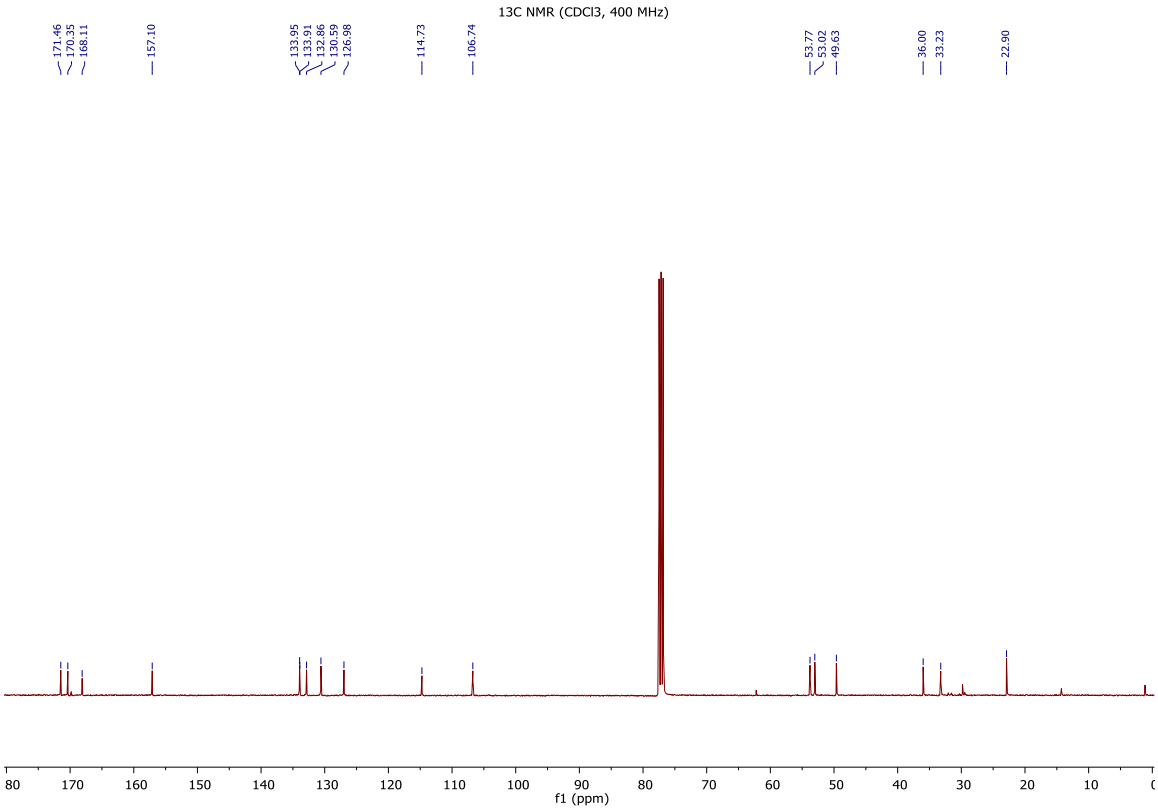


Figure S235: 13C NMR spectrum of **10.**

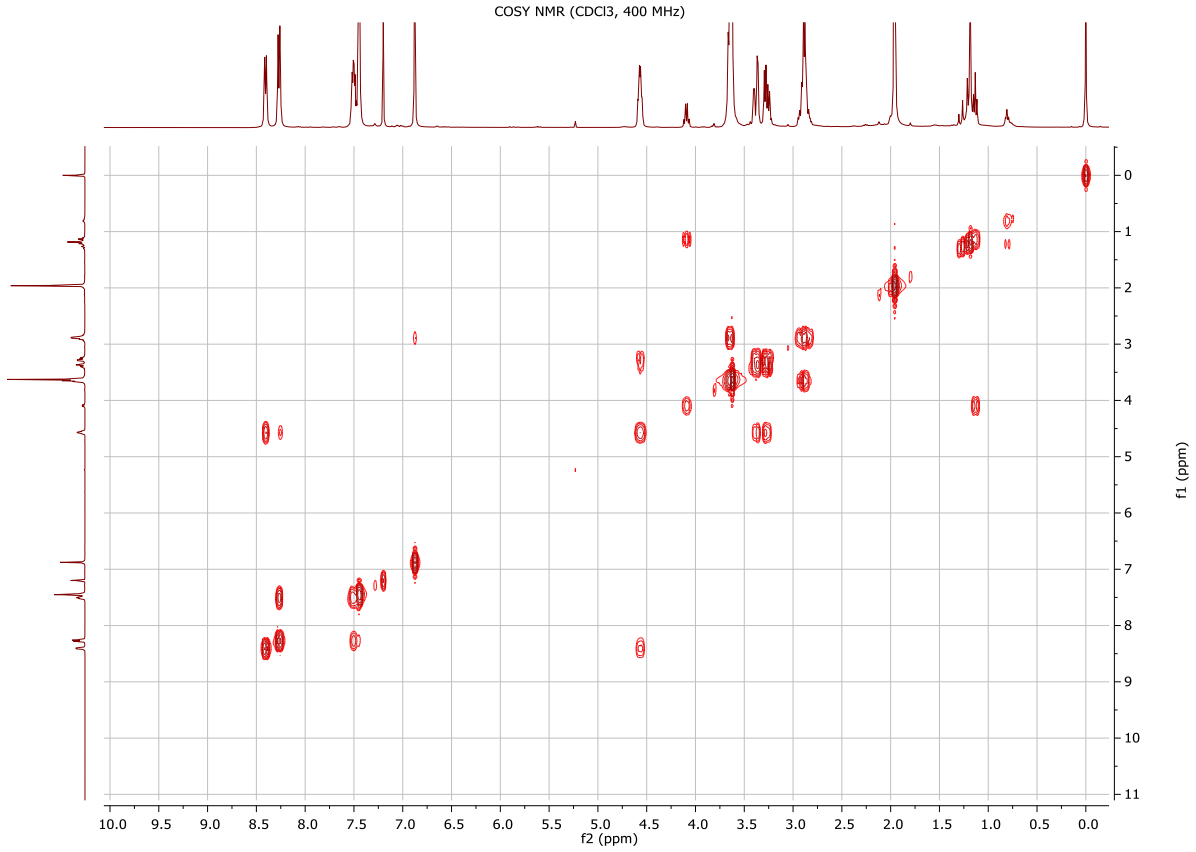


Figure S236: COSY NMR spectrum of **10.**

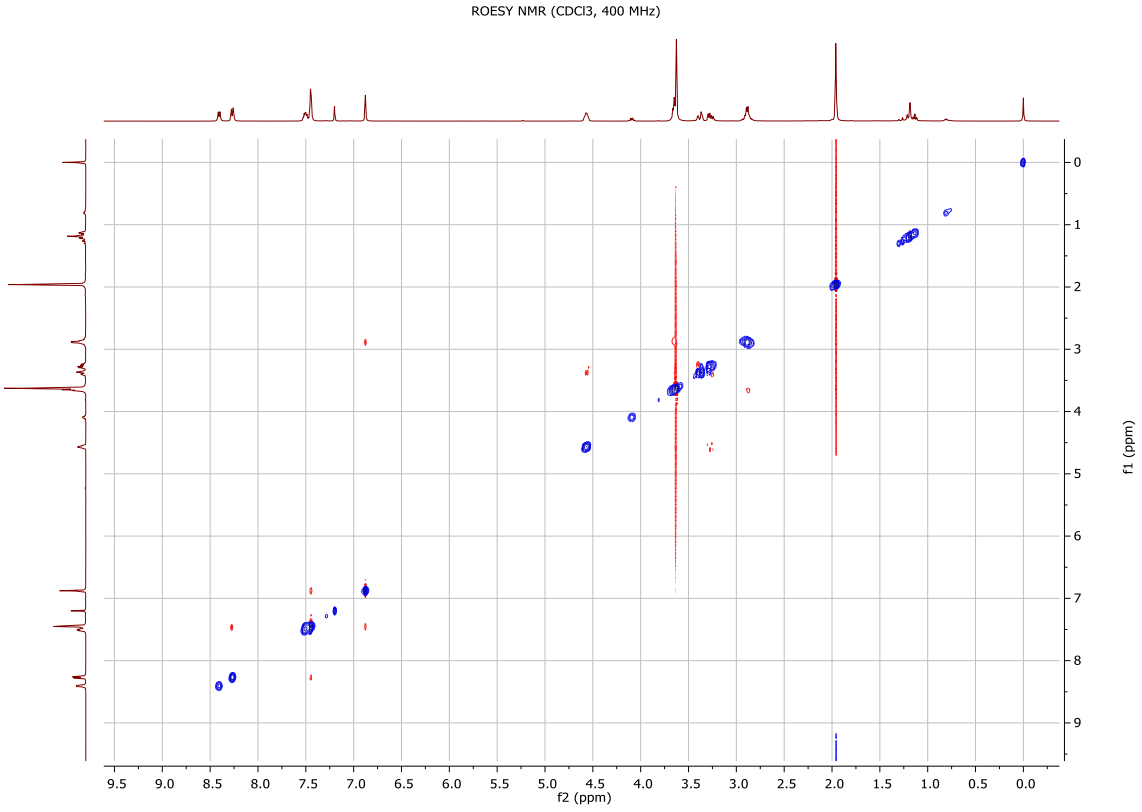


Figure S237: ROESY NMR spectrum of **10.**

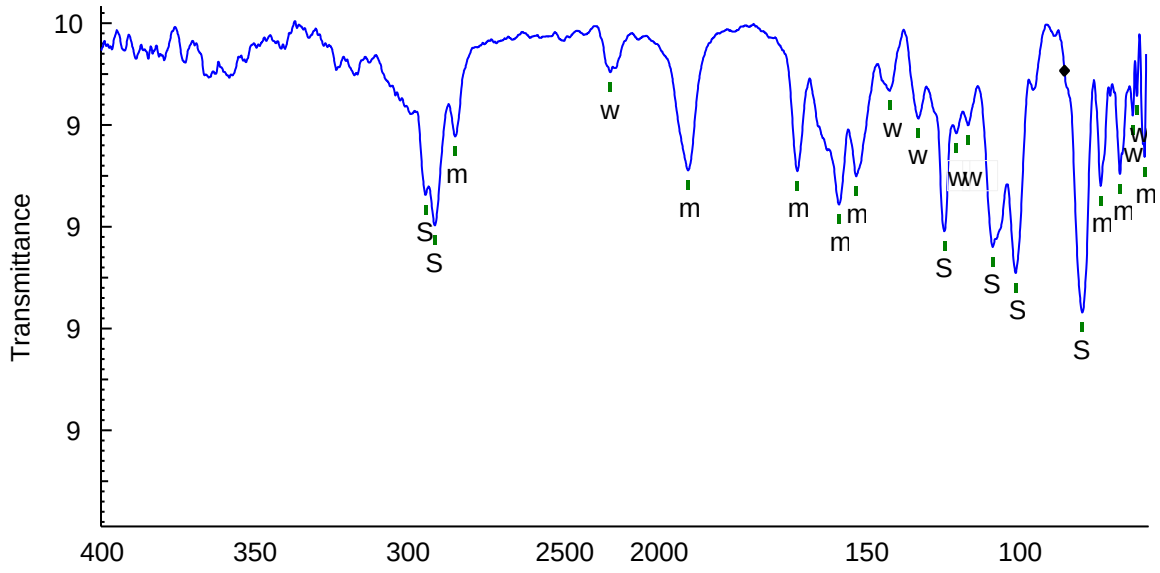
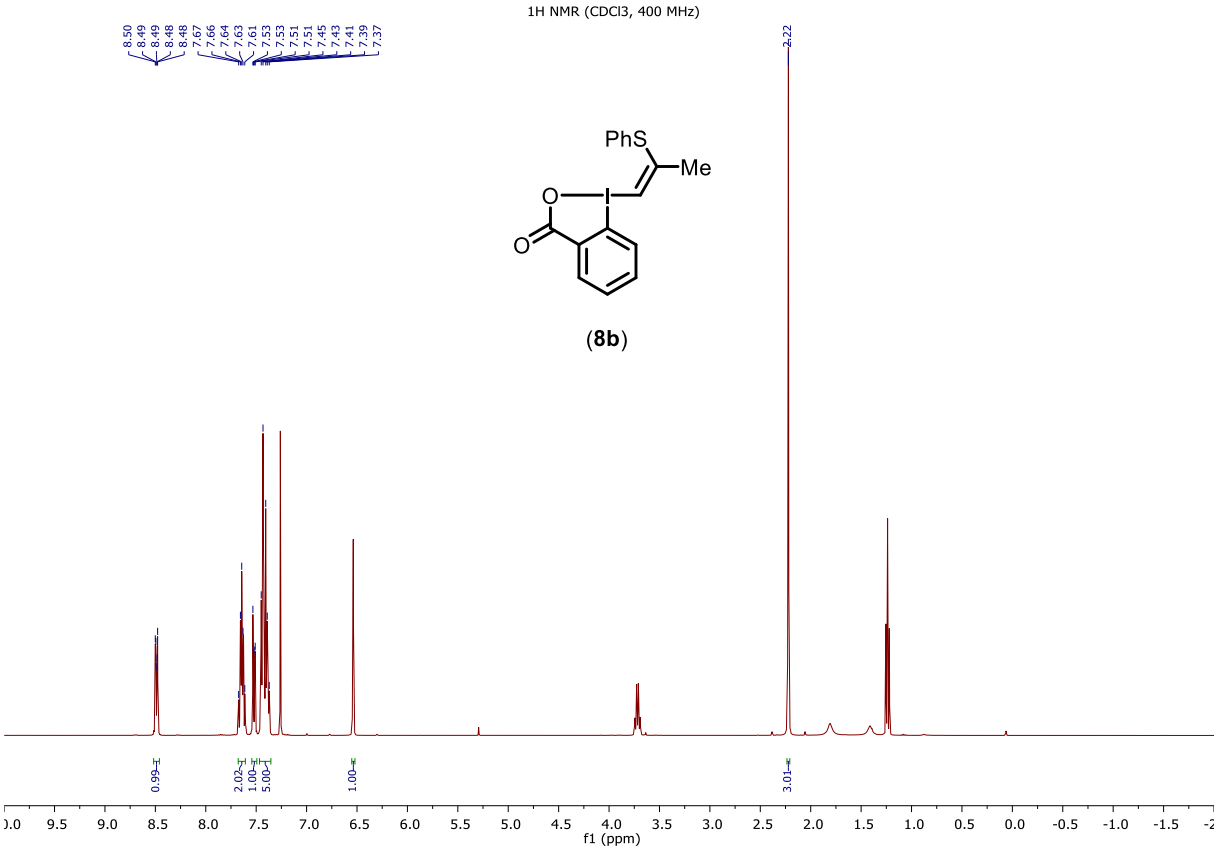


Figure S238: IR spectrum of **10**.



(**8b**)

Figure S239: 1H NMR spectrum of **8b.**

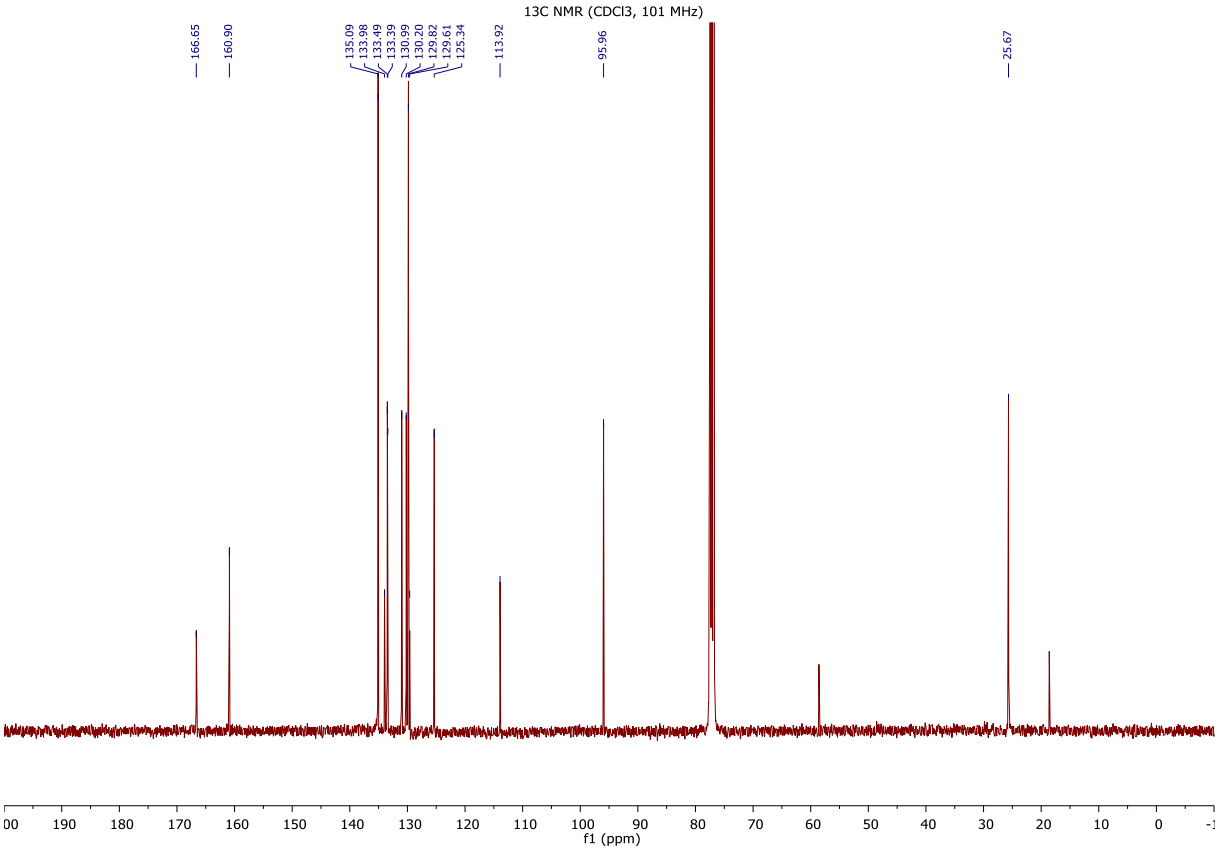


Figure S240: 13C NMR spectrum of **8b.**

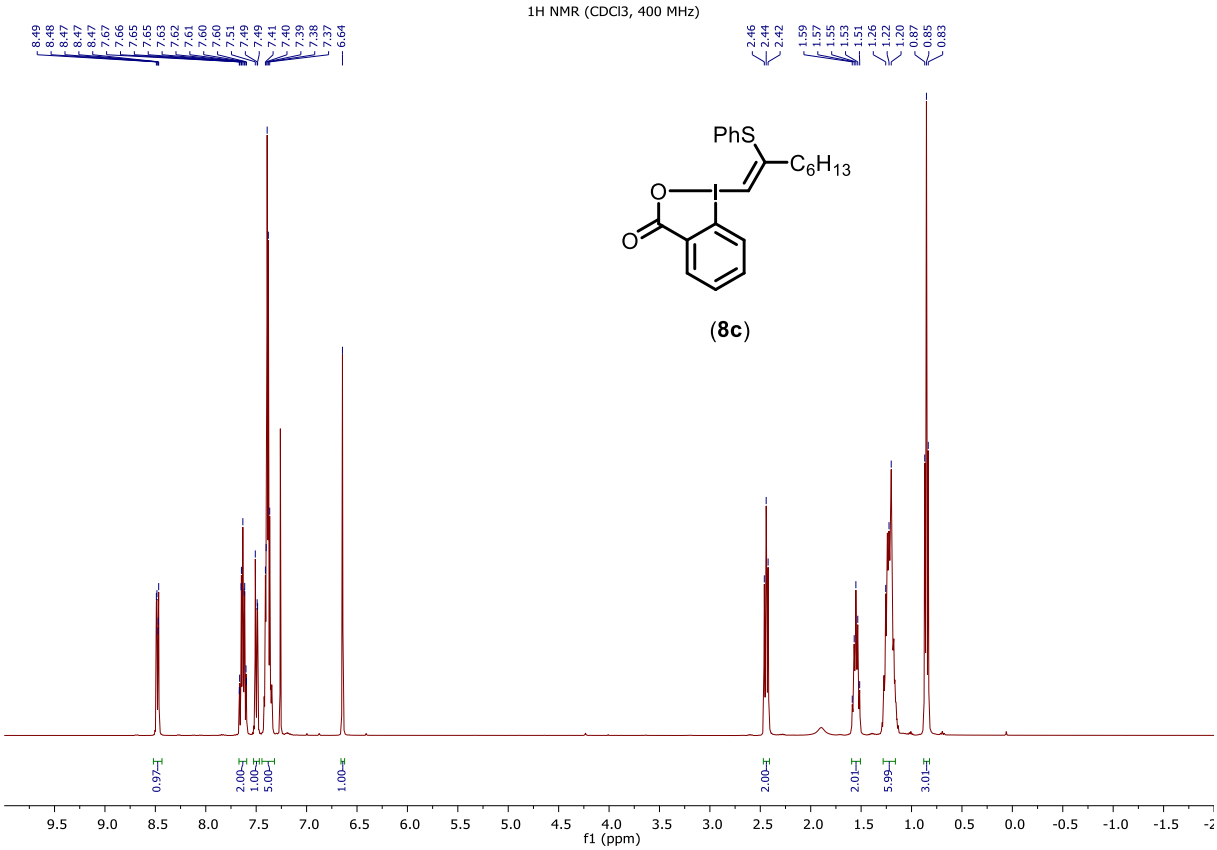


Figure S241: 1H NMR spectrum of **8c.**

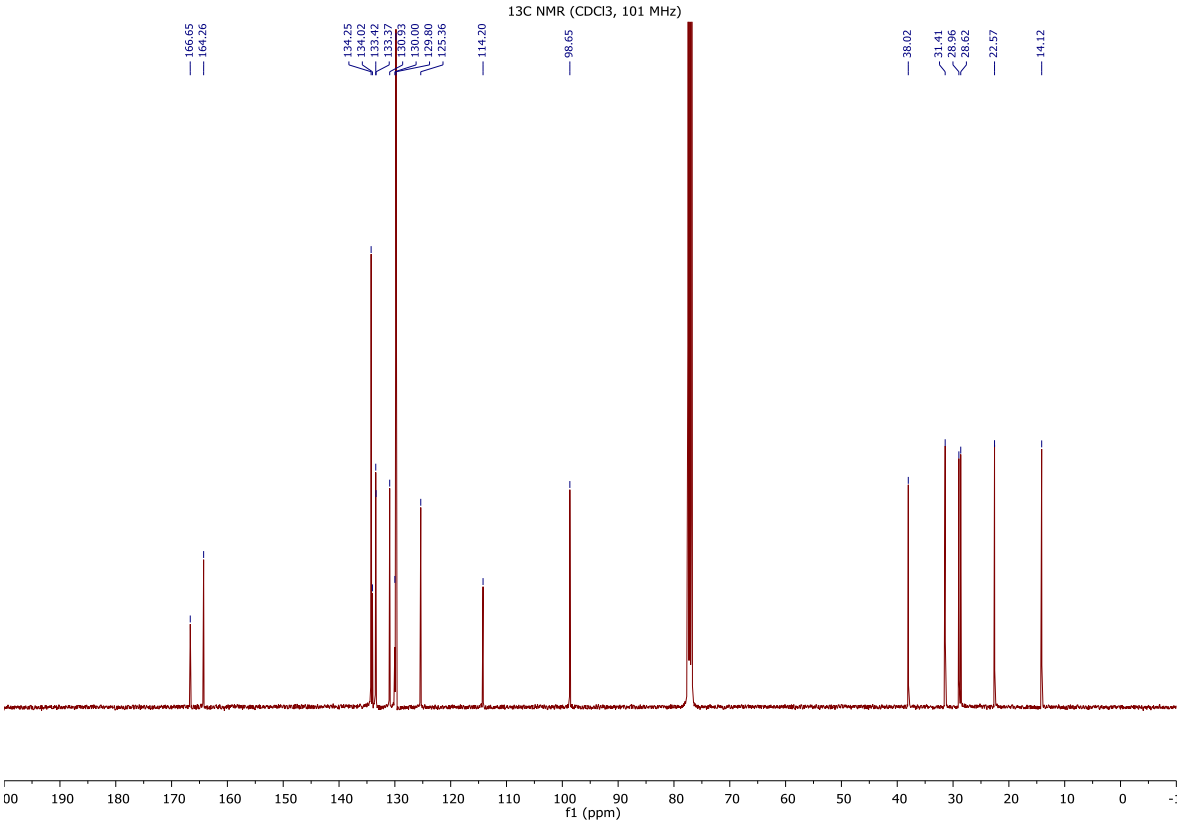


Figure S242: 13C NMR spectrum of **8c**.

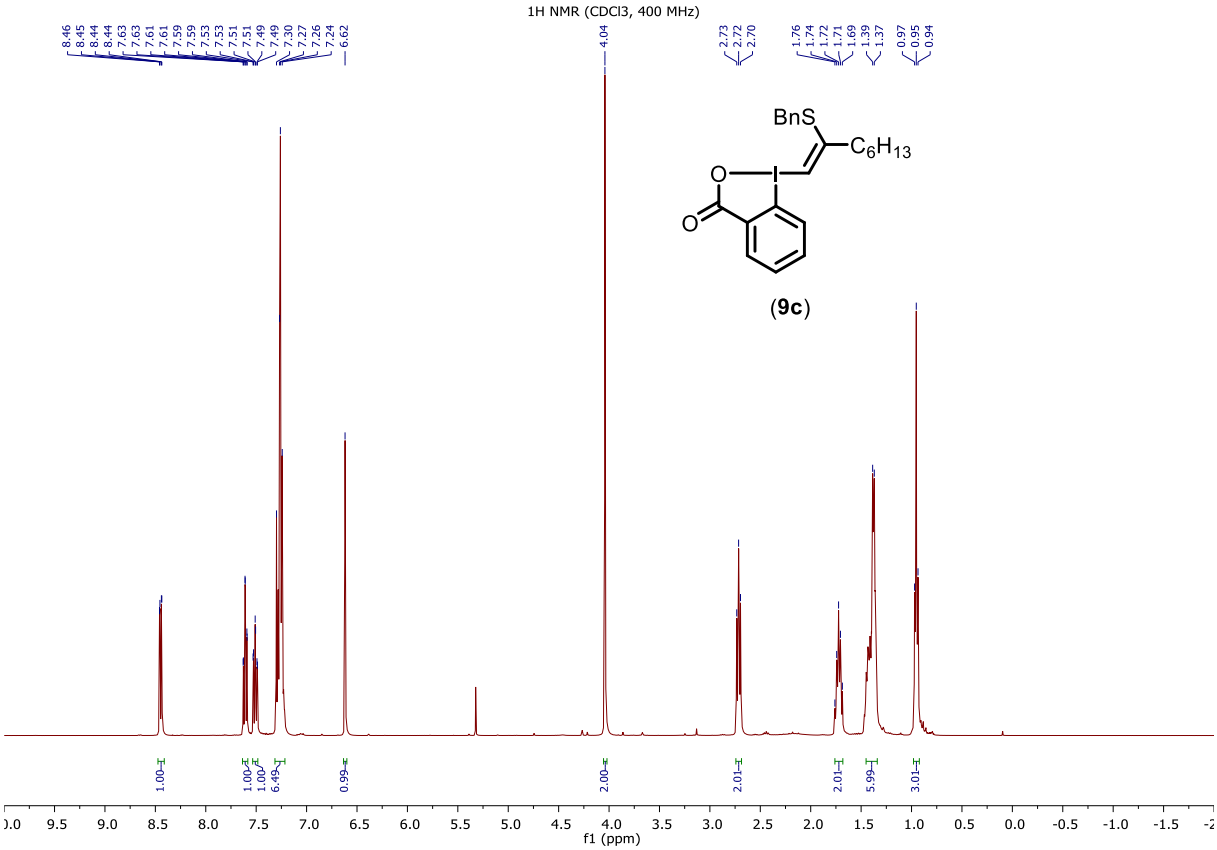


Figure S243: 1H NMR spectrum of **9c**.

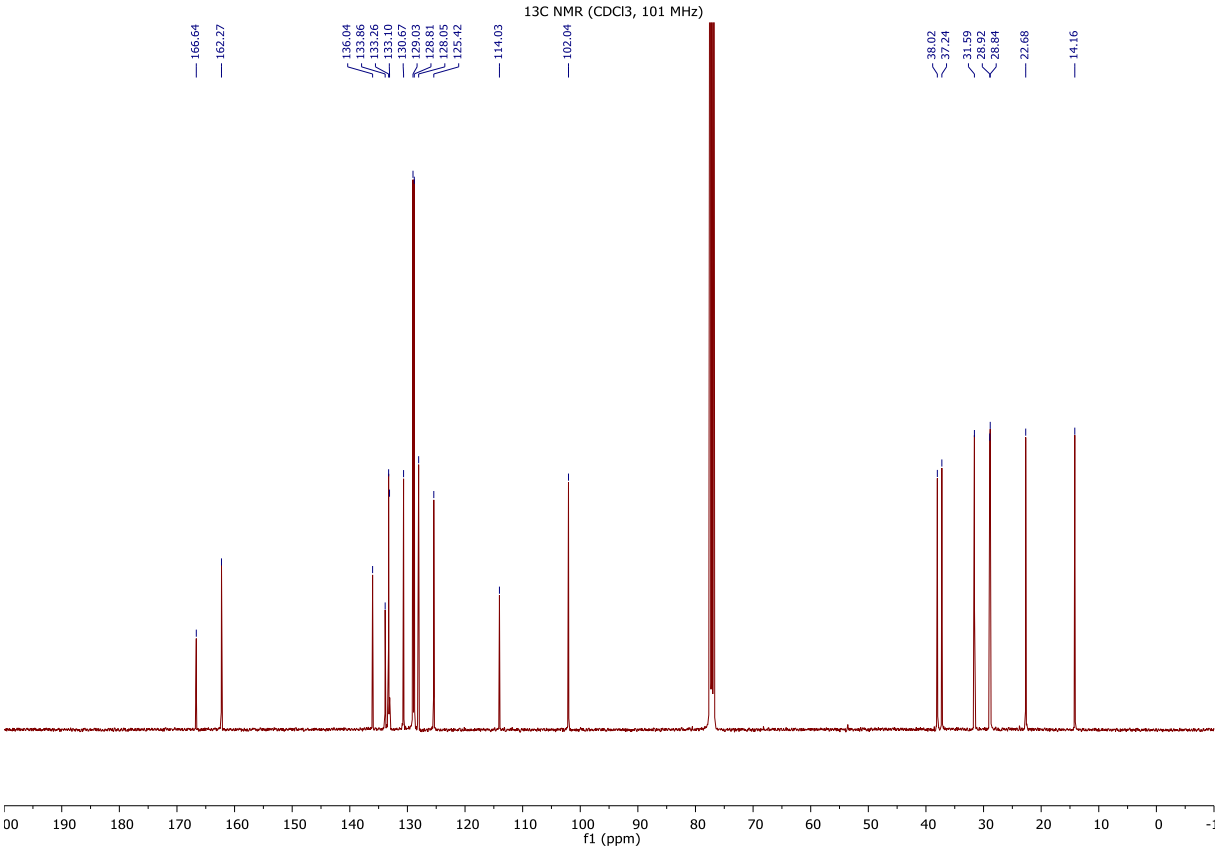
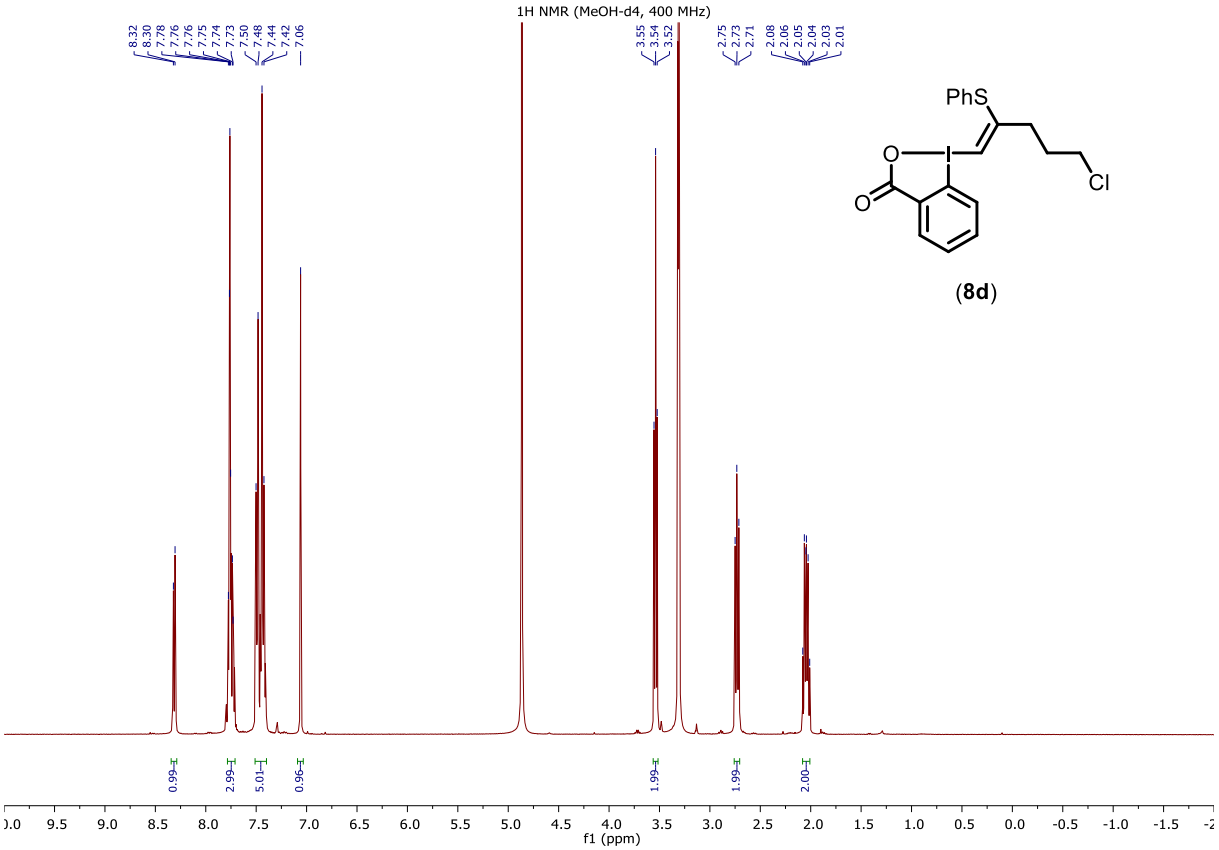


Figure S244: 13C NMR spectrum of **9c**.



(**8d**)

Figure S245: 1H NMR spectrum of **8d**.

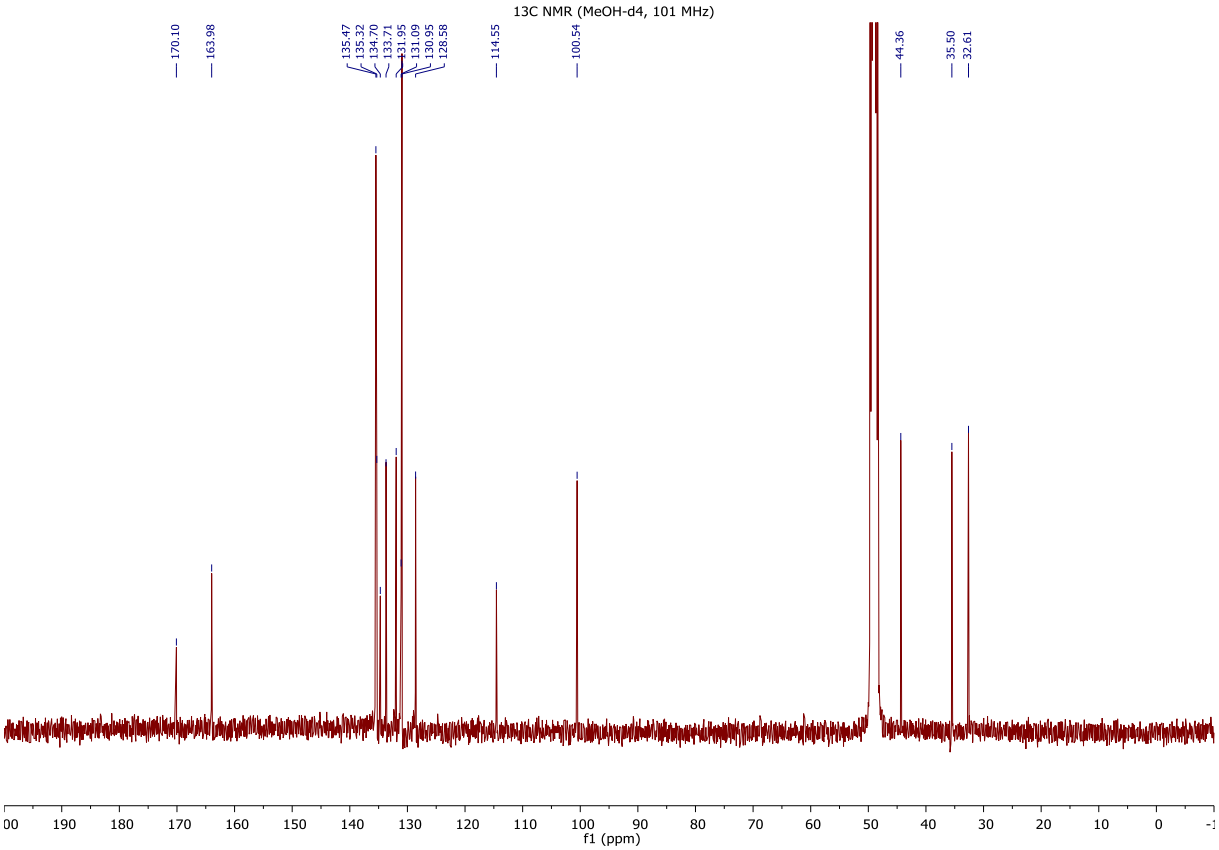


Figure S246: 13C NMR spectrum of **8d**.

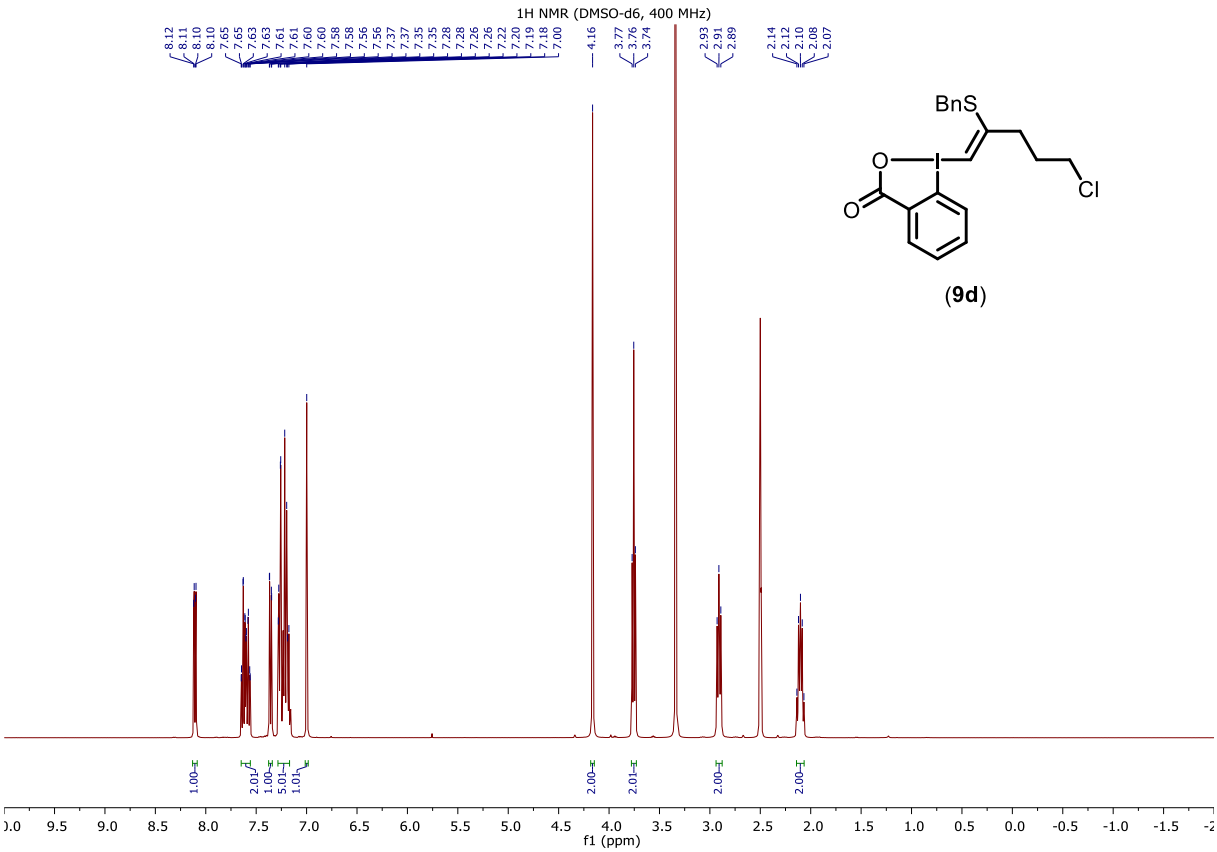


Figure S247: 1H NMR spectrum of **9d**.

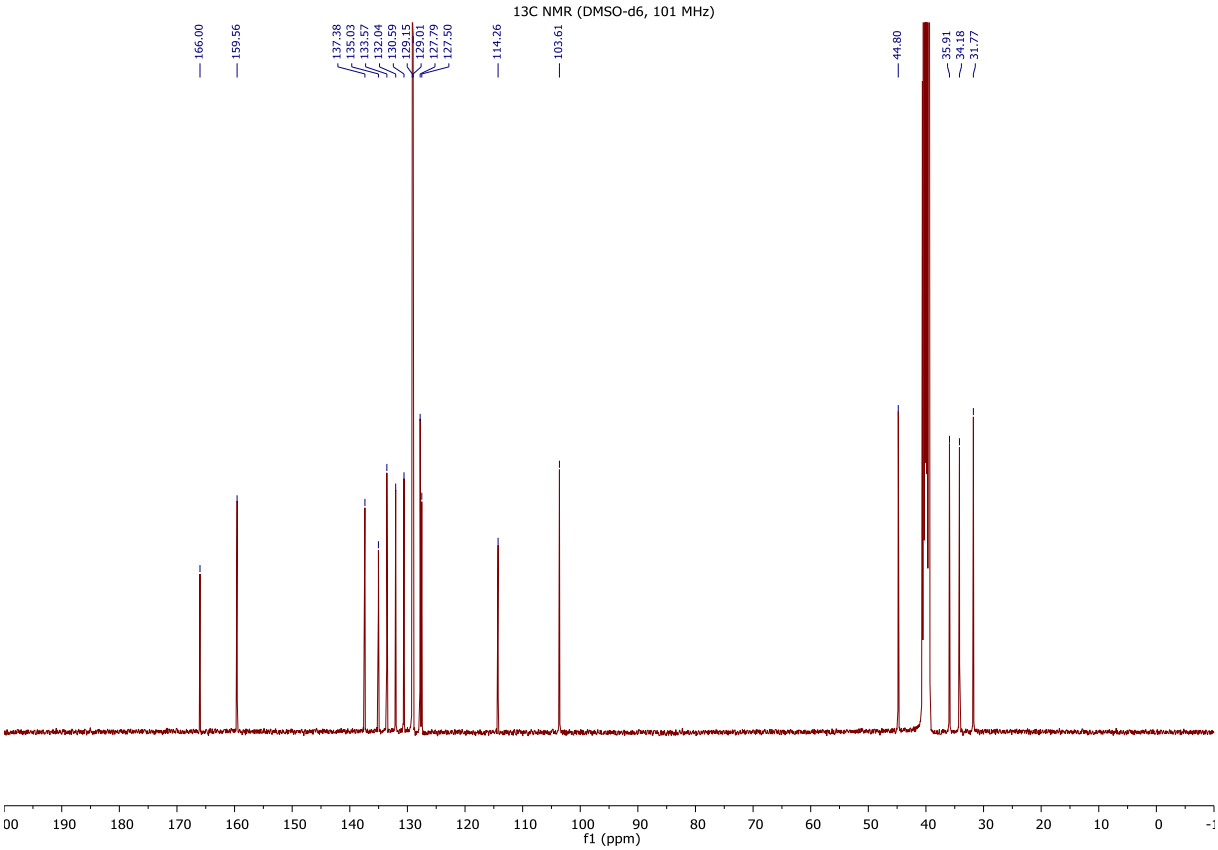


Figure S248: 13C NMR spectrum of **9d**.

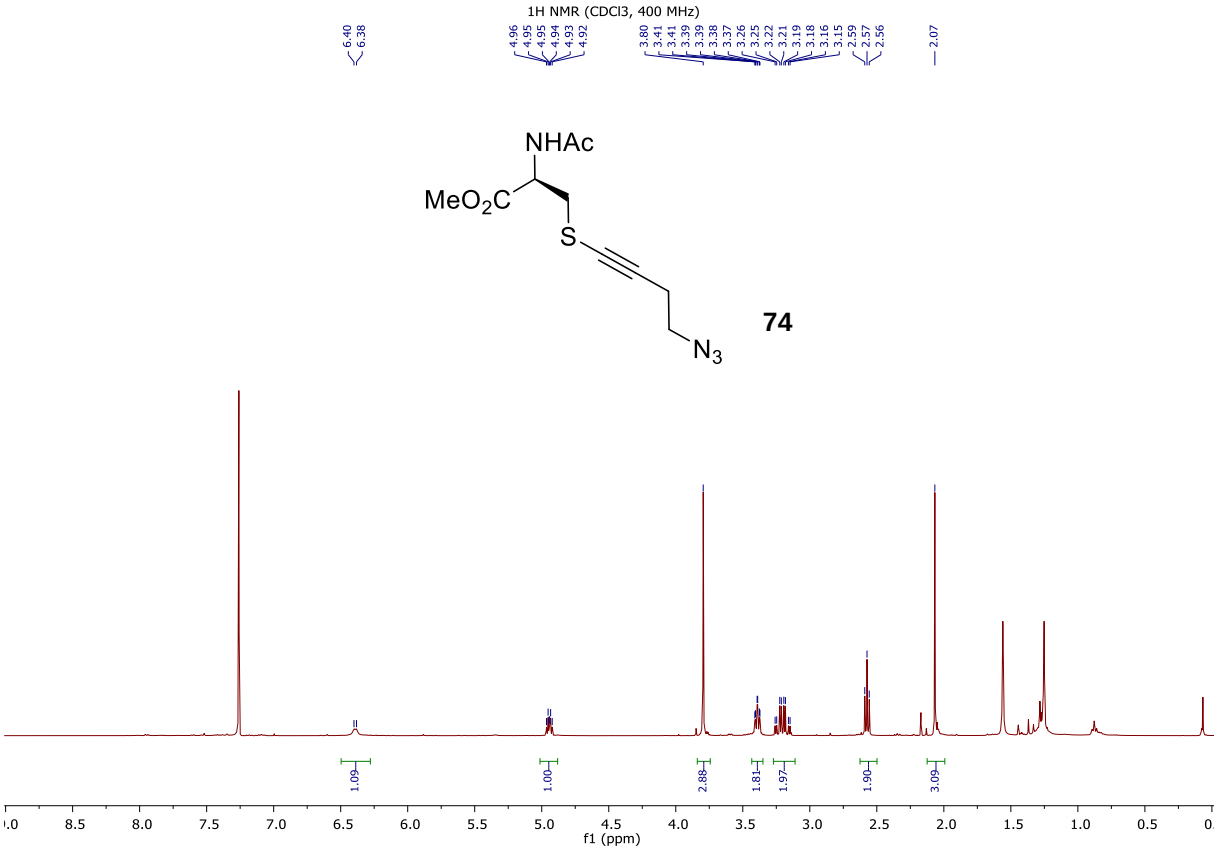


Figure S249: 1H NMR spectrum of **74**.

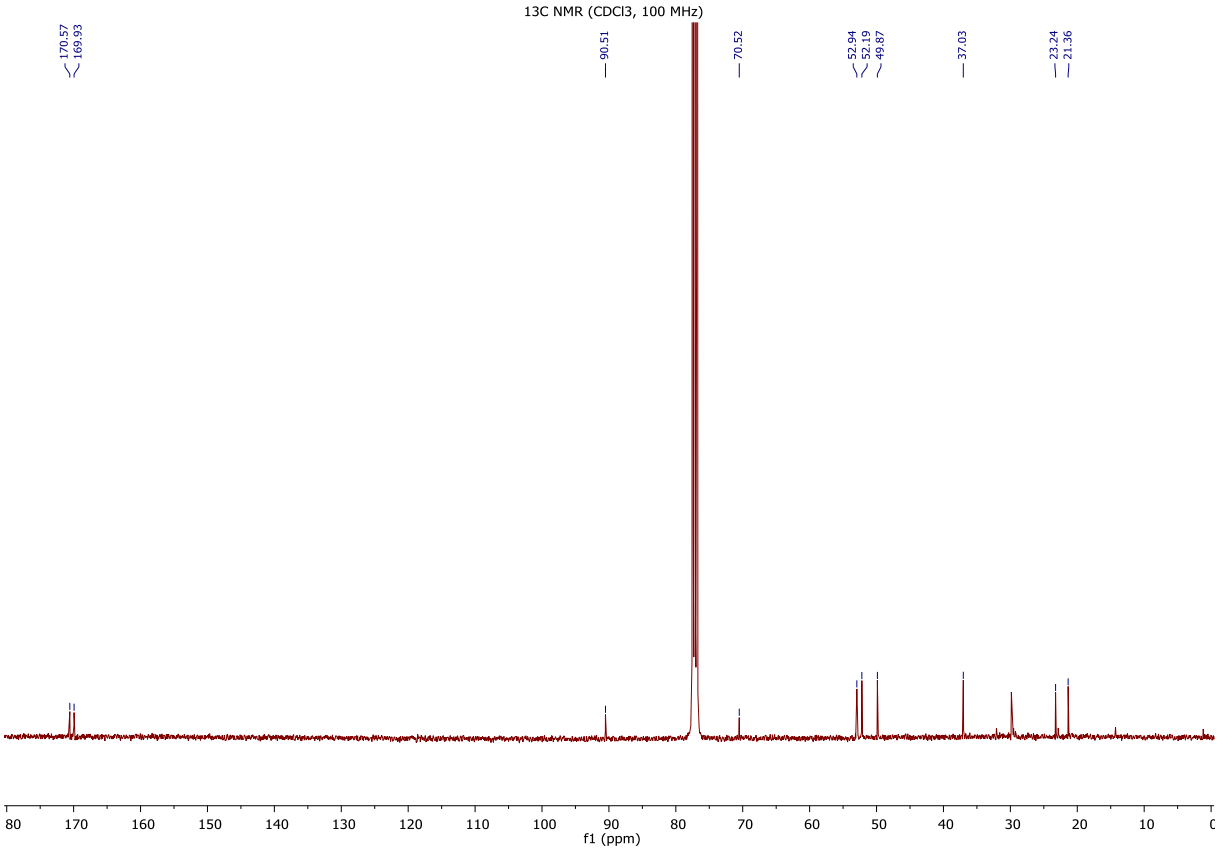


Figure S250: 13C NMR spectrum of **74**.

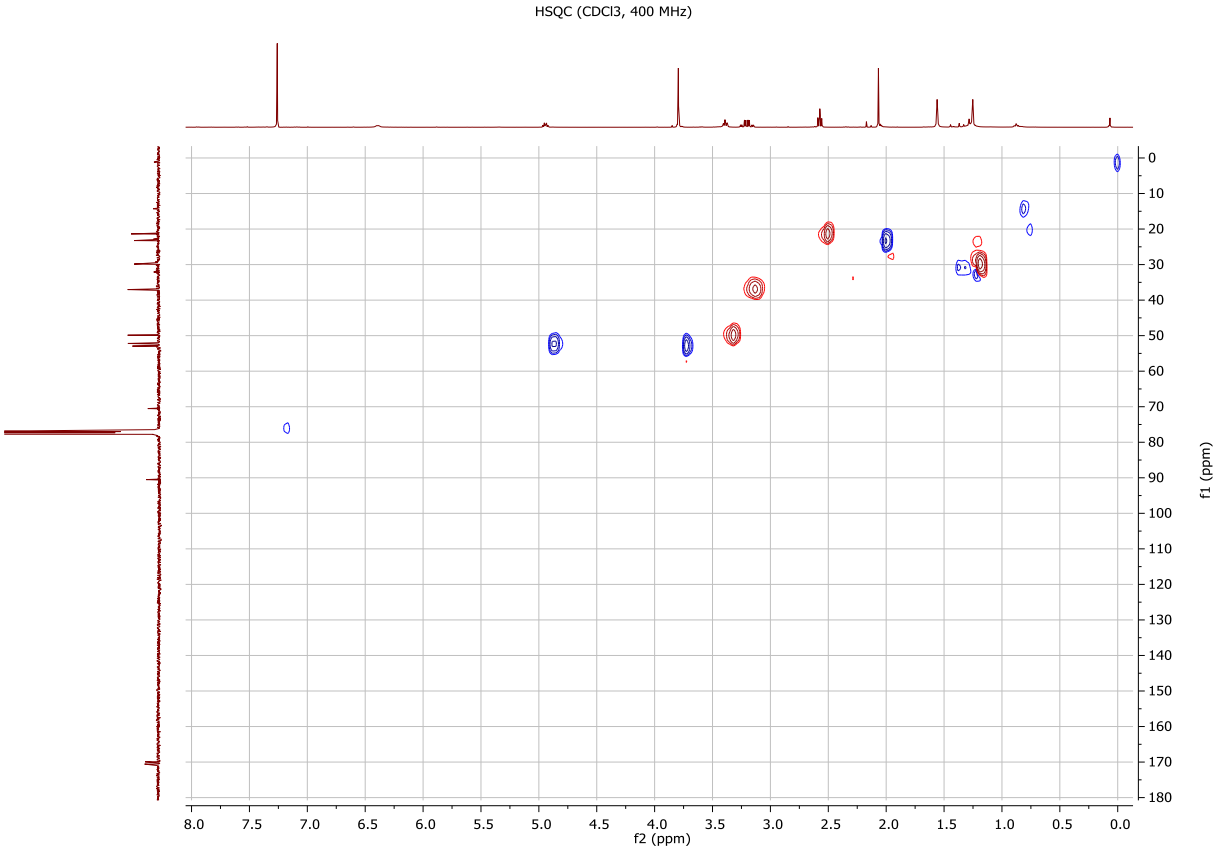


Figure S251: HSQC NMR spectrum of **74**.

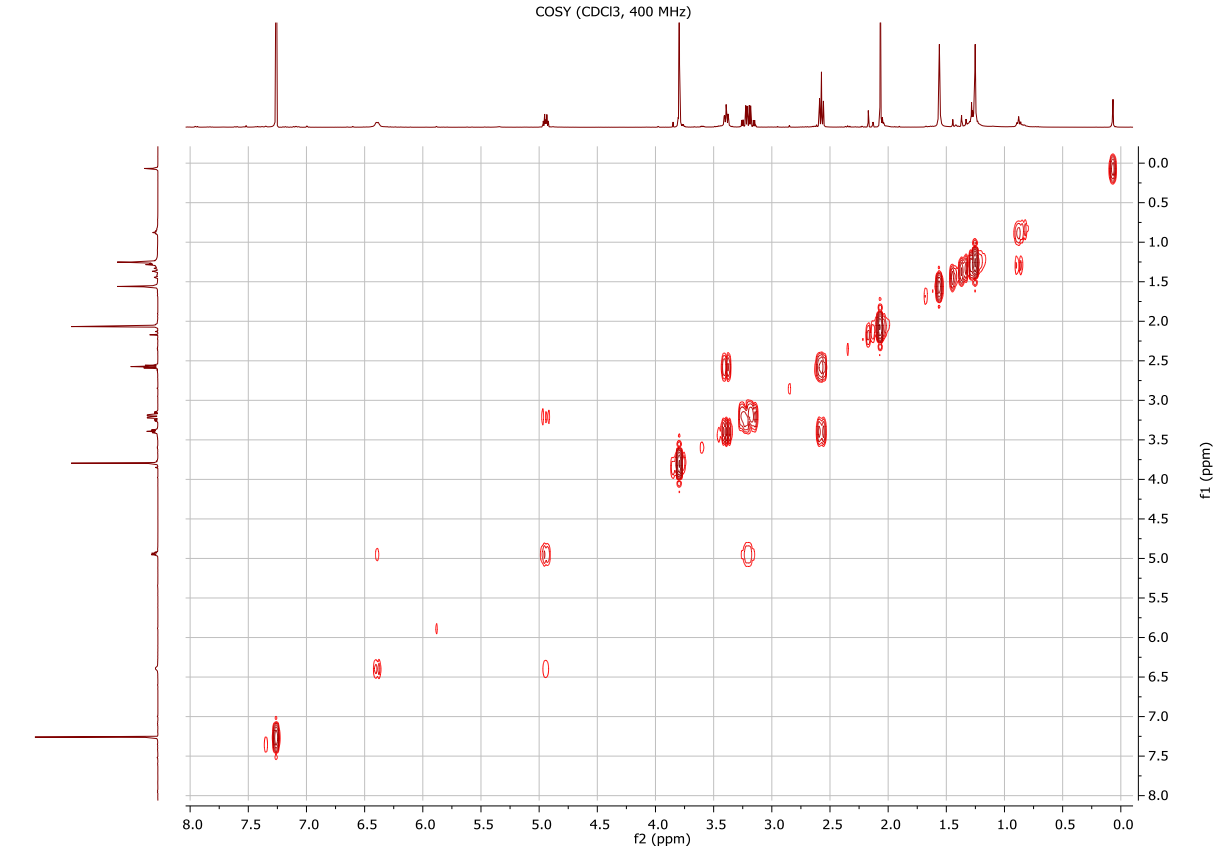


Figure S252: COSY NMR spectrum of **74**.

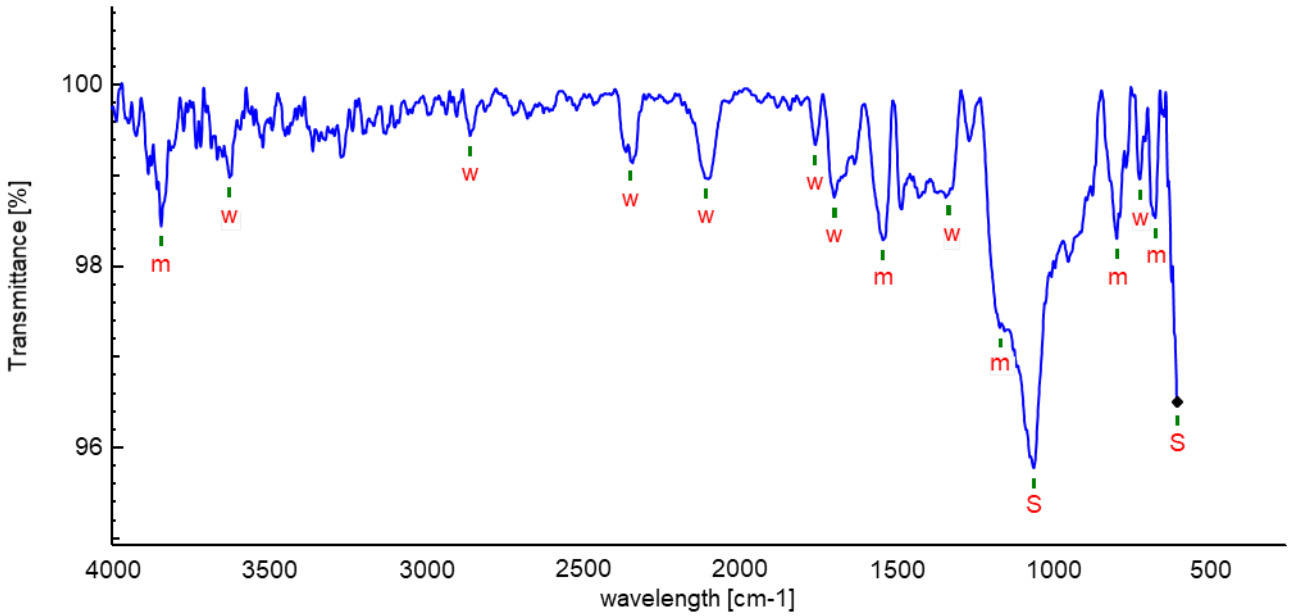


Figure S253: IR NMR spectrum of **74**.

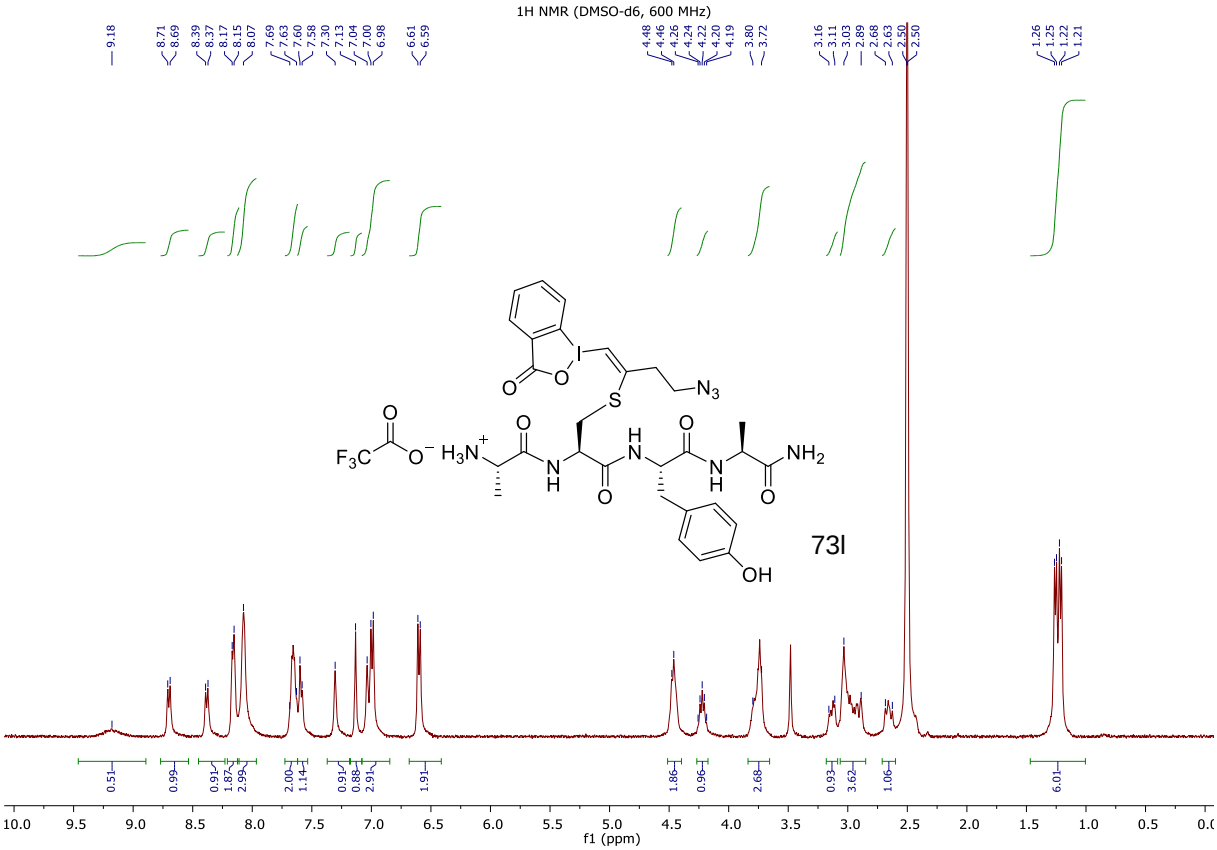


Figure S254: 1H NMR spectrum of **73l**.

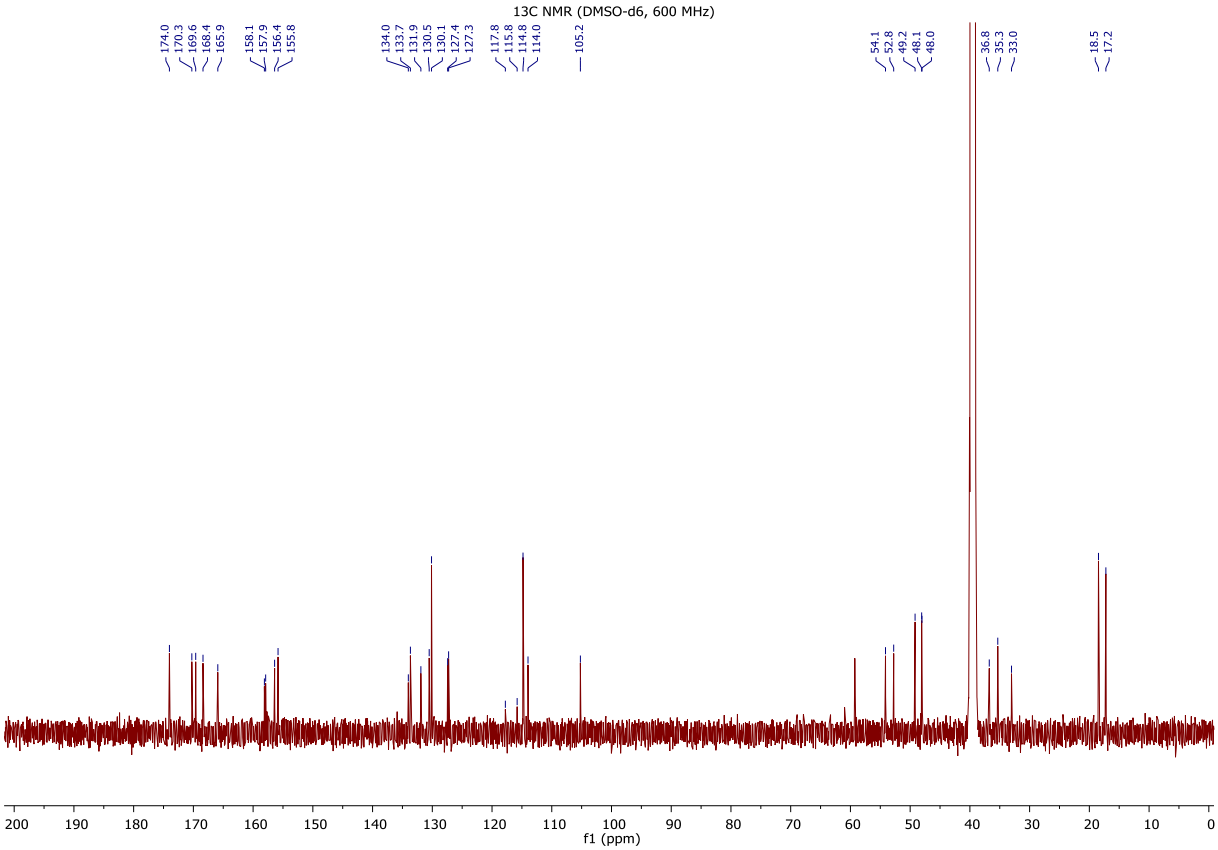


Figure S255: 13C NMR spectrum of **73l**.

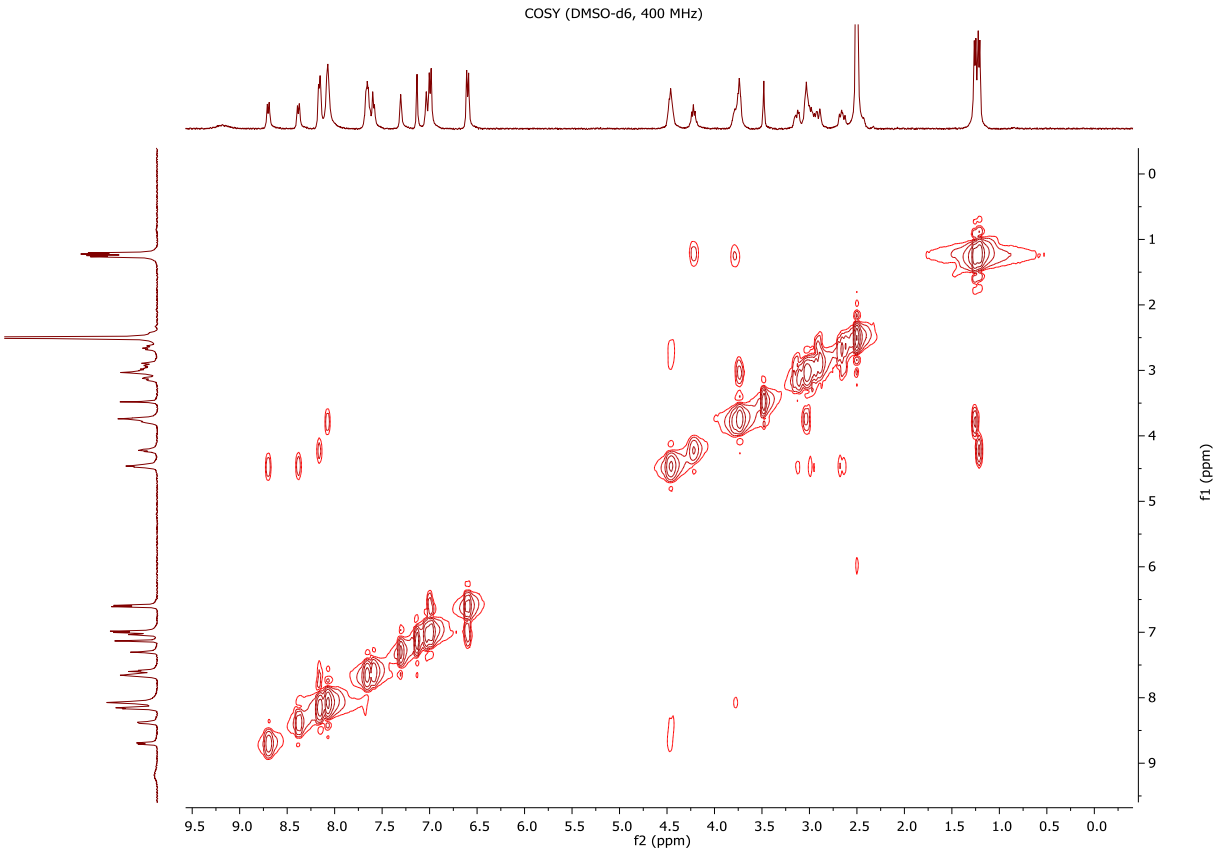


Figure S256: COSY NMR spectrum of **73l**.

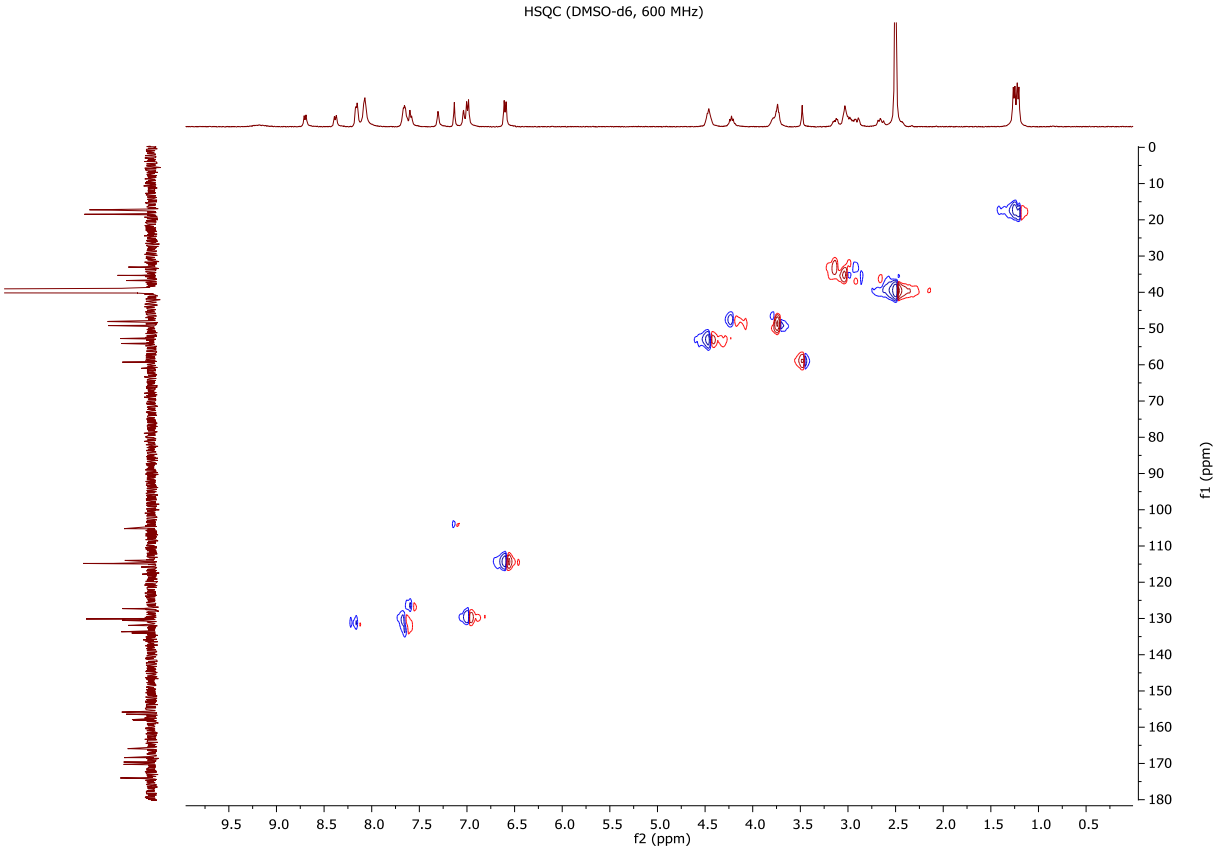


Figure S257: HSQC NMR spectrum of **73l**.

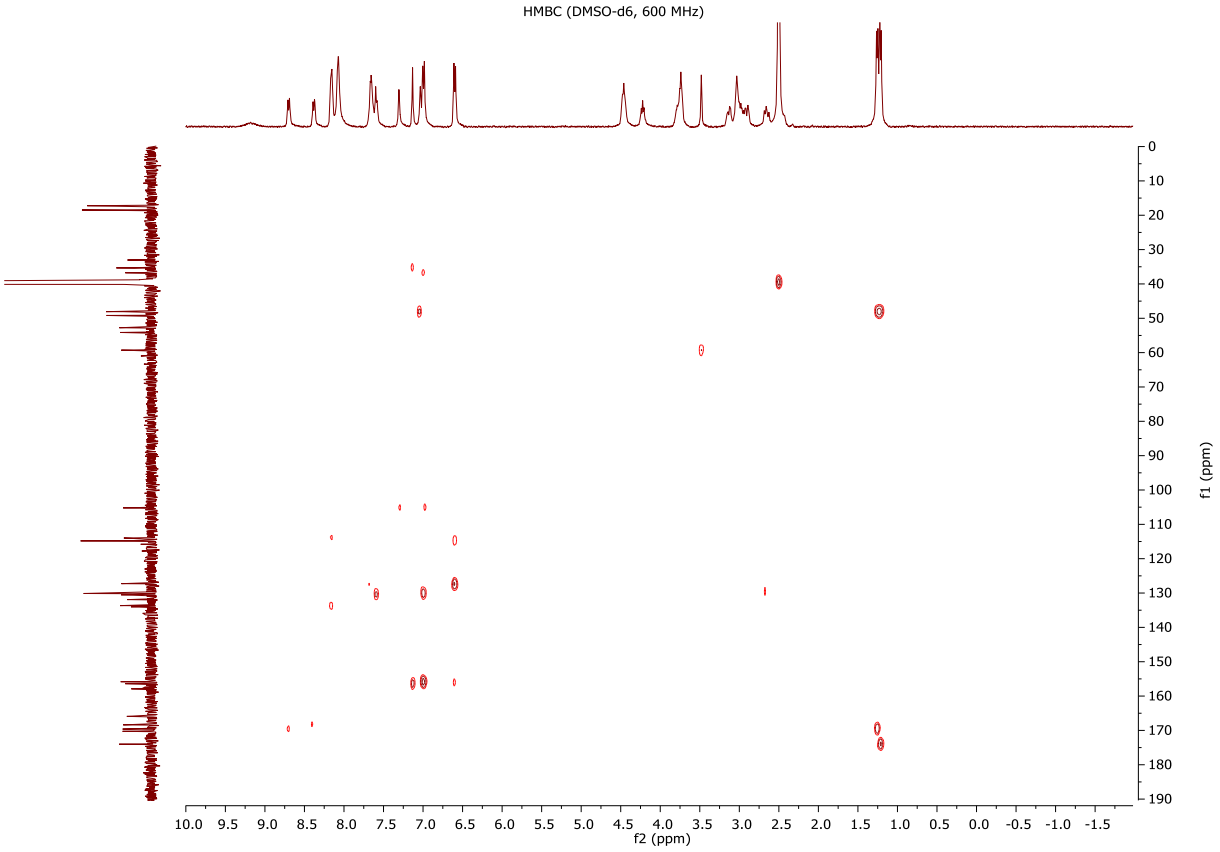


Figure S258: HMBC NMR spectrum of **73l**.

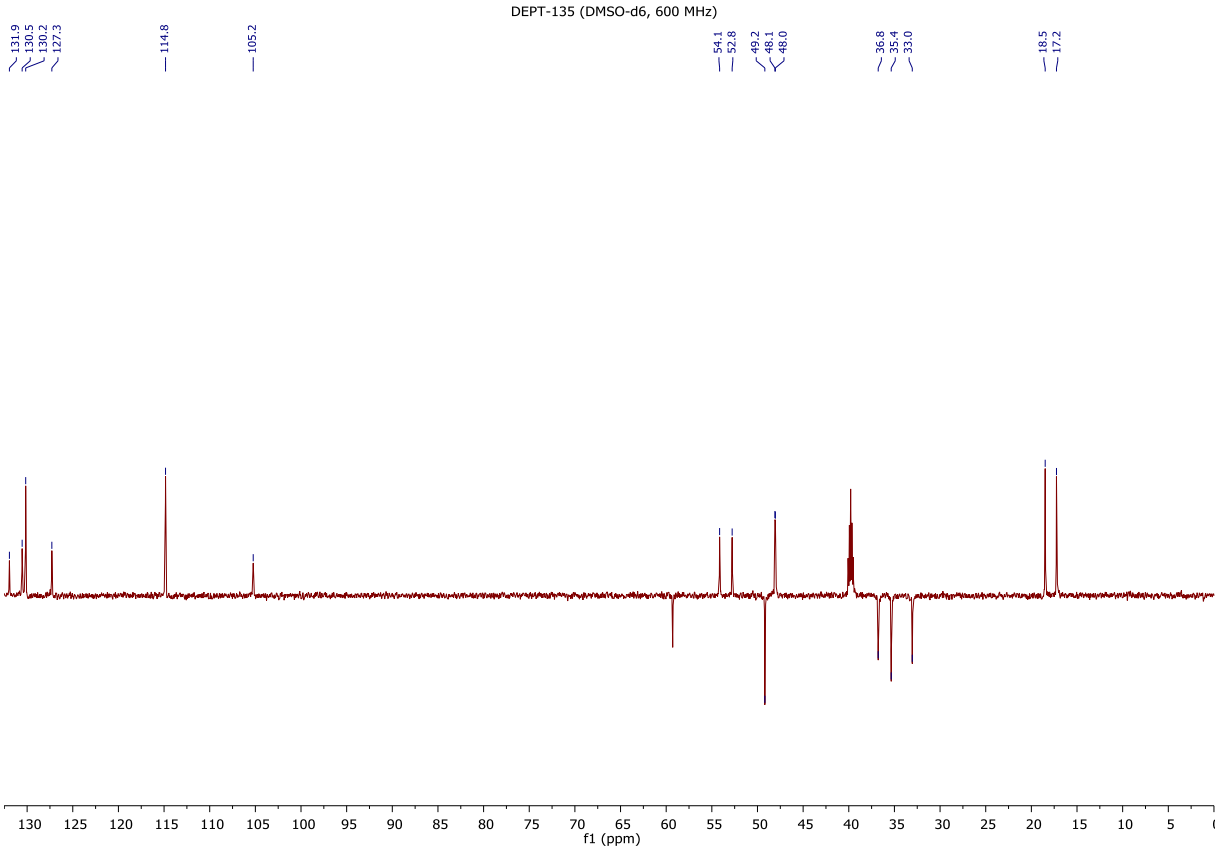


Figure S259: DEPT-135 NMR spectrum of **73l**.

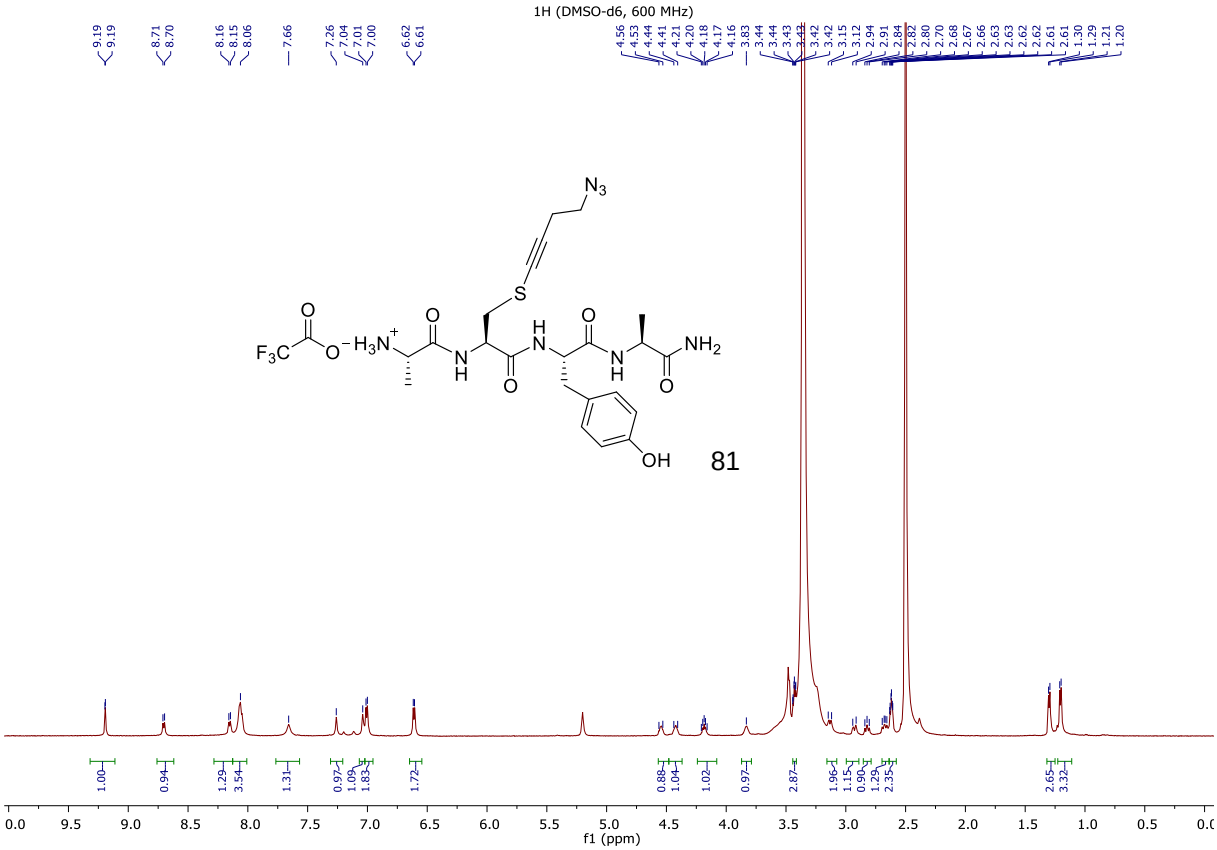


Figure S260: 1H NMR spectrum of **81**.

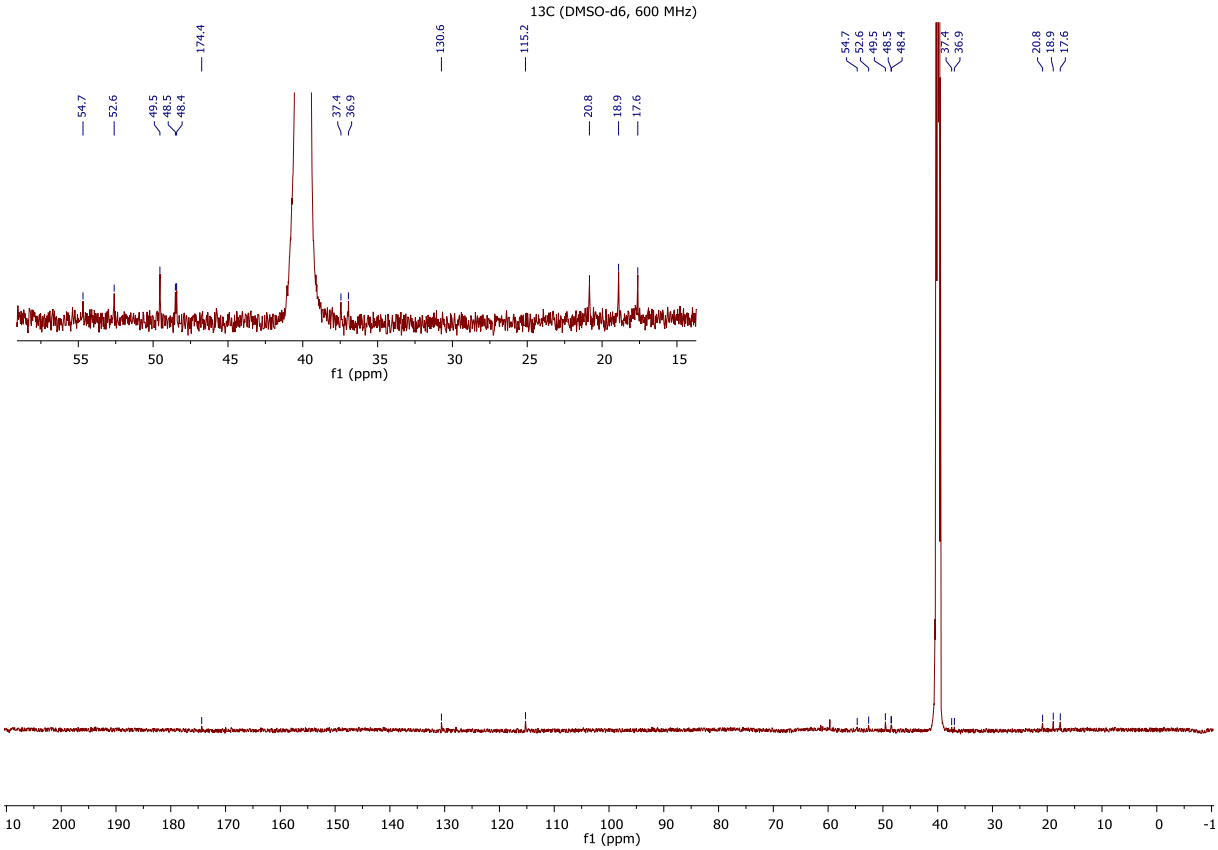


Figure S261: 13C NMR spectrum of **81**.

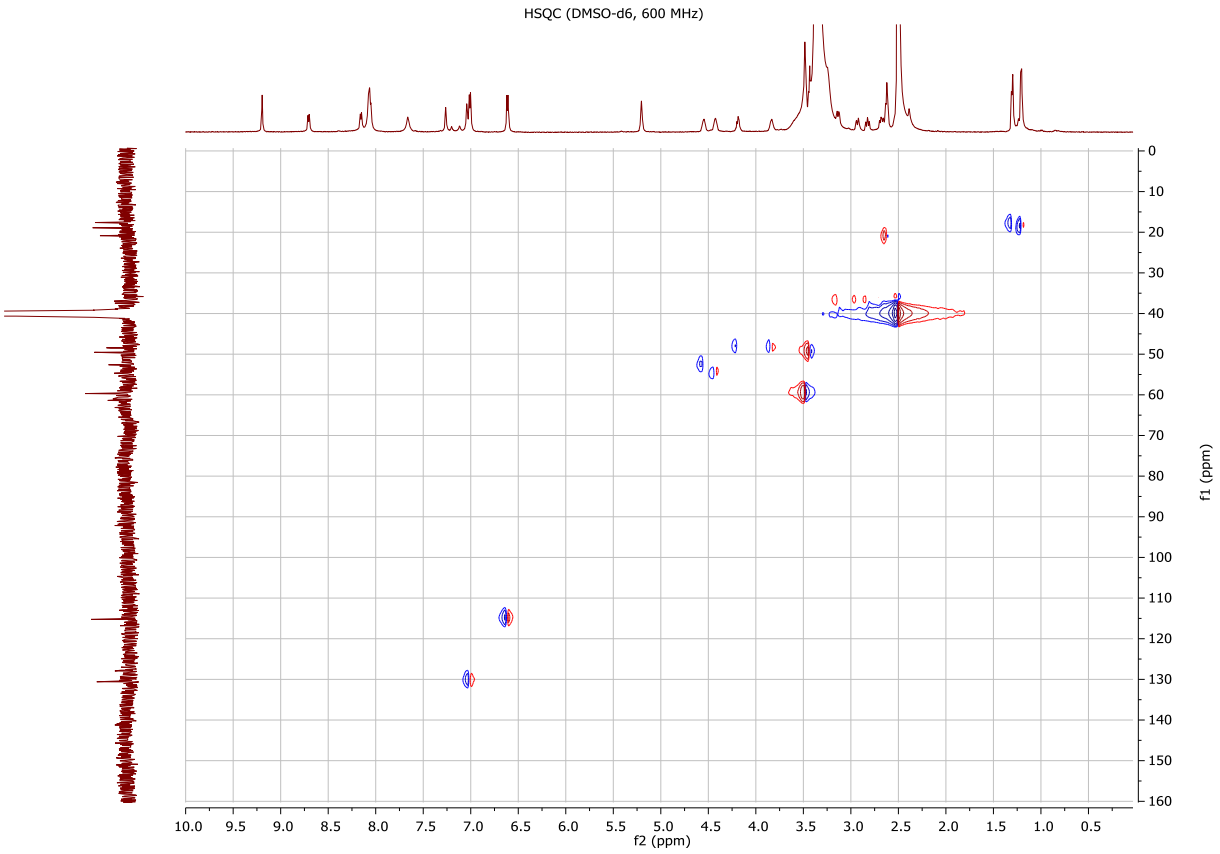


Figure S262: HSQC NMR spectrum of **81**.

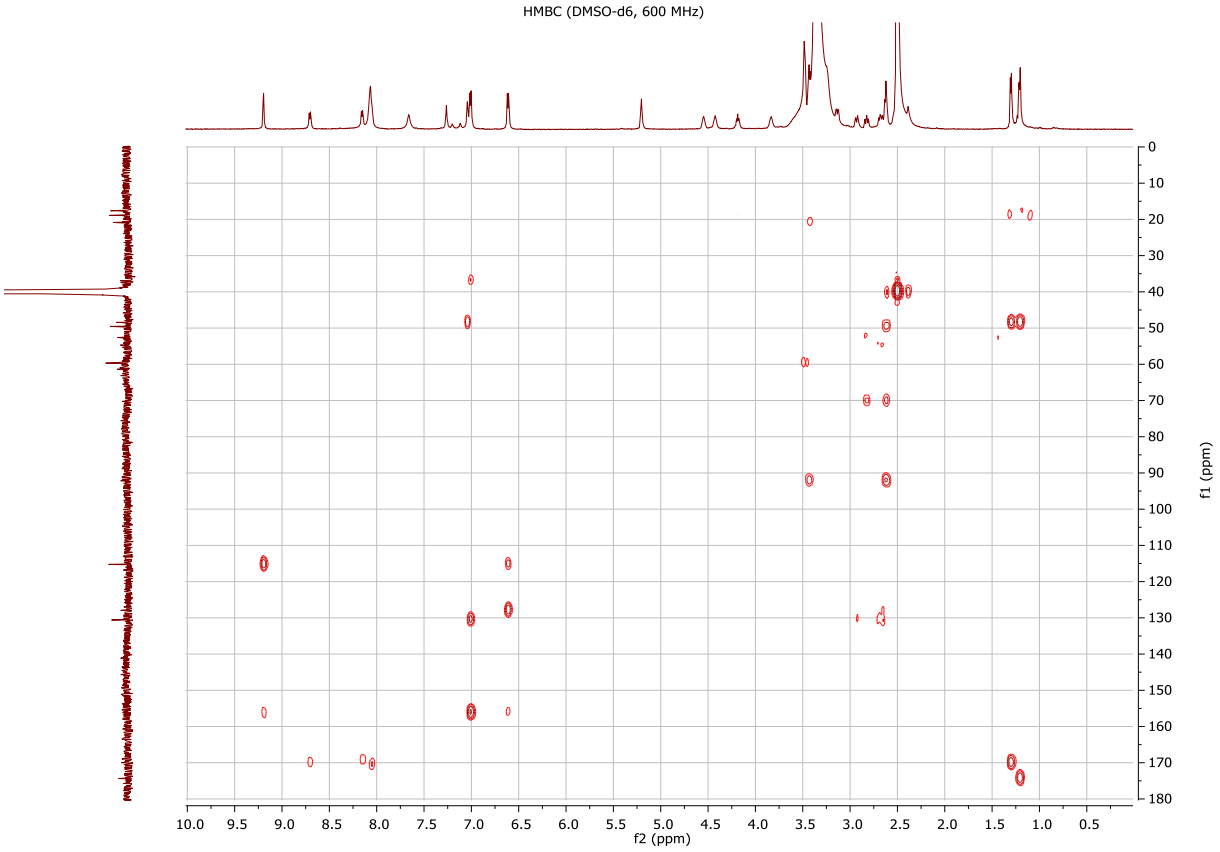


Figure S263: HMBC NMR spectrum of **81**.

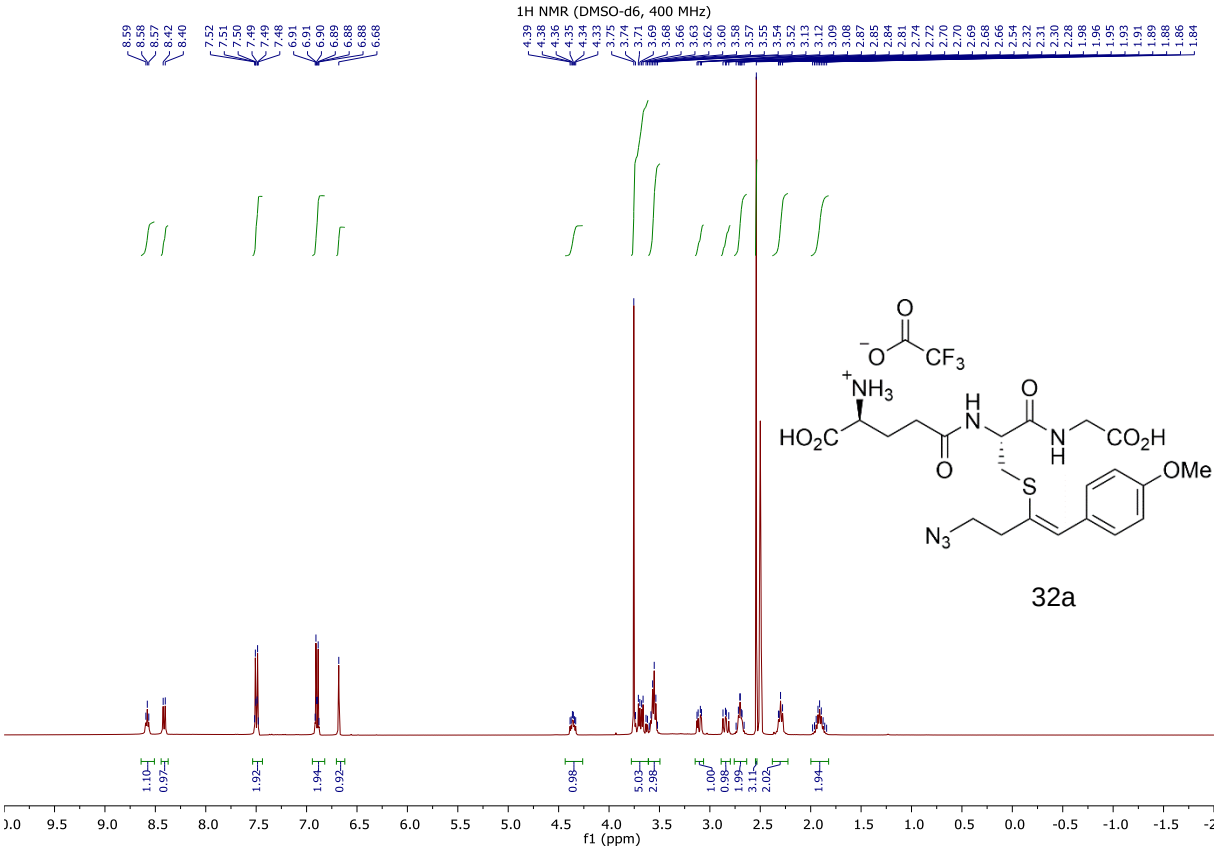


Figure S264: 1H NMR spectrum of **32a**.

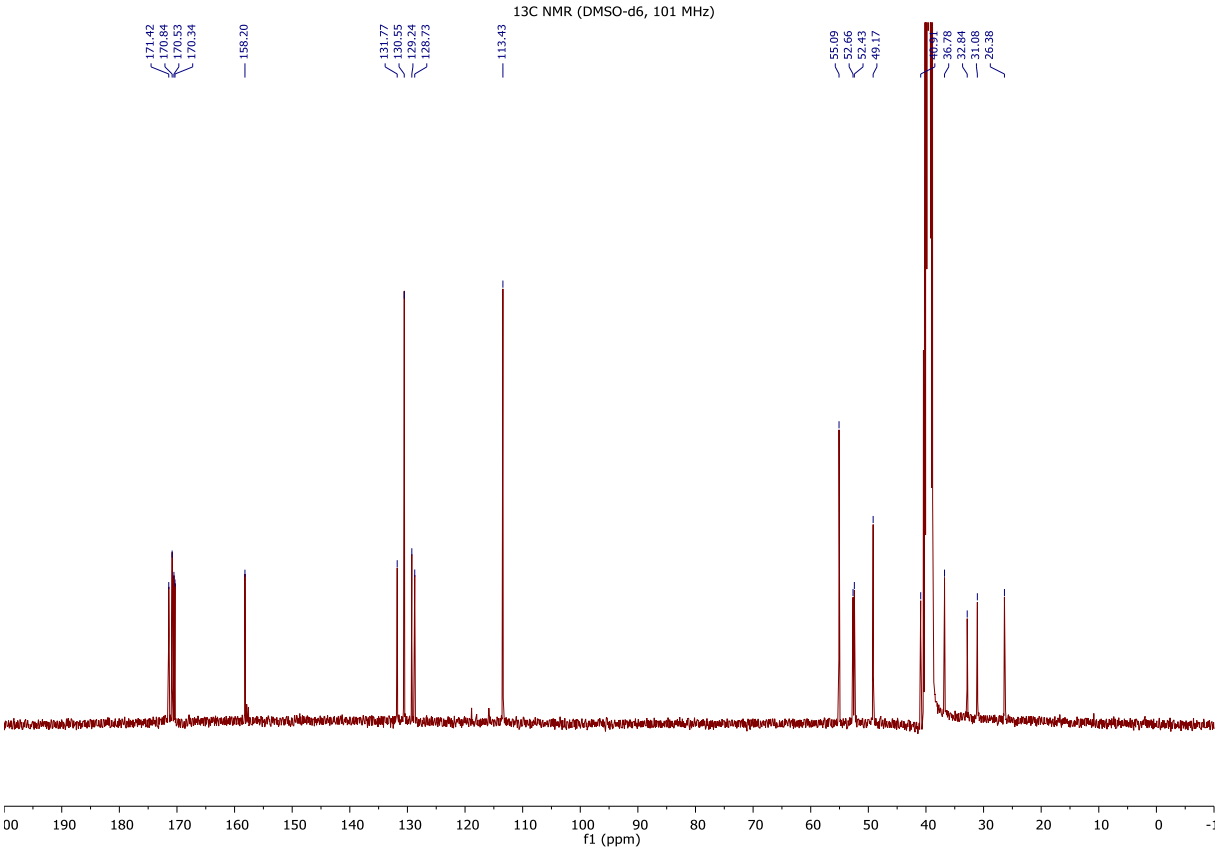


Figure S265: 13C NMR spectrum of **32a**.

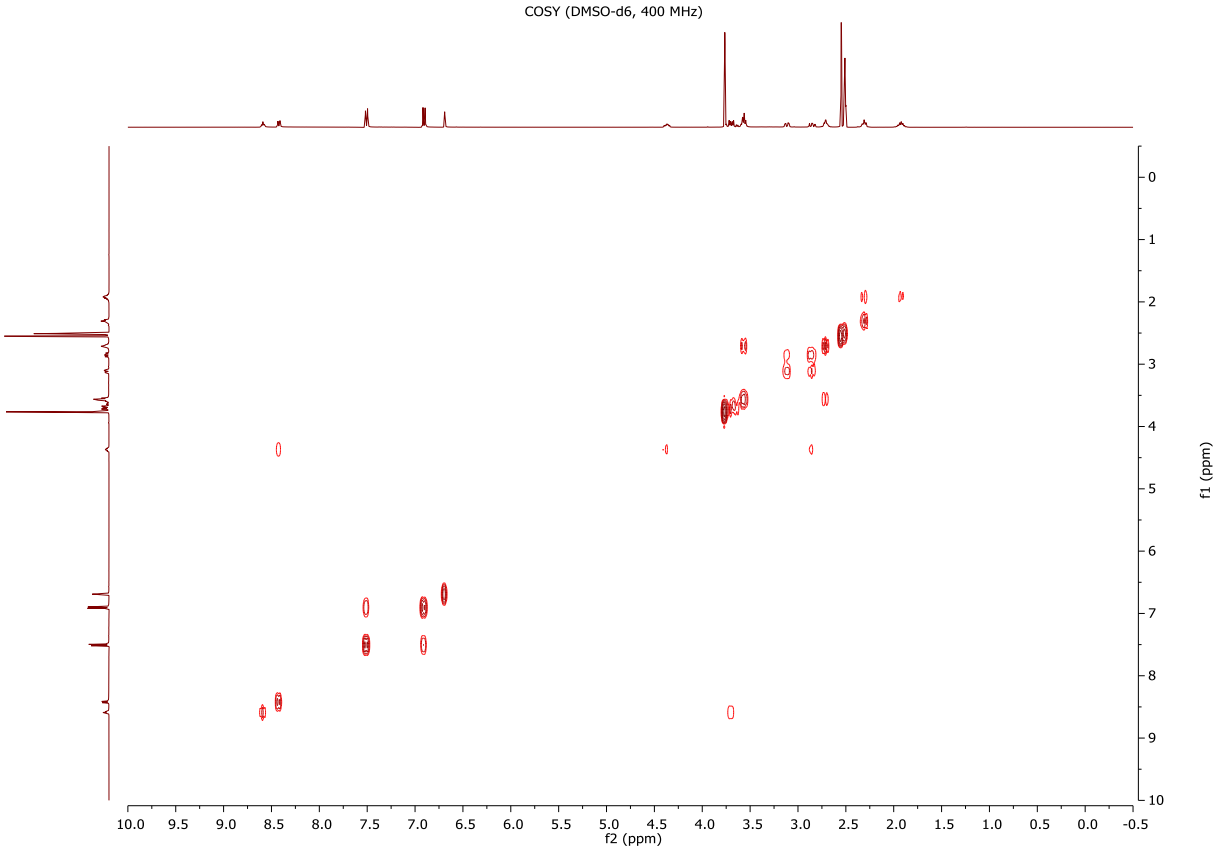


Figure S266: COSY NMR spectrum of **32a**.

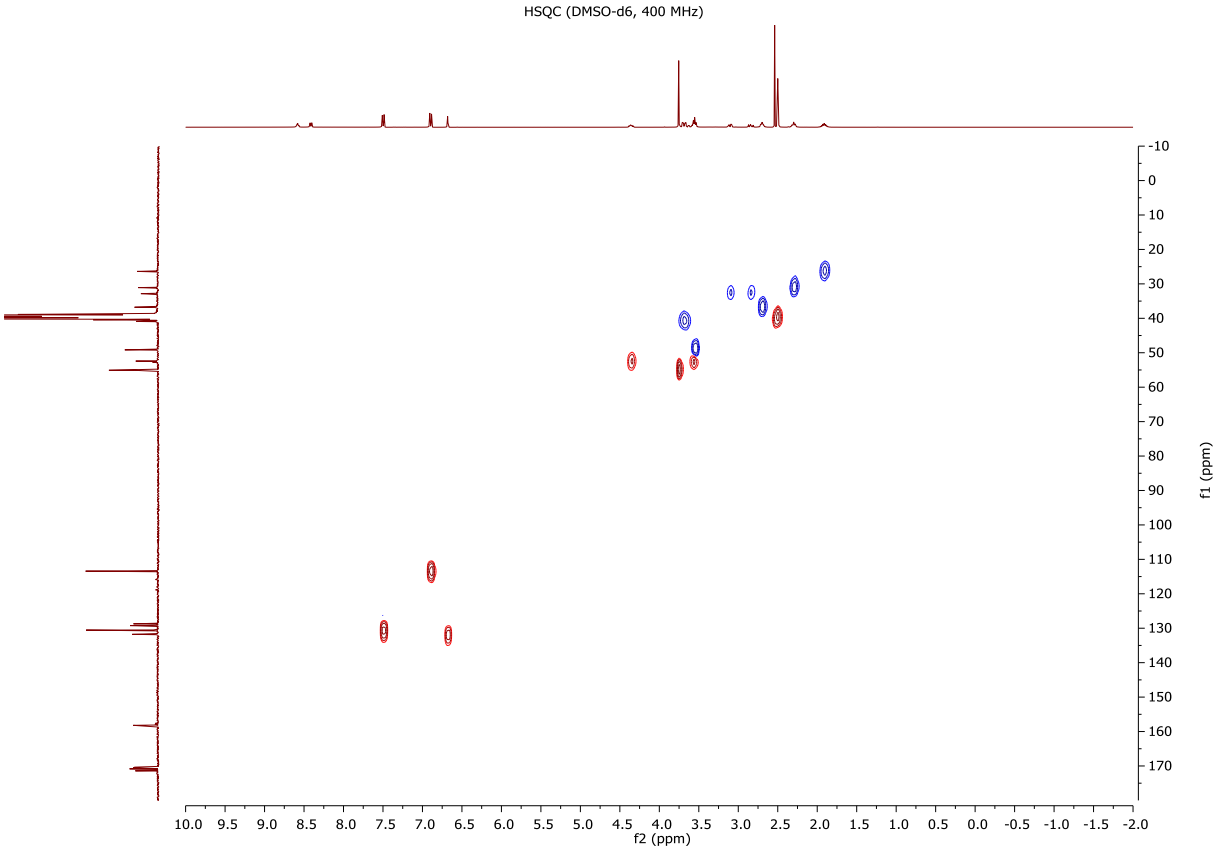


Figure S267: HSQC NMR spectrum of **32a**.

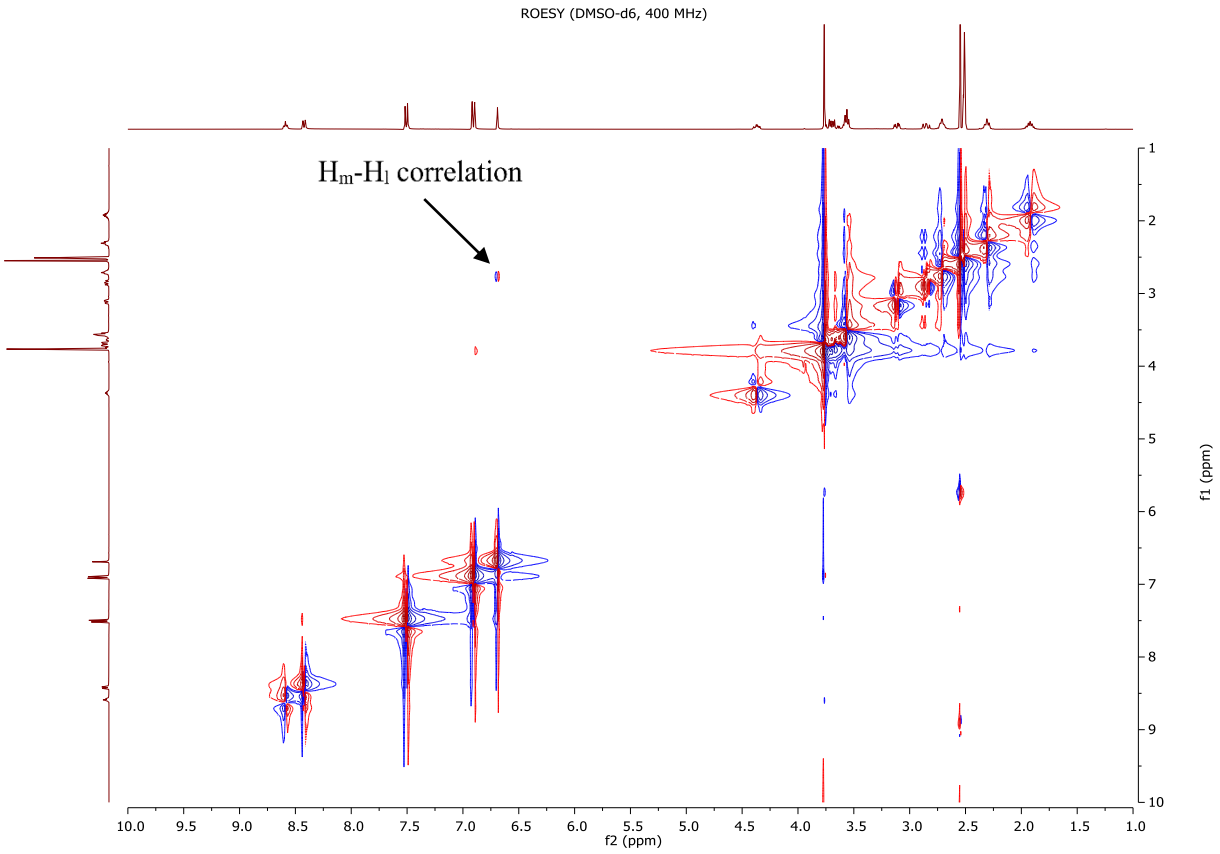


Figure S268: ROESY NMR spectrum of **32a**.

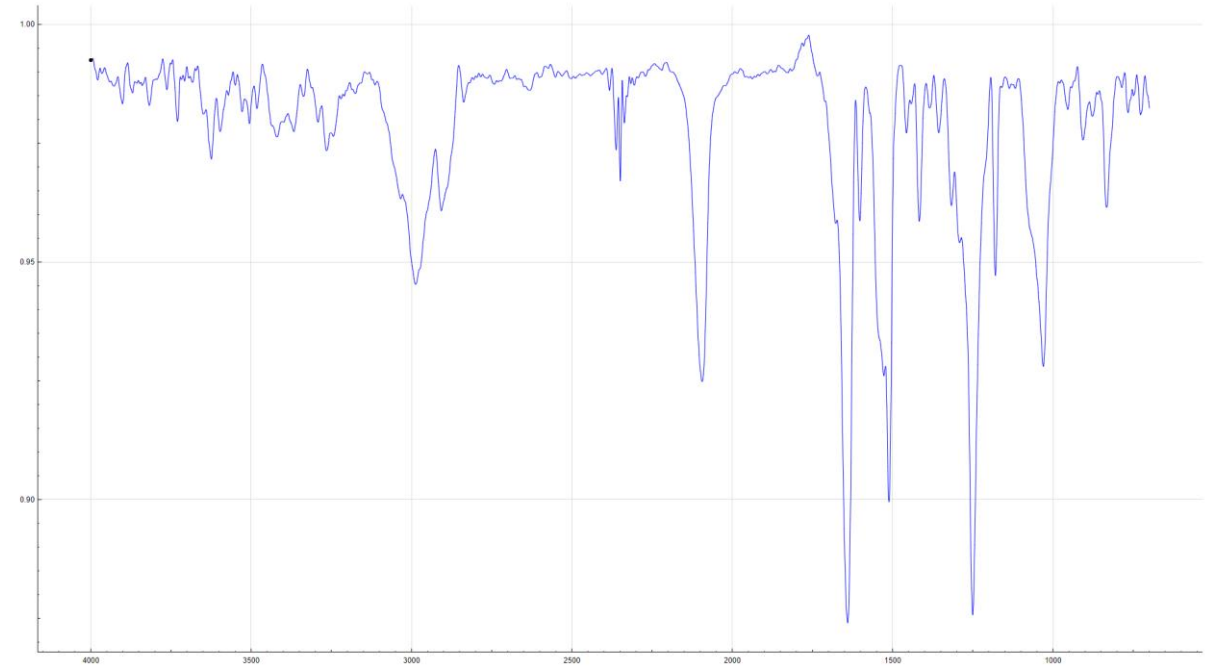


Figure S269: IR spectrum of **32a**.

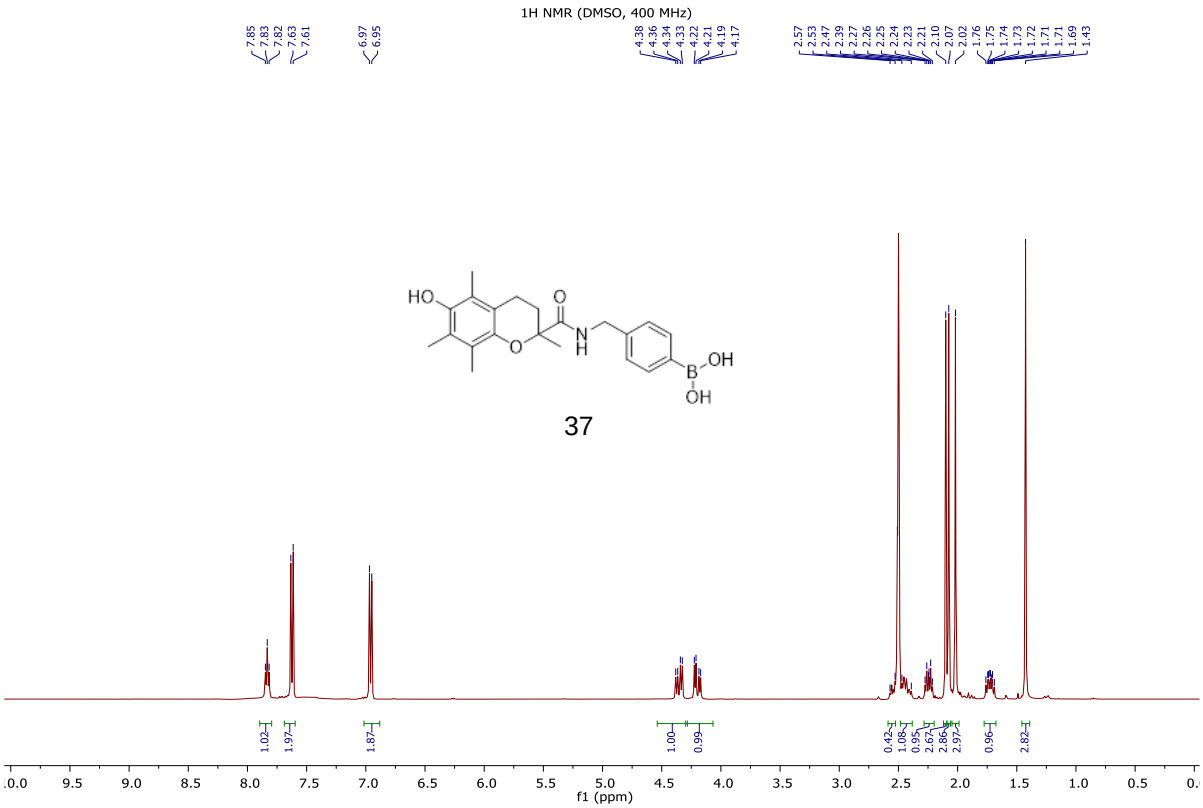


Figure S270: 1H NMR spectrum of **37**.

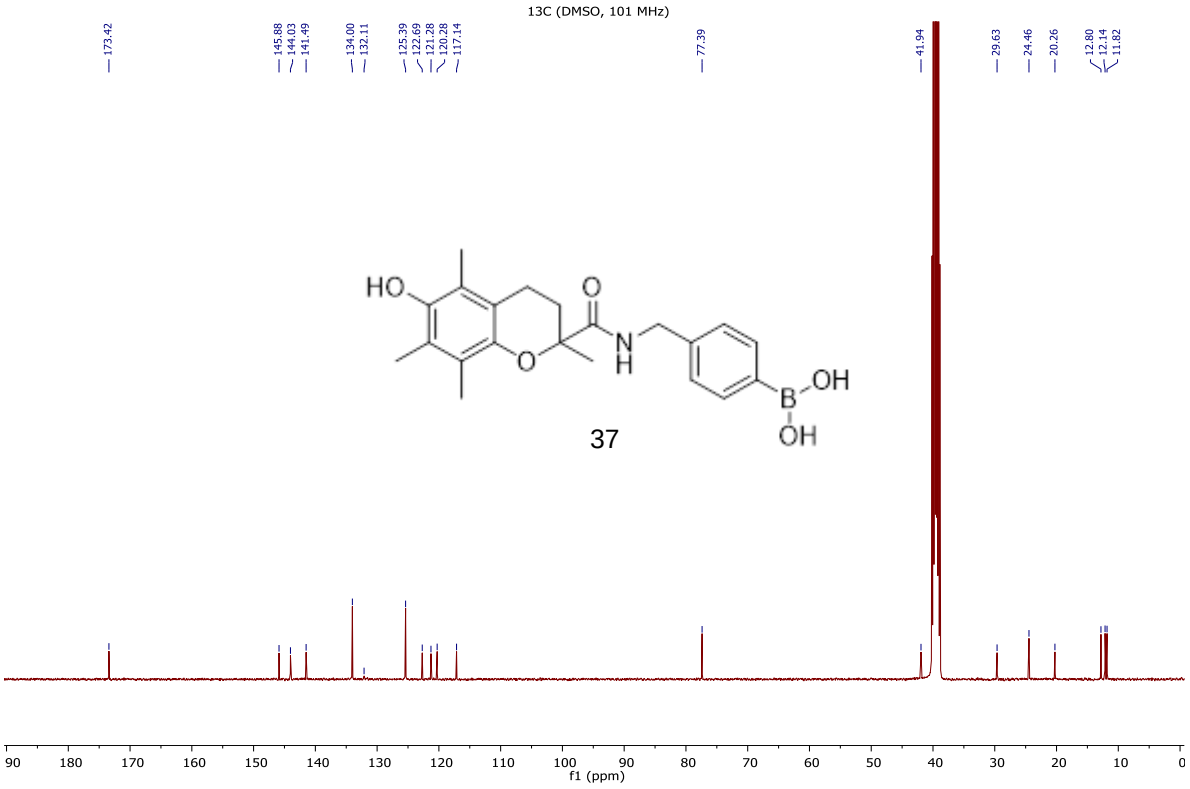


Figure S271: 13C NMR spectrum of **37**.

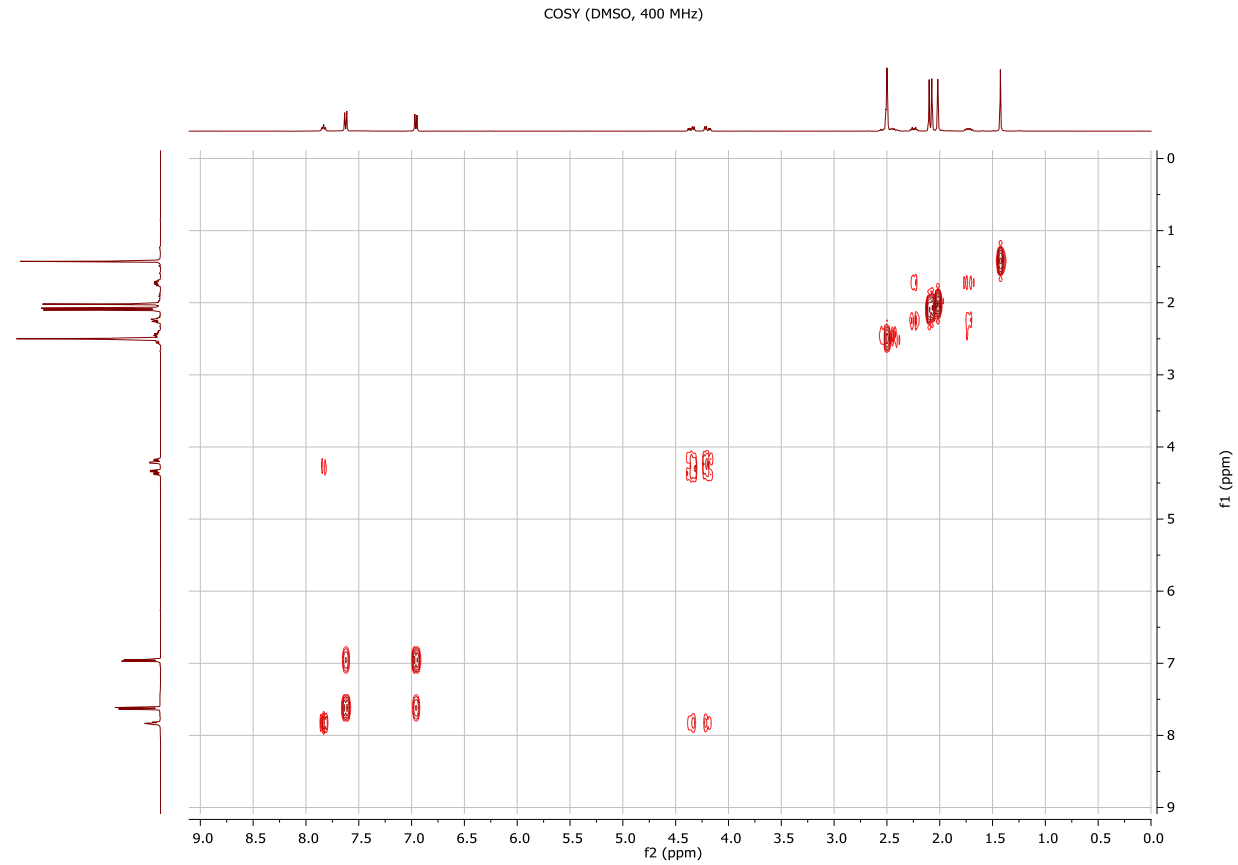


Figure S272: COSY NMR spectrum of **37**.

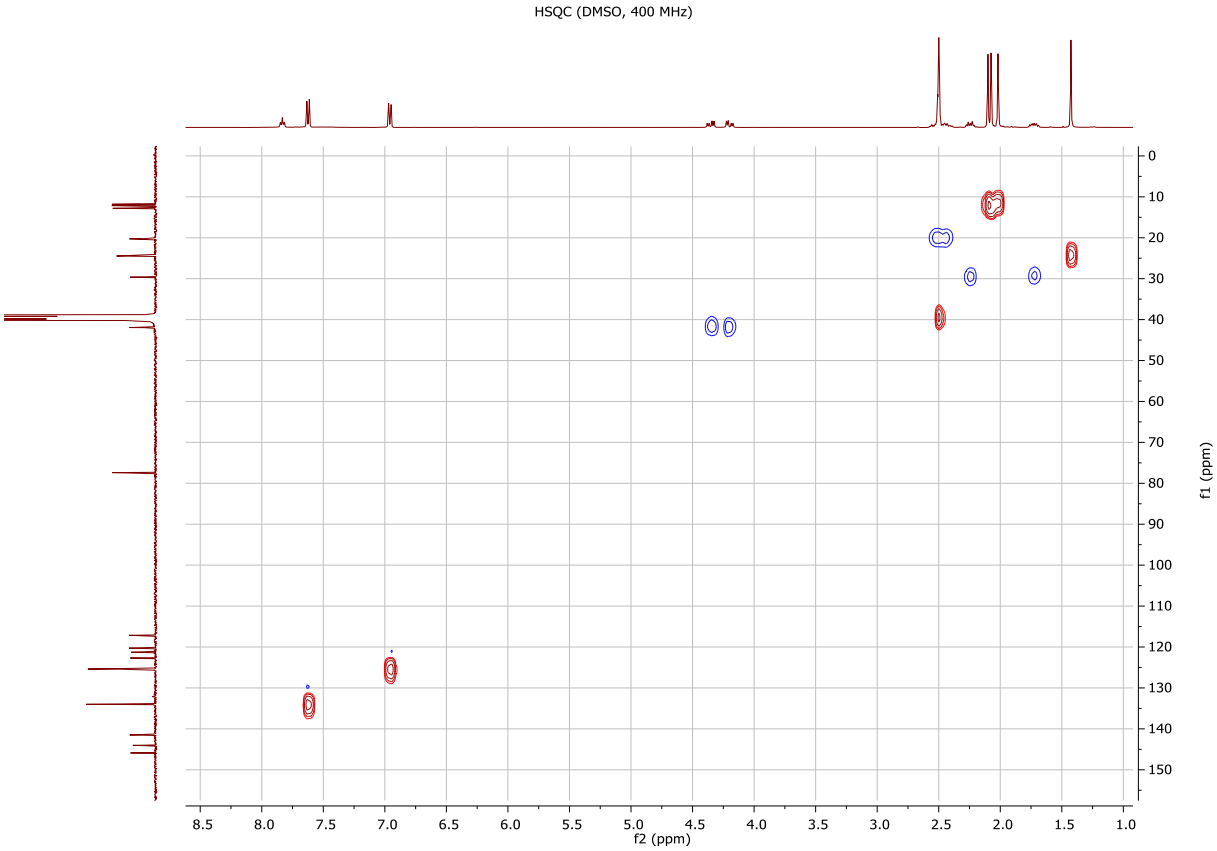


Figure S273: HSQC NMR spectrum of **37**.

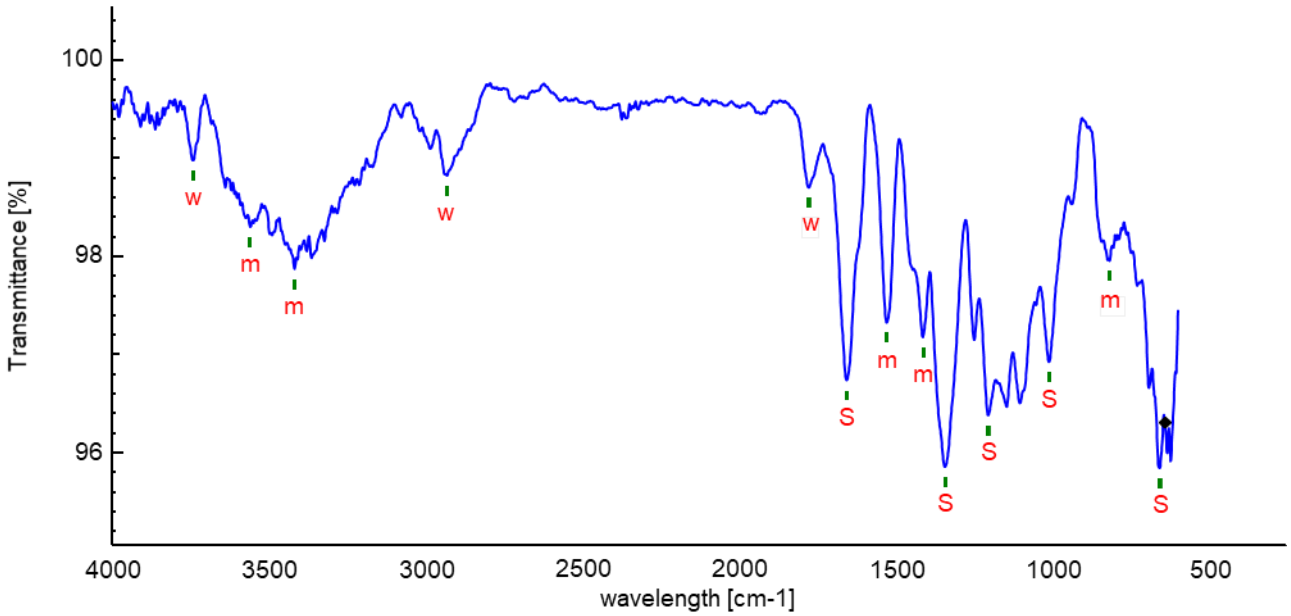


Figure S274: IR spectrum of **37**.

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